Modulation of p145<sup>c-kit</sup> Function in Cells of Patients with Acute Myeloblastic Leukemia<sup>1</sup>

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ABSTRACT

The function of the steel factor receptor, p145<sup>c-kit</sup>, in patient-derived acute myeloblastic leukemia (AML) cells was investigated. Steel factor stimulation of AML cells coexpressing p145<sup>c-kit</sup> and the progenitor cell antigen CD34 resulted in complete receptor down-regulation, a marked decrease of CD34 antigen expression, and the induction of the granulocytic lineage antigen CD15. These changes in surface marker expression paralleled morphological differentiation to granulated blasts and promyelocytes. Interestingly, the same phenotype was achieved by IL-3 stimulation of AML cells. p145<sup>c-kit</sup> extracellular domain-specific antibodies had either blocking or enhancing effects on ligand binding, receptor phosphorylation and down-regulation, and induction of cell proliferation. Correlations of these phenomena with distinct effects of antibody stimulation on cell substrate phosphorylation provide clues for the dissection of the p145<sup>c-kit</sup> signal and the analysis of its relevance for AML treatment.

INTRODUCTION

The product encoded by the protooncogene c-kit (p145<sup>c-kit</sup>) is a cell surface RTK<sup>1</sup> (1, 2) with structural homologies to the receptors for PDGF (3, 4) and macrophage CSF-1 (5–7). p145<sup>c-kit</sup> is activated by binding a dimeric ligand, which is known as kit ligand, SCF, MGF, or SLF, and is thought to mediate receptor dimerization (8–11). In addition to other functions in melanocytes, germ cells, and possibly cells of the nervous system, SLF supports the growth of hemopoietic progenitor and stem cells when combined with other growth factors (12–15). Recent studies have shown that SLF can directly induce proliferation and maturation of progenitor cells (16). Moreover, cultures of blasts from patients with AML respond to added SLF with an increase in spontaneous and terminal differentiation (17).

Binding of ligands to RTKs triggers diverse reactions in the cell, including receptor and substrate phosphorylation, activation of protein kinases, changes in ion fluxes, activation of enzymes involved in phospholipid metabolism, increased transcription of different protooncogenes, and rearrangements of the cytoskeleton. These and other events, which are still rather poorly characterized in molecular terms, ultimately result in cell division, differentiation, or other responses (2, 18). Most of our current knowledge regarding the mechanisms of signal generation by RTKs is based on studies in model cell systems such as mouse or rodent fibroblasts. Only recently, partly due to the insights obtained by the connection of the well-characterized genetic phenotypes of the mouse "white spotting" or "W" locus and the

1 This work was supported by the Deutsche Forschungsgemeinschaft (SFB 120, projects B5b and C5) and SUGEN, Inc. (to J. S. and A. U.).
2 To whom requests for reprints should be addressed.
3 The abbreviations used are: RTK, receptor tyrosine kinase; PDGF, platelet-derived growth factor; CSF, colony-stimulating factor; SCF, stem cell factor; MGF, mast cell growth factor; SLF, steel factor; AML, acute myeloblastic leukemia; MAb, monoclonal antibody; FAB, French-American-British; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter; rh, recombinant human; FITC, fluorescein isothiocyanate; EGF, epidermal growth factor; EGF-R, EGF receptor; SLF-b, biotinylated SLF; FGF, fibroblast growth factor; IGF, insulin-like growth factor.
4 Unpublished observations.
Thereafter, cells were incubated with the designated antibody and stained after washing with the F(ab')2 fragment of an FITC labeled conjugate anti-mouse (IgG + IgM) serum (Dianova). Alternatively, in indicated cases the fluorochrome conjugates consisted of isotype-specific goat anti-mouse antibody fragments coupled with R-phycoerythrin (Southern Biotechnology). Back ground staining of FITC-labeled cells was performed with the monoclonal antibody W6/32.HK, an inactive variant of W6/32.HL, directed against a common determinant of all HLA-ABC heavy chains (25). Background staining of Phycoerythrin-labeled cells was performed with isotype-matched control antibodies (Southern Biotechnology).

**Staining of Cells with Biotinylated SLF.** Cells were incubated with saturating concentrations of SLF-b and with Streptavidin-Phycoerythrin (Dianova). To indicate background staining, cells were labeled with Streptavidin-Phycoerythrin only.

**DNA Labeling of Cells.** Cell pellets were treated with a hypotonic solution containing 0.05 mg/ml propidium iodide (Sigma) and 0.1% sodium citrate (26). The DNA content of the resulting nuclei were analyzed on a flow cytometer.

**Cytogloumometric Analysis.** Fluorescence analysis was performed as described elsewhere (27). In brief, cells were analyzed on a FACSVantage cell sorter (Becton Dickinson) and the fluorescence was excited at 488 nm by an argon laser. The green fluorescence of the FITC-labeled cells was measured through a 530-nm band pass filter, and the red fluorescence of propidium iodide-stained nuclei was measured through a 610-nm band pass filter. Signals were analyzed by a data lister and evaluated on an IBM 386 computer using in-house programs. The percentage of proliferating cells (S + G2-M) was estimated by applying the rectangular method (28).

**Proliferation of AML Cells Stimulated with MAbs and/or Growth Factors.** 5 x 10^5 - 1 x 10^6 cells were preincubated either with 0.5 ml of PBS or 2 µg/ml of MAbs for 30 min at 4°C. After washing, cells were suspended in 1 ml of RPMI containing either no growth factor or 100 ng/ml of SLF and incubated for the indicated number of days. Triplicate cultures were cultured in 16-mm diameter 24-well culture plates at 37°C in a fully humidified atmosphere containing 5% CO2. The proliferation rate of collected cells was determined by disruption of cells with hypotonic buffer and subsequent flow cytometric cell cycle analysis of propidium iodide-labeled nuclei as described above.

**MAb/SLF-Mediated Down-Regulation of p145c-kit on AML Cells.** 5 x 10^5 - 1 x 10^6 AML cells were preincubated either with 0.5 ml of PBS or 2 µg/ml of MAbs for 30 min at 4°C. After washing, cells were suspended either in 0.5 ml of PBS or 100 ng/ml of SLF and incubated in 15-ml Falcon tubes, which were placed in a gently shaking water bath warmed to 37°C. After defined time intervals, the collected cells were washed and labeled with MAbs, and fluorescence was analyzed on a FACSVantage sorter using a linear amplification. The mean fluorescence value obtained by PBS-treated cells labeled with the control antibody W6/32.HK was subtracted from the values obtained by cells labeled with p145c-kit recognizing antibodies (Δ fluorescence). The percentage of down-regulation was calculated as follows:

\[
100 - \left( \frac{\text{Δ fluorescence of SLF/MAb-treated cells}}{\text{Δ fluorescence of untreated cells}} \right) \times 100
\]

**Cell Lysis and Immunoprecipitation.** Cells were lysed on ice with 0.2 ml "lysis" buffer (50 mM 4-4'-dithiothreitol)-1-piperazineethanesulfonic acid, pH 7.5, containing 150 mM NaCl, 1.5 mM MgCl2, 1 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N',N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 0.5 mM p-nitrophenyl-phosphate, 1 mM MnCl2, and 100 µM ATP). Lysates were mixed vigorously for 10 s, incubated for 20 min on ice, and then centrifuged at 12,500 g for 15 min at 4°C.

For analysis of total cellular proteins, 50 µl of sample buffer were added directly to 100 µl lysate and the mixture was incubated for 5 min at 95°C.**

**Immunoblotting.** Proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose filters. For immunoblotting analysis, nitrocellulose filters were first incubated with a 5% milk powder solution in TBST buffer (20 mM Tris, pH 7.5, containing 10 mM NaCl and 0.02% Tween-20), incubated for 2 h at room temperature with the primary antibody, washed 3 times with TBST, and then incubated for 1 h with a horseradish peroxidase-conjugated goat anti-mouse antibody. Immunoreactive proteins were visualized by using a horseradish peroxidase-catalyzed chemiluminescence reaction (enhanced chemiluminescence; Amersham, Arlington, IL).

**RESULTS**

AML is thought to develop from hemopoietic stem cells or early progenitor cell types (29, 30). Both AML blast cells and subpopulations of early hemopoietic progenitor cells have been shown to express the RTK p145c-kit (31). To assess the functional role of the c-kit system in AML and to explore possibilities for intervention with pathogenic mechanisms potentially involving this receptor, we investigated the effects of the p145c-kit ligand, SLF, in combination with IL-3 and three monoclonal antibodies, 3D6, 14A3, and 17F11, on cells isolated from the blood of five AML patients. AML cell samples were isolated which contained a high percentage of leukemic blasts (>80%), the majority of which (><95%) expressed high levels of p145c-kit. According to the FAB classification (32), acute leukemias can be distinguished by morphological and cytochemical criteria. AML cells are grouped into subtypes FAB M0, M1, and M2 were used, which represent the more undifferentiated maturational stages of hematopoiesis.

p145c-kit Down-Regulation Parallels Differentiation of AML Cells. The AML cells used contained about 80% blasts, which were highly positive for p145c-kit, and the recently cloned hemopoietic progenitor cell antigen CD34 (33), but did not express antigens that are restricted to the granulocyte (CD15; 6F3-epitope) or monocyte (CD14) lineages. Morphologically, these cells could not be assigned to any of the prominent myeloid lineages.

To study the regulatory effect of SLF and its potential synergy with IL-3, AML cells were stimulated with either 100 ng/ml of recombinant human IL-3, recombinant human SLF, a combination of both, or medium. After 3 days, cells were labeled with antibodies and analyzed by FACS. In comparison with untreated control cells (Fig. 1, panel 1A), SLF-treated AML cells no longer expressed p145c-kit on the surface, indicating that the receptor had been down-regulated (Fig. 1, panel 1C). While SLF stimulation also led to a reduction of the early stem cell antigen CD34 (Fig. 1, panel 2C) and induced the appearance of granulocyte-restricted CD15 epitopes on a subpopulation of cells (Fig. 1, panel 3C), there was no detection of the monocyte lineage antigen, CD14 (Fig. 1, panel 4C). The reduced expression of CD34 and the appearance of a CD15-positive subpopulation suggested the potency of SLF to induce differentiation of immature AML cells to the granulocyte lineage.

Interestingly, as shown in Fig. 1, IL-3 induced a very similar change in the surface antigen expression pattern of AML patient cells, including the down-regulation of p145c-kit. Combined stimulation with IL-3 and SLF, however, resulted in only a slight increase of the CD15-positive population (49% compared to 39% and 43% after stimulation with IL-3 and SLF, respectively) and showed no significant difference in the antigen expression profile obtained by each factor alone (Fig. 1, panel 4D). This suggested that either factor alone was able to induce the same differentiation pathway with equal potency.

To determine the time course and dose requirements of SLF-induced p145c-kit down-regulation, we stimulated AML cells with varying concentrations of the ligand in medium-free buffer (PBS) at 37°C and analyzed receptor cell surface expression at various time points. As shown in Fig. 2, SLF concentrations of 100 and 10 ng/ml triggered a rapid decrease of p145c-kit within 40 min of incubation, at which time most of the receptors had disappeared from the cell surface. In
Fig. 1. Stimulation of p145c-kit-positive AML M 
1. control 2. IL-3 100 ng/ml 3. SLF 100 ng/ml 4. IL-3+SLF 100 ng/ml 

The changes in AML cell surface antigen expression were paralleled by distinct morphological changes. Cells stimulated either with medium, SLF, IL-3, or a combination of both were centrifuged on slides and stained with May-Grünwald. While medium alone had no effect on the undifferentiated FAB M1 subtype morphology of the leukemic cells (Fig. 4A), exposure to 100 ng/ml SLF for 18 h resulted in partial differentiation to granulated blasts and promyelocytes (Fig. 4B). Consistent with the immunophenotypic analysis (Fig. 1), stimulation with IL-3 or a combination of IL-3 and SLF resulted in analogous changes of morphological features (data not shown), which confirmed that the activation of presumably differential signalling pathways by SLF or IL-3 resulted in differentiation of AML cells to a virtually identical phenotype.

Comparative experiments with five different samples showed no significant differences regarding p145c-kit downmodulating activity and cell differentiating capacity of SLF (data not shown).

Monoclonal Antibodies Differentially Modulate p145c-kit Functions. The potency of monoclonal antibodies recognizing surface proteins of cancer cells to influence their growth and differentiation has been demonstrated (see Ref. 34). In the case of RTK MAbs, various functions may be modulated that could result in interruption of cancer progression. p145c-kit extracellular domain-specific MAbs were generated by immunizing mice with c-kit-transfected NIH-3T3 cells (3D6 and 14A3) or with leukemic cells (17F11). To determine whether SLF binding affects interaction of antibodies 3D6, 14A3, and 17F11 with p145c-kit, AML cells were first incubated for 10 min with 100 ng/ml of SLF at 0°C to avoid receptor down-regulation. Subsequently, cells were stained with saturating concentrations of MAbs and fluorescence intensities were compared with the values obtained from untreated cells. Table 1 shows that treatment of cells with SLF reduced the binding capacity of MAbs 3D6 and 14A3 to 31 and 14%, respectively. This suggested that the respective epitopes were either within the ligand binding domain of p145c-kit or were conformationally disturbed by ligand binding. In contrast, the binding capacity of MAb 17F11 was significantly enhanced on cells exposed to SLF (156% compared to untreated cells), indicating a SLF-induced conformational alteration of the receptor or, alternatively, preferential recognition of dimeric forms of p145c-kit.

In the next experiment, cells were preincubated with MAbs and subsequently the binding capacity of SLF-b was analyzed. While MAb 3D6 almost completely abrogated binding of SLF-b (Table 2), preincubation of MAb 14A3 decreased the binding capacity of SLF-b to a level of 71% compared to untreated cells, and MAb 17F11 had no significant effect even at a concentration of 20 μg/ml.

The influence of p145c-kit antibodies on SLF-dependent induction of proliferation of AML cells was investigated by preincubating cells with MAbs (2 μg/ml) for 30 min prior to stimulation with SLF or...
Fig. 2. Influence of ligand concentration and incubation time on SLF-mediated receptor down-regulation. AML cells were incubated in ice-cold sodium azide containing PBS buffer at SLF concentrations of 100, 10, and 1 ng/ml, respectively. After defined time intervals, washed cells were labeled with MAb 17F11 and analyzed as described in Fig. 1. The relative fluorescence intensity of FITC-labeled cells (Y-axis, four decade log scale) is plotted versus the relative number of cells labeled with isotype-matched control antibodies.

Medium, respectively. After 1–4 days of ligand stimulation, the proliferation rate was determined by disruption of cells with hypotonic buffer and subsequent flow cytometric cell cycle analysis of propidium iodide-labeled nuclei. Fig. 5 shows the weak but significant response of an AML cell sample to stimulation with SLF (4–5% proliferating cells after 3–4 days ofstimulation). No induction of proliferation was observed if only the MAbs were added. However, when MAb 14A3 or 17F11 were combined with SLF, the proportion of cycling cells within the population increased dramatically to 21 and 27%, respectively, after 2 days of incubation. In contrast, MAb 3D6 blocked the stimulating effect of SLF on AML cell proliferation.

Similarly, receptor down-regulation induced by SLF was completely inhibited by MAb 3D6, while MAb 14A3 caused moderate attenuation of this ligand-induced function after 1 h of incubation (data not shown). Interestingly, MAb 17F11 was almost as potent in its capacity to down-regulate p145c-kil as SLF itself (Fig. 6). This effect could not be further enhanced by costimulation with the ligand.

To investigate the biochemical basis of anti-p145c-kil MAb activities, we analyzed their effects on receptor and substrate phosphorylation in AML cells (Fig. 7). For this purpose, AML cells from peripheral blood were incubated with or without MAbs 14A3, 3D6, or 17F11 and then stimulated with SLF or vehicle. The cells were lysed and the cleared lysate was directly mixed with sample buffer. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose, and tyrosine-phosphorylated proteins were detected by immunoblotting with MAB 5E2 (23). In the absence of MAbs, SLF stimulated autophosphorylation of p145c-kil, which migrated as a doublet of Mr 144,000 and Mr 152,000, on tyrosine residues (Fig. 7, Lanes 1 and 2) and also resulted in enhanced phosphorylation of cellular proteins with apparent molecular weights of 54,000, 60,000, 89,000, and 114,000. Reprobing of the blot with the polyclonal Ab LJ11 (24) confirmed that the Mr 144,000 and Mr 152,000 bands represented presumably differentially glycosylated forms of the SLF receptor p145c-kil and that equivalent amounts of receptor were present in each sample (data not shown). When cells were preincubated with MAb 3D6, a slight increase in the basal phosphorylation level of the Mr 144,000 variant of p145c-kil was observed (Fig. 7, Lanes 1 and 5). A ligand-dependent further increase in receptor phosphorylation was completely inhibited by MAb 3D6 (Fig. 7, Lane 6). Interestingly, preincubation of cells with MAb 3D6 led to an increased and ligand-independent phosphorylation of a Mr 54,000 substrate, while the ligand-dependent phosphorylation of all other proteins was completely inhibited by this MAb. In contrast, MAb 14A3 had little influence on receptor phosphorylation, but enhanced ligand-induced phosphorylation of a Mr 30,000 protein in AML cells.

Fig. 3. Restimulation of AML cells treated with SLF. After stimulation for 2 days with 100 ng/ml SLF, cells were washed twice and restimulated for an additional 3 days with either medium or SLF. Control cells were stimulated with medium only. Labeling with antibodies was performed as described in Fig. 1. The relative fluorescence intensity of FITC-labeled cells (Y-axis, four decade log scale) is plotted versus the relative number of cells labeled with isotype-matched control antibodies.
ANTIBODIES MODULATE c-kit RECEPTOR-LIGAND INTERACTIONS

Based on matching results obtained in experiments with five different AML samples, the activity of the MAbs can be summarized as follows: MAb 3D6 inhibits SLF-mediated receptor phosphorylation and down-regulation and induction of cellular proliferation; MAb 14A3 enhances proliferation but has little influence on receptor down-regulation and phosphorylation; and the semiagonistic MAb 17F11 causes ligand-independent receptor down-regulation and autophosphorylation and enhanced SLF-dependent induction of cell proliferation.

DISCUSSION

Signals generated by tyrosine kinases regulate normal cell proliferation, and there is compelling evidence that aberrations in this process, either by mutations in the genes of signal transducing molecules or their abnormal expression, can lead to the development of cancer. While mutated forms of genes encoding receptor-type tyrosine kinases, such as c-erbB/EGF-R, c-fms/CSF-1R, and c-kit/SLF-R, are found in the genome of acutely transforming retroviruses and have been shown to be responsible for their dominant tumorigenic actions, the major aberration found in certain human malignancies is RTK overexpression (2). In the case of the EGF receptor and the HER2/neu protooncogene product, it is well established that overexpression in mammary and ovarian carcinoma correlates with aggressive disease progression and poor prognosis (35). Extracellular domain-specific

Table 1 MAb binding to p145 c-kit is modulated by SLF

<table>
<thead>
<tr>
<th>MAb</th>
<th>% mean fluorescence intensitya</th>
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<tbody>
<tr>
<td>3D6</td>
<td>100</td>
</tr>
<tr>
<td>14A3</td>
<td>31</td>
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<td>17F11</td>
<td>14</td>
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<td>156</td>
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a AML cells were incubated for 10 min on ice in PBS containing either medium or 100 
µg/ml SLF. Subsequently, cells were incubated with the indicated antibodies and stained
with isotype-specific secondary antibodies. Mean fluorescence intensities (linear scale) of
medium- and SLF-treated cells were compared.

Consistent with its effect on AML cell proliferation, MAb 17F11 enhanced phosphorylation of p145 c-kit, even in the absence of the ligand (Fig. 7, Lanes 7 and 8). In combination with SLF, tyrosine phosphorylation of the receptor increased severalfold as compared to the control (Fig. 7, Lanes 2 and 8). Similarly, SLF-stimulated phosphorylation of selected substrates of M, 60,000 and M, 114,000 was enhanced. Furthermore, 17F11 stimulation alone was sufficient to induce phosphorylation of a M, 27,000 polypeptide.

Table 2 Binding of biotinylated SLF to AML is influenced by MAbs

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<tr>
<th>MAb</th>
<th>% mean fluorescence intensitya</th>
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<tbody>
<tr>
<td>3D6</td>
<td>100</td>
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<tr>
<td>14A3</td>
<td>71</td>
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<tr>
<td>17F11</td>
<td>107</td>
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a AML cells were incubated with MAb concentrations of about 2 µg/ml. Experiments
were performed on ice in PBS containing 0.01% NaN3. After 30 min of incubation,
biotinylated SLF (125 ng/ml) was added and cells were stained with Streptavidin-Phy-
cerythrin. Mean fluorescence intensities (linear scale) of treated and untreated cells were
compared.

Fig. 5. Effect of MAbs on the SLF-mediated proliferation of AML cells. Cells were incubated at 37°C either with medium, 100 ng/ml SLF, 2 µg/ml of MAb 3D6, 14A3, or 17F11, respectively. Alternatively, cells were preincubated with the indicated MAbs for 30 min at 4°C prior to stimulation with SLF. After 1, 2, 3, and 4 days of incubation, the proliferation rate of cells was determined as follows. Cells were disrupted with a hypotonic solution containing 0.05 mg/ml of propidium iodide. The DNA content of the resulting nuclei was analyzed on a FACS and the proliferation rate was estimated according to the method (28). Percentage of cells in S- and G2-M-phase was plotted versus incubation time. On day 0, the proliferation rate of freshly prepared cells was estimated.

Fig. 4. May-Gruenwald-Giemsa staining of AML M1 cells stimulated with either me-
dium (A) or SLF (B) for 3 days at 37°C. Bars, 10 µm.

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estimated.

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cells under conditions that are as close to the in vivo situation as possible. After 1 or 2 h of incubation, the cells were labeled with MAb and analyzed on a FACS sorter. Mean fluorescence intensities (linear scale) are plotted versus incubation time.

antibodies to EGF-R or HER2/neu have been shown to inhibit the growth of tumor-derived cell lines and primary tumors in nude mouse models, which demonstrated that disruption of an oncogenic signaling cascade at the level of the RTK may be a strategy for suppression of cancer cell proliferation (36).

In order to assess the significance of p145c-kin functions in AML cells under conditions that are as close to the in vivo situation as possible, we used blood samples from leukemia patients and selected immature nonproliferating cells with high p145c-kin density for further analysis. We used this cell population because, apart from mature mast cells and their precursors (37), a direct stimulating effect of SLF had been observed exclusively on very primitive hemopoietic progenitor cells, while progenitors committed to defined lineages appear to require combinations of SLF with other growth factors (15).

Although SLF is thought to be a weak stimulator by itself but to act as a potent costimulating agent when combined with other growth factors (8), recent studies have demonstrated an intrinsic capacity of SLF to induce proliferation and maturation of normal hemopoietic cells and to affect the growth of clonogenic AML blasts (16, 17). By comparing differentiation patterns and morphological features of unstimulated AML cells with those of SLF- or IL-3-treated cells, we demonstrated that, similar to IL-3, SLF alone was able to induce differentiation of very immature blasts (FAB M0) into cells of the granulocyte lineage (Figs. 1 and 3). Moreover, we showed that SLF treatment down-regulated its cognate receptor on AML cells with kinetics similar to those determined for other members of the RTK family, such as the EGF-R (38, 39). Down-regulation of p145c-kin apparently led to degradation, as is known for other RTKs such as EGF-R, CSF-1R, and PDGF-R, since it took approximately 18 h of incubation in SLF-free medium until cell surface receptor levels were completely restored, presumably due to de novo biosynthesis. Interestingly, IL-3 treatment of AML cells also led to p145c-kin down-regulation with an efficiency comparable to that of SLF, which suggested cross-talk between the two signal transduction systems. This finding is in agreement with data published by Welham and Schrader (40), who demonstrated that IL-3 down-regulates c-kit mRNA in mast cells and stem cell progenitors.

These observations indicated that although SLF was able to induce receptor activation and down-regulation, resulting in proliferation and differentiation of AML cells, this did not result in a state of the leukemic cell that was sufficiently altered to be independent of p145c-kin signals. Alternatively, one must consider the possibility that regulation of c-kit gene expression is deregulated in the leukemic cells and therefore cannot be suppressed even in states of advanced differentiation. These observations are similar to those made for mammary carcinoma cells overexpressing HER2/neu, which could be differentiated to some extent by neu differentiation factor (41).

As an alternative to ligand-induced terminal differentiation for experimental AML therapy, we investigated the properties of monoclonal antibodies to the p145c-kin extracellular domain. A number of growth factor receptor MAbs have been reported to inhibit ligand binding (42–47), which indicates that the ligand binding site itself or epitopes surrounding this site are highly antigenic.

Interestingly, MAbs 14A3, 3D6, and 17F11 exhibited very different effects on different receptor functions. While MAbs 3D6 and 14A3 appeared to recognize closely related epitopes on p145c-kin (data not shown), the functional properties of the MAbs differed considerably. MAb 14A3 exhibited no effect by itself in our experimental systems but enhanced most SLF-mediated signals. This synergistic effect was observed on receptor phosphorylation (Fig. 7) and induction of AML cell proliferation (Fig. 5). In contrast, receptor down-regulation was weakly but significantly attenuated (Fig. 6), indicating that enhancement of phosphorylation and proliferation is not related to accelerated internalization of receptors.

Since p145c-kin is closely related to CSF-1R, which in its noncovalently dimerized form was shown to possess higher tyrosine kinase activity than in its covalently connected dimeric state (48), one could speculate that MAb 14A3 potentially decreases the rate of covalent receptor dimer formation and therefore promotes prolonged tyrosine kinase activity of noncovalently associated receptor dimers. In support of this interpretation, we recently found that the alkyllating agent, iodoacetic acid, which selectively inhibits covalent dimerization of CSF-1 receptors and their internalization but enhances protein tyrosine phosphorylation (48), also blocks SLF-mediated internalization

Fig. 6. Modulation of SLF-mediated receptor down-regulation. AML cells were incubated at 37°C either with medium, 100 ng/ml SLF, or 2 μg/ml of MAb 17F11. Alternatively, cells were preincubated with this antibody for 30 min prior to stimulation with SLF. After 1 or 2 h of incubation, the cells were labeled with MAb and analyzed on a FACS sorter. Mean fluorescence intensities (linear scale) are plotted versus incubation time.

Fig. 7. Influence of MAbs 14A3, 3D6, and 17F11 on SLF-mediated p145c-kin and substrate phosphorylation in AML-cells. Five × 10⁶ cells/sample were incubated in 0.5 ml RPMI (+0.5% fetal bovine serum) with or without 10 μg/ml of MAbs 14A3, 3D6, or 17F11 for 30 min on ice. Cells were washed once with medium and incubated for 10 min at 37°C in fresh medium containing SLF (150 ng/ml) or buffer. Cells were lysed and the precleared lysate was directly mixed with sample buffer. Proteins were separated by a 7 to 15% gradient gel and electrophoretically transferred to nitrocellulose. Phosphotyrosine-containing proteins were detected with MAB SE2 (α-PY) and visualized by a horseradish peroxidase-catalyzed chemiluminescence reaction.

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of p145c-kit, suggesting that covalent dimers are also formed by p145c-kit. Experiments are currently underway to confirm these results.

In comparison with MAbs 14A3, MAb 17F11 exhibited a much more dramatic synergistic effect with SLF. In the AML cell system, MAb 17F11 enhanced SLF-mediated receptor phosphorylation (Fig. 7) and proliferation (Fig. 5) by 4–5-fold. Moreover, it displayed an intrinsic capacity to induce p145c-kit phosphorylation to a degree significantly above that achieved by the ligand under identical conditions (Fig. 7) and to down-regulate receptors with kinetics comparable to SLF (Fig. 6). Interestingly, however, MAb 17F11 alone was unable to induce proliferation of p145c-kit-positive AML cells (Fig. 5).

In combination with SLF, this signalling function surpassed that of the ligand by a factor of 4–5, similar to MAb 14A3 (Fig. 5). Such a synergistic effect was not observed, however, on receptor down-regulation which, in contrast to MAb 14A3, MAb 17F11 was able to stimulate alone and could not further enhance in cooperation with the ligand. This suggested that other rate-limiting factors were involved in the latter function, while the mitogenic signal could be amplified.

In contrast to MAbs 14A3 and 17F11, binding of SLF was almost completely abrogated when AML cells were preincubated with MAb 3D6. Consequently, MAb 3D6 blocked SLF-induced p145c-kit phosphorylation (Fig. 7) and down-regulation (Fig. 6) and AML cell proliferation (Fig. 5). These effects were similar to MAb SR-1 (45), which blocks binding of SLF to p145c-kit and causes substantial inhibition of colony formation of hematopoietic progenitor cells. Unlike these reagents, MAb Y5B8 does not influence ligand binding but nevertheless causes a significant decrease of colony formation (47, 49–51).

Unfortunately, the differential effects of the MAbs used in this study on p145c-kit functions did not allow direct correlations or, based on our current view of RTK signalling mechanisms, suggest clear conclusions. Most importantly, the extent of receptor phosphorylation did not correlate with the efficacy of inducing a proliferative response in AML cells. Furthermore, in addition to their differential effects on receptor phosphorylation, the MAbs had distinct effects on the tyrosine phosphorylation pattern of AML cell polypeptides. Remarkably, MAb interaction with different p145c-kit extracellular epitopes resulted in strong phosphorylation of characteristic substrates and differential modulation of SLF-induced phosphorylation, independent of their effects on receptor phosphorylation itself. At this point it is unclear whether the strong mitogenic signal-enhancing activities of MAbs 14A3 and 17F11 can be explained by the common feature of enhanced phosphorylation of a M, 61,000 substrate, whether the inhibitory activity of 3D6 is connected to ligand-independent and specific hyperphosphorylation of the M, 53,000 band (Fig. 7), or whether the receptor down-regulating activity of 17F11 is related to the pronounced effect on SLF-independent phosphorylation of a M, 27,000 polypeptide. Our findings clearly demonstrate, however, that RTK signals may be significantly and distinctly modulated by agents interacting with extracellular ligand-binding domains. This may allow different natural or artificial ligands to transmit distinct signals into the target cell and could be the basis for differential activities of EGF versus TGF-α, IGF-1 versus IGF-II, acidic FGF versus basic FGF, and the different forms of PDGF mediated by the EGF-R, FGF-receptor, IGF-IR, and α and β PDGF-R. Moreover, the insights provided by our experimental results indicate that in natural nontransformed cells, receptor phosphorylation may not be the only prerequisite for signal definition and that MAbs may provide valuable tools for the dissection of cellular signalling pathways.

While the molecular mechanisms underlying AML development and progression, and possible connections with aberrations in the p145c-kit signalling system are still poorly understood, our findings provide a basis not only for further understanding the mechanisms of RTK signal generation and definition in relevant cell systems but for the development of interventional strategies in cancer therapy.

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