Identification Through Chemical Cross-Linking of Distinct Granulocyte-Macrophage Colony-Stimulating Factor and Interleukin-3 Receptors on Myeloid Leukemic Cells, KG-1

By Thomas Gesner, R. Allan Mufson, Katherine J. Turner, and Steven C. Clark

Granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) each bind specifically to a small number of high-affinity receptors present on the surface of the cells of the acute myelogenous leukemia line, KG-1. Through chemical cross-linking of IL-3 and GM-CSF to KG-1 cells, we identified distinct binding proteins for each of these cytokines with approximate molecular masses of 69 and 93 Kd, respectively. Although these two binding proteins are distinct, GM-CSF and IL-3 compete with each other for binding to KG-1 cells. Other cell lines, which express receptors for either factor but not for both do not display this cross-competition for binding with IL-3 and GM-CSF. These findings imply that distinct IL-3 and GM-CSF binding proteins are expressed on the cell surface and that an association exists between these proteins on KG-1 cells.

THE HEMATOPOIETIC growth factors interleukin-3 (IL-3) and granulocyte/macrophage colony-stimulating factor (GM-CSF) support growth and development of hematopoietic progenitor cells through binding with high-affinity surface receptors on the respective target cells. These two factors have distinct but overlapping biologic activities reflecting the relative levels in the hematopoietic developmental system at which they act: IL-3 on the average supports proliferation and development of earlier, more primitive progenitors than does GM-CSF. One model to explain these observations is that more primitive progenitors acquire receptors for IL-3 and subsequently, as they undergo differentiation, acquire receptors for the later-acting growth factors such as GM-CSF or granulocyte (G)-CSF. During the intermediate stages of development, many of the progenitors respond to multiple hematopoietic growth factors and presumably have several different growth factor receptors expressed on their surfaces simultaneously. In both human and murine systems, at least some of the differentiating committed progenitors gradually lose responsiveness to IL-3, suggesting that expression of the IL-3 receptor within several cell lineages may decrease as the cells differentiate.

Simultaneous expression of receptors for distinct growth factors on hematopoietic cells raises the possibility of associations between these cell surface molecules that could influence their interactions with their respective ligands. With normal murine target cells, complex receptor interactions are manifested by the ability of several of the CSFs to downmodulate expression of the receptors for other CSFs. In our initial studies of binding of human IL-3 with its receptor, we observed that GM-CSF competed significantly with IL-3 for binding to the acute myelogenous leukemic cells KG-1 under conditions shown to prevent receptor internalization. We now report that these two hematopoietins can be chemically cross-linked to distinct polypeptides on KG-1 cells and that either IL-3 or GM-CSF can significantly inhibit the cross-linking of either factor to its respective receptor. These interactions suggest that the receptors for IL-3 and GM-CSF on KG-1 cells are associated on the cell surface.

MATERIALS AND METHODS

Growth factors. Purified recombinant human IL-3 (1 x 10^6 U/mg), GM-CSF (4 x 10^6 U/mg), and erythropoietin (EPO) (4 x 10^7 U/mg) were provided by the Genetics Institute Process Development Laboratory (Cambridge, MA).

Cell culture and cell preparation. KG-1 cells were maintained in Iscove’s medium supplemented with 10% fetal bovine serum (FBS) and 5 U/mL each penicillin and streptomycin (P/S) in 1L or 3L spinner flasks (Bellco, Vineland, NJ) at cell densities between 1 and 10 x 10^6/mL. KG-1 cells are biologically responsive to GM-CSF, and we showed that IL-3 enhances their cloning efficiency in soft agar. HL-60 cells were cultured in RPMI 1640 medium supplemented with 15% FBS and P/S at similar cell densities. HL-60 cells were induced to differentiate with dimethylsulfoxide (DMSO) as described by other investigators. Blin-1 cells were maintained in RPMI 1640 medium supplemented with 15% FBS and P/S and cultured at cell densities between 0.75 and 3.0 x 10^6/mL.

Preparation of radiolabeled IL-3 and GM-CSF. [35S]IL-3 (375 cpm/fmol) was prepared through metabolic labeling of Chinese hamster ovary (CHO) cells engineered to express high levels of human IL-3 as described previously. Human GM-CSF also derived from CHO cells was labeled using a modification of the Bolton-Hunter iodination method. The benzene contained in 1 mCi Bolton Hunter reagent (New England Nuclear, Boston, MA) was evaporated to dryness, and the dried reagent was incubated at 0°C for 3 hours. GM-CSF (27 µg in 10 µL phosphate-buffered saline (PBS) was added, and the incubation was continued at 4°C for 18 hours. The reaction was terminated by addition of 240 µL 1 mol/L Tris-HCl, pH 8.5. [35S]GM-CSF was isolated by passage over a PD-10 column (Pharmacia, Piscataway, NJ) pretreated with PBS containing 5% bovine serum albumin (BSA) followed by PBS alone. The labeled GM-CSF was estimated to have a specific activity of 800 cpm/fmol. Both GM-CSF and IL-3 expressed in CHO cells have an approximate molecular mass of 25 Kd.

Specific binding assays. The specific binding assays were performed as described previously. Precooled HL-60, KG-1, or Blin-1 cells were incubated at 4°C with radiolabeled ligand in α-medium (Hazelton, Denver, PA) containing 10 mmol/L HEPES, pH 7.2,
10% FBS with or without a 500-fold excess of IL-3, GM-CSF, or EPO for 18 hours in a volume of 0.35 mL. The samples were layered over FBS, centrifuged, and quick-frozen at -70°C. The pellets were collected by cutting off the centrifuge tube tips. 

Chemical cross-linking and analysis of the IL-3 and GM-CSF receptor complexes. Radiolabeled ligands and competitors were permitted to interact simultaneously with KG-1 cells as described for specific binding. Typically, 2 to 4 x 10⁶ KG-1 cells were incubated in a 2-mL binding reaction with 200 to 400 pmol/L ligand overnight at 4°C (all subsequent steps were performed at this temperature). The cells were processed by centrifugation through FBS, and the pelleted cells were resuspended in 10 mL PBS. The cross-linking was initiated by addition of 0.1 mL disuccinimidyl suberate (DSS) (Pierce, Rockford, IL) (100 mmol/L) in DM50. The reaction was stopped after 30 minutes by addition of 2 mL 1 M Tris-HCl, pH 8.5 followed by centrifugation of the cells at 800 g. The cell pellets were washed twice with 10 mL 50 mmol/L glycine, pH 3.0, 15 mmol/L NaCl to remove the ligand that was not cross-linked to the cells.

To analyze the cross-linked complexes, the cell pellets were solubilized for 30 minutes in 2 to 3 mL wheat germ agglutinin (WGA) buffer (20 mmol/L Tris-HCl, pH 7.4, containing 0.1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L n-ethylmaleimide, 0.1% sodium azide, and 6 U/mL aprotinin (all from Sigma, St Louis, MO) had been added. The lysate was clarified by centrifugation at 15,000 g for 30 minutes and the supernatant was collected and added to 1 mL WGA-Sepharose 6MB (Pharmacia, Piscataway, NJ) preequilibrated in WGA buffer. The resin and lysate were incubated for 2 to 4 hours with end-over-end mixing. The slurry was placed in a 10-mL Econo-column (BioRad, Richmond, CA), and the resin was washed with 100 mL WGA buffer. The WGA-bound complexes were released by resuspending the resin in 5 mL 0.1% SDS for 30 minutes at room temperature, and the resin was removed by centrifugation at 1,000 g for 5 minutes. The supernatant was lyophilized and reconstituted in 0.2 to 0.3 mL H2O and diluted with an equal volume of sample buffer: 100 mmol/L Tris-HCl, pH 6.8, 20% glycerol, 0.02% bromophenol blue, 1% SDS for analysis by SDS polyacrylamide gel electrophoresis (SDS-PAGE). The gels were fixed, impregnated with En3Hance (New England Nuclear) and exposed to x-ray film (Kodak, Rochester, NY) (preflashed for 1/2 second) with two intensifying screens (DuPont, Wilmington, DE) at -85°C. Exposure times of 1 to 3 weeks were required to visualize the cross-linked complexes. Relative molecular weights (mol wt) were estimated from the mobilities of the high mol wt protein standards (New England Nuclear).

RESULTS

The cross-linking of [35S]IL-3 bound to KG-1 cells with DSS yielded a major cross-linked species with an approximate mol wt of 94 Kd as well as a much less abundant species with an apparent mol wt greater than 200 Kd (Fig 1). The presence of either of these species was blocked by incubation with excess unlabeled IL-3, and the mobility of the cross-linked species did not dramatically change on reduction. The latter indicates that the complexes are not composed of disulfide-linked subunits, although under reducing conditions there did appear to be a minor shift in electrophoretic mobility. The presence of the ligand–polypeptide complex was completely dependent on the presence of the cross-linking reagent (DSS), and the amount of complex formed depended on the DSS concentration (Fig 2). Concentrations of 1 to 3 mmol/L gave optimal complex formation. Subtraction of the mol wt of the human IL-3 (25 Kd) yielded an estimate of 69 Kd for the size of the predominant IL-3–binding protein on the surface of KG-1 cells; the size of the larger species, which barely migrated into the gel, could not be measured reliably.

Analysis of the chemical cross-linking of human [125I]GM-CSF to KG-1 cells gave similar results to those obtained with IL-3 (Fig 3B). A predominant 120-Kd cross-linked species was readily detected, as was a less abundant complex with a mol wt greater than 200 Kd. (In this experiment, considerable free GM-CSF, mol wt 22 to 25 Kd, was also evident in the gel.) From these data, we estimated that the major GM-CSF binding protein on the surface of KG-1 cells is approximately 93 Kd (range 85 to 100 Kd), significantly larger than the major IL-3 binding protein. This difference in size is clear when the electrophoretic mobilities of the two complexes are compared with the relative mobility of the reference protein phosphorylase b (97.4 Kd): the
cross-linked GM-CSF receptor complex migrated more slowly than did phosphorylase b (Fig 3B), and the corresponding IL-3 complex migrated slightly more rapidly than did the reference protein (Fig 3A). These data establish that the predominant binding proteins for IL-3 and GM-CSF on KG-I cells are different.

Our previous analysis of binding of IL-3 to KG-I cells revealed that binding of this ligand was significantly competed for by the presence of excess GM-CSF at 4°C with or without 0.1% sodium azide.11 Furthermore, there was no change in the equilibrium level of bound IL-3 between 5 and 18 hours of incubation at 4°C.11 The experiments clearly established that no ligand–receptor internalization was occurring during our 4°C 18-hour incubation and that under these conditions GM-CSF and IL-3 cross-compete for binding. We confirmed and extended these findings by comparing the effects of each factor for the ability to prevent the chemical cross-linking of the alternate factor to its receptor when present in large excess during the binding reaction. As shown in Fig 3A, GM-CSF partially inhibited the chemical cross-linking of IL-3 to its receptor, whereas another hematopoietin, EPO, had no effect. Similarly, inclusion of excess IL-3 but not EPO substantially inhibited the cross-linking of GM-CSF to its receptor at 4°C under conditions that prevent receptor downmodulation (Fig 3B).

To determine whether the cross-competition required the presence of both hematopoietin receptors, experiments were performed using cell lines that contain only GM-CSF or IL-3 receptors but not both receptor species. As reported by other investigators,14,18 DMSO-induced HL-60 cells have receptors for GM-CSF but few if any IL-3 receptors. IL-3 did not inhibit binding of GM-CSF to these cells (Table 1). Similarly, the Blin-1 cells, a pre-B-lymphocytic leukemia cell line which specifically binds IL-324 did not specifically bind GM-CSF; neither did the two factors cross-compete for binding on Blin-1 cells. In contrast, the binding of excess IL-3 or GM-CSF largely prevented binding of either labeled ligand to KG-1 cells. At least in the cases studied, the cross-competition between IL-3 and GM-CSF for target cell binding appears to require simultaneous expression of both hematopoietin receptors.

DISCUSSION

Our analysis of the binding and chemical cross-linking of radiolabeled IL-3 and GM-CSF to KG-1 cells showed a 69-Kd IL-3 binding protein and a distinct 93-Kd GM-CSF binding protein on the surface of these leukemic cells. Our estimate for the size of the human GM-CSF receptor on KG-1 cells is slightly larger than the 84 Kd estimated by other investigators,16,21 but this difference probably is within the error limits of our determination. Several investigators have identified a 68-Kd IL-3–binding protein on the surface of murine IL-3–dependent cell lines, in good agreement with our estimate for the human receptor.22–24 More recently,
IL-3 AND GM-CSF RECEPTORS

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<th>Table 1. Competition Between GM-CSF and IL-3 for Binding to Different Cell Lines</th>
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<td>Specific Binding (fmol/10^7 cells)</td>
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<td>[35]SIL-3 binding</td>
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Liquid binding was performed as described in the Materials and Methods section with [35]SIL-3 (160 pmol/L) or [125]I]GM-CSF (370 pmol/L) with or without a 500-fold excess of competitor. Specific binding was determined by subtracting the binding observed in the presence of an excess isologous ligand from the total binding in the absence of competitor. The percentage of competition represents the percentage by which the total binding was reduced when an excess of the alternate ligand was included in the binding reaction.

however, higher mol wt species have also been identified from one murine cell line, including a prominent IL-3–binding protein with an apparent mol wt of 140 kDa that is phosphorylated on tyrosine residues after IL-3 binding. The smaller IL-3 binding protein may represent a proteolytic degradation product of the 140-kDa protein, a possibility that our experiments with the human KG-1 cells cannot fully exclude.

Our finding with KG-1 cells implies that interaction of IL-3 with the IL-3 receptor polypeptide at 4°C significantly reduces binding and cross-linking of GM-CSF to its receptor polypeptide and conversely implies an association between these distinct receptor polypeptides because downmodulation is blocked under these conditions. The requirement for this association of binding proteins to demonstrate cross-competition is further supported by the observation that such cross-competition does not occur on cells that express receptors for only one of the hematopoietins. A receptor association of this type could result from the physical proximity of the receptor proteins in the membrane or, alternatively, from coupling of both receptors with yet other proteins expressed on the surface of KG-1 cells. Further studies will be necessary to determine the exact nature of the association between the IL-3–and GM-CSF–binding proteins.

Complex interactions have been demonstrated between the various CSF receptors on the surface of unfractionated normal murine hematopoietic cells at 37°C but not of the type that we observed with KG-1 cells at 4°C. In the murine system, each CSF modulates the surface expression of its own receptor on normal cells at 37°C. In addition, IL-3 which acts on earlier cells in the pathway of hematopoiesis is capable of simultaneously downmodulating expression of receptors for all of the later-acting CSFs that may be coexpressed on the IL-3–responsive target cell. Similarly, GM-CSF, which acts at intermediate stages of development, downmodulates the receptors for the late-acting G-CSF and M-CSF but not the early-acting factor IL-3. In the human system, GM-CSF downmodulates surface expression of G-CSF receptors on blood neutrophils. This hierarchy of receptor downmodulation, which implies the existence of rather complex interactions between the different receptors on the cells, has been proposed to be part of the mechanism controlling normal hematopoietic cellular differentiation.

The operation of this receptor downmodulation requires active cellular metabolism and relatively high CSF concentrations. Blocking cell metabolism either by treatment with sodium azide or by lowering the temperature inhibits both receptor internalization and receptor downmodulation. Under these conditions, none of the CSFs display cross-competition with one another for binding with normal murine bone marrow cells in contrast to our findings with KG-1 cells. This raises the possibility that either the associations of the IL-3 and GM-CSF receptors are very different in the human system or that KG-1 cells display aberrant interactions that result from their leukemic transformation. In either case it will be important to investigate the interactions of IL-3 and GM-CSF with other human normal and leukemic blood cells to elucidate the interactions between their receptors.

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