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Reassessment of interactions between hematopoietic receptors using common beta-chain and interleukin-3–specific receptor beta-chain–null cells: no evidence of functional interactions with receptors for erythropoietin, granulocyte colony-stimulating factor, or stem cell factor

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Mice lacking both the gene encoding the shared receptor for granulocyte macrophage–colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), and IL-5 common β-chain (*Bc*) and the gene for the IL-3 specific receptor *(BIL3)* were generated. This was achieved by targeting the *Bc* locus in embryonic stem cells that were heterozygous for a null mutation of *BIL3*. Cells from mice generated with the doubly targeted embryonic stem cells were unresponsive to all 3 cytokines. Considerable previous data suggested a role for common beta-chain (β_c) in modulating signaling of cytokines including erythropoietin (EPO), G-CSF, and stem cell factor (SCF). However, bone marrow cells from mice lacking β_c and β_{IL3} showed normal responsiveness to these cytokines. Thus, there was no evidence for a biologically significant interaction between signaling via β_c or β_{IL3} and signaling by EPO, G-CSF, or SCF. Previously documented biochemical phenomena, including receptor transmodulation, receptor transphosphorylation, and even direct physical interaction, involving the β_c/βIL-3 receptor systems do not reflect genuine interactions of physiological significance in primary hematopoietic cells. This study provided results that challenge conclusions previously established using a variety of biochemical assays. (Blood. 2000;96:1588-1590)

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Introduction

Interleukin (IL)-3 has numerous effects on hematopoietic cells including actions on precursors and mature cells.[1-4] The receptor for IL-3 consists of a unique specific α-chain, IL-3Rα, which binds IL-3 with low affinity,[5,6] and the common β-chain (β_c) which is also used by granulocyte macrophage–colony-stimulating factor (GM-CSF) and IL-5. Following the binding of IL-3 to IL-3Rα, β_c converts the interaction to one of high affinity.[7] In mouse cells, but not human cells, an additional IL-3–specific β-chain (β_{IL3}) is used in preference to β_c for signaling by IL-3[8] and, unlike β_c, uses low affinity to directly bind IL-3.

Mice lacking β_c (β_c null mice), have an eosinopenia and, like mice deficient in GM-CSF, develop lung disease reminiscent of human pulmonary alveolar proteinosis.[9-12] Cells from these mice lack high-affinity binding for GM-CSF and IL-5. Mice that lack $\beta_{IL\text{-}3}$ ($\beta_{IL\text{-}3}$ null mice)[8,10] show decreased biological responsiveness of cells to IL-3 (via intact β_c signaling).[8] Ablation of $\beta_{IL\text{-}3}$ explained the conflicting results observed for hierarchical receptor interactions in mouse cells versus human cells. Although GM-CSF was not able to "transmodulate" IL-3 receptors and alter IL-3 binding in wild-type murine cells (because of the availability to IL-3 of $\beta_{IL\text{-}3}$), it was able to trans-down-modulate IL-3 binding in $\beta_{IL\text{-}3}$ null cells, as a result of competition between GM-CSF and IL-3 for binding to βc chains.[8]

Accumulated evidence suggests a role for β_c in modulating signaling of other hematopoietic cytokines including G-CSF, erythropoietin (EPO), and stem cell factor (SCF). Both GM-CSF and IL-3 transmodulated binding of G-CSF and M-CSF in normal cells.[13] IL-3 also showed this effect in both β_c null and in β_{IL3} null cells.[8] However, transmodulation by GM-CSF required a functional β_c receptor. Based on these results we proposed that β_c and $\beta_{IL\text{-}3}$ interacted with the G-CSF receptor (G-CSFR) and M-CSFR and/or that the GM-CSF or IL-3 activation of cellular signaling pathways modified G-CSFR and M-CSFR, perhaps resulting in their internalization. However, this phenomenon remains unexplained.[8]

Interaction between β_c and the EPO receptor (EPOR) has been demonstrated. EPO stimulated tyrosine phosphorylation of β_c in the UT-7 erythroleukemia cell line,[14] although neither GM-CSF[14] nor IL-3[15] stimulated tyrosine phosphorylation of EPOR. It was therefore suggested that EPO might activate the GM-CSF signaling pathway by phosphorylating β_c.[14] Functional and physical interactions between β_c and EPOR were demonstrated using the murine IL-3–dependent cell line (Ba/F3), which expresses IL-3Rα, β_c, and $\beta_{IL\text{-}3}$. The Ba/F3 cells transfected with murine EPOR acquired responsiveness to EPO, and increased expression of murine β_c resulting in heightened responsiveness to EPO. Conversely, inhibition of murine β_c function in Ba/F3/EPOR cells inhibited both IL-3–dependent and EPO-dependent cell growth. Moreover, an

From The Walter and Eliza Hall Institute of Medical Research, The Cooperative Research Centre for Cellular Growth Factors, and the Rotary Bone Marrow Research Laboratories Factors, PO Royal Melbourne Hospital, Victoria, Australia.

Submitted December 13, 1999; accepted April 10, 2000.

Supported in part by the Anti-Cancer Council of Victoria and the Cooperative Research Centre for Cellular Growth Factors, Victoria, Australia; the Bone Marrow Donor Institute; the Sylvia and Charles Viertel Charitable Foundation; grant HL62275 from the National Institutes of Health, Bethesda, MD; and the National Health and Medical Research Council of Australia.

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EPO-independent physical interaction between β_c and EPOR was demonstrated by coimmunoprecipitation.[16] We sought to address interactions involving the $\beta_c/\beta_{IL\text{-}3R}$ system using cells from $\beta_c/\beta_{IL\text{-}3}$ null mice.

Study design

Generation of B_c/*BIL3* null mice

The B_c and $\beta_{IL\text{-}3}$ loci are closely linked on mouse chromosome 15.[17] To generate mice with a mutation in both loci, the embryonic stem (ES) cell line 3.15, which contains a targeted mutation of one allele of the *BIL3* locus, was electroporated with a linearized targeting construct for the B_c locus. This construct was as previously described,[9] except that a cassette containing the hygromycin-resistance gene[18] was inserted in exon 7, and a thymidine kinase cassette[19] was ligated to the 5′-end of the construct. Selection and screening of hygromycin and FIAU-resistant ES cell clones were performed as previously described. To detect homologous recombinants, *BamHI*-digested DNA was hybridized with probe A (Figure 1) and a 3′-probe. Correctly targeted clones were further analyzed by Southern blot analysis for the presence of the *BIL3* mutation.[8] We used 3 ES cell lines to create chimeric mice, which were screened for cosegregation of the *BIL3* and B_c mutations. Control mice were C57BL/6 or 129/Sv, and the experiments were conducted on mice 6 to 14 weeks of age.

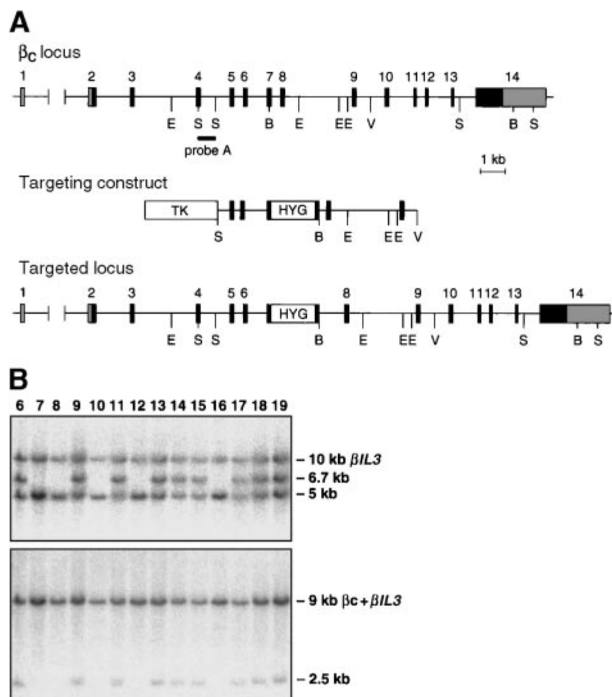

Figure 1. Targeting the B_c and $\beta_{IL\text{-}3}$ loci. (A) Partial map of the B_c locus, targeting construct, and predicted alteration of the B_c locus after homologous recombination. Coding exons are numbered and shown as black boxes. Noncoding exons are shaded gray. The position of probe A is indicated. This probe was used to identify homologous recombinants and to genotype mice by Southern blotting. Restriction enzyme sites are shown, where B indicates *BamHI*; E, *EcoRI*; S, *SacI*; V, *EcoRV*. (B) Southern blot analysis of tail DNA from offspring of a chimera generated by injection of ES cells containing targeted mutations of the B_c and *BIL3* loci. DNA was digested with *BamHI*. Blots were probed with probe A (top panel) or a probe that detects the targeted mutation of the *BIL3* locus (bottom panel).[8] In the top panel, the targeted B_c allele is a 6.7-kb (kilobase) band, and the wild type allele is a 5-kb band. The probe cross-hybridizes with the *BIL3* locus, which is seen as a 10-kb band. In the bottom panel, the 9-kb band represents hybridization of the probe to the B_c and *BIL3* wild type alleles. The targeted *BIL3* allele is seen as a 2.5-kb band. In the ES cell line used to generate these mice, the targeted mutations in the B_c and *BIL3* loci are always observed in the same offspring, indicating that the homologous recombination events in the 2 loci lie on the same chromosome.

Progenitor cell assays

Bone marrow (BM) progenitor cells were assayed in clonal culture as previously described.[9] Semisolid 1-mL agar cultures containing 5×10^4 BM cells or 10^5 spleen cells in 0.3% agar in Dulbecco's modified Eagle's medium (DMEM) with 20% newborn calf serum were plated in triplicate and stimulated by multiple combinations of purified recombinant growth factors. To determine cytokine responsiveness, BM cells from β_c/β_{IL3} null mice and control mice were cultured in agar using serial dilutions of G-CSF (initial G-CSF concentration, 500 U/mL) or SCF (initial SCF concentration, 100 ng/mL) for 7 days of incubation at 37°C in a fully humidified atmosphere of 10% carbon dioxide (CO_2) in air. The colonies were enumerated using a dissection microscope. For colony-forming unit–E (CFU-E) assays, bone marrow cells were cultured using serial dilutions of EPO (initial EPO concentration, 4 U/mL) in methylcellulose cultures incubated for 2 days in 5% CO_2 in air. The colonies were enumerated using an inverted microscope. Cultures were scored by an investigator blinded to the genotype of the cells.

Results and discussion

Baseline hematopoiesis in $\beta_c/\beta_{IL\text{-}3}$ null mice was no different than that seen in β_c null mice (C.L.S., L.R., and C. G. B., unpublished observations). This was in keeping with previous reports of mice lacking either β_c,[9,10] $\beta_{IL\text{-}3}$,[8,10] or the combination of β_c and $\beta_{IL\text{-}3}$.[20,21] Thus, in mouse cells and in spite of the presence of an additional β_c specific for IL-3, which might imply an important function, IL-3 did not have an essential role in steady-state hematopoiesis.

Hematopoietic progenitor cells from $\beta_c/\beta_{IL\text{-}3}$ null mice were examined. The lack of responsiveness to IL-3 and GM-CSF was confirmed. There was no proliferation when BM cells from β_c/β_{IL3} null mice were stimulated by either cytokine. In contrast, colony formation in response to stimulation with other hematopoietic cytokines, including SCF and the combination of SCF, G-CSF, and IL-6, was normal. Analysis of erythroid colonies in methylcellulose cultures revealed that the number of erythroid progenitor cells (both BFU-E and CFU-E) was also normal (C.L.S., L.R., and C.G.B., unpublished observations).

We have previously demonstrated that IL-3 was able to transmodulate both G-CSFR and M-CSFR in the absence of either β_c null or β_{IL3} null cells.[13] However, transmodulation of G-CSFR and M-CSFR by GM-CSF required the presence of β_c.[8] These results predict that a biochemical analysis of transmodulation of G-CSFR and M-CSFR by IL-3 in the absence of both β chains would show a lack of transmodulation capacity by IL-3. Indeed, in keeping with the lack of high-affinity IL-3 binding that results from generating $\beta_c/\beta_{IL\text{-}3}$ null cells, we did not see transmodulation of G-CSF receptors by IL-3 on BM cells from $\beta_c/\beta_{IL\text{-}3}$ null mice, even at doses of 100-nmol/L IL-3. This was in contrast to the transmodulation of G-CSF receptors by IL-3 that was observed on normal BM cells in the same experiments (N.A.N., unpublished observations).

The corollary of the G-CSFR transmodulation results predicted that the response to G-CSF may, in part, be mediated by β_c or $\beta_{IL\text{-}3}$, although neither receptor was directly engaged by G-CSF. This issue was addressed using β_c/β_{IL3} null cells. We would have predicted that the absence of B_c rendered cells less sensitive to stimulation with G-CSF. However, as shown in Figure 2, β_c/β_{IL3} null cells showed normal responsiveness to G-CSF. This indicated that biological responsiveness to G-CSF was not dependent on β_c or β_{IL3}, implying that the biochemical phenomenon of transmodulation was of no genuine biological significance.

Several different observations have suggested a role for β_c in signaling by EPO. In addition to the phosphorylation and physical

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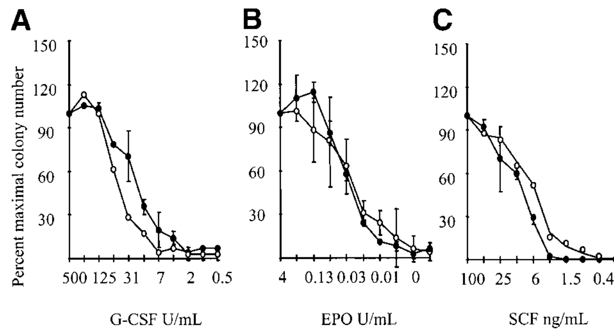

Figure 2. Cytokine responsiveness of the β_c null and β_c/β_{IL3} null mice. Responsiveness to (A) 500 U/mL G-CSF (initial concentration), (B) 4 U/mL EPO, and (C) 100 ng/mL SCF with serial 2-fold dilutions. Results are the colony number (the mean plus or minus SD) at each cytokine dilution expressed as a percentage of maximal colony number, using 1-2 mice per genotype. Similar results were seen in 3 independent experiments. A minimum of 3-4 mice were examined per genotype. Wild type is indicated by open circles and β_c/β_{IL-3} null by closed circles.

interaction data described above, IL-3 and GM-CSF are known to cooperate with EPO in erythropoiesis in vitro,[22,23] and common signal transduction pathways involving STAT5 are used by their receptors.[24] In addition, β_c has been implicated in signaling by SCF via its receptor, c-kit, and SCF is able to induce serine/threonine phosphorylation of β_c.[25]

To determine the physiological significance of these observations, responsiveness of β_c/β_{IL-3} null BM cells to EPO and SCF was examined. However, there was no observed difference in responsiveness to EPO (Figure 2). Nor was there a difference in responsiveness to SCF for BM cells cultured from β_c/β_{IL-3} null mice compared to wild-type mice (Figure 2).

We therefore conclude that previously documented biochemical phenomena, including receptor transmodulation and receptor transphosphorylation, are not physiologically relevant in the context of hematopoietic cell growth responses to individual cytokines. Moreover, even the demonstration of direct physical interaction involving the β_c/β_{IL-3} receptor systems in cell lines did not extrapolate to an interaction of physiological significance in primary hematopoietic cells. This result is important for interpreting the significance of biochemical interactions between receptor molecules, particularly in studies in which cell lines are employed.

Acknowledgment

The authors thank Louise Barnett for her work in generating the ES cell line.

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