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Two different pathways link G-protein-coupled receptors with tyrosine kinases for the modulation of growth and survival in human hematopoietic progenitor cells

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Abstract

The G-protein-coupled receptor agonists CXCL12 (SDF-1, a chemokine) and thrombin showed opposite effects on growth and survival of multipotent and erythroid human hematopoietic progenitor cells. CXCL12 promoted growth in multipotent cells by activating the RhoA–Rho kinase pathway. Its effect was largely blocked by Y-27632, a specific inhibitor of Rho kinase, and by clostridial toxin B, a specific inhibitor of Rho family proteins. Rho activation required a G_i-mediated stimulation of tyrosine kinases, which was blocked by PP2 and tyrphostin AG 490, inhibitors of Src and Jak type kinases, respectively. By contrast, in erythroid cells, inhibitors of Src family and c-Abl tyrosine kinases (tyrphostin AG 82, PP2, imatinib) enhanced protein kinase C (PKC)-dependent cell growth and antagonized thrombin-promoted apoptosis by specifically stimulating PKCβ activity. The PKC activating phorbol ester PMA (a growth factor in erythroid cells) induced the activation of Lyn and c-Abl tyrosine kinases, thus establishing a feedback inhibition of PKCβ. Hence, developmental stage-specific crosstalk between PKC subtypes and tyrosine kinases appear to determine whether growth and survival of hematopoietic cells are promoted or inhibited by G-protein-coupled receptor agonists.

Keywords: CXCL12; Chemokines; Thrombin; Hematopoietic progenitor cells; G protein signalling; Tyrosine kinases; Survival; Proliferation

1. Introduction

Proliferation and differentiation of human hematopoietic progenitor cells are primarily regulated by a set of cytokines with multi-lineage (e.g. SCF, TPO, IL-3), or lineage-restricted (e.g. GM-CSF, G-CSF, Epo) activities. These growth factors share the activation of either cytosolic or receptor-linked tyrosine kinases as an initial step in signal transduction. Prominent among additional factors modulating cytokine-driven hematopoiesis are chemokines, in particular CXCL12 (SDF-1), which act by binding to G-protein-coupled receptors (GPCR) [1]. Hematopoietic cells are known to carry an array of GPCR, but with the exception of thrombin (acting on PAR-1) and CXCL12 (acting on CXCR4), most of the associated ligands have not been tested systematically for effects on progenitor cell

Abbreviations: AG 82, AG 490, tyrphostin tyrosine kinase inhibitors; BFU-E, burst forming unit-erythroid; CXCR4, CXC-chemokine receptor 4, specifically recognizing CXCL12; Epo, erythropoietin; Flt-3L, ligand of flt-3 receptor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; GPCR, G-protein-coupled receptors; IL-3, interleukin-3; Jak, Janus tyrosine kinases; NF-κB, nuclear factor kappa B; PAR-1, protease-activated receptor-1; PTPα, phosphotyrosine phosphatase α; PDGFR, platelet-derived growth factor receptor; PI 3-kinase, phosphatidyl inositol 3-kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PP2 (tyrosine kinase inhibitor), 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo [3,4-*d*]pyrimidine; PTX, pertussis toxin; p160ROCK or ROCK, Rho-associated coiled-coil kinase; SDF-1, stroma cell-derived factor; SCF, stem cell factor; Src kinase, sarcoma virus tyrosine kinase; TPO, thrombopoietin; Y-23632 (ROCK inhibitor), *trans*-4-[(1*R*)-1-aminoethyl]-*N*-4-pyridinylcyclohexanecarboxamide dihydrochloride.

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development [2–4]. CXCL12 that is produced in bone marrow stroma cells exerts a chemotactic stimulus in early hematopoietic progenitors and plays an important role in the homing reaction of circulating progenitors [5,6]. More recently, CXCL12 has been shown to promote survival and proliferation of progenitor cells in synergy with cytokines [7,8]. By contrast, thrombin was shown to antagonize the growth-promoting effect of erythropoietin in erythroid progenitors [3].

GPCR differ in their preferred trimeric G protein partner(s) and, consequently, intracellular effector systems. While the chemotactic effect of CXCR4 seems to be exclusively mediated by G_i-type G proteins, thrombin-mediated effects on cell growth have usually been linked to stimulation of G_q and $G_{12/13}$ [9]. Even though not all of the subsequent transduction steps are completely understood, subtypes of protein kinase C (PKC) and several members of the Src tyrosine kinase family and PI 3-kinase have been identified as common targets for synergistic interactions between GPCR- and cytokine receptor-stimulated growth signals [10–13]. However, on the basis of these observations, it is difficult to explain why thrombin and CXCL12 have opposite effects on growth and survival. Using primary human hematopoietic progenitor cells, we show that a growth-regulating negative feedback cycle involving c-Abl and PKCβ is established during erythroid commitment, but is not active in multipotent cells. This mechanism could account for developmental stage-specific effects of GPCR agonists on cell proliferation.

2. Materials and methods

2.1. Cell culture

Human $CD34^+$ hematopoietic stem cells were isolated after informed consent from the peripheral blood of G-CSF-challenged myeloma patients in remission or from cord blood. The project was approved by the appropriate Ethical Committee. $CD34^+$ cells from the two sources yielded comparable results with respect to the properties analyzed in this study.

Mononuclear cells were isolated by density gradient centrifugation in Ficoll Paque (Amersham Biosciences, d=1.077). The resultant cell suspension was washed and incubated for 1 h in Iscove's medium supplemented with 100 U/ml DNAse I (Sigma type IV). After coating with magnetically labeled CD34 antibodies (Miltenyi Biotec, Bergisch-Gladbach, Germany), the cells were separated in a magnetic field according to the instructions of the manufacturer. Purified $CD34^+$ cells were grown for 5–7 days in serum-free medium (Iscove's modification of Dulbecco's minimal essential medium, IDMEM), supplemented with 20% BIT-9500 (Stem Cell Technologies, Vancouver, BC, Canada) and a cocktail of cytokines, promoting either multipotent (SCF [50 ng/ml], TPO [20 ng/ml], IL-3 [20 ng/ml]) or erythroid (SCF [50 ng/ml], Epo [0.5 U/ml]) progenitor cell expansion. Erythroid cell cultures also contained dexamethasone (1 μM) to retard terminal differentiation. In addition, the culture medium (IDMEM-BIT) was enriched with pyruvate (1 mM) mercaptoethanol (100 μM), human LDL (35 μg/ml), MEM essential amino acids, MEM nonessential amino acids, MEM vitamins, penicillin/streptomycin (50 U+50 μg/ml) and amphotericin B (1 μg/ml).

2.2. Cell proliferation assays

2.2.1. ^{3}H-Thymidine incorporation

Cells, aliquoted in 96-well plates, were first starved for 12–14 h in IDMEM-BIT medium in the absence of growth factors. Cytokines or other factors were subsequently added individually or in combination. After a 5-h incubation period, ^{3}H-thymidine (1 μCi/ml) was added and the cells were kept in culture for a further 18-h period. ^{3}H-Thymidine incorporation was measured as described previously [3]. The relative stimulation by growth factors remained somewhat smaller than expected because basal thymidine incorporation had to be maintained at 2×10^5–1×10^6 cpm/10^6 cells. A more rigorous starvation protocol seriously compromised cell survival.

2.2.2. MTT assay

This method uses the metabolic conversion of a tetrazolium dye (3,-[4,5-2-yl]-2,5-diphenyltetrazolium bromide) into a formazan product to measure the number of living cells. The assay was performed according to the protocol provided by the manufacturer (Promega, Wallisellen, Switzerland). Although this method is significantly less sensitive than the thymidine assay, it was used as an independent control that was not subject to interference of kinase inhibitors with transmembrane nucleotide transport [14].

2.3. Measurement of RhoA activation

Cytokine-starved multipotent cells were incubated in IDMEM-BIT at 37 °C under various conditions for predetermined time periods as given in the legends of the corresponding figures. The reaction was terminated by adding ice-cold phosphate-buffered saline (PBS) supplemented with a cocktail of protease inhibitors (Complete Mini, Roche Applied Science, Rotkreuz, Switzerland). The amount of GTP-bound Rho was quantified by the Rhotekin pull-down assay [15] using glutathione agarose linked to the glutathione *S*-transferase (GST)-tagged rhotekin Rho binding domain and following the protocol of the manufacturer (Upstate Biotechnology, Charlottesville, VA, USA).

2.4. Immunoblotting and immunoprecipitation

Cellular proteins were solubilized as previously described [16] and subjected to sodium dodecylsulfate polyacrylamide