**ABSTRACT**

Interleukin-5 (IL-5) and granulocyte macrophage-colony stimulating factor (GM-CSF) are important cytokines for the proliferation, differentiation, and activation of myeloid lineages. The JAK/STAT pathway is one of the signaling pathways implicated in mediating biological responses induced by these cytokines. Previous studies have demonstrated that these cytokines predominantly activate an 80 kDa STAT5 isoform in mature granulocytes. To better understand the role of STAT proteins during growth and differentiation of granulocytes, we evaluated differentiation of human CD34+ hematopoietic stem cells ex vivo toward eosinophils and neutrophils. Bandshift experiments showed that in an early stage of both differentiation pathways (14 days), the 94 kDa STAT5B protein was activated by both IL-5 (eosinophil lineage) and GM-CSF (neutrophil lineage). However, during maturation of both lineages (days 21 and 28), increased expression of a functionally distinct 80 kDa STAT5 isoform was observed, resulting in heterodimer DNA-binding complexes containing both the 94 and 80 kDa STAT5 proteins. The finding that functionally distinct isoforms of STAT5 are activated during the early and late differentiation stages of granulocytes suggests that they might be involved in regulating different biological functions in these cells. *Stem Cells* 1998;16:397-403

**INTRODUCTION**

Signal transducers and activators of transcription (STAT) proteins are a recently identified class of transcription factors involved in mediating many cytokine-induced responses [1]. These proteins reside in a latent form in the cytoplasm and become phosphorylated by the Janus kinase (JAK) family of tyrosine kinases following cytokine-receptor activation. Once phosphorylated, STAT proteins dimerize, translocate to the nucleus, and bind specific DNA sequences present in promoter regions to regulate gene transcription [2, 3].

The STAT proteins may play an important role in hematopoiesis. Recent persuasive data suggest a role for STAT proteins in both proliferation and differentiation processes. For example, STAT4 is important for both the proliferation and differentiation of Th1 lymphocytes [4, 5], and STAT6 for the proliferation and differentiation of Th2 lymphocytes [6-8]. Furthermore, it has been shown that STAT3 plays a role in the differentiation of the myeloid M1 cell line into macrophages [9]. Constitutive activation of STAT5 in T-cells transformed by the HTLV-1 leukemia virus [10] and in chronic myelogenous leukemia (CML) cells transformed with BCR-ABL [11] suggests that activation of STAT5 correlates with mitogenic responses.

Of the presently known STAT proteins, STAT1α, STAT3 and several isoforms of STAT5 have been reported to be activated by the cytokines interleukin-3 (IL-3), IL-5, and GM-CSF [12-17]. Of this cytokine family, the activity of IL-5 is restricted to the differentiation and subsequent activation of...
eosinophils and basophils [18-21]. In contrast, IL-3 and GM-CSF have broader and overlapping roles in the growth, differentiation, and activation of multiple myeloid lineages [22].

Despite recent progress in unraveling the signaling events activated by IL-5 and GM-CSF, the mechanism by which these cytokines utilize the JAK-STAT signaling to accomplish their distinct spectrum of biological activities remains largely unknown. We have recently shown that in contrast to the activation of full-length STAT5 proteins in myeloid cell lines, IL-5 and GM-CSF activated a carboxyl-truncated p80 STAT5 protein in human granulocytes (Caldenhoven et al., submitted). These observations prompted us to study the activation of STAT proteins by IL-5 and GM-CSF during the differentiation of CD34+ hematopoietic stem cells (HSCs) into eosinophils or neutrophils. HSCs expressing the CD34 antigen (CD34+ cells) mainly reside in the bone marrow and are capable of extensive self-renewal and of differentiation into committed progenitors of the different myeloid and lymphoid compartments [23]. The growth, differentiation and survival of hematopoietic cells is at least in part regulated by a network of hematopoietic growth factors (HGFs) [22]. Whereas cytokines such as IL-3, GM-CSF, stem cell factor (SCF), fms-like tyrosine kinase-3 (Flt-3) ligand, and IL-6 are early and multilineage growth factors [24-26], terminal differentiation is accomplished by unilineage cytokines such as G-CSF (neutrophil lineage), erythropoietin (erythrocyte lineage), thrombopoietin (megakaryocyte lineage), and IL-5 (eosinophil lineage) [21, 22].

In this report we have explored the activation of STAT proteins in CD34+ HSC isolated from cord blood (CB), and induced in liquid suspension culture to gradual differentiation along the neutrophil (G-CSF) and eosinophil lineage (IL-5).

**MATERIALS AND METHODS**

**Cell Culture and Reagents**

Blood was obtained from healthy volunteers from the bloodbank, Utrecht, The Netherlands. Human polymorphonuclear neutrophils (PMNs) were isolated from theuffy coat of 500 ml blood anticoagulated with 0.4% trisodium citrate (pH 7.4) as previously described [27]. Eosinophils were subsequently isolated by the method described by Hansel et al. [28]. Human interleukin-5 (huIL-5) was a kind gift of Dr. D. Fattah (Glaxo Wellcome group research; Stevenage, UK). Human GM-CSF and huL-3 were obtained from Genzyme (Cambridge, MA), and human G-CSF was obtained from Amgen (Thousand Oaks, CA). Human fms-like tyrosine kinase-3 (FLT-3) ligand and SCF were obtained from Pepro Tech (Rocky Hill, NJ).

**Isolation of Human CD34+ Stem Cells by Magnetic Cell Sorting (MiniMacs)**

Cord blood was collected in 50 ml tubes containing trisodium citrate (pH 7.4, 0.4% final concentration). Blood was then diluted twofold with Iscove’s modified Dulbecco’s medium (IMDM) (GIBCO; Paisley, UK) containing 0.4% trisodium citrate. Mononuclear cells (MNC) were isolated from umbilical cord blood cell (CBMC) samples by density centrifugation (2,000 rpm, 20 min) over Ficoll-Hypaque solution (density 1.077 g/ml). The resulting MNC were subjected to hypotonic lysis of red cells. MNC were resuspended at 10⁶ per 150 µl in cold PBE buffer (phosphate buffered saline [PBS], 0.5% bovine serum albumin [BSA], 5mM EDTA). Blocking reagent (50 µl) and the antibody reagent from the CD34 isolation kit (50 µl), (MACS, Miltenyi Biotec; Bergisch Gladbach, Germany) were added simultaneously to the cell suspension, mixed well and incubated for 15 min at 4° C on a rotating wheel. After incubation, cells were washed with PBE, resuspended in 200 µl PBE, and 50 µl of the colloidal suspension of submicroscopic magnetic beads were added to the cells, mixed and incubated for 15 min at 4° C on a rotating wheel. The cells were washed carefully with PBE, resuspended in 0.5 ml PBE and applied to the prefilled column which was placed in a magnetic field. Cells which did not bind CD34 antibody passed through the column while CD34+ cells were retained. The CD34+ cells were eluted by removing the column from the magnetic field and flushing the column with PBE. The entire procedure was performed according to manufacturer’s instructions.

**Culturing and Differentiation of CD34+ Cells**

Between 2 x 10⁵ and 5 x 10⁶ CD34+ cells/ml were cultured in 24-well plates in IMDM. The medium was supplemented with 10% fetal calf solution (FCS), SCF (100 ng/ml), FLT-3 ligand (100 ng/ml), IL-3 (0.1 nmol/l), GM-CSF (0.1 nmol/l), and IL-5 (0.1 nmol/l) for eosinophil differentiation or G-CSF (30 ng/ml) for neutrophil differentiation. After three days, cells were counted and resuspended in IMDM containing IL-3 and IL-5 (eosinophils) or IL-3 and G-CSF (neutrophils). Cells were maintained at a density between 0.5 x 10⁶ and 3 x 10⁶ cells. From day 21, IL-3 was also omitted from the media. Growth and viability were determined by light microscopic evaluation with 1% trypan blue exclusion. Viability remained >95% throughout the 28-day experiment period.

**Antibodies and Synthetic Oligonucleotides**

The polyclonal antibodies directed against STAT5 (N-20, aa9-24), and STAT5B (C-17, aa711-727), were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The following oligonucleotides were used in this study (only the upper strands are shown below): β-casein STAT5 binding site (5’-AGCTTAGAT TTCTAGGAATTCATCA-3’).
and human ICAM-1 IRE (5'-AGCTTAGTT TCCGGGAAGCAC-3').

**Gel Retardation Assay**

Twenty-four hours prior to nuclear extract preparation, cells were washed three times and resuspended in fresh IMDM (2 x 10^6 cells per ml) without any cytokines. Nuclear extracts were prepared from unstimulated cells or cells stimulated with either IL-5 (0.1 nmol/l) or GM-CSF (0.1 nmol/l) for 15 min following a previously described procedure [29]. Oligonucleotides were labeled by filling in the cohesive ends with [α-32P]dCTP using Klenow fragment of DNA polymerase I. Gel retardation assays were carried out according to published procedures with slight modifications [30]. Briefly, nuclear extracts (10 µg) were incubated in a final volume of 20 µl, containing 10 mM HEPES, pH 7.8, 50 mM KCl, 1 mM EDTA, 5 mM MgCl2, 10% (v/v) glycerol, 5 mM dithiothreitol, 2 µg poly(dI-dC) (Pharmacia; Roosendaal, The Netherlands), 20 µg BSA and 1.0 ng of 32P-labeled oligonucleotide for 20 min at room temperature. Subsequently, samples were electrophorized for three to four hours on 5% nondenaturing polyacrylamide gels at room temperature. Supershift analyses were performed by pre-incubating 10 µg of nuclear extract with 1 µg of anti-STAT5 antibody for 30 min on ice prior to the addition of the binding buffer and 32P-labeled probe.

**Whole Cell Extracts and Western Blotting**

Whole cell extracts were prepared by lysing the cells directly in boiling Laemmli sample buffer. Protein samples were separated on 8% SDS polyacrylamide gels, and electropherated to Immobilon-P membranes (Millipore; Bedford, MA). Ponceau S staining was used to check for variations in protein loading, which were never higher than 1.5-fold between different lanes. Membranes were blocked in TBST-buffer (150 mmol/l NaCl, 10 mmol/l Tris pH 8.0, 0.3% Tween 20) containing 5% BSA for 30 min and probed with a polyclonal antibody against human STAT5 (aa 5-24) for one h. After three washes with TBST the membranes were incubated for one hour with either antiperoxidase-conjugated swine-anti-rabbit antibodies (DAKO; Glostrup, Denmark), followed by five washes with TBST. Proteins were visualized with enhanced chemiluminescence (ECL), Amersham, Buckinghamshire, UK.

**RESULTS AND DISCUSSION**

**Ex Vivo Expansion and Differentiation of Isolated CD34+ Stem Cells**

CD34+ hematopoietic stem cells were enriched from cord blood by using an immunomagnetic system as described in **Materials and Methods**. To induce ex vivo expansion, CD34+ progenitor cells were cultured in the presence of human SCF, FLT3-ligand, IL-3, and GM-CSF and with either IL-5 or G-CSF to induce eosinophil or neutrophil maturation, respectively. After three days, SCF, FLT3-ligand and GM-CSF were omitted from the media, while after 21 days IL-3 was also omitted. The number of cells in culture and their morphological features were monitored from day 0 to day 28. The CD34+ cell population (purity 95%) consists of small cells with very little cytoplasm (Fig. 1A). After three days of culture in the presence of SCF, FLT3-ligand, GM-CSF, and with either IL-5 or G-CSF, cell numbers increased two- to fourfold (Fig. 1B), and the morphology and size of the cells changed dramatically (Fig. 1A). Further culture to day 28 with the addition of IL-3 and IL-5 or IL-3 and G-CSF resulted in phenotypic maturation toward mature eosinophils or neutrophils, respectively (Fig. 1A). The growth of human CD34+ cells for 28 days resulted in a 300-fold increase in cell number for the eosinophil lineage, and a 500-fold increase for the neutrophil lineage (Fig. 1B). Although at three, seven and 14 days the cultures were heterogeneous, eosinophilic and neutrophilic cells were clearly observed in increasing numbers. After 28 days, the cultures with G-CSF contained about 90% neutrophils, while the IL-5-treated cultures contained 90% eosinophils as judged by microscopic analysis (data not shown).
Activation of Functionally Distinct STAT5 Proteins by IL-5 and GM-CSF During Eosinophil and Neutrophil Differentiation of CD34+ Cells

We and others have recently shown that several different STAT5 isoforms are activated by IL-3, IL-5, and GM-CSF in distinct populations of cells [15-17] (Caldenhoven et al., submitted). Moreover, it was shown that activation of these functionally distinct STAT5 isoforms is correlated with the differentiation stage of the cell [15]. We used our ex vivo CD34+ stem cell differentiation system to analyze STAT activation by IL-5 and GM-CSF during eosinophil and neutrophil development. Since the other cytokines used in our differentiating culture system (e.g., IL-3 and G-CSF) also affect STAT proteins, the cells were washed three times and resuspended in fresh IMDM (2 × 10^6 cells per ml) without any cytokines 24 h prior to nuclear extract preparation. Cells were then stimulated with IL-5 (eosinophils) or GM-CSF (neutrophils) for 15 min, after which nuclear extracts were prepared and STAT activation was analyzed in a bandshift assay using the S^2P-labeled β-casein element as a probe. As can be seen in Figure 2, IL-5 and GM-CSF induce the activation of a DNA-protein complex in cells of different developmental stages (day 14, day 28, and mature cells) of both the eosinophil and neutrophil lineages. However, we observed along the maturation of both lineages an increase in migration of the DNA-protein complexes, indicating that there might be differences in the components in these complexes (Fig. 2). Although the cells at day 14 are still somewhat heterogeneous, it is likely that the IL-5 effects observed represent eosinophilic cells, since we have never observed basophilic in our cultures, which are the only other human cells expressing a functional IL-5 receptor. Similarly, the effects of GM-CSF in early (14 day) neutrophil cultures are likely to represent neutrophilic cells, although we cannot rule out that some pre-eosinophilic or monocytic cells contribute to the observed GM-CSF-induced STAT5 band. However, since the cultures at day 28 contain 90% mature granulocytes, here we can be quite sure that the effects observed with IL-5 and GM-CSF represent eosinophils and neutrophils, respectively.

To evaluate whether the observed differences in migration are due to the activation of different STAT5 isoforms, we pre-incubated these nuclear extracts with an amino- and carboxyl-terminal (N) anti-STAT5 antibody (aa 5-24) recognizing all STAT5 isoforms and a carboxyl-terminal (C) anti-STAT5B antibody (aa 750-769) recognizing only full-length STAT5B. To distinguish the differences between these and the DNA-protein complexes, we performed a long-run gel retardation assay (16 h). As can be seen in Figure 3A, the DNA-protein complex activated by IL-5 and GM-CSF in cells of both lineages after a 14-day culture was supershifted by both the C- and N-terminal antibodies indicating that the full-length 94 kDa STAT5B protein is activated (lanes 1-3 and 10-13). In contrast, in mature eosinophils and neutrophils a faster migrating DNA-protein complex was activated by IL-5 and GM-CSF which was only recognized by the N-terminal anti-STAT5 antibody (lanes 7-9 and 16-18). This observation is consistent with our previous findings that a carboxyl-terminal truncated 80 kDa STAT5 protein is activated in these cells (Caldenhoven et al., submitted). Therefore, we analyzed the different STAT5 complexes for binding to the ICAM-1 IRE. As shown in Figure 3B, the full-length STAT5 homo-heterodimer which is activated on day 14 was not able to bind to the ICAM-1 IRE, whereas the heterodimer p94/p80 (day 28) and the p80 homodimer (mature cells) did bind to this sequence. In addition to the difference in DNA-binding specificity, it is also suggested...
that carboxyl-truncated STAT proteins are transcriptionally inactive. This has been shown not only for the naturally carboxyl-truncated STAT1 protein [31, 32], but also for carboxyl-truncated STAT5 proteins generated by polymerase chain reaction [33, 34]. Furthermore, it has been shown that in FdTrk cells in which only the truncated p80 STAT5 protein is activated, IL-3 fails to induce several immediate early genes, including CIS, Pim-1 OSM and c-fos, suggesting that p80 STAT5 is transcriptionally inactive [17].

Taken together, this might indicate that by generation of functionally distinct STAT5 proteins during granulocyte differentiation, different genes might be affected, potentially eliciting different biological responses.

**Expression of STAT5 Proteins in CD34⁺ Stem Cells Undergoing Eosinophil or Neutrophil Differentiation**

To determine the expression levels of the short and long STAT5 isoforms during CD34⁺ differentiation, we prepared whole cell extracts from cells (5·10⁵ per lane) at different time points during eosinophil or neutrophil differentiation. These whole cell lysates were subjected to SDS-PAGE, and analyzed by Western blotting with the N-terminal anti-STAT5 antibody recognizing both full-length and carboxyl-truncated STAT5 proteins. Our results show that CD34⁺ hematopoietic stem cells express mainly the long forms (96/94 kDa) of STAT5 (Fig. 4, lane 4). Interestingly, during eosinophil and neutrophil differentiation increased expression of the 80 kDa STAT5 is observed (lanes 1-3 and 5-7). The expression of the 80 kDa STAT5 protein in the neutrophil lineage was somewhat higher than in the eosinophil lineage. Although there was some variation in the amount of p80 and the time course of p80 generation between different experiments (probably due to donor variation), enhanced expression of p80 was consistently observed during both eosinophil and neutrophil differentiation (data not shown).

**Taken together, our results show that during the differentiation of CD34⁺ stem cells into mature eosinophils or neutrophils, the p80 STAT5 protein is upregulated and subsequently activated by IL-5 and GM-CSF. Recently, it has been suggested that the carboxyl-truncated STAT5 isoforms are generated by a unique protein-processing event from the full-length STAT5 proteins by a specific protease [17]. Our observation that during granulocyte differentiation the expression of the carboxyl-truncated STAT5 protein is increased suggests that this protease might be differentially regulated in granulocytes. In contrast to our observations, Azum et al. [15, 17] have reported that the carboxyl-terminal truncated STAT5 protein is activated in immature murine myeloid cells and the long forms of STAT5 in mature myeloid cells. An explanation for this discrepancy could be that their studies were done in murine...**
myeloid cell lines and not in human primary cells. Although the functional relevance of the switch between p96 and p80 STAT5 remains to be determined, the generation of functionally distinct STAT5 isoforms during granulocyte differentiation might well be involved in the regulation of distinct biological responses elicited by these cytokines during granulopoiesis, such as proliferation versus differentiation/activation.

**SUMMARY AND CONCLUSIONS**

While IL-5 and GM-CSF stimulate the proliferation of early myeloid progenitors, they are also involved in the terminal differentiation and maturation of granulocytes. We have shown that IL-5 and GM-CSF activate wild-type STAT5 in immature human myeloid cells cultured from CD34+ stem cells. By contrast, these cytokines induce the activation of a C-terminally truncated p80 STAT5 protein during later stages of eosinophil and neutrophil differentiation and in mature peripheral granulocytes. Since the p80 STAT5 has different functional properties compared with wild-type STAT5, the switch between these proteins during granulocyte differentiation might well contribute to the different responses elicited by IL-5 and GM-CSF in immature compared to mature granulocytes.

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**REFERENCES**

20. Clutterbuck EJ, Hirst EM, Sanderson CJ. Human interleukin-5 (IL-5) regulates the production of eosinophils in human bone marrow cultures: comparison and interaction with IL-1, IL-3, IL-6, and GM-CSF. Blood 1989;73:1504-1512.


30 Fu XY, Kessler DS, Veals SA et al. ISGF3, the transcriptional activator induced by interferon alpha, consists of multiple interacting polypeptide chains. Proc Natl Acad Sci USA 1990;87:8555-8559.


