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INTERLEUKIN-13 INDUCES RAPID TYROSINE PHOSPHORYLATION AND ACTIVATION OF RAF-1 KINASE IN HUMAN MONOCYTIC PROGENITOR CELL LINE U937

Samuel Evans Adunyah[1], Melvin L. Pegram and Roland S. Cooper

Dept. of Biochemistry and Comprehensive Sickle Cell Center, Meharry Medical College, Nashville, TN

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IL-13 is a pleiotropic cytokine produced by Th0, Th1-like, and CD8 T cells in response to antigen stimulation. Its biological effects include suppression of cytotoxic activity of monocytes/macrophages and suppression of pro-inflamatory cytokine production. However, the mechanism of IL-13 remains unknown. In this study we investigated the effects of rhIL-13 on tyrosine phosphorylation in U937 monocytic progenitor cells by immunoblotting and immunocomplex kinase assays. We demonstrate that rhIL-13 stimulates dose-dependent tyrosine phosphorylation of several proteins of Mr. 93, 80, 74, 49.5, 42, 30, 22 and 18 kDa within 30 sec. The effect of IL-13 was blocked by the tyrosine kinase inhibitor erbstatin. Furthermore, IL-13 induces tyrosine phosphorylation and rapid activation of raf-1 kinase. These findings provide the first evidence that the mechanism of IL-13 involves rapid tryrosine phosphorylation and activation of raf-1 serine/threonine kinase.

Interleukin-13 (IL-13) is a newly identified cytokine of molecular mass of 12 kDa which elicits broad pleiotropic biological responses (1-3). The effects of IL-13 includes induction of B cell activation and suppression of pro-inflammatory responses and cytotoxic activities of monocytes/macrophages including cytokine (IL-1a, IL-1 beta, IL-6, IL-8, Il-10, GM-CSF and M-CSF) production (4-8). It is produced by activated ThO, Th1-like and CD8 T cells (1,9). IL-13 was originally isolated by the differential screening of anti-CD28 activated human peripheral blood mononuclear cell cDNA library. The original posulated murine homologue of IL-13 was cloned as the P600 gene, which is richly expressed by the Th2

[1]**Address Communications To Dr. Samuel E. Adunyah,** Fax: (615)327-6442.

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subset of T lymphocytes (1, 2, 10). Further studies indicated that the IL-13 gene is located on chromosome 5 q 23-31, adjacent to IL-4 and that it shares a 25 % sequence homology with IL-4 (11, 12).

Several reports suggest that the biological effects of IL-13 strongly resembles that of IL-4 and IL-10 in that like these two cytokines IL-13 is cabale of inhibiting production of procoagulant activity in endothelial and monocytic cells (13, 14). Also, current evidence indicate that IL-13 exhibits selective induction of vascular cell adhesion molecule-1 and amplification of IL-6 production (15). Furthermore, it has been shown that IL-13 inhibits the proliferation of both normal and leukemic human B-cell precursors (16). Based on these observations many investigators have predicted that IL-13 has potential for great clinical application (13-16). However, no information on the signal transduction mechansims of this new cytokine is available. Therefore, a better understanding of the biological effects of IL-13 requires an identification of the signal molecules and the pathways involved in IL-13 signalling.

This study was undertaken to determine whether tyrosine phosphorylation plays any role in the IL-13 mechanism of action and to identify some of the signal transduction molecules involved in IL-13 signalling. Our results indicate that upon binding to its receptor, IL-13 stimulates rapid tyrosine phosphorylation of a number of proteins in both time and dose-dependent manner and that IL-13-induced stimulation of tyrosine phosphorylation is inhibited by erbstatin. This implies that activation of tyrosine kinases occur in response to IL-13 stimulation. Furthermore, we demonstrate that stimulation by IL-13 results in increases in tyrosine phosphorylation and kinase activation of raf-1, imlpying that the mechanism of IL-13 involves initial activation of tyrosine kinases coupled to downstream activation of raf-1 kinase.

In conclusion, we have provided evidence that tyrosine as well as serine/threonine kinases may play important role in transducing the IL-13 signal to the nucleus.

MATERIALS AND METHODS

Human U937 cells were purchased from ATCC and maintained in RPMI 1640 medium containing 10 % fetal bovine seruim (FBS) and 50 U/ml each of streptomycin and penicillin in 5 % CO2 at 37^0C. The viable cell population was estimated to be greater than 96 % by trypan blue exclusion test. Human recombinant IL-13 was purchased from R & D Systems, MN and diluted into tissue culture medium. Tyrosine kinase inhibitor, erbstatin is a product of CalBiochem.

Erbstatin solution was prepared in DMSO and diluted into tissue culture medium such that the final DMSO concentration was less than 0.05 %. Anti-phosphotyrosine antibody coupled to horse reddish (RC-20) was purchased form Signal Transduction Laboratory, Lexington, KY. Monoclonal antibody to c-Raf-1 was purchased from NCI Repository (Quality Biotech), Camden, NJ and ECL detection reagent was purchsed from Amersham Life Sciences, Arlington Heights, IL.

Western Blotting and Detection of Phosphotyrosine Bands:

U937 cells (3×10^6) were pretreated with orthovanadate (5 mM) for 30 min at 37^0C to inhibit endogenous phosphatases followed by exposure of cells to various doses of rhIL-13 for various periods of time. In some experiments erbstatin (50 ng/ml) was included in the preincubation medium for 15 min prior to addition of IL-13. Cells were then packed by centrifugation at 13,000 x g for 20 sec, media removed by aspiration and cells were washed in PBS. Cell pellets were resuspended in SDS gel sample buffer containing 0.1 % SDS (250 µl/pellet) and vortexed extensively to completely lyse the cells. Solubilized proteins were submitted to analysis on 10 % or 12 % polyacrylamide SDS gels run at 30 to 35 mA for 4 hrs (17). Protein bands were transferred to nitrocellulose filters by electrotransfer (18) under a constant current of 142 mA for 3 hrs at 4^0C. The efficiency of transfer was verified by rapid ponceau (0.4 % in 5% TCA) staining and destaining of the filters. The destained membrane filters were incubated in a blocking buffer, TBST (TBS containing 0.1 % Tween 20 and 1.0 % BSA) at 37^0C for 20 min. Next, the filters were immunoblotted in a blocking solution containing anti-phosphotyrosine antibody RC20 (1/2500 dilution) for 30 min at 37^0C. The filters were washed in a wash buffer (blocking buffer minus BSA) for 30 min with three changes of buffer. Finally, the filters were incubated in ECL detection reagent (1:1 mixture) for 1 min followed by exposure to X-ray films (0.5 to 5min) for autoradiography.

IMMUNOPRECIPITATION OF RAF-1 AND IMMUNOCOMPLEX KINASE ASSAY:

To determine whether stimulation of cells causes tyrosine phosphorylation of raf-1, we employed specific monoclonal anti raf-1 antibody in immunoprecipitation reaction to detect raf 1 protein from cell extract. Briefly, cells were lysed in lysis buffer (TBS containing 0.2 % NP-40, 2 mM EDTA, 0.5 mM PMSF and 1 µg/ml each of protease inhibitors, leupeptin, aprotinin and pepstatin A) by extensive vortexing. Next, unbroken cell debri and unsolubilzed membraneous materials were removed by microcentrifugation at 13,000 x g for 10 min. Cell lysate (supernatant) was incubated with monoclonal antibody to raf-1 (20:1 ratio) for 3 hrs with gentle chaking at 4^0C. Protein A agarose was then added to capture the immunocomplex during a 1 hr incubation. The protein A agarose-immunocomplex mixture was pelleted and washed for 30 min in lysing buffer (with three changes of buffer). The final protein A agarose-immunocomplex pellet was resuspended in SDS-gel sample buffer, vortex extensively and boiled for 3 min to solubilize the bound protein for SDS-gel analysis. In order to determine whether the immunoprecipitated protein was phosphorylated on tyrosine residue, Western blot analysis using RC20 was performed after immunoprecipitation.

To investigate whether treatment of cells with IL-13 causes activation of raf-1 kinase activity, following immunoprecipitation, the protein A-immunocomplex pellet was dissolved in a kinase reaction buffer (25 mM Tris-HCl, pH 7.4, 0.1 % NP-40, 3 mM $MnCl_2$, 5 mM vanadate, 50 µM Na_2ATP containing histone H1 (10 µg) and

0.2 mCi of [^{32}P]-ATP. After a 30 min incubation at 37^{0}C, the kinase reaction was terminated by addition of ice cold 30 % TCA followed by incubation on ice for 30 min. The amount of ^{32}P incorporated into histone H1, was determined by membrane filtration and liquid scintillation analysis.

RESULTS

To investigate whether tyrosine phosphorylation plays a role in the mechanism of IL-13, we treated U937 cells with recombinant human IL-13 for various periods of time and at the indicated times, samples were analyzed for phosphotyrosyl bands. The results in Fig. 1 show that IL-13 is capable of eliciting rapid phosphorylation of a number of proteins (indicated by arrows) on tyrosine residues. As can be seen, IL-13 elicited a maximum effect

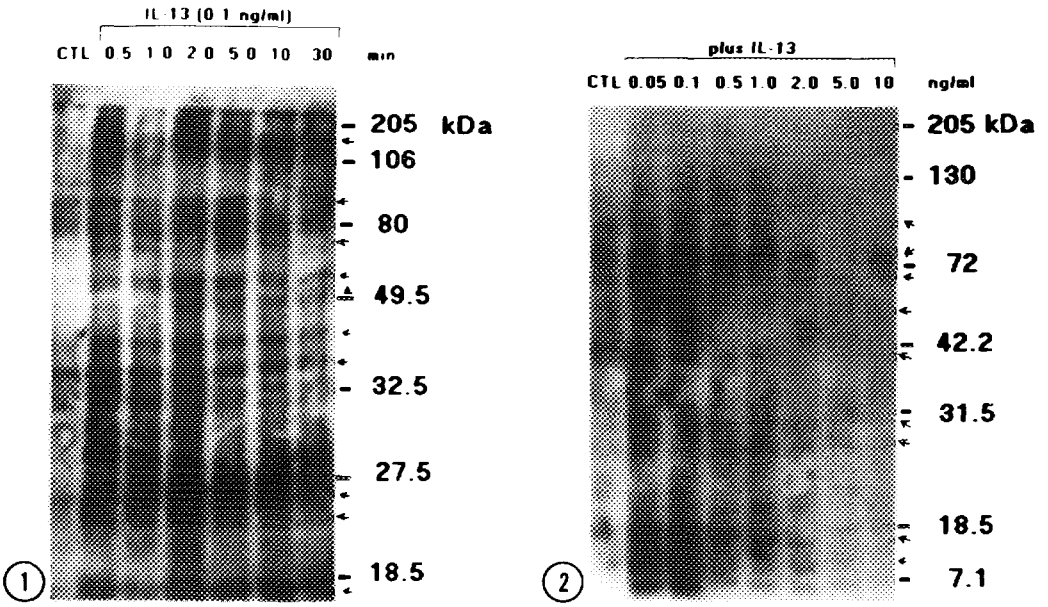

Fig. 1. Time course of stimulation of tyrosine phosphorylation by IL-13. Human U937 leukemic cells (3 x 10^{6}) were pretreated with vanadate (5 mM) for 30 min prior to treatment with 0.1 ng/ml rhIL-13 for various periods of time. Cells were immediately pelleted by microcentrifugation for 20 sec, washed in PBS and lysed in SDS-gel sample buffer (17). Solubilized proteins were separated on 10 % polyacrylamide gels, transferred to membrane and Western Blotted to anti-phosphotyrosine antibody RC20. Phosphotyrosine bands were deteced by ECL detection reaction and autoradiography.

Fig. 2. Concentration dependence of IL-13-induced tyrosine phosphorylation. Vanadate pretreated U937 cells (3 X 10^{6}) were treated with various doses of rhIL-13 for 2 min and total cell lysates were analyzed for tyrosine phosphorylation as described under Fig. 1.

on tyrosine phosphorylation of bands of Mr. 93, 80, 74, 49.5, 30, 22 and 18 kDa within 2 min. Beyond 2 min, the effect of IL-13 on tyrosyl phosphorylation showed a gradual decline.

As seen in Fig. 2, treatment of cells with IL-13 resulted in a dose-dependent stimulation of tyrosine phosphorylation of proteins (indicated by arrows). The optimum effect of IL-13 was attained at concentration corresponding to 0.1 ng/ml. A gradual decline in the effect of IL-13 on tyrosine phosphorylation occurred as the IL-13 concentration was increased beyond 0.1 ng/ml. At 10 ng/ml, IL-13 caused a slight inhibition of tyrosine phosphorylation in comparison with the untreated control (lane 8 compared with lane 1). Stimulation of increase in the levels of tyrosine phosporylation induced by IL-13 was almost completely blocked by the tyrosine kinase inhibitor erbstatin (50 μg/ml) in the preincubation medium (Fig. 3). This indicates that the effect of IL-13 on tyrosine phosphorylation is mediated through activation of tyrosine kinases.

A number of reports have implicated raf-1 kinase as a major signal transduction molecule involved in serine/threonine

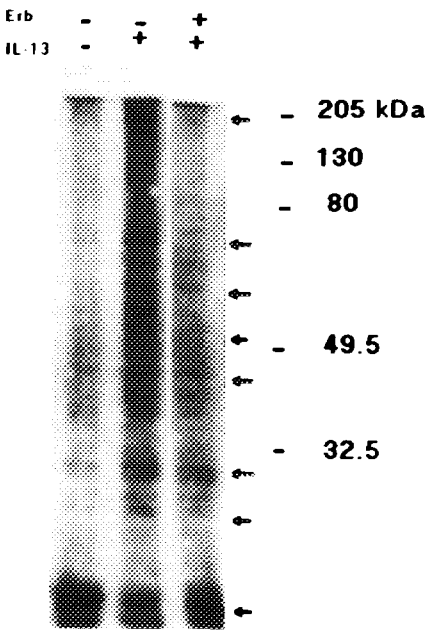

Fig. 3. Inhibition of IL-13-induced tyrosine phosphorylation by Erbstatin. Vanadate pretreated U937 cells (3 X 10^6) were preincubated with erbstatin (50 μg/ml) for 15 min prior to exposure to rhIL-13 (0.1 ng/ml) for 2 min. Total cell lysates were analyzed for tyrosine phosphorylation as described in Materials and Methods.

phosphorylation of key cellular proteins including histone H1 during activation of cells by cytokines, growth hormones or mitogens (19-24). Therefore, to investigate whether raf-1 kinase plays a role in the signal transduction events evoked by IL-13, we compared the status of tyrosine phosphorylation of raf-1 in untreated and IL-13 treated cells. We exposed cells to IL-13 (0.1 ng/ml) for various times, immunoprecipated raf-1 protein with anti raf-1 monoclonal antibody followed by Western Blotting with antiphosphotyrosine antibody. In Fig. 4A, we demonstrate that an initial transient decrease in the level of tyrosine phosphorylation of raf-1 occurs within 30 sec in response to IL-13. The initial decrease in tyrosine phosphorylation of raf-1 in response to IL-13 was followed by a 2-fold increase in phosphorylation 10 min following the addition of IL-13 (0.1 ng/ml).

Because the level of tyrosine phosphorylation of raf-1 changed in response to IL-13, it was important to investigate whether modulation of tyrosine phosphorylation of raf-1 by IL-13, results

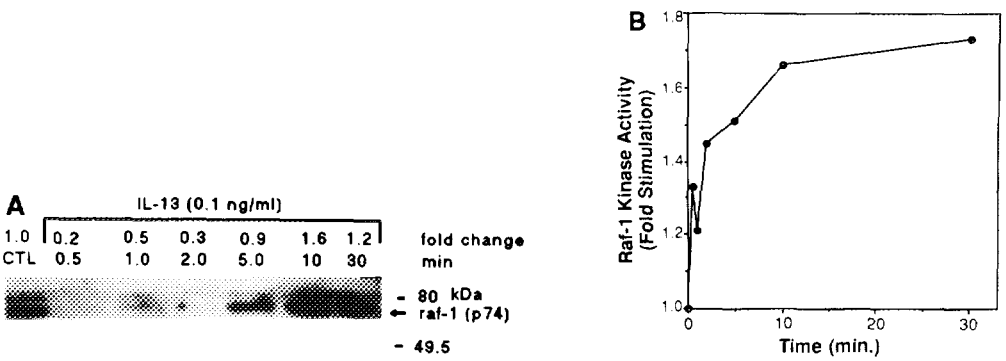

Fig. 4A. IL-13-induced modulation of tyrosine phosphorylation of raf-1. Vanadate pretreated cells (10 X 10^6) were treated with rhIL-13 (0.1 ng/ml) for 2 min. Cells were lysed in lysing buffer and cell extracts (120 to 250 μg/ml protein) were incubated with anti raf-1 monoclonal antibody to immunprecipitate raf-1. Immucomplexes were captured on protein A agarose matrix and subjected to Western Blot analysis. Raf-1 phosphotyrosyl protein was detected by Western Blot hybridization with RC20 as described in Materials and Methods.

Fig. 4B. Stimulation of activation of raf-1 kinase activity by rhIL-13. Vanadate pretreated U937 cells (10 X 10^6) were treated with rhIL-13 (0.1 ng/ml). Raf-1 protein was immunoprecipitated from cell lysates with anti-raf-1 monoclonal antibody. Immunocomplexes were captured on protein A agarose matrix, dissolved in raf-1 kinase reaction mixture containing 10 μg H1 histone and raf-1 kinase-induced ^{32}P incorporation into histone H1 was assessed as described in Materals and Methods. Data are expressed as fold stimulation of control (4,008 cpm) representing an average of two determinations with a standard error of less than 10 %.

in alteration of raf-1 kinase activity. Immunocomplex kinase assays were performed on an immunoprecipited raf-1 protein by following the degree of incorporation of ^{32}P into histone H1 in extracts from untreated or IL-13 treated cells. The results in Fig. 4B indicate that IL-13 stimulates rapid but progressive activation of raf-1 kinase activity as demonstrated by the time-dependent increase in ^{32}P incorporation into H1 histone in IL-13 treated cells. This is in contract to tyrosine phosphorylation of raf-1 which showed an initial negative response to IL-13. Approxiamtely 2-fold increase in the level of activation of raf-1 kinase activity was elicited by IL-13 within 2 min, indicating that IL-13 induced modulation of tyrosine phosphorylation of raf-1 is associated with activation of the phosphotransferase acivity of raf-1 kinase.

DISCUSSION

In this report we have provided the first evidence that the mechansim of IL-13 invloves rapid activation of tyrosine phosphorylation of several proteins. IL-13-induced stimulation of tyrosine phosphorylation was detected on major tyrosine phosphoproteins corresponding to Mr. of 93, 74, 49.5, 42 and 18 kDa. The effect of IL-13 on tyrosine phosphorylation was detected within 30 sec, reached a maximum level in 2 min, after which there was a gradual decline to the basal level. The maximum effect of IL-13 on tyrosine phosphorylation was attained with as low as 0.1 ng/ml of IL-13. Because the ability of IL-13 to stimulate increases in tyrosine phosphorylation was almost completely inhibted by erbstatin, our data indicate that activation of tyrosine kinases probably occurs in response to IL-13.

The biological effects of IL-13 in suppression of pro-inflammatory responses and cytotoxic activities of monocytes/macrophages are considered to resemble those induced by IL-4 (4-9, 15-16). According to a recent report (10), the receptors for IL-13 and IL-4 share a novel component that is important for signal transduction. In addition, many authors have shown that the mechansims of IL-4 involves initial activation of tyrosine kinases coupled to downstream activation of serine/threonine kinases (19, 20). Therefore, the ability of IL-13 to stimulate tyrosine phosphorylation and activation of raf-1 kinase reported here further strengthens the notion that perhaps the similarities between the biological effects of IL-13 and IL-4 arise from the possiblity that some aspects of the pathways used by these

molecules may be interrelated (10, 19-20). Such a possibility is yet to be established.

Evidence is also provided that one of the proteins which experience changes in tyrosine phosphorylation in response to IL-13 is raf-1 kinase. Furtheremore, IL-13-induced modulation of tyrosine phosphoprylation of raf-1 is associated with activation of the kinase activity of raf-1. This is demonstrated by a 2-fold increase in raf-1 kinase-mediated incorporation of ^{32}P into histone H1 in IL-13 treated cell. Cellular activation by cytokines, growth hormones and mitogens involve stimulation of tyrosine phosphorylation and activation of key signal transduction molecules including c-fes, raf-1, MAP kinase kinase and MAP kinase (21-24). Thus, the mechanism of IL-13 may be similar to that of other cytokines (19-24).

In conclusion, we have provided the first evidence that tyrosine phosphorylation and activation of raf-1 are important early events in the signal transduction mechanism of IL-13. Further studies are in progress to elucidate the roles of other protein kinases in mediating IL-13 signal to the nucleus.

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