Eis (Enhanced Intracellular Survival) Protein of Mycobacterium tuberculosis Disturbs the Cross Regulation of T-cells*

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The pathogenesis of tuberculosis is complex and its manifestations diverse, reflecting a lifetime of dynamic interactions between mycobacterial virulence factors and the human immune system. The pathogenic mycobacteria have developed strategies to circumvent the major killing mechanisms employed by macrophages and take advantage of the enclosed environment within its host cell to avoid humoral and cell-mediated immune responses. Secretory proteins play a major role in host-pathogen interactions. The eis (Rv2416c) gene has been identified as a secretory protein, and it has been shown that it enhances intracellular survival of Mycobacterium smegmatis in the macrophage cell line. The main aim of this study was to gain insight into the biological role of Eis in the host. Stimulation of T-cells with Eis recombinant protein of Mycobacterium tuberculosis inhibits Con A-mediated T-cell proliferation in vitro. Treatment of T-cells with Eis inhibits ERK1/2, JAK pathway, and subsequent production of tumor necrosis factor-α and interleukin-4. On the contrary, there is increased production of interferon-γ and interleukin-10, which indicates that immunity in response to Eis treatment is skewed away from a protective Th1 response and Eis disturbs the cross regulation of T-cells.

Virulence of mycobacteria is a multifaceted phenomenon based on the expression of multiple genes involved in various stages of host-pathogen interactions including adhesion, invasion, intracellular replication, and dissemination to other sites. Although both virulent and avirulent mycobacteria are internalized by monocytes and macrophages (1, 2), only pathogenic mycobacteria survive and replicate intracellularly (3). Innate and T-cell-mediated immunity plays a major role in the control of bacterial propagation and protection against disease (4–6). Animal studies indicate that acquired protection against tuberculosis is mediated by sensitized T lymphocytes. Besides other bacterial determinants, proteins that are actively secreted have regained center stage in host-pathogen interactions as they are involved in mounting of specific protective immune responses (7–10).

Mycobacterium-induced production of proinflammatory cytokines, such as TNF-α, IL-1, and monocyte chemoattractant protein 1, is dependent on MAPK activation (11–14). In addition, the extent of MAPK phosphorylation in human monocyte-derived macrophages determines the intracellular growth control of Mycobacterium avium, which indicates an essential role for macrophage activation (11). Understanding the specificity of the human cytokine response and exploring the intracellular signaling pathways that relate to the individual mycobacterial antigens are critical for defining the mechanisms responsible for host defense and pathogenesis during tuberculosis (15).

Wei et al. (16) have recently identified the eis gene of Mycobacterium tuberculosis that was responsible for the enhanced intracellular survival of Mycobacterium smegmatis in the human macrophage cell line U-937. The eis gene has no significant homology to other known genes. Roberts et al. (17) have recently characterized the eis promoter and shown that a 200- and 412-bp region of the promoter was necessary for maximum expression of gfp in M. smegmatis and M. tuberculosis H37Ra, respectively. Eis is found to be a non-glycosylated protein. Eis appears primarily in the cytoplasm and in modest amounts in the cell envelope and in the culture supernatant (18).

As of now, little is known about the structure and function of Eis and the immunological response to Eis. For gaining insight into the biological role of Eis in the host, we carried out cloning and overexpression of Eis from M. tuberculosis H37Rv. The overexpressed protein was purified to homogeneity, and its immunological properties were studied using T-cell proliferation assays. We further examined the role of Eis in MAPK signaling pathways and the subsequent production of proinflammatory cytokine-inducing activities. We found that the purified Eis recombinant protein inhibits the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) and subsequent production of TNF-α and IL-4. Furthermore, the production of IFN-γ and IL-10 by Eis stimulated T-cells was also found to be enhanced.

EXPERIMENTAL PROCEDURES

Materials—Escherichia coli host strain BL21 (AΔDE3) pLysS and the plasmid vector pET28a were purchased from Novagen.

1 The abbreviations used are: TNF-α, tumor necrosis factor-α; Con A, concanavalin A; ERK1/2, extracellular signal-regulated kinase 1/2; IAK1, Janus kinase 1; STAT1, signal transducers and activators of transcription 1; MAPK, mitogen-activated protein kinase; IL, interleukin; IFN-γ, interferon γ; IPTG, isopropyl-β-D-thiogalactopyranoside; Ni-NTA, nickel-nitrilotriacetic acid; ELISA, enzyme-linked immunosorbent assay; LPS, lipopolysaccharide.

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IPTG and imidazole were purchased from GE Healthcare. Anti-His antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Ni-NTA Superflow was procured from Qiagen. Sodium phosphate buffer and buffer components for Western blotting were obtained from SRL Ltd. Molecular weight markers for SDS-PAGE were purchased from MBI Fermentas, Hanover, MD.

Cloning of Eis—Genomic DNA of M. tuberculosis H37Rv was prepurified using the method described by Kremer et al. (19). The eis gene was amplified by polymerase PCR using forward primer 5′-CGGGATCCAGATCGGATCT-3′ and reverse primer 5′-GCTCTAGATCGAATTCTGCGG-GTC-3′. The nucleotides underlined in the forward and reverse primers correspond to BamHI and XbaI restriction sites, respectively. The amplified PCR product was cloned in pGEM-T Easy vector at the TA cloning site. The eis gene was further subcloned into pET28a prokaryotic expression vector using BamHI and NotI to produce Eis protein with a hexahistidine tag at the N terminus. The cloning strategy described above added a sequence of 34 additional amino acid residues (including 6 histidines) at the N terminus of Eis. The cloned eis gene with correct orientation was verified by DNA sequencing.

Overexpression and Purification of Eis Protein—The vectors containing the eis gene were transformed into E. coli expression hosts like BL21 (ADE3), Rosetta, BL21 (ADE3) pLYsS, C41 (ADE3), and Codon + cells. Out of these strains, pLYsS gave the highest soluble protein expression at 0.5 mM IPTG induced for 6.0 h at 28 °C. A single colony was inoculated into LB broth containing 50 μg/ml kanamycin and 34 μg/ml chloramphenicol and grown overnight at 37 °C. The 1.0 ml of overnight culture was inoculated into 1.0 liter of LB medium containing antibiotics and grown at 37 °C. Cells in logarithmic phase (A 600 of 0.6) were induced with 0.5 mM IPTG and grown further for 6.0 h at 28 °C. After induction, cells were harvested, and the pellet was resuspended in phosphate-buffered saline (pH 7.4). The cell suspension was sonicated on ice until it was optically clear. The insoluble debris was removed by centrifugation at 14,000 rpm for 30 min. The clear supernatant of N-terminal hexahistidine-tagged Eis was directly loaded onto a pre-equilibrated Ni-NTA superflow (5.0 ml, Qiagen) column. The column was pre-equilibrated sterile nylon wool column and incubated for 1.0 h at 37 °C, 5% CO2, in a humidified chamber. After incubation, T-cells were eluted slowly by adding RPMI 1640 and 10% fetal bovine serum. The purity of T-cells was analyzed through fluorescence-activated cell sorter by labeling with fluorophore conjugated α-CD3, α-CD4, α-CD8, and α-CD14 (supplemental Fig. S1). The purity of T-cells was found to be >90%

3.5 × 10^5 cells/well were cultured for 96 h in 96-well culture plates in RPMI medium supplemented with 10% fetal bovine serum at 37 °C, 5% CO2. T-cells were not treated with phytohemagglutinin/IL-2. No antibiotics or fungicides were used during the entire period of the culture of the cells. The viability of cells was measured using trypan blue exclusion after 96 h of treatment with Eis. Cells were either stimulated with an increased amount of purified recombinant Eis protein (2.5, 5.0, and 10 μg) or left untreated. Con A (2.5, 5.0, and 10 μg) was used as mitogen for positive control. T-cell proliferative responses were assayed by [3H]thymidine incorporation during the last 24 h of 96-h cultures. 0.5 μCi of [3H]thymidine, specific activity 5.0 Ci/mmol (Bhabha Atomic Research Centre), was added to each well. The cells were harvested and processed by washing on glass fiber filters using an automated cell harvester (Nunc), and [3H]thymidine incorporation was measured with a scintillation counter. Results are expressed as the means of the cpm of triplicate cultures and ± S.E.

Cytokine Assay—The supernatant from cultured and stimulated T-cells was sampled for cytokines TNF-α, IL-4, IFN-γ, and IL-10. Cytokine enzyme-linked immunosorbent assay kits (Pharmingen) were used for detecting TNF-α, IL-4, IFN-γ, and IL-10 in culture supernatant. Assays were performed as specified by the manufacturer, and samples were run in triplicates. Cytokine concentrations in the samples were calculated using standard curves generated from recombinant cytokines, and the results were expressed in picograms or nanograms per milliliter. The difference between triplicate wells was always less than 10% of the mean. The detection limits of the four ELISA assays were: TNF-α, 7.8 pg/ml; IL-4, 7.8 pg/ml; IL-10, 7.8 pg/ml; IFN-γ, 4.7 pg/ml. Graphs were generated using Microsoft Excel software, and error bars referred to standard deviation.

As overexpressed recombinant protein of Eis was purified from E. coli, therefore, to rule out the possibility of significant LPS contamination in Eis protein, we studied the production of IL-10 cytokine from T-cells in response to Eis, LPS, and polymyxin treatment either alone or in combination. Polymyxin binds to the lipid A portion of bacterial lipopolysaccharides (22). As expected, polymyxin inhibits the production of IL-10 in response to LPS, but there is no change in the production of IL-10 in response to polymyxin and Eis treatment (supplemental Fig. S2).

Determination of MAPK Phosphorylation—A total of 3 × 10^5 T-cells were treated with the 10 μg of Eis protein for 96 h. Then cells were washed twice with phosphate-buffered saline. Washed cells were boiled with 50 μl of 1× Laemmlli sample buffer, and Western blot analysis was performed with specific primary antibodies (ERK1/2, pERK1/2, JAK1, STAT1, and pSTAT1) as per the manufacturer’s instructions. Membranes were developed using a chemiluminescence assay (ECL). Finally, the results were visualized by autoradiography and quantitated using Quantity One® software from Gel Doc 2000
RESULTS

Expression and Purification—We amplified the eis gene from the *M. tuberculosis* H37Rv genomic DNA (Fig. 1A) and prepared N-terminally hexahistidine-tagged Eis using the strain BL21 (DE3) pLysS of *E. coli*. Induction of *E. coli* BL21 (DE3) pLysS pET28a/eis by the addition of IPTG resulted in production of recombinant Eis protein predominantly (>90%) in the soluble form (data not shown). Time course studies to optimize IPTG-induced protein expression, as analyzed by SDS-PAGE, demonstrated maximum protein yield to be obtained through induction for 6.0 h at a concentration of 0.5 mM IPTG, 28 °C (Fig. 1B). Further, the expression of Eis protein was confirmed with anti-His antibodies (Fig. 1C). Overexpressed recombinant Eis protein was purified to apparent homogeneity from extracts of *E. coli* BL21 (DE3) pLysS pET28a/eis cells using Ni-NTA Superflow column. A single band on SDS-PAGE confirmed the homogeneity of the protein (Fig. 1D).

Eis Inhibits T-cell Proliferation—The effect of Eis on the response of T-cells is shown in Fig. 2A. As expected, Con A stimulated proliferations of T-cells, but the inhibition of T-cell proliferation by Eis is very prominent. Effect of Eis was checked by titrating the various concentrations of Eis (Fig. 2A). Cell viability was not compromised on exposure to Eis as confirmed by the trypan blue exclusion method (data not shown). Further, we checked whether Eis suppresses Con A-induced T-cell proliferation. As shown in Fig. 2B, Eis inhibited the Con A-mediated proliferation of T-cells in a dose-dependent manner, and the maximum inhibition observed is ~3-fold.

Cytokine Production and MAPK Phosphorylation in Response to Eis by T-cells—The levels of cytokines produced by T-cells following Eis treatment are shown in Fig. 2, C–F. Con A treatment produced significantly higher levels of TNF-α and IL-4. In contrast, there were extremely low levels of TNF-α and IL-4 production in response to Eis treatment (much below the detection limit of ELISA), which shows clearly that Eis inhibits the production of TNF-α and IL-4. Although Eis could inhibit Con A-mediated TNF-α production by only 1.5-fold, the effect of Eis on Con A-mediated IL-4 production is very dramatic (Fig. 2, C and D). The production of TNF-α is regulated by IL-10 (23). Therefore, we studied the effect of Eis on T-cells for the synthesis of IL-10. Eis induced the production of IL-10 by 20-fold in comparison with untreated cells (Fig. 2E). Unexpectedly the production of IFN-γ was also found to be enhanced in response to Eis stimulation (Fig. 2F). To rule out the variation in the production of cytokines between individuals, the actual cytokine data points from 15 healthy volunteers were also graphed for TNF-α, IL-10, and IFN-γ production (Fig. 3). The profile of TNF-α, IL-10, and IFN-γ cytokine productions in

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response to Eis treatment in different individuals was found to be similar. The IL-4 production in response to Eis treatment in all the individuals was found to be below detection limit of ELISA (data not shown).

Given that MAPKs are critical factors mediating cellular responses to external stimuli and because of the importance of these pathways in inflammatory and other immune responses, we examined the role of Eis protein of *M. tuberculosis* in the induction of MAPK signaling pathways. Human T-cells were stimulated with 10 μg of purified recombinant Eis protein, and the phosphorylation of ERK1/2, JAK, and STAT was analyzed (Fig. 4). Fig. 4A shows that lower levels of phosphorylated ERK1/2 were observed in Eis-treated lanes. Fig. 4B shows the level of total ERKs. Similarly, JAK1 is not detectable in response to Eis treatment (Fig. 4C). Analysis of pSTAT and total STAT did not reveal significant difference in the level of activation upon Eis treatment (Fig. 4, D and E). A control immunoblot probed with standard actin antibodies demonstrated equal loading in each lane (Fig. 4F).

**DISCUSSION**

The identification of Eis has recently been reported. Eis is involved in the survival of mycobacteria during the infection of macrophages (16). It has been shown that Eis is expressed only when the clinical isolate was replicating in activated macrophages (24). However, nothing is known about how Eis mediates its functional activity. Our main goal here was to characterize the biological role of Eis protein. The main question we posed in this study was whether Eis could affect the host immune response. The data presented here showed that (i) the stimulation of human T-cells with the Eis protein of *M. tuberculosis* has an inhibitory effect on Con A-mediated T-cell proliferation in vitro, (ii) stimulation of T-cells in vitro with the Eis protein inhibits ERK1/2 and JAK activation, and (iii) production of TNF-α, IL-4, IFN-γ, and IL-10 is modulated by Eis-treated T-cells. Although secretion of TNF-α and IL-4 is inhibi-
ited by Eis-treated T-cells, the IFN-γ and IL-10 production is higher in Eis-stimulated T-cells. Down-regulation of TNF-α expression in response to Eis treatment shows that Eis is able to impair the host defense of tuberculosis.

MAPKs are evolutionarily conserved signal transduction pathways that play important roles in the transduction of signals in the innate immune responses of plants, insects, and mammals (25). The activation of intracellular signaling pathways and subsequent inflammatory cytokines can be induced by different stimuli in different cell types (26). We found that the Eis-dependent down-regulation of ERK and JAK pathway in T-cells impairs the secretion of TNF-α and IL-4. The production of IFN-γ and IL-10 was found to be more. Demissie et al. (27) have reported the elevated levels of IL-4 and reduced levels of IFN-γ. There are several reports that link inverse correlation between TNF-α and IFN-γ levels (28, 29). Eis is found to elicit strong T₄₁ response, i.e., elevated IFN-γ expression associated with lower expression of IL-4. The 38-kDa lipoprotein is also reported to elicit a strong T₄₁ response (30). Although IFN-γ inhibits the proliferation of T₄₂ cells (31, 32), IL-4 preferentially stimulates growth of T₄₂ cells (32, 33). IL-10 inhibits the synthesis of cytokines by T₄₁ cells. Increased production of IFN-γ and IL-10 by Eis-stimulated T-cells shows that there is an Eis-mediated disturbance of cross regulation of T-cells, which may impair the balance between T₄₁ and T₄₂ response, and this imbalance could be the main action of Eis, which could be a factor of the pathogenesis of tuberculosis. Gaining mechanistic insight into the Eis-mediated altered T₄₁/T₄₂ imbalance may help in understanding the implications for enhanced survival of M. tuberculosis in host, which may further contribute toward development of a myriad of strategies for fighting tuberculosis.

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