Effect of mycobacterial secretory proteins on the cellular integrity and cytokine profile of type II alveolar epithelial cells

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ABSTRACT

Background: Pulmonary tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (*M. tb*). In lungs, alveolar macrophages and type II alveolar epithelial cells serve as a replicative niche for this pathogen. Secretory proteins released by actively replicating tubercle bacilli are known to interact with host cells at the initial stages of infection. To understand the role of these cells in TB pathogenesis, it is important to identify the mycobacterial components involved in interaction with alveolar epithelial cells. Materials and Methods: We fractionated the whole secretory proteome of *M. tb* H₃₇Rv into 10 narrow molecular mass fractions (A1-A10; <20 kDa to >90 kDa) that were studied for their binding potential with A549; type II alveolar epithelial cell line. We also studied the consequences of this interaction in terms of change in epithelial cell viability by MTT assay and cytokine release by ELISA. Results: Our results show that several mycobacterial proteins bind and confer cytolysis in epithelial cells. Amongst all the fractions, proteins ranging from 35-45 kDa (A5) exhibited highest binding to A549 cells with a consequence of cytolysis of these cells. This fraction (A5) also led to release of various cytokines important in anti-mycobacterial immunity. Conclusion: Fraction A5 (35-45 kDa) of mycobacterial secretory proteome play an important role in mediating *M. tb* interaction with type II alveolar epithelial cells with the consequences detrimental for the TB pathogenesis. Further studies are being carried out to identify the candidate proteins from this region.

KEY WORDS: Cytokines, cytotoxicity, *Mycobacterium tuberculosis* (*M. tb*), type II alveolar epithelial cells

INTRODUCTION

*Mycobacterium tuberculosis* (*M. tb*), the causative agent of tuberculosis (TB), claims nearly 2 million deaths annually. *M. tb* primarily infects lungs after inhalation of bacilli-laden droplet nuclei via respiratory route. In the lung microenvironment, alveolar macrophages constitute the principal host cells involved in *M. tb* invasion and replication.[1] They function to engulf and mount the foremost line of defense to tubercle bacilli; however, *M.tb* resists the phagocytic cell’s bactericidal mechanism and manages to replicate within.[2] Although numerous facets of phagocytic cell- *M. tb* interactions have been studied, there is mounting evidence that bystander alveolar epithelial cells (AEC) are also cellular targets for *M. tb* infection. Various studies have documented mycobacterial invasion and replication potential within type II AEC.[3-5] It is considered that mycobacterial invasion of AEC possibly creates a niche for the bacterium to replicate and establish the infection, avoiding potentially hostile environment of macrophages. Additionally, epithelial cell cytolysis owing to mycobacterial infection results in alveolar barrier disruption and subsequently contributes to the dissemination of both free and macrophage-ingested mycobacteria from the lungs.[6-8]

Formerly, AEC were looked upon as bystander cells performing only their physiological roles, while it has
also been documented that type II AEC are ideally suited to play a role in local regulation of inflammatory response within alveolar space.[9] These cells produce inflammatory mediators such as interleukin (IL)-8,[10] prostaglandins,[11] nitric oxide,[12] and complement proteins[13] capable of modulating local immune response.

Secreted proteins by M. tb are believed to mediate important biological functions by interacting with host cells and are potentially important for virulence and pathogenesis of the bacterium.[14,15] Though the complete biology of mycobacterial antigens and their role in directing the fate of an infection is not deciphered, yet it is a well-established fact that extracellular proteins secreted by actively growing bacilli rather than mycobacterial subcellular components are the key molecules to come in contact of host cells at the initial stages of infection both in human[16] and in various experimental animal models.[17] These proteins have been studied for immuno-dominant antigens as these were considered to be rapidly recognized by host lymphocytes.[18] However, till now, no systematic study has been carried out to dissect the components of mycobacterial secretory proteome in order to identify the candidate proteins involved in the interaction with AEC. Therefore, present study has been designed to elucidate the interaction of mycobacterial short term culture filtrate (STCF) components with A549 cells, with the consequences imperative for the dissemination or control of disease. As it is not possible to elute and evaluate all the secretory proteins present in the culture filtrate, here fractionation of the mycobacterial secretory proteome into narrow molecular mass fractions was performed, and the interaction of these fractions with type II AEC was investigated.

**MATERIALS AND METHODS**

**Cell Line Culture and Maintenance**

A549 (ATCC CCL185): a human type II AEC line, was obtained from ATCC (American Type Culture Collection, Rockville, MD) and maintained in RPMI-1640 supplemented with 25 mM HEPES, 1 mM L-glutamine, and 1 mM sodium pyruvate and 10% heat inactivated fetal bovine serum (FBS, Sigma chemical, St Louis, MO) at 37°C and 5% CO₂ in T50 and T250 tissue culture flasks (Grenier Bio-one Bioscience, Germany). Before use in experiments, the cells were released from the culture flasks with trypsin-EDTA solution, washed with incomplete media (RPMI without FBS), seeded onto tissue culture plates (Nunc, Waltham, MA) as needed, and were allowed to grow to 80-90% confluency.

**Fractionation of M. tb STCF into Narrow Molecular Mass Fractions**

Mycobacterial crude STCF was obtained from the M. tb H₃₇Rv log phase cells grown as stationary culture for 3-4 weeks as described earlier.[19] 5 mg of STCF was separated on 16% preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) overnight on protein II xi cell (BioRad). Constant current of 10 mA was applied for stacking of protein samples, followed by resolution at 30 mA. After the electrophoresis run, the gel was pre-equilibrated in tris borate buffer (pH 8.7; 50 mM tris and 250 mM boric acid) and were transferred to whole gel elutor and electroeluted at 250 mA for 30 min, and the current was reversed for 10 sec at the end of the elution period to loosen molecules sticking to the cellophane membrane at the bottom of the wells. Fractions were harvested from the unit with the help of vacuum pump and designated as A1 to A10 in the order of increasing molecular mass and were kept frozen at -20°C until use. All fractions were analyzed by separation on SDS-PAGE (16%) followed by silver staining. Protein concentration of various fractions, obtained upon fractionation of STCF, was estimated using bicinchoninic acid (BCA) kit (Sigma chemicals, Ltd.) according to manufacturer’s instructions.

**Binding of STCF Fractions with Host Cells**

A549 cells cultured for 4 days were harvested and were washed 3 times with RPMI-1640 after centrifuging at 400 × g for 10 min, suspended in PBS and were counted by trypan blue dye exclusion method. 5 × 10⁵ cells/tube/L were taken for each test and were treated with different culture filtrate’s fractions at optimized concentration of 20 µg/mL and kept for incubation for 30 min at 37°C. After 3 washings with PBS (containing 0.1% bovine serum albumin), the cells were incubated with anti-STCF antisera (available in lab) at the dilution of 1:100 for 45 min at 4°C. Subsequently 6 washings with RPMI-1640 were given, and the cells were again incubated with anti-mouse FITC (1:250) for 20 min at 4°C. The pelleted cells after 3 washings with RPMI-1640 were suspended in 300 µL of fixative (1% paraformaldehyde in phosphate buffered saline). Flow cytometric analysis was performed in FACS scan flow cytometer (Becton Dickinson, Mountview, CA). Data analysis was done using WinMDI 2.8 software. Debris in the cellular suspension was excluded from the analysis by suitable gating. The cell population was identified on the basis of forward vs. side scatter and gated appropriately to determine the percentage of positively stained cells. Appropriate antibody control, cell control were included, and the data obtained was represented as percent binding. The experiments were conducted thrice in duplicate.

**Cytotoxicity Assay**

For MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, 4 × 10⁵ A549 cells/mL were cultivated, counted with trypan blue dye exclusion method, and 100 µL of cell suspension was dispensed in each well of 96-well plates. Cells were maintained in a 5% CO₂ incubator at 37°C. After 72 hr, supernatant was removed leaving adhered cells, and 20 µg/mL of each of the fractions (culture filtrate protein) was added to the wells, followed by 72 hr incubation in a 5% CO₂ incubator at 37°C. Supernatant was removed, and 110 µL MTT reagent was added to each well. After 4 hr incubation at 37°C, 85 µL of MTT reagent was removed and to remaining 25 µL, 50 µL of dimethyl sulfoxide was added for cell lysis and
solubilization. After 10 min incubation, the absorbance was read at 545 nm, and the results were represented as percent cytotoxicity, taking control (cells without proteins, with MTT) as reference standard. The experiment has been conducted twice in triplicate wells.

**Cytokines Release Assay**

4 × 10^5 A549 cells/mL were cultured, in 96-well plates in 5% CO₂ at 37°C. Concentration of various cytokines; interferon (IFN)-γ, tumor necrosis factor (TNF)-α, IL-6, IL-12(p40) was measured in the culture supernatants removed from the cell monolayers stimulated with 20 µg/mL of each of the fractions for 72 hr by ELISA kits (BD Pharmingen) according to manufacturer’s instructions.

**RESULTS**

**Fractionation of STCF in Narrow Molecular Mass Fractions**

Short-term culture filtrate was obtained from the stationary culture of *M. tb* H₃₇Rv, which was allowed to grow for 4-5 weeks. The protein concentration of STCF was found to be 4.44 mg/mL. The protein profile of STCF resolved on 16% SDS-PAGE [Figure 1] showed the presence of numerous bands with ~30 prominent protein bands ranging in molecular weight from 3 kDa to more than 100 kDa.

Fractionation of STCF by whole gel elutor resulted into 10 narrow molecular mass fractions (A1 to A10) ranging from <20 kDa to >90 kDa of molecular mass [Table 1] and each fraction consisting of about 2-5 visible protein bands [Figure 1]. The fractionation was carried out using the combination approach of preparative high resolution SDS-PAGE, followed by whole gel electro-elution. The elutor acts as an electro-dialyzer and removes SDS from protein, leaving culture filtrate protein fractions in a non-toxic physiological buffer, which was then used directly in various cellular assays.

**Binding of STCF Fractions with Host Cells**

To identify proteins from the mycobacterial secretory proteome that could have some role in pathogenesis of *M. tb* disease, the binding of STCF fractions with AEC was checked by flow cytometric analysis, and binding capacity was recorded as the percentage of positively stained cells by anti-mouse FITC [Figure 2]. The background staining observed in control i.e. without protein treatment, was ~5.1 % [Figure 2]. The minimal cut-off for the binding capacity was taken to be 15% (3 times the background fluorescence) and has been subtracted from the fluorescence obtained after treatment with each fraction. The fractions A1, A2, A4, A5, A6, A7, and A8 exhibited good binding with A549 cells, with fraction A5 (35-45 kDa) showing maximum binding, followed by fraction A6 (45-55 kDa) [Figure 2]. These observations clearly demonstrate that the 35-55 kDa region of mycobacterial STCF predominantly contains the proteins involved in binding and interaction with host AEC.

**Cytotoxicity Assay**

The effect of *M. tb* secretory proteins interaction with AEC was measured in terms of cell viability by MTT assay. Wells containing medium alone (without cells) and MTT

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**Figure 1:** Protein profile of STCF of narrow molecular mass fractions obtained from the fractionation of *M. tb* H₃₇Rv on 16% SDS-PAGE

**Figure 2:** Percent binding of mycobacterial secretory proteins with A549 cell line. Bars represent mean values ± SD, n = 3

**Figure 3:** Percent cytotoxicity to A549 cells by mycobacterial secretory proteins. Bars represent mean values ± SD; n = 6
Cytokine Release

To determine the immuno-modulatory effect of *M. tb* secretory proteins on AEC, cytokine profile of A549 monolayer treated, with narrow molecular mass fractions of mycobacterial STCF, was determined by sandwich ELISA. Amongst various narrow molecular mass fractions of the mycobacterial secretory proteome, A1 (<20 kDa), A5 (35-45 kDa), A6 (45-55 kDa), and A7 (55-65 kDa) resulted into higher IFN-γ secretion [Figure 4a]. Only proteins corresponding to 35-45 kDa (A5) were able to induce prominent release of TNF-α [Figure 4b]. Similar to IFN-γ and TNF-α, high levels of IL-6 [Figure. 4c] were observed in response to A5 fraction corresponding to region of 35-45kDa. However, two more regions containing the proteins of 20-30 kDa were also identified as potent stimulator of IL-6 secretion from A549 cells. Maximum level of secretion of IL-12(p40), a Th1 cytokine considered to be important for protective immunity against mycobacterial infection presented in Figure 4d (> 180 pg/mL), was observed in response to A5 fraction.

The comparative analysis [Table 1] clearly indicated the significance of proteins present in 35-45 kDa region to be important in the interaction with A549 cells with a consequence of effect on cellular viability as well as cytokine release. Therefore, there is a need to characterize various proteins present in this molecular range (35-45kDa) to have an insight in the significance of these proteins in host AEC interaction and pathogenesis of tuberculosis.

**DISCUSSION**

The secretory proteins released by actively replicating mycobacteria have been suggested to play an important role in anti-mycobacterial immunity as infection with killed mycobacteria failed to evoke cellular immunity.[20] Both in human and murine models, *M. tb* secretory proteins have been studied for immuno-dominant antigens based on their potential to generate protective immune responses during *M. tb* infection.[19,21-23] While the roles for various *M.tb* secretory proteins are ascribed based on their interaction with lymphocytes/macrophages, no study has clearly investigated the interaction of these proteins with lung epithelia. Although alveolar macrophages are believed to be principal host cells for the intracellular multiplication of tubercle bacilli, various studies have also reported type II AEC as one of the major portals of mycobacterial entry as well as replicative niche in the lung parenchyma.[4,5,24]

In the present study, we fractionated STCF into 10 fractions to explore their role in interaction with type II AEC. We studied various aspects of host-pathogen interactions such as binding, cytokotic, and immuno-modulatory potential of mycobacterial proteins on A549 cells. The elution pattern

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**Table 1: Combined overview of the interaction of STCF fractions with A549 cells**

| Fractions | Molecular weight | Percent binding | Percent cytotoxicity | IFN-γ (pg/mL) | TNF-α (pg/mL) | IL-6 (pg/mL) | IL-12 (pg/mL) |
|-----------|-----------------|----------------|----------------------|---------------|---------------|--------------|---------------|
| A1        | <20 kDa         | 28.025         | 35.64273             | 20.7          | 29.4          | 8.47         | 93.1          |
| A2        | 21-25 kDa       | 16.445         | 37.164               | 6.3           | -             | 16.2         | 9.8           |
| A3        | 25-30 kDa       | 6.86           | 13.46                | 3.15          | 3.676         | 19.22        | 63.726        |
| A4        | 30-35 kDa       | 18.54          | 21.7333              | -             | 5.08          | -            | -             |
| A5        | 35-45 kDa       | 71.26          | 34.4975              | 31.53         | 106.6         | 18.46        | 196           |
| A6        | 45-55 kDa       | 67.025         | 14.775               | 39.63         | 3.76          | 5.46         | 58.8          |
| A7        | 55-65 kDa       | 34.145         | 16.9                 | 11.26         | -             | -            | 19.6          |
| A8        | 65-75 kDa       | 19.665         | 42.88571             | -             | -             | -            | -             |
| A9        | 75-90 kDa       | 3.11           | 13.4                 | -             | 7.352         | 5.46         | -             |
| A10       | >90 kDa         | 0.295          | 44.125               | -             | -             | -            | -             |
of culture filtrate fractions on SDS-PAGE employed in the current study was found to be nearly similar as described previously by Andersen and Heron[19] and reported from our lab.[25] Bacterial pathogens typically gain entry to host tissues using some adherence molecules that bind host cells.[26] In vitro studies using cultured epithelial cells have also shown that mycobacteria are able to bind type II AEC.[27] However, till date, only a few mycobacterial proteins have been explored for their role in interaction with non-phagocytic cells. Studies of interaction of M. tb with human respiratory epithelial cells (Hep-2) have shown binding of a 28 kDa protein, which was found to be heparin-binding hemagglutinin (HBHA). HBHA has also been demonstrated to mediate bacterial attachment to epithelial cells[30] and is further suggested to involve in the extra pulmonary dissemination of these bacteria.[29] Mycobacterial cell entry protein (Mce-1A, 48 kDa),[30] mycobacterial DNA-binding protein 1 (MDP1, 21 kDa)[31] are some other proteins identified as a mycobacterial adhesins employed in the mycobacterium-epithelial cell interaction. Another mycobacterial protein, culture filtrate protein-10 (CFP-10) has been shown to bind murine macrophage cell line,[32] but it failed to bind to A549 monolayers.[33]

The present study shows that various mycobacterial secretory proteins are capable of binding with A549 cells and predominantly proteins in the regions of <20-25 kDa, 35-55 kDa, and 55-65 kDa are involved in binding and interaction with type II AEC. Thus, mycobacterial secretory proteome contains numerous proteins, including some of the well-characterized proteins as well as some other yet uncharacterized proteins that might aid mycobacteria to bind AEC. Epithelial cells have been suggested to play a significant role in dissemination of mycobacterial infection. Infection of A549 cells with virulent M. tb strains Erdman and CDC1551 demonstrated significant cell monolayer clearing,[34] suggesting that mycobacteria cause epithelial cell cytotoxicity, which may result in breaching of the single-layered alveolar barrier. Barrier disruption allows the passage of both, free and macrophage-ingested mycobacteria, and thus facilitates dissemination.[33,35] Till date, only two well-characterized proteins ESAT-6 and HBHA have been implicated in disrupting alveolar epithelial barrier. Our previous work with ESAT-6 has identified this protein to be a bacterial toxin as it causes contact lysis after binding to AEC lines.[33] The results of the present study demonstrate that mycobacteria secrete certain proteins that following binding to epithelial cells result in cytotoxicity of these cells. A5 fraction comprising 35-45 kDa proteins was found to exhibit both maximum binding as well as cytotoxicity to A549 cells. Surprisingly, A10 fraction lead to cytolysis of A549 cells, even without exhibiting any binding to these cells. This could be due to the presence of one or more high molecular weight protein(s) having the inhibitory effect on the binding of other proteins in A10 fraction with A549 cells. However, further studies are required to isolate and purify all the proteins of this fraction and study their interaction with A549 cells individually.

Earlier during experimental TB in mouse model, two regions (4-11 kDa and 26-35 kDa) of mycobacterial secretory proteins have been reported to be good IFN-γ inducers in T-lymphocytes.[23] Phagocytic cells, especially macrophages, are programmed to mediate host defense by releasing pro-inflammatory cytokines such as TNF-α[36] and IL-6[37] that are important for control of mycobacterial growth. Type II AEC has also been shown to contribute to lung immunity by presenting mycobacterial antigens to immune CD4+ T cells by using the class II MHC pathway.[38] A549 cells have been reported to express iNOS, release nitric oxide under the influence of tubercle bacilli conditioned media[39] or cytokine stimulation,[40] and IFN-γ[41] contributing to the control of microbial infection. Our results show that various mycobacterial proteins in narrow molecular mass fractions (A1, A5, A6, and A7) resulted into higher IFN-γ secretion. While, only proteins corresponding to 35-45 kDa (A5) were able to induce prominent release of TNF-α, IL-6, and IL-12. Thus, our study demonstrates that proteins from 35-45 kDa region modulate epithelial cells to release pro-inflammatory and immuno-regulatory cytokines, which could further aid in mycobacterial infection control.

In summary, the present study illustrates that amongst various narrow molecular mass fractions, A5 fraction comprising 35-45 kDa proteins is predominantly involved in the interaction with AEC, thereby leading to modulation of their cellular integrity as well as immune status. Further studies to identify the proteins from this region that are either involved in the disruption of AEC barrier or in the immuno-modulation of these cells are needed to have the better understanding of the pathogenesis of TB. At the same time, host epithelial cell surface molecules utilized for the interactions with mycobacteria also need to be investigated, and such information could be important to develop the novel anti-mycobacterial therapeutic strategies to block this interaction.

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