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MINIREVIEW

Impact of Proteomics on Anti-*Mycobacterium tuberculosis* (MTB) Vaccine Development

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Abstract

Tuberculosis is a serious infection disease which causes more than two million deaths annually. The TB pandemic has continued despite widespread use of the only available licensed TB vaccine – Bacillus Calmette-Guerin (BCG). Additionally, the increasing incidences of multidrug resistant strains and coinfection with HIV mean that tuberculosis constitutes a growing global threat. Thus, improvement of the vaccination strategy against TB is an urgent need, requiring international cooperation of the research community. The completion of many mycobacterial genome sequences has greatly facilitated the global analysis at the transcriptome and proteome level. This in consequence has accelerated progress in the vaccinology field resulting in identification of a large numbers of antigens with potential in TB vaccines. This review concentrates on the proteomic contribution to TB vaccinology. At the end of the article some recent achievements of structural proteomics and developing an epitope-driven tuberculosis vaccine are presented.

K e y w o r d s: *Mycobacterium tuberculosis*, epitope-driven vaccine, proteomic for TB vaccinology, structural proteomics, subunit vaccines

Introduction

Tuberculosis (TB) is a re-emerging disease that remains one of the leading causes of morbidity and mortality in humans. According to the World Health Organization data, about one-third of the world's population is infected with *M. tuberculosis* and 8 to 9 million of new cases are reported each year. More than 2 million people die every year of tuberculosis**.** (World Health Organization, 2006). The only available vaccine against TB is BCG (*Mycobacterium bovis* Bacillus Calmette-Guerin) vaccine, widely used in the world. The expression BCG describes a family of more than 10 daughter strains derived from original BCG. BCG vaccines are divided into the early strains such as BCGs Japan, Birkhaug, Sweden and Russia and the late strains including BCGs Pasteur, Danish, Glaxo and Prague (Hsu *et al.*, 2003). It is estimated that more than 3 billion people were immunized with BCG since it was introduced in 1921. Although BCG is effective as a pediatric vaccine administered to newborns, it provides significant protection only for a limited period of time, about 10 to 20 years. BCG immunization showed highly variable efficacy (0–80%) in trials conducted in different geographical regions, being ineffective as a vaccine against adult pulmonary TB, mainly in tropical and subtropical part of the world. Globally, it is estimated that BCG immunization prevents only ~5% of all deaths due to TB. Additionally, emergence of many multi-drug resistant (MDR) *M. tuberculosis* strains and the fact that TB is currently the leading cause of death in HIV-infected individuals mean that tuberculosis constitutes a growing global threat. Thus, improvement of the vaccination strategy against TB is an urgent need, requiring international cooperation of the research community. Several avenues have recently been explored to generate novel effective TB vaccine. The leading candidates can be assigned into two groups: live vaccines (attenuated *M. tuberculosis* or rBCG overexpressing novel antigens) or subunit vaccines (DNA vaccines, protein vaccines administrated with specific adjuvant stimulating Th1 immune response, fusion molecules consisting of selected immunodominant antigens or antigens delivered by adenovirus vectors). The first category is designed to replace BCG,

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while the latter should be rather used as a heterologous prime-boost vaccination strategy employing BCG or rBCG for priming and novel vaccines as the boosting regimen, for details concerning new TB vaccines see (Andersen *et al.*, 1991; Brennan *et al.*, 2007; Dietrich *et al.*, 2006; Doherty and Andersen, 2005; Skeiky and Sadoff, 2006).

Proteomics *vs* genomics and transcriptomics

Proteomics, together with genomics and transcriptomics, are emerging, complementary approaches which have led, in combination with bioinformatic tools, to enormous progress in basic as well as applied biology. Other than vaccine development, applied aspects of proteomics are reviewed in references (Lee and Jeoung, 2007; Matharoo-Ball *et al.*, 2007; Simpson *et al.*, 2008; Sleno and Emili, 2008) In contrast to genomics, which is a stable technology, transcriptomics and proteomics are considered to be dynamic analyses. Proteomics, large-scale analysis of cellular proteins, is a powerful tool for protein identification, study of their localization, modifications, functions and possible interactions or complexes they can form. Proteome databases of diverse pathogenic microorganisms, including *Mycobacterium. tuberculosis*, *Helicobacter pylori*, *Salmonella enterica*, *Bacillus anthracis* and many others, were established by different research groups using various technologies. The information accessible *via* Internet significantly supports research community working on identification candidates for the next generation of vaccines (Nesvizhskii, 2007; Pleissner *et al.*, 2002; Pleissner *et al.*, 2004; Zhang *et al.*, 2008).

Rapid growth of proteomics is evidently grounded on the progress made in the genomics field, where interpretation of the proteomic analysis is based on data created by annotation of the sequenced genomes. In return, proteomics is often employed for validation of bacterial genome annotations (de Souza *et al.*, 2008; Jungblut *et al.*, 2001; Maillet *et al.*, 2007). Additionally, analysis of the surface proteomes allows verification of the membrane protein topology predicted with PSORT (Rodriguez-Ortega *et al.*, 2006)

A proteomic analysis usually consists of two steps: protein separation and protein identification. There are two major proteomic approaches: 2-DE for protein separation combined with MALDI-TOF-MS for protein identification and 1-or 2-D LC for protein separation followed by ESI or MS/MS for protein identification. Proteomics has achieved tremendous progress during the past few years. The progress could be observed at all steps of the proteomic analysis: sample preparation protocols, peptide/protein separation methods, mass spectrometry (MS) data collection, data analysis and interpretation. All these aspects have been frequently reviewed (Canas *et al.*, 2006; Domon and Aebersold, 2006; Malmstrom *et al.*, 2007; Smith, 2006).

Mycobacterium tuberculosis global analysis

The availability of the whole genome sequence of *M. tuberculosis* laboratory strain H37Rv (Cole *et al.*, 1998), followed by elucidation of the genome of clinical isolate *M. tuberculosis* CDC1551 (Fleischmann *et al.*, 2002) *M. bovis* AF2122 (Garnier *et al.*, 2003) and *M. bovis* BCG Pasteur 1173P2 (Brosch *et al.*, 2007) as well as the genomes of several other mycobacterial species belonging to the *M. tuberculosis* complex, enable the global comprehensive analysis of the pathogen. The list of *M. tuberculosis* complex genomes which have been sequenced up to now comprises 29 genomes (7 completed genomes and 22 genomes in progress) and most of the information is available to the general public www.ncbi.nih.gov/genomes/lproks.cgi. Among these microorganisms several are uniquely pathogenic to humans (*M. tuberculosis*, *M. africanum* and *M. canettii*) whereas other species have broad host ranges. This progress is feasible due to the development and commercialization of new sequencing strategies (Medini *et al.*, 2008). In consequence it enables the development of *Mycobacterium* comparative genomics, transcriptomics and proteomics. Recently performed comparative genomic experiments changed the commonly accepted view that TB in humans came into being as a result of host range alteration of *Mycobacterium* strain specific to cattle. It is now acknowledged that all *Mycobacterium* species diverged about 20 000 years ago from single species which resembled *M. tuberculosis* or *M. cannetti* (Brosch *et al*., 2002). Analysis of the complete genome sequence of *M. bovis* BCG Pasteur 1173P2 followed by comparative genomic and transcriptomic studies, performed by Brosch *et al*. allowed them to refine the genealogy of BCG strains (Brosch *et al.*, 2007).

Studying global changes in bacterial gene expression is crucial for understanding the pathogenesis process – microorganism ability to survive in the host and its interaction with eukaryotic cells. The availability of complete genome sequences of numerous mycobacterial strains allows the development of comparative transcriptomics. The experiments comprised monitoring MTB global gene expression under different growth conditions, upon exposure to various drugs or studying gene expression profiles of MTBs directly obtained from patients. Comparative transcriptomic analysis revealed that the transcriptomes of MTBs taken from patients are significantly different from those obtained *in vitro* (Rachman and Kaufmann,

2007). Knowledge of the host cells response to pathogenic microorganisms is important for understanding the disease process. Toward this end, the DNA microarray technology was also employed to clarify the process of cytokine gene expression of eukaryotic cells (macrophages and monocytes) in response to MTB infection (Smit van Dixhoorn *et al.*, 2008). As mentioned above, proteomics is a strategy complementary to and generally compatible with genomics and transcriptomics. Out of the three global approaches, proteomics is the most informative one. Many research groups generated protein maps of *M. tuberculosis* strains using diverse proteomic technologies (Jungblut *et al.*, 1999; Mollenkopf *et al.*, 2002; Rosenkrands *et al.*, 2000a; Schmidt *et al.*, 2004).

It is generally accepted that an antigen expressed or up-regulated during infection and relevant to pathogenesis is likely to be a more effective antigen. As the changes in the transcript level due to different growth conditions not always correspond with the abundance of the relative gene product, the transcriptome analysis aimed at identification of new vaccine candidates should be verified by proteome analysis. Many analyses of the level of MTB gene expression under different conditions on the proteome level have also been recently performed. The research concentrates on identification of MTB proteins that are up-regulated during latency period of infection or are unique to the intraphagosomal phase of infection (Betts *et al.*, 2002; Cho *et al.*, 2006; Florczyk *et al.*, 2001; Mattow *et al.*, 2006; Starck *et al.*, 2004).

The preparation of protein extracts containing many proteins in a form that would be soluble and compatible with further analysis is still a critical starting point for proteomic analysis. Proteins desirable for subunit vaccine generation are largely surface, membrane or cell wall located and secreted proteins. Much work has been carried out to improve the extraction method for *M. tuberculosis* membrane proteins, which are hydrophobic and low in abundance. A survey is presented by Zheng *et al.*, 2007 and Mattow *et al*., 2007 in the introduction sections of two recently published papers. Generally the strategies relied on cell membrane and cell wall fractionation and the use of different washing techniques and different detergents for membrane protein extraction, followed by 2D MS identification. Recently Malen *et al.*, 2008 showed that combining Triton X-114 extraction with LC/MS is an efficient method for *Mycobacterium bovis* BCG membrane proteins identification that allows skipping the step of cell envelope fractionation. As outer membrane proteins (OMPs) are considered as potential candidates for effective vaccine construction, the development of new strategies to isolate mycobacterial OMPs might be of a great importance in order to identify new vaccine candidates. However, analysis of this bacterial subproteome is difficult, mainly due to the proteins' hydrophobic nature. Two new methods have been described recently to isolate MTB OMPs (Rezwan *et al.*, 2007; Song *et al.*, 2008). Although they both have advantages and shortcomings, their application on a large scale, combined with novel bioinformatic tools might result in identification of new immunogenic proteins. Effectiveness of the methodology was proved by Western blot analysis and proteinase K test using specific antibodies against two OMPs: Rv1698 and Rv1973.

Proteins secreted by *M. tuberculosis* play an important role in pathogenesis and some of the secreted MTB components recognized by host immune system could be protective antigens. Thus, many attempts have been undertaken to define MTB secretome. Standard technology, introduced by Andersen *et al*., 1991 relays on ammonium sulphate precipitation of the proteins from filtered culture supernatant. This protein fraction enriched in secreted proteins, mainly derived from log-phase MTB culture growing in synthetic medium is called short-term culture filtrate (ST-CF) (Andersen *et al.*, 1991; Okkels *et al.*, 2004; Rosenkrands *et al.*, 2000a; Rosenkrands *et al.*, 2000b). Several modifications such as culturing cells as a surface pellicle without shaking have been introduced into original methodology to avoid contamination of the secretome with cytoplasmic proteins (Malen *et al.*, 2008).

Development of new tuberculosis vaccines: impact of proteomics

Although many novel protective *M. tuberculosis* antigens were isolated by conventional biochemical methods, global analyses such as genomics and proteomics absolutely accelerate the research at the vaccine level, leading to the confirmation of the usefulness of previously described antigens and allowing expansion of the list of novel candidates for subunit vaccine construction. With respect to searching for novel protective antigens, during the initial proteomic era, comparative proteomic analyses of *M. tuberculosis* isolates of various virulence as well as virulent *vs* attenuated strains were performed. Examination of the proteome of *M. tuberculosis* H37Rv (laboratory strain isolated in 1905) *vs* new clinical isolate *M. tuberculosis* CDC 1551, where 1,750 protein spots were visualized on the gel, did not show significant differences in the protein expression profile between two analyzed strains (Betts *et al.*, 2000). Similarly, comparative proteomic studies conducted by Pheiffer *et al.*, 2005 between *M. tuberculosis* H37Rv and two clinical isolates belonging to Beijing and 23 families did not discover huge differences which could explain why Beijing is more prevalent than other clinical

strains. Western blot analysis, using plasma from TB patients, showed differential level of expression of some antigens and documented patient-to-patient variation in humoral immune response to infection (Pheiffer *et al.*, 2005). Comparative secretome analysis (laboratory H37Rv strain vs attenuated H37Ra strain) has revealed that subtle differences in amino acid composition of the proteins might play role in strain attenuation (He *et al.*, 2003). A proteome comparison was also carried out between *M. tuberculosis* and *M. bovis* BCG, aimed at identification of proteins unique for *M. tuberculosis* proteome (Jungblut *et al.*, 1999; Mattow *et al.*, 2001). More than 60 proteins differentially expressed were detected, some of them previously described by genomic analysis, others discovered for the first time. Those which are also present in wild-type *M. bovis* are taken into account as candidates for vaccine. Interestingly, out of 36 genes encoding the proteins examined by Mollenkopf *et al.*, 2004 only seven were absent from the BCG genome, whereas the rest were not detected at the proteome level. One of them, Rv3407, encoding a 10 kDa protein of unknown function, used as DNA vaccine significantly improved the efficacy of BCG immunization in a prime-boost vaccination protocol in mice (Mollenkopf *et al.*, 2004).

The most extensively examined DNA region, which is missing in all *M. bovis* BCG substrains but present in all members of *M. tuberculosis* complex causing human TB, is extRD1 (extended region of difference 1), containing twelve ORFs. Some of them encode strongly immunogenic proteins serving as potent T-cell antigens such as ESAT-6 (6 kDa early secreted antigenic target) and CFP-10 (culture filtrate protein 10). Both abundantly secreted proteins are recognized as effective vaccine-relevant antigens. Recently, it has been determined that proteins encoded by RD1 locus constitute a novel secretion apparatus named ESX-1 (ESAT-6 system 1), required for ESAT-6 and CFP-1 secretion, and play an important role in virulence (Berthet *et al.*, 1998; Brodin *et al.*, 2004; Guinn *et al.*, 2004). Understanding the ESX-1 secretion system would facilitate rational design of subunit vaccine containing proteins encoded by the RD1 region. Proteomic analysis of the culture filtrate of wild-type H37Rv and H37Rv lacking the RD1 region led to discovery a novel effector protein of the ESX-1 secretory apparatus. The 40 kDa protein, product of the Rv3616c gene, designated EspA, is necessary for *M. tuberculosis* virulence and for ESAT-1 secretion. Thus, the experiments proved that similar to secretion systems III and IV of gram-negative bacteria, the ESX-1 apparatus also exports the effector proteins encoded by genes unlinked to RD1 region (Fortune *et al.*, 2005). Proteomic analysis of the ST-CF (short-term culture filtrate) allows determination of post-translational modification of ESAT-1 and elucidation of its interaction with CFP-1. Monoclonal antibodies detected eight ESAT-1 protein species resolved by 2-DE. Their further analysis showed acetylation of N-terminal threonine and proteolytic cleavage of their C-termini. Additionally, using 2-DE blot overlays, it was documented that CFP 10 recognized only non-acetylated ESAT-1, revealing a new regulatory mechanism (Okkels *et al.*, 2004).

As secreted *M. tuberculosis* proteins were proven to be key T-cell antigens for a protective immune response, a lot of effort has been put into understanding the pathogen secretome. Comparative analysis of culture supernatant (CSN) proteins of *M. tuberculosis* and *M. bovis* BCG Copenhagen done in 2003 resulted in identification of 22 novel secreted proteins, present only in the *M. tuberculosis* subproteome. The analysis also identified five proteins encoded by ORFs which had been previously described as deleted in *M. bovis* BCG as compared to *M. tuberculosis* proving the usefulness of the applied technology (Mattow *et al.*, 2003). The recent work of Malen *et al.*, 2007 who used two complementary proteomic technologies, revealed 257 secreted *M. tuberculosis* H37Rv proteins. More than 50% of them are probably secreted by the general secretory pathway; some are lipoproteins and some have transmembrane domains. Interestingly, apart from previously described, abundantly secreted proteins, such as antigen complex 85 or the ESAT-1 family, a large portion of identified secreted proteins have unknown functions. Thus, the screening of their immunological features might uncover new protective antigens (Malen *et al.*, 2007). It was documented that multicomponent subunit vaccine composed of five highly immunogenic secreted proteins (CFP-25, CFP-20.5, Ag85B, Ag85A and CPF-32) stimulates both humoral and cellular immune responses and induces the degree of protection comparable to classical BCG vaccine (Sable *et al.*, 2005). Also some β-barrel outer-membrane *M. tuberculosis* proteins containing T-cell epitopes are considered to be putative novel vaccine candidates (Pajon *et al.*, 2006).

Thus, the proteomic experiments expanded the list of novel, potentially protective antigens. The challenge now is to select and test the most promising antigens, especially in terms of their ability to stimulate T-cells. Covert *et al.* developed a strategy which enables fast screening of pathogen proteins for T-cell antigens. The method combines protein resolving by 2D-LPE (two-dimensional liquid phase electrophoresis) followed by analysis of γ-interferon production by splenocytes of mice infected with *M. tuberculosis* previously stimulated by separated proteins (Covert *et al.*, 2001). McMurry *et al.* 2005 applied bioinformatic tools to search for MTB secreted proteins of MTB H37Rv and CDC 1551. Subsequently the selected proteins were screened for the presence

of MHC II binding motifs. Based on the performed *in silico* analysis twenty nine peptides containing MHC II binding motifs were selected and 17 were synthesized. Epitopes identified in this study were derived from among eight proteins which have defined functions and nine hypothetical proteins. They were evaluated for their antigenicity using PBMC (peripheral blood mononuclear cells) derived from healthy MTB-infected patients. Overall, some of the analyzed epitopes which induced strong IFN-γ secretion in PBMC may be considered as candidates to be included into multi-epitopes vaccine (McMurry *et al.*, 2005). Similar technology, screening of *M. tuberculosis* proteome for protein sequences containing specific motifs that permit them binding to human MHC molecules, resulted in generation multi-epitope DNA constructs which are highly immunogenic in mice (De Groot *et al.*, 2005).

The current leading-edge of research is moving towards structural genomics/proteomics; understanding 3D structures of the selected antigens using X-ray crystallography, NMR or modelling (Gayathri *et al.*, 2007). This will guarantee progress in creating new vaccines based on fusion protein molecules. It has been demonstrated that two fusion molecules, one consisting of Ag85B and ESAT-6 and the second comprised of Ag85B and TB10.4 (Rv0288) induced protective immune response in different animal models when delivered in cationic liposomes (Dietrich *et al.*, 2005; Olsen *et al.*, 2004). The final goal of TB Structural Genomics Consortium (TBSGC) which was formed in 2000 and now has 432 active members from 76 institutions in 15 countries is to eradicate TB by the development of novel intervention (immunoprophylaxis, diagnosis and effective therapy) against MTB. The join forces of TBSGC and SPinE (Structural Proteomics in Europe) resulted in resolving the structure of more than 200 proteins unique to MTB (Arcus *et al.*, 2006; Baker, 2007). However, even when international consortia are involved in the initiatives, the introduction of new effective vaccines into the market is a long-term undertaking. It is estimated that licensing a new effective vaccine against tuberculosis by the year 2015 will require at least 20 new vaccine prototypes entering into phase I safety clinical trials (Raviglione, 2007; Young and Dye, 2006) It is estimated that over 130 new candidate vaccines against tuberculosis including DNA vaccines, polyproteins, subunits, recombinant BCGs and live attenuated vaccines have been tested to date. The list of new-generation tuberculosis vaccines being now under pre-clinical or clinical trials is given in the reference (Skeiky and Sadoff, 2006). The progress in TB vaccine-research field indicates that the availability of effective TB vaccine by the year of 2015 and worldwide tuberculosis elimination by 2050 might be a realistic goal.

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