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Review Article

Proteomics – The research frontier in periodontics

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ABSTRACT

Periodontitis is an inflammatory condition resulting from the interplay between the infectious agents and host factors. Various protein molecules play a vital role in the initiation, progression and severity of periodontal diseases. The study of proteins as biomarkers in periodontal diseases has been highlighted during the last few years. In periodontitis multiple bacteria derived (e.g. collagen degrading enzymes, elastase like enzymes etc) and host derived mediators (eg. PGE₂, TNF, IL1, IL6, MMP's etc) expressed in the saliva and gingival crevicular fluid, can be utilized as diagnostic markers for the disease. Another significant development regarding human genes and proteins has been the discovery of potential new drugs for the treatment of periodontal diseases. Therefore the information of the proteins involved in the pathogenesis of periodontal diseases can be utilized for its diagnosis, prevention and treatment.

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1. Introduction

Proteins, the building blocks of life play a crucial role in nearly all biological metabolic processes including structural support, catalysis & signal transmission and therefore they are regarded as the “working horses” of a cell. The word protein came from a Greek word “Proteios”, meaning “holding the first place”. This name was introduced by Jons Jakob Berzelius in the year 1838.

The word “proteome”, first coined by Mark Wilkins in 1986¹ is an amalgamation of two words that is “protein” and “genome”. The proteome is defined as the collection of all proteins in a cell, including their alterations which will vary with time and specific requirements, or stresses that a cell or

organism undergoes. In 1997² the term “proteomics” was coined to make an analogy with genomics, the study of genes & their functions.

Proteomics is the study of the entire set of proteins expressed by an organism in a given environment and at a specific stage in the cell cycle. It also encompasses their relative abundance, distribution, functions & interaction with other macromolecules & post-translational modifications.³ Proteins play a crucial role in nearly all biological processes and this arises from the existence of thousand of proteins, each folded into a peculiar three dimensional pattern that enable it to interact with one or more of a highly heterogenous array of molecules. Proteomics research has boost up our understanding of cellular behavior by studying the different patterns of protein content & activity & how these variations

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occur during development or in response to disease process. Proteomics has also given new ray of hope as far as the identification of new target drugs and development of new diagnostic markers are concerned.

2. Proteomics – The post-genomic science

Genomics includes the complete genome of cell whereas proteomics with added advantage & tremendous potentiality over genomics, directly employs the entire gene products of a cell in a given state encompassing its cellular activities & interaction with other macromolecules.³ Since proteomics gives a better understanding of an organism than genomics, researchers are very keen in exploring it. Firstly, the transcription of a gene into consequent proteins gives only a rough estimate owing to the rapid degradation of mRNA or inappropriate translation. Secondly, several proteins undergo post-translational modifications (i.e. phosphorylation, acetylation, glycosylation). This can have a significant effect on their activities. Phosphoproteomics and glycoproteomics are the sciences used to study such post-translational modifications. Thirdly, alternative splicing and post-translational modifications can produce more than one protein from one transcript. Lastly, many proteins form complexes and function only in presence with other macromolecules.

3. Periodontal challenges

Analyzing the protein expression of entire genome rather than one protein at a time was proposed by Mark Wilkins in 1994.⁴ But the complexity of the proteome seems to be the major obstacle in achieving Wilkin's idea. In addition, there is exceedingly high abundance of proteins in a cell & its function may depend not only on its abundance, post-translational changes but also its cell location & association with other proteins, and all these may change in a fractional second. Further for the isolation of proteins, we are entirely dependent on the samples due to the unavailability of techniques like polymerase chain reaction as we have for the amplification of nucleic acid sequences.

Periodontium has a complex tissue structure & to understand it in depth, a better knowledge of total set of cellular and matrix proteins is absolutely essential for futuristic advances. Bosshardt conducted an introspective study on cemental subtypes & he concluded that some of its subtypes have structural similarity to alveolar bone.⁵ The definite set of proteins present in these structures and their post-translational modifications are still unknown and therefore it is extremely difficult to understand the exact mechanism that determine the development, formation & remodeling of cemental subtypes. In order to overcome this problem, researchers have employed traditional gene to protein approach that would include isolation of gene of importance first, followed by determining the function of each gene. In another proteomic approach the entire set of expressed gene products is examined including its function (Fig. 1). Hence it is clear that proteomics is an increasingly difficult field and it is only since the introduction of new methods in mass spectrometry in the

mid 1990s that an attempt could be made to analyze the proteome.

4. Proteomics is technology driven

In short proteomics is technology based science involving fractionation & separation of large number of cells or tissue derived proteins that are analyzed by mass spectrometry followed by identification & characterization with the help of bio-informatics approaches. These steps are briefly described as follows.

1) Cell & tissue fractionation

The first step required prior to protein analysis, for the dissected periodontal tissues is fractionation of cells & matrix. Production of an ideal homogenate in which all the organelles and other cellular constituents are released as a free suspension, appears to be the major hindrance in achieving successful fractionation. Mineralized tissues or tissue in between mineralized tissue (e.g. periodontal ligament) has an additional limitation since it is almost impossible to fractionate them without contamination from the adjacent tissues. Fractionation can be accomplished by a wide array of parameters including post-translational modifications like the sub-cellular organelle localization.

2) Protein separation

It is performed at protein or peptide level and involves separation of the very large number of proteins in cells or tissue prior to analysis by mass spectrometry which is then followed by recognition and characterization with bioinformatics approaches (Fig. 2).

Fig. 1 – Flow diagram showing the contrast between traditional, gene-to-protein analysis and global analysis of gene & protein expression.

Fig. 2 – The flow chart illustrates critical steps along the midline, from protein separation, identification followed by sequence analysis. Along with the major steps, analysis of post-translational modifications, as well as functional and interactive proteomics provides a new direction for the future possibilities for the biologists focusing on the periodontium.

Several pre-fractionated methods including one and two dimensional gel electrophoresis, one or two dimensional chromatographic separation or ion exchange and reversed phase resins can be utilized for the identification of hundreds of new proteins that remain undetected in unfractionated samples. These methods remove the high abundance proteins from the samples thereby facilitating the detection of low abundance proteins. For analyzing a complex mixture of peptides, a single chromatographic or electrophoretic procedure seems inefficient. Therefore two or more procedures can be combined to enhance the detection of a larger number of peptides.⁶

For complex protein mixtures derived from biological samples, two dimensional polyacrylamide gel electrophoresis⁷ remains an important tool for its analysis, in which proteins are separated in two sequential steps according to molecular weight and isoelectric points. Capillary electrophoresis is considered to be a substitution to two-dimensional gel electrophoresis for protein separation and to chromatography for peptide separation.

3) Mass Spectrometry

Amongst the advances in proteomic researches, mass spectrometers was a breakthrough as it can detect and characterize low amounts of proteins in biological samples with femtomole sensitivity. Analysis of an unknown mixture involves firstly the separation of proteins from the biological sample, followed by tryptic digestion of the proteins into peptides and then mass spectrometer analysis of such peptides (Fig. 3)

4) Quantification & sequence analysis

Two-dimensional electrophoresis used for comparison of different proteome is technically challenging, so an easier

approach for quantification of different sets of proteins or peptides is the use of different fluorescent dyes in one single gel (i.e. two-dimensional difference gel electrophoresis).⁸ During mass spectrometric analysis, different peptides have

Fig. 3 – Flow diagram emphasizes the basic steps prior to mass spectrometry which includes biological sample preparation, protein separation, protease digestion, peptide separation and analysis.

peculiar intensity of signals that vary as a function of the chemical & physical properties of the analyte and the solvent. Hence signals arising from two peptides, even though from same protein, will demonstrate unique intensities by which they can be identified.

Another technique called Tandem mass spectrometry is used for protein recognition, structure elucidation & characterization of post-translational modifications. It facilitates sequencing peptides in complex mixtures or for sequencing of proteins blocked at the amino terminus. This is in contrast to Edman degradation technique, which is useful for N-terminal sequencing of large amounts of relatively pure peptides.

5. Proteomics in modern periodontics

Proteomics offers a new ray of light not only in understanding the complex interactions that the periodontal pathogens undergoes with the host both in the health and disease but also the role of periodontal ligament fibroblast & various disease related protein markers that offers an insight of the physiology of periodontal ligament.

5.1. Role in periodontal pathogens

The oral ecology is a wide array of diversified communities of microorganisms that undergoes complex interactions with the host in both the healthy and diseases state. Proteomics offers an entirely new way for understanding the changes occurring as the oral microorganisms adapt to changes in the environment within their habitat in the mouth.

5.2. Role in periodontal ligament (PDL) fibroblasts

To understand PDL physiology and disease related protein markers, the analysis of the entire complement of PDL fibroblast proteome is of utmost importance. Although immunological methods have been tried to study the protein expression of PDL fibroblast, but this is limited to only previously identified proteins for which antibodies are available. In total 117 proteins have been identified from PDL fibroblasts which can act in reference mapping for further clinical & basic research work.⁹

6. Proteomic analysis of periodontogenic microorganisms

6.1. Proteomics of *Porphyromonas gingivalis* within a model of oral microbial community

Oral microbial communities are considered to be complex dynamics biofilms that develop through distinct microbial colonization. With the extension of biofilm subgingivally, there is colonization of more pathogenic anaerobic gram negative microorganisms including *P. gingivalis*.¹⁰ The most remarkable property of *P. gingivalis* is that its pathogenicity is expressed only in mixed microbial communities. Substratum and metabolic support is provided by the pioneering microorganisms which ultimately help in the establishment of

succeeding organisms. A complex consortium is thus formed through interspecies communication and signaling molecules like short range soluble mediators, AI-2, or nutritional stimuli.¹¹ *P. gingivalis* can accumulate into both single species and mixed species consortia with *Streptococcus gordonii* and other related oral streptococci. This can be very well appreciated by the exclusive localization of *P. gingivalis* in areas of streptococcus rich plaque.¹² In the supragingival plaque due to the aerated environment, development of more complex multi-species communities occur with predominance of *Fusobacterium nucleatum*, due to its oxygen scavenging property.¹³ *F. nucleatum* is also able to coaggregate with *P. gingivalis* and with oral streptococci.

7. Proteins and functions differentially regulated by *P. gingivalis* in a community

7.1. Cell envelope and cell structure

In oral biofilms significant surface–surface contact occurs both within and among the bacterial communities, as was also observed in *P. gingivalis* – *F. nucleatum* – *S. gordonii* consortia. *P. gingivalis* regulates its outer membrane constituents in a community which can be analyzed by proteomics results. In totality, 84 proteins were detected to be involved in the cell envelope, out of which 40 showed reduce abundance in the three species consortia. Only 4 proteins showed increased abundance, one of which was a bacterial actin homologue that plays a role in determining the cell–shape. The stress related proteins were generally reduced in *P. gingivalis* cells in a community.¹²

7.2. Protein synthesis

Drastic changes were seen in ribosomal proteins and translation, elongation and initiation proteins. There was overall reduction in proteins in the three species consortia with the exception of the translation machinery that exclusively shows an increased abundance. Out of 49 ribosomal proteins detected, 27 showed an increased abundance, with only one showed decrease abundance.¹²

7.3. Vitamin synthesis

Pathways for synthesizing several vitamins showed reduced protein abundance in the three species community. Most of the proteins involved in Vitamin B1 biosynthesis were down regulated. Thiamine (vitamin B1) acts as a cofactor in anaerobic pentose phosphate pathways. The decrease in the vitamin pathways could be due to a decreased utilization of these cofactors. However, in case of thiamine, the proteins that utilize this cofactor showed no decrease. The likely explanation could be nutrient transfer, meaning either one or both of the other microorganisms in the three species community could be providing *P. gingivalis* with cofactors, allowing reduced cofactor synthesis without reduction in the expression of the cofactor dependent pathways. Cross-feeding of nutrition among members of oral biofilms is well

established, and perhaps *P. gingivalis* has been found to utilize succinate synthesized by *Treponema denticola*.¹⁴

7.4. Nucleotide synthesis

The production of finished pyrimidine nucleotides appeared to decrease in the three species consortia because of reduction in the biosynthesis of many proteins that produces pyrimidine. But the proteins that help in incorporation of finished ribonucleotides into RNA remain unchanged or have shown increased abundance.¹²

7.5. Quantitation strategy for *P. gingivalis* proteins by 15 nitrogen labeling

For tracing the fate of *P. gingivalis* proteins in the gingival epithelial environment (GEC), stable isotope dilution mass spectrometry gives a more detailed quantitative data regarding the protein expression.¹⁵ Prior to exposure to GEC, *P. gingivalis* were grown in a uniformly labeled 15 N ammonium sulfate & uniformly 15 N labeled peptone nitrogen sources in minimal media. 15 N that gets incorporated into the polypeptide backbone is easier to trace in this regard. Provided that the level of isotope incorporated is high, the breakdown products from *P. gingivalis* that are utilized in the metabolism of the GECs can easily be identified using this approach.

7.6. Spectral counting

Gao, Hefta & co-workers¹⁶ proposed spectral counting methods that refers to a process in which the discrete number of peptides observed for a given protein is used to estimate the relative abundance of that protein, as opposed to the strength of the observed signals associated with the protein. However for the proteins which produce smaller number of proteolytic fragments or are present at low abundance, spectral count

gives limited quantitative reproducibility. Under these circumstances metabolic labeling procedures produce more reliable information.

8. Biological relevance

Proteomic analysis has the potentiality of unveiling a large number of differentially regulated proteins but the major hurdle is how to interpret the data in a biologically meaningful way. Owing to the relatively small size of prokaryotic genomes, it is possible to assess the importance of individually regulated proteins in the context of the overall expression pattern. For organisms like *P. gingivalis*, the proteomic analysis has shown marked increase in series of proteins of the Clp family i.e. ClpC, ClpP and ClpX. Further in *Listeria*, the ClpC ATPase is required for adhesion and invasion of host cells and the expression of other virulence factors are modulated by proteins. ClpC also helps in the early escape of *Listeria* from the phagosomal compartment of macrophage.¹⁷ This has led to prediction that the Clp proteins may play an important role in *P. gingivalis* invasion and intracellular survival.

Therefore proteomic analysis helps in understanding the biology of the organism in depth. Further the next logical step for identifying new drug targets for antimicrobial therapy will be the usage of the global proteomic approaches (Table 1).

9. Biomarkers for periodontal diseases by proteomic approaches

An understanding of the human salivary proteome is a prerequisite to gain insight into the physiological and pathological processes relevant to oral health, and is crucial for the identification of meaningful biomarkers for oral diseases.

Table 1 – Cells of periodontal interest studied by proteomic approaches and their relevance.

Cells studied by proteomic analysis	Proteins identified	Relevance significance
1) PDL fibroblasts ¹⁸	Cytoskeleton proteins- actin, tubulin, vimentin; cellular mobility protein; membrane trafficking protein; stress folding enzymes; chaparonone; metabolic enzymes.	Related to periodontal ligament fibroblast function and homeostasis
2) PDL cell undergoing mineralization ¹⁹	Cytoskeleton proteins; cytoskeleton associated proteins; nuclear protein; cell membrane bound protein	Maintain periodontal tissue homeostasis
3) <i>Porphyromonas gingivalis</i> ²⁰	PG1089; PG1385; PG2102	PG1089, PG1385 & PG2102 are involved in <i>P.gingivalis</i> virulence
4) <i>Fusobacterium nucleatum</i> ²¹	Various cytoplasmic proteins are found, a) Regulated by acidic pH eg. NADP specific glutamate dehydrogenase, anthranilate synthase etc. b) Regulated by alkaline pH eg. fructose bisphosphate aldolase, enolase, pyruvate kinase etc.	Upregulation of NADP specific glutamate dehydrogenase & anthranilate synthase that increase ATP production & maintains internal homeostasis. Upregulation of fructose bisphosphonate aldolase & downregulation of enolase & pyruvate kinase which increase the pH seen in diseased gingival sulcus.
5) Saliva in periodontitis subjects ²²	S100 proteins; haptoglobin, prolactin inducible protein and parotid secretory protein	Associated with host defense; New potential biomarker for monitoring disease activity in periodontitis

Periodontopathic bacteria produce virulence factors that cause degradation of host tissue either directly or activate a host response resulting in the release of biological mediators from host cells. These mediators in case of exaggerated response lead to host tissue destruction.²³ Host & bacteria derived enzymes, proteins and other inflammatory mediators appear to be hopeful salivary diagnostic biomarkers for periodontal diseases. Bacterial lipopolysaccharide & other microbial components (like bacterial DNA) trigger the innate host defense resulting in recruitment of neutrophils, monocytes & activated macrophages at the site. These host cells in turn release numerous cytokines such as prostaglandins (PGE_2), tumor necrosis factor (TNF), interleukins IL-1 & IL-6 which direct the inflammatory process further. As a result, collagen destroying enzymes called matrix metalloproteinases (MMP's) are produced by alveolar bone & polymorphonuclear leukocytes. Pyridinoline cross-linked carboxyterminal telopeptide and osteocalcin are then released into the surrounding area & transported through gingival crevicular fluid into the periodontal pocket.^{24,25}

Numerous mediators of diagnostic value involved in this disease process have been shown to be correlated with the disease onset & activity. (Fig. 4). Among these mediators, host derived MMP's are considered to be key initiators of the extracellular matrix degradation associated with periodontal diseases.²⁶ Both MMP-1 (interstitial collagenase) & MMP-8 (polymorphonuclear leukocyte derived collagenase) appear to be associated in periodontitis.^{25,26} Polymorphonuclear

leukocyte derived MMP-8 has been identified as a major tissue destructive enzyme during active stages of periodontitis. Therefore quantification of level of MMP-8 in saliva is a promising candidate for diagnosing and predicting the progression of this episodic disease. Likewise significant increase in levels of salivary IL-1 β & TNF- α level were observed in patients with active periodontal disease sites, as these cytokines mediate osteoclastogenesis and bone breakdown.²⁴

Also gingival crevicular fluid (GCF) reflects the condition of the gingiva and contains proteins passed from serum or cells at inflamed sites. In a study²⁷ conducted to discover potential protein biomarkers for periodontitis in GCF proteome, for the first time azurocidin was identified as an upregulated protein in the periodontitis patients and was verified for its increased expression during periodontitis by ELISA (Enzyme linked immunosorbent assay) using the GCF of the classified periodontitis patients compared to the healthy individuals. In addition, it was found that azurocidin inhibited the differentiation of bone marrow-derived macrophages to osteoclasts thus, a protective role in alveolar bone loss during the early stages of periodontitis.

10. Clinical applications

The use of rapid point-of-care oral diagnostics will greatly advance periodontal surveillance and disease diagnosis over the coming years.²⁸ Novel technologies such as lab-on-a-chip and microfluidic devices have the potential to manage complex oral fluids such as saliva and gingival crevicular fluid, and helps in determining the patient's periodontal disease-risk profile, current disease activity and response to therapeutic interventions. This approach should acts like a catalyst for clinical decision-making and monitoring of episodic disease progression in a chronic infectious disease such as periodontitis.

While the future of periodontal disease diagnosis using salivary diagnostics looks encouraging, hurdles to these approaches may be encountered in the clinical setting. These novel periodontal diagnostic technologies need to be validated & benchmarked with existing gold standards of disease, such as alveolar bone levels and clinical attachment levels, in large patient populations.

11. Conclusion

The use of proteomics and gene expression will advance the diagnosis and treatment of periodontal diseases. Advances in tissue engineering, drug delivery, gene therapy and biopharmaceuticals will present new therapeutic opportunities. However, its application into the field of dentistry depends on how best oral health care practitioners will incorporate this into their practice as it requires a thorough knowledge of human genetics and application of new diagnostic and therapeutic technologies.

Conflicts of interest

All authors have none to declare.

Fig. 4 – Overview of pathogenesis of periodontal disease and the role of biological mediators. LPS – Lipopolysaccharide from gram negative bacteria on tooth surface. PMN – Polymorphonuclear leukocyte. PDL – Periodontal ligament. PGE_2 , TNF- α & IL-1 β – Prostaglandin E₂, Tumor necrosis factor & Interlukin. RANKL – Receptor activator of nuclear factor kappa B ligand mediates osteoclastogenesis. ICTP – Pyridinoline cross-linked carboxyterminal telopeptide of type I collagen (bone specific markers).

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