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Journal Scan

Proteomics, Saliva, and Sjögren's Syndrome

Robert I. Fox, MD; Carla M. Fox, RN

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Arthritis and Rheumatism

Proteomic Study of Salivary Peptides and Proteins in Patients with Sjögren's Syndrome Before and After Pilocarpine Treatment.

Peluso G, De Santis M, Inzitari R, et al.
***Arthritis Rheum*. 2007;56(7):2216-2222.**

Introduction

Proteomics, as defined in MedicineNet, is the study of the proteome, the complete set of proteins produced by a species, using the technologies of large-scale protein separation and identification. Wikipedia.com defines Proteomics as the large-scale study of proteins, particularly their structures and functions.

The proteome of an organism is the set of proteins the organism produces during its lifetime, and its genome is its set of genes. The proteome set of proteins is expressed and modified following its expression by the genome. The term "proteome" was coined from the PROTEin complement of the genOME in 1994 by Marc Wilkins, a graduate student at Macquarie University in Australia, who defined it as "the study of proteins, how they are modified, when and where they are expressed, how they are involved in metabolic pathways, and how they interact with one another."

Proteomics is often considered the next step in the study of biologic systems, after genomics. It is much more complicated to study than genomics, mostly because while an organism's genome is rather constant, a proteome differs from cell to cell and constantly changes through its biochemical interactions with the genome and the environment.

One organism has radically different protein expression in different parts of its body, different stages of its life cycle and different environmental conditions. Another major difficulty is the complexity of proteins relative to nucleic acids. This increased complexity derives from mechanisms such as alternative splicing, protein modification (glycosylation, phosphorylation), and protein degradation.

Relevance to Rheumatologists

Proteomics is important to rheumatologists for several reasons. We have all seen numerous reports of genetic markers associated with particular autoimmune disorders, yet few new therapeutic leads have derived from this exhaustive gene hunt. Therefore, the search for new therapies now has moved to "gene expression" and "posttranslational modification" as a method to understand the interplay of genetic and environmental factors. This new area of research is termed "proteomics" and is actively being applied to other fields of endocrine and oncologic diseases.

More recently, these methods have been applied to autoimmune diseases that involve a complex array of different cell types. As an initial dip into the proteomic pool, investigators have begun by studying the relatively simple system of saliva and have taken advantage of the safety of obtaining biopsy specimens from the salivary glands.

In addition to briefly reviewing the molecular gymnastics of the proteomics technology, the recent reports described below have also revealed some surprising results that may help clarify pathogenesis or provide new leads to therapy.

Peluso and colleagues[1] recently applied the methods of proteomic analysis to compare changes in saliva in patients with Sjögren's syndrome (SS) and those of normal patients. The researchers then evaluated changes in the salivary profile of low-molecular-weight proteins following a specific therapy using a cholinergic agonist. The investigators then decreased the levels of defensins in the salivary gland of patients with SS saliva compared to the normal patients. This decrease in saliva production was unexpected, since the defensins are made in situ both by the inflamed ductal glands and by the lymphocytes that surround these glands. However, the defensins are not being transported/secreted into the SS patient's saliva. This observation was not predicted by "genomics" (i.e., the gene sequences are normal) but reveals the "microenvironment" of the gland revealed by "proteomics."

The second observation by Peluso and colleagues[1] was that a neurotransmitter (pilocarpine) increased the level of defensin in the saliva. The investigators studied small peptides including proline-rich peptide, defensins, and other small molecules involved in mucosal defense such as statherin, cystatin, and histatins.

It has been shown that in SS patients, 60% of the small salivary proteins in samples from primary SS patients were at significantly lower levels than those in healthy controls. However, 30-60 minutes following pilocarpine treatment, many of the less represented proteins returned to the level in non-SS controls.

Although there is relatively limited interest in mucosal immunity in general (or salivary defensins in specific) among rheumatologists, there is increasing recognition of the importance of the interaction of cytokines, neurokines, and the endocrine systems in autoimmune disease. For example, our traditional pathogenetic models do not provide much insight into chronic fatigue -- one of our most common and difficult to treat problems in patients with systemic lupus erythematosus or SS.

Perhaps an understanding of the interactions of the neuro-endocrine-immune axis that operates in an easily measured system such as tear or saliva flow may provide insight in poorly understood conditions such as chronic fatigue syndrome. The role of gender (androgen/estrogen) has been unclear in SS and systemic lupus erythematosus, and proteomic studies have recently demonstrated a role for androgen that is required for post-translational modification of the salivary protein cysteine-rich secretory protein 3 (CRISP3, discussed below).

If past decade was the era of understanding/treating imbalance of the acquired/innate immune system with tumor necrosis factor inhibitors or B-cell depleters, perhaps the next decade need to focus on the complex interactions leading to fatigue and interaction of the immune system with the neural system.

These studies on a process as simple as salivation help us expand the studies conducted a hundred years ago by Pavlov, who showed the interaction of cortical function and salivation as he taught dogs to salivate as a conditioned response to ringing a bell.

Most rheumatologists suspect that fibromyalgia will ultimately be more like a conditioned response to stress that involves the hypothalamic-adrenal axis, literally an extension of the Hans Selwyn famous experiments on the role of the adrenergic/cholinergic responses to stress, and we must now reinterpret the "stress axis" in the newer knowledge of the important influence of cytokines and neurokines.

The article by Peluso and colleagues[1] emphasizes several general points about mucosal immunity, an area of research that is not generally considered by rheumatologists. We have come to accept the parallel and overlapping existence of "acquired" (adaptive, human leukocyte antigen [HLA]-DR linked lymphocyte responses) and "innate" (HLA-DR independent responses mediated by Toll receptors) immune systems. However, the full breadth of the innate immune system (ie, immediate response to particular environmental antigen motifs) also includes mucosal defense mechanisms. This has been emphasized in current research in inflammatory bowel disease (ie, the NOD/card system), but has received less attention in rheumatology journals. Small molecules such as defensins represent an important link between the environment and the "innate" immune system.

Salivary Production in Normal and Sjögren's Syndrome Glands

The rate of salivary protein secretion is controlled mainly by noradrenalin, which is released from the sympathetic terminals and acts through the alpha-adrenergic receptors.[2] The rate of fluid and electrolyte secretion is controlled by acetylcholine, which is released from the parasympathetic terminals and acts through the muscarinic cholinergic

receptors. Thus, the finding of a cholinergic stimulator as increasing the level of defensin protein is surprising. Some hypotheses have been suggested by Peluso and colleagues:[1] (1) that increased innervations of the gland by cholinergic nerves may be partially inhibited by cytokines that inhibit the stimulation of the muscarinic receptors, and (2) that the augmentation of cholinergic neurotransmitters may help restore secretory function.

This would be analogous to the small intestine where Paneth cells at the base of the crypts of Lieberkühn secrete alpha-defensins and additional antimicrobial proteins at high levels in response to cholinergic stimulation.[3]

This stimulation of Paneth cells to secrete defensins plays a critical role in inflammatory bowel disease since these molecules protect mitotically active crypt cells from colonization by potential pathogens and confers protection from enteric infection.[4] By analogy in SS, decrease in small antimicrobial proteins may alter the micro flora of the mouth and predispose to the oral complications of SS. In addition to this study of small molecules, several other recent studies on the salivary proteosome have been published.

Amado and colleagues[5] discussed the different techniques and methodologies applied to the separation and identification of salivary proteins. Nowadays, proteomic techniques are the state-of-the-art for the analysis of biologic materials and saliva is no exception. Two-dimensional (2D) gel electrophoresis and tryptic digest analysis by mass spectrometry are the typical methodology, but new approaches using "fast" 2D liquid chromatography/mass spectrometry methods are now being supplemented by bioinformatics technology to also examine posttranslational modification of proteins including glycosylation and phosphorylation as they relate to dental problems and even cancer diagnosis.[6-8] They demonstrated in SS patients a series of new proteins related to oxidative injury and well as proteins induced in response to pro-inflammatory cytokines.

Beklen and colleagues[9] demonstrated that changes in metalloproteinase and biofilm constituents play a role in periodontitis and dental caries. Of interest, salivary components are different in response to different taste challenges.[10]

Results of the study by Peluso and colleagues[1] dealt with only small proteins (defensins), while Ryu and colleagues[11] examined larger salivary protein biomarkers using surface-enhanced laser desorption/ionization time-of-flight-mass spectrometry (SELDI-TOF-MS).

Examining the proteins in the 10-200 kilodalton (kDa) size range, they found eight peaks with > 2-fold change in quantity/concentration between the SS group and non-SS. They demonstrated that:

- Proteins with sizes 11.8, 12.0, 14.3, 80.6, and 83.7 kDa were increased; and,
- Proteins with sizes 17.3-, 25.4-, and 35.4-kDa peaks were decreased in SS samples.

2D-gel electrophoresis identified significant increases of beta-2-microglobulin, lactoferrin, immunoglobulin (Ig) kappa light chain, polymeric Ig receptor, lysozyme C, and cystatin C in all stages of SS. Two presumed proline-rich proteins, amylase and carbonic anhydrase VI, were reduced in the patient group.

Laine and colleagues[12] demonstrated a decreased level of the androgen-regulated CRISP3 in saliva of SS patients. Serum and salivary levels of dehydroepiandrosterone sulfate levels were low, suggesting a relative androgen deficiency as the cause for the decreased CRISP3 levels.

Porola and colleagues[13] have also suggested that imbalance of secretory proteins in SS patient's saliva results in part from deficient androgen action at the level of the salivary glands.

Antimicrobial Proteins

The study by Peluso and colleagues[1] also brings the area of small antimicrobial proteins and peptides to the "radar screen" of rheumatologists because it is an area of increasing research and therapeutic interest in fields such as inflammatory bowel disease.

Therefore, it is important to review the major classes of the antimicrobial proteins and peptides.

Defensins

Two classes of antimicrobial proteins and peptides that have received the most attention in recent years are the

defensins and cathelicidins.

Defensins[4,14,15] and cathelicidins[4] perform several functions to preserve the "barrier" and epithelial integrity of the skin, lips, tongue, intestine, and rectum.

In settings such as the moist airways, gastrointestinal tract, and urinary tract, defensins are also secreted into the biofilm covering the epithelial surface, where they create a barrier that is chemically lethal to microbes.[14] Certain of these antimicrobial peptides promote epithelial growth and angiogenesis.

Defensins are small (15-20 amino acid residue) cysteine-rich cationic proteins found in both vertebrates and invertebrates. They are active against bacteria, fungi, and enveloped viruses.

They consist of 30 to 45 amino acid precursors that are processed to final size 15-20 amino acids, including 6 to 8 conserved cysteine residues.

Defensins are very diverse with respect to amino acid sequence and secondary structure, but share certain properties, such as an affinity for the negatively charged phospholipids that are present on the outer surfaces of the cytoplasmic membranes of many microbial species.

Most defensins function by penetrating the microbial cell membrane by way of electrical attraction, and once embedded, forming a pore in the membrane that allows efflux.

The defensins are divided into 2 classes, alpha (a-) and beta (b-), on the basis of the differences in their secondary structures. More recently, a third class (theta defensins) was reported, but there is relatively little information about their role in normal health or disease.

- a-Defensins were first discovered in the granules of white cells. Several years later, they were found in Paneth cells, the granule-containing cells that lie in the base of the crypts of the small intestine:
- In white cells, a-defensins contribute to nonoxidative killing; and
- In Paneth cells, they are secreted into the crypts and control the growth of bacteria in the small bowel.
- b-Defensins: Four human b-defensins have been identified (although more than 20 genes appear to exist in our genome):
- Human b-defensin 1 (HBD-1) is generally produced constitutively, whereas the others are inducible. In humans, b-defensins are synthesized by all epithelial tissues.

Cathelicidins

Another family of antimicrobial proteins is cathelicidins, which is a large and ancient class, traced from hagfish through humans. They are secreted in neutrophils and can be found in the bloodstream after infection. On epithelial surfaces, they function like defensins.

Salivary Proline-rich Proteins, Cystatins, and Histatins

The salivary protein gene complex consists of a series of loci coding for related but distinct proline-rich proteins found chiefly in saliva, and to a lesser extent in respiratory tract secretions.

Human glandular salivary secretions contain several acidic proline-rich phosphoproteins. Amino acid sequences have shown that the two 150-residue molecules, proline-rich phosphoproteins-1 and proline-rich phosphoproteins-2 are closely related, while 2 related 106-residue proteins (proline-rich phosphoproteins-3 and proline-rich phosphoproteins-4) also show similarity. These proteins have important biologic functions related to providing a protective environment for the teeth, and appear to possess other activities associated with modulation of adhesion of bacteria to oral surfaces.

Studies of the physical properties of the proteins of human parotid saliva together with their inheritance patterns have led to the description of at least 10 discrete but genetically linked loci controlling the synthesis of a group of proline-rich proteins. These proteins also occur in the respiratory tract, suggesting that they could have some general functions unrelated to those conventionally ascribed to saliva.

The proline-rich proteins are characterized by a predominance of the amino acids proline (25% to 42%), glycine, and glutamic acid or glutamine. Together these 3 amino acids account for 70%-88% of the total amino acids in the proteins.

Antimicrobial Proteins and Peptides and Immune Disease

Rheumatologists are well acquainted with the acquired (HLA-DR dependent) and innate (HLA-DR independent) arms of the immune system. Most of the recent attention on the innate immune system has derived from recognition of Toll receptors expressed on dendritic and other cells that play a role in instruction of B-cells, T-cells, and release of cytokines such as type I interferon. However, other defense factors such as C-reactive protein and complement are part of the innate system's response to "danger." The antimicrobial proteins and peptides should be considered as part of that immediate response, and their activity plays a role in immune disorders.

Defensins have been most clearly studied for their role in inflammatory bowel disease (IBD). For example, linkage studies of members of multiply affected kindreds have identified a number of genomic areas of linkage with IBD, including putative IBD genes ("IBD1") that have been identified as encoders for the nucleotide-binding oligomerization domain protein 2 (NOD2), which is a sensor for bacterial muramyl dipeptide.

Activation of NOD2 stimulates expression of alpha-defensins and promotes cytokine and chemokine production by immunocytes.[16] Defective NOD2 signaling pathway and impaired expression of defensins are linked to the pathogenesis of Crohn's disease, to instruct adaptive immune response in the gut micro-environment.

Summary

The article by Peluso is called a "proteomic study."

Perhaps the most available fluid is saliva (or tears), and analysis of these fluids in SS may provide clues to changes in pathogenesis. Certainly the unexpected change in SS saliva may seem modest at first, but it does demonstrate the existence of complicated pathways.

The article also points out several other important, emerging areas of pathogenesis and therapy:

- The new techniques of proteomics and the role of mucosal immunity may help us retard complications to the eye and mouth/teeth; and
- The interaction of cytokines, neurokines of the hypothalamic-adrenal axis and estrogen pathway that influences salivation, may provide insight into one of most difficult problems; namely, the chronic fatigue and "brain fog" that accompanies so many autoimmune disorders.

Thus, several exciting studies are being pursued to apply the science of proteonomics and develop new therapies for inflammatory diseases to enhance patient quality of life.

Abstract

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Robert I. Fox, MD, PhD, Professor/Member, Scripps Memorial Hospital and Research Foundation; Rheumatologist, Scripps Memorial Hospital, La Jolla, California

Carla M. Fox, RN, clinical and research coordinator, Scripps Memorial Hospital and Research Foundation; La Jolla, California

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