[Frontiers in Bioscience 14, 1505-1515, January 1, 2009]

Proteomics of tendinopathy

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1. ABSTRACT

Recent advances in the basic understanding of both human biology and animal models have enabled an unprecedented level of information to be gathered about the genetic regulation of human disease. However, a corresponding insight into functional interactions between genetic information and protein expression has been slower to accumulate. Measuring mRNA transcripts does not yield a complete set of information about the protein dynamics involved in tissue injury and repair. Proteomics methods applied to unravel these relationships rely upon the identification and analysis of the proteins present within selected tissues and cells. Our understanding of complex biological processes can be enhanced by exploring the differences in protein structure and function between normal and injured tissues. This review concentrates on the development and application of proteomics techniques to uncover the specific processes and pathways involved in tendon injury and chronic tendinopathy.

2. INTRODUCTION

Tendinopathy is a descriptive term for the collection of clinical conditions arising from tendon problems, and is characterized by pain, edema, and impaired performance (1).

Tendinopathy is a frequent consequence of sudden, direct injury, or repetitive strain. Over two-thirds of all workplace injuries in the U.S. and half of the 100 million musculoskeletal injuries that occur each year worldwide are tendon and ligament problems (2). The outcome from such pathology is loss of performance and decreased productivity in the workplace, both resulting in a negative impact for a significant proportion of the population annually.

Chronic tendon problems associated with overuse injury plague nearly 40% of athletes including runners and tennis players (3). The tendency of a given individual to suffer from either abrupt tendon rupture or

chronic pain and tenderness is thought to be related to a combination of both genetic and nongenetic factors, including structural abnormalities that are predisposing to injury (4). It is likely that complex interactions among factors like age, fitness and body mass, anatomical variations, and occupation, overuse/overload and environmental conditions contribute to the genetic tendency of an individual to experience tendinopathy (5). Recent studies have identified genetic variants associated with Achilles tendon pain, lending credibility to this hypothesis (6). Polymorphisms in the genes encoding several tendon proteins are associated with variable risk for tendon injury (7,8). In one study, individuals having the A2 allele of the alpha-1 type V collagen gene (*COL5A1*) were less likely than control subjects to experience tendon injury (8). Several retrospective studies found that variations in the tenascin C and ABO blood group glycosyltransferase genes were associated with more frequent Achilles tendon injuries (5,7). Further analysis of unique genetic susceptibility to injury could lead to customized exercise recommendations for specific patient populations. Prevention strategies like avoidance of weight-bearing and high-impact sports for individuals who have risk profile genotypes would take advantage of this information.

Proteomics is a relatively new technology which could provide an understanding of the expression of a genotype at the phenotypic level in a target tissue such as tendon. Proteomics methods applied in the analysis of individual variations in susceptibility could provide information about the protein profiles of tendon injury, healing, and the effectiveness of therapeutic interventions including potential gene therapies (7). In particular, proteomics holds promise for the identification of biomarkers which may reveal the role of cytokines, growth factors, and other cellular mediators of tendinopathy and repair. Uncovering the tendon proteome which matches specific genotypes is an area ripe for investigation.

2.1. Tendon, tendon proteins and tendinopathy

Tendon is the tough, fibrous band of connective tissue which forms the termination of a muscle, attaching it to bone (9). When a muscle contracts, it pulls on the tendon, and facilitates movement of the bone. Thus, tendon is uniquely positioned to transmit force from muscle to bone. Healthy tendon under normal conditions demonstrates high mechanical strength, acting as a buffer to prevent muscle damage (10).

Proteins that comprise tendons include type I collagen (65 – 80% of tendon mass), elastin (2% of mass), and extracellular matrix proteins (ECM), including tenascin-C (abundant in the tendon body and at the osteotendinous and myotendinous junctions), proteoglycans, glycosaminoglycans, and glycoproteins (9). Tendinopathy changes the expression of many of these tendon proteins (11). Tenascin-C is regulated by mechanical strain and it is upregulated with tendinopathy. Glycoproteins such as fibronectin and thrombospondin participate in tendon repair and as such should be expressed at higher levels during tendon regeneration. Proteoglycans enable the rapid diffusion of water-soluble molecules and the migration of cells into areas of tendon injury. Several studies have indicated that the ratio of type III to type I collagen may be a key factor in non-healing tendinopathy profiles (12,13). Type III collagen is overexpressed during repair and it is replaced by type I collagen during post-injury remodeling. Achilles tendon fibroblast cells (tenocytes) produce a disproportionate amount of type III collagen in response to microtrauma (13). An abnormal accumulation of type III collagen could predispose the tendon to rupture by affecting tendon tensile strength and resistance to injury. In addition, numerous cytokines and growth factors such as vascular endothelial growth factor (VEGF) regulate the microenvironment of the tendinopathy. An appropriate regulatory microenvironment leads to injury resolution and return to normal tendon function, features that may be influenced by appropriate therapeutic intervention.

Although several physical modalities are commonly used in the management of tendinopathy, there is limited evidence to support the clinical use of many methods. In addition, many patients suffer from ongoing pain and disability despite therapy (11). Indeed, in tendinopathy the damaged tissues often respond poorly to commonly used management modalities which include immobilization and rest (14). A quick improvement in tensile strength would allow a more rapid return to mobility. Early mobility would in turn decrease morbidity by reducing painful adhesions at the repair site. This would be a major advance in therapy for tendinopathy (15). Accelerated healing has been the focus of many studies which evaluate therapies for tendinopathy. Proteomics analysis has a unique potential in this area because it would enable researchers to profile alterations in the biochemical milieu that promotes a healing environment.

2.2. Growth factors and regulatory molecules

In a mode similar to wound healing and the repair process following bone injury, tendon healing entails 1) inflammation; 2) angiogenesis; 3) cellular proliferation; 4) collagen synthesis; and 5) remodeling. Matrix metalloproteinases (MMPs) are activated, and collagen degradation occurs early in the repair process, followed by synthesis of type III collagen and proteoglycans in the initial stages prior to remodeling (16). Damaged tendon has a low level of vascular infiltration to promote healing relative to other sites. Therefore, a better understanding of the overall process through proteomic evaluation would improve the design of new therapies.

Changes in the pattern of growth factor expression are particularly important to the process of tendon repair. Growth hormone, insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), bone morphogenic protein-2 (BMP-2) and mechano growth factor (MGF) have all been implicated in the regulation of repair in tendon healing after injury (11). In addition, MMPs, proteolytic enzymes

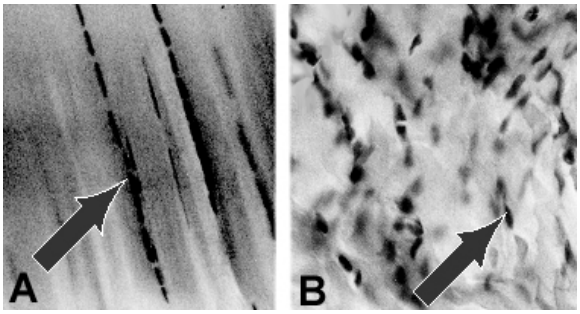

Figure 1. Photomicrographs of control (A) and injured (B) rat tendons. Tendons were embedded in paraffin, sectioned at 10 μm, stained with hematoxylin and eosin, and imaged at 400 X magnification. Arrows indicate fibroblast cells, positively identified by immunohistochemistry.

which mediate degradation and facilitate remodeling and repair, play a key role in tendon regeneration (16).

Information about regulatory molecules has been uncovered using a wide range of approaches. Tendon fibroblast explant cultures have provided information about the relative concentrations of MMP-1 and MMP-3 (17). In addition, tissue inhibitors of MMP-1 and -2 as well as other MMPs could be implicated in the regulation of tendon repair. The contribution of MMPs and their inhibitors to the process of tendon remodeling has been studied in extracts from ruptured compared with intact, normal tendon using enzyme hydrolysis of fluorogenic substrates and zymography (16). Quantitative gene arrays in one study of Achilles tendinopathy showed that chronic tendon problems were associated with lower than normal levels of MMP-3, while levels of type I and type III collagens remained higher than in normal tendon controls (6). Plasma/serum proteomics directed toward analysis of the regulators of tendinopathy could yield ideas about systemic factors that contribute to the control of localized enzyme and protein levels within injured tendon.

2.3. Characteristics of tendinopathy

Much of what is understood about tendon problems comes from histological studies. Repetitive strain resulting from repeated microtraumas which induce mechanical overload are at the heart of tendinopathy. Injury manifests at the light microscopic level as disruption of collagen fibers, with reports of early hypocellularity and tissue necrosis (18). Healing descriptions include increases in cellularity, with a fibroblastic, vascular appearance emerging as the cells that will produce regulatory molecules and structural proteins infiltrate the injured area (Figure 1). Fibroblast cells are responsible for the synthesis of both type III collagen that predominates during early stages (Figure 2), and for the type I collagen that replaces it during the final remodeling repair stage (11).

The entire process is regulated by a variety of protein modulators, several of which are described above. In addition, neuropeptides such as substance P and calcitonin-related gene peptide are released by nerve fibers in the vicinity of injured tendon (19). These peptides and other molecules including nitric oxide may act as chemical mediators of the healing process (17,20).

Imbalances in the regulation of the tendon protein composition may contribute to the pathogenic process in tendinopathy. Cells with upregulated protein synthesis from diseased human tissues placed in culture deposited less collagen III and fibronectin than control cells from normal tissue in related systems (21). Decreased deposition of collagen III and fibronectin was not correlated with a decrease in the mRNA expression for these proteins, suggesting post-translational dysregulation which can only be uncovered with protein-level analysis. Likewise, the balance of synthesis between collagens I and III is critical in determining the mechanical properties of tendon. In one study, the tendon insertions in rotator cuff tendons were shown to contain a higher proportion of type III collagen than the midtendon substance (22). In another, intratendinous tearing due to shears within the tendon was demonstrated via light microscopy (18). Similar findings may be elucidated more clearly using the tools of proteomic analysis.

3. PROTEOMIC METHODS

Wasinger *et al.* first introduced the concept of the proteome as "the protein equivalent of the genome" (23). The functional proteome is the physiological state of a tissue, represented by its entire protein complement at a given time, including all protein isoforms and post-translational modifications which are present (24). Twenty-first century proteomics uses wholesale analysis of proteins to establish functional relationships and provide information about complex biological processes (25). When the entire tendon is removed and rapidly processed for proteomic studies, all the components that might contribute to the process are sampled simultaneously. Damaged proteins and fragments of proteins, as well as the molecules produced by the body in response to the damaged state can be detected and characterized using the methods of proteomic analysis.

Ultimately, differences in genetic composition which give rise to tendinopathy, such as structural abnormalities, could be described on a protein level by developing a complete tendon proteome. Diminished protein function and distorted information flow within diseased cells, and their interconnected tissue microenvironments undoubtedly create preexisting conditions which lead to injury when tendon is exposed to stress. By understanding the causal mechanisms, novel pathologies could be elucidated, leading to improved therapies to relieve pain and enhance patient healing outcomes.

Several schema have been developed that are included within the proteomics genre. Figure 3 is a diagrammatic representation of one method using tendon as a specific example: two-dimensional gel electrophoresis (2DE). Regardless of the schema used (see sections 3.1

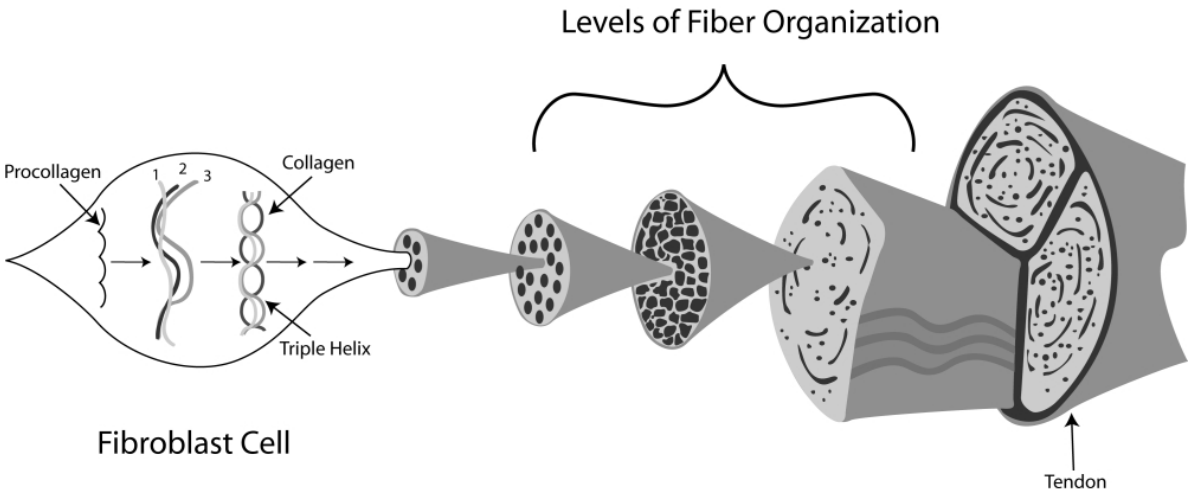

Figure 2. The fibroblast is the site of collagen synthesis. Procollagen molecules are produced individually and then developed into triple helical fibrils (tropocollagen) through a series of posttranslational modifications in association with other procollagen molecules. Fibrils are organized into fibers, which comprise primary fiber bundles (subfascicles), surrounded by an endotenon layer. Successive levels of fiber organization (secondary and tertiary bundles) culminate in the tendon substance which is itself surrounded by the epitenon sheath (11).

through 3.5, below), amino acid sequence identification using mass spectroscopy followed by bioinformatic analysis is the final stage of proteomics research. Bioinformatic analysis utilizes databases of peptide/protein amino acid sequences available on the worldwide web, such as the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics, including SWISS-2DPAGE (26), or the BLAST (Basic Local Alignment Search Tool) sequence alignment tool provided by the U.S. National Center for Biotechnology Information of the National Library of Medicine (27). This database analysis yields the information needed for proteins to be identified based on comparison with the published sequences for proteins and peptides submitted by researchers in all areas of proteomics. This last step is fundamental to each of the methods described in the following sections: 2DE, MALDI, SELDI, ICAT and peptide mapping.

Sample preparation is one of the most critical steps in proteomic analysis. Careful preparation ensures that the proteins retain their original primary structure so that an unbiased and accurate representation of all proteins in the sample is accomplished. The integrity of tendon proteins is preserved by flash freezing in liquid nitrogen within the shortest possible time after tendon removal by surgery (or experimental procedure in the case of an animal model). The preparation must be optimized to minimize the protein modification and proteolytic damage which would compromise subsequent analysis. Proteases released during the process can be suppressed by including non-protein inhibitors such as phenylmethylsufonyl fluoride in the buffer solution.

Tendon is a tough tissue with distinct protein populations which must be isolated selectively and analyzed separately. Soluble noncollagenous proteins can typically be removed by aqueous extraction in a buffer with low osmolarity. Lysis of the cells and maceration of the tissue in a chilled mortar with pestle or homogenization device (such as a polytron or ultrasound unit) finishes the extraction process. The collagens which constitute the second, major fraction of the tendon proteins are left behind in this initial purification. The aqueous extract is enriched in cellular, matrix, and regulatory proteins which are present in much lower amounts than the structural molecules. Interfering substances such as nucleic acids, and insoluble materials must be removed by high speed centrifugation. Pepsin treatment in the presence of acetic acid has been used extensively to release the subunit proteins in the collagen protein fraction. Precipitation of the several collagens present in pepsin digests, using increasing concentrations of sodium chloride followed by high speed centrifugation, produces further separation of the isolated structural proteins. For each extract, a final precipitation step using ice-cold acetone cleans the proteins for further manipulations.

These methods produce purified extracts which become substrates for further analysis using the various proteomics methods described in the following sections. Cyanogen bromide, trypsin and other highly purified proteolytic enzymes are used to reduce the fractionated tendon extracts to peptide subunit digests for many of the procedures. An exception would be 2DE where proteins are separated based on intact features of size and charge.

3.1. Two-dimensional gel electrophoresis

The proteomics boom began with 2DE. This technology uses separation of proteins and peptides based on a combination of their molecular mass and p*I* (isoelectric point). Differential patterns of protein spots

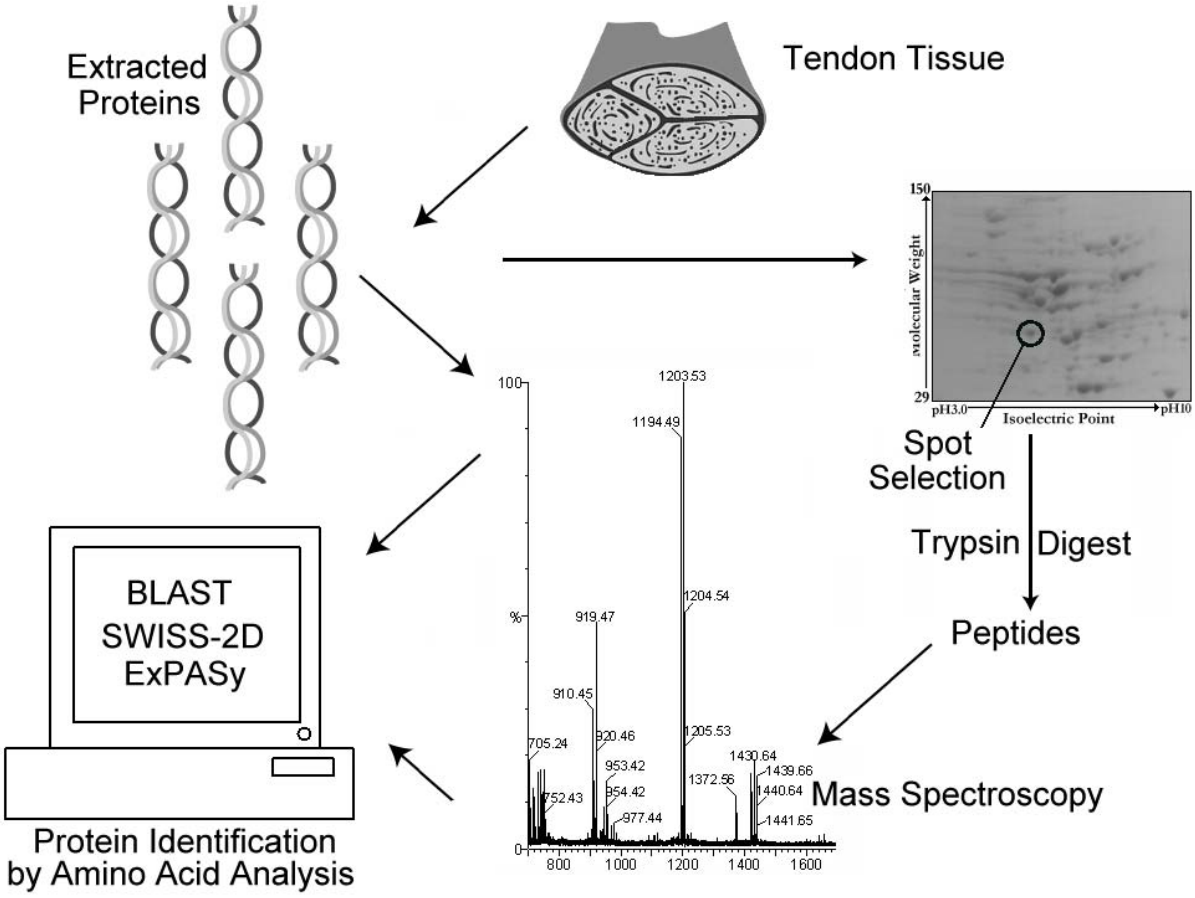

Figure 3. Overview of the process for tendon proteomics. Tendon tissue is isolated and flash frozen to preserve protein integrity. Proteins are extracted using methods described in the text, then subjected to 2DE electrophoretic separation, followed by spot selection, trypsin digestion, and peptide isolation using tandem mass spectroscopy. A final bioinformatics step allows protein identification from amino acid sequence information (28).

from two different protein extracts (for control and injured tendon in the present example) can be analyzed to produce expression profiles (the appearance or increase/decrease of protein spots). Spots are visualized using colloidal coomassie brilliant blue, silver staining or fluorescent dyes (28). The method of staining is not trivial, since compatibility with subsequent mass spectroscopy procedures is crucial. Noteworthy spots can be excised and then digested with proteolytic enzymes such as trypsin, and characterized using peptide mass fingerprinting. Mass fingerprinting relies upon a comparison of peptide mass/charge spectra obtained using mass spectroscopy (MS) of prepared protein samples with information stored in proteome databases populated with information obtained in previous experiments and reported by laboratories across the world (29).

The 2DE method separates proteins in the first stage by isoelectric focusing. Originally described by O'Farrel using mobile carrier ampholytes to establish labile pH gradients in tube gels (30), the method has matured over the last decade. Immobilized pH gradient (IPG) strips have been developed with a gradient of charge embedded in acrylamide (31,32). Commercial availability of IPG strips prepared with narrow p*I* ranges has facilitated the widespread use of this protein separation technique for proteome research. Such innovations have enhanced the reliability and reproducibility of the 2DE method. Systems which accommodate 11 cm or 17 cm IPG strips used in conventional separations, as well as 24 cm strips for large format separations are available (33). Some investigators have found that the longer IPG strips improve sample resolution. Coupled with prepoured SDS slab gels specialized to separate proteins by discrete molecular mass ranges in the second dimension, 2DE remains a powerful tool for proteomic analysis which has not been used extensively for tendon protein analysis.

Tris-tricine SDS-PAGE separation in the second dimension is a 2DE variation which enhances the identification of low molecular weight proteins (34). Many regulatory molecules fall in this narrow mass range where proteins cannot be analyzed using traditional Laemmli SDS-PAGE formulations (35). Combining IPG first

dimension with the Tris-tricine system in the second dimension provides another tool for the analysis of tendon injury and healing.

Several persistent problems limit 2DE's promise as an analytical tool. One is the difficulty in quantifying low abundance proteins, given the small amount of sample which can be loaded onto each IPG strip in the first dimension. This problem has been addressed through prefractionation of samples to selectively remove high abundance proteins which provide little new information, such as immunoglobulins and albumin, or, in the case of tendon, collagens. Aqueous extraction of low copy number proteins to selectively enrich them relative to the nonsoluble collagens is a feature of tendon biology which lends itself to proteomics. Many companies have developed both narrow range (p*I* of 4-7 or 6-9) and extremely narrow range IPG strips (p*I* of 3.5-4.5; 4-5; 4.5-5.5 and so on). A much larger volume of sample can be applied to these strips, greatly enhancing the resolving power for the detection of proteins present in low amounts (36).

Another issue is the low-throughput and time-intensive nature of the 2DE method. This partially results from the labor involved in staining and image analysis of differential spot patterns from matched samples after the electrophoresis is completed. To ensure that any observed differences are real, multiple gels must be run for each sample, with a suggested minimum of 3-5 replicates (37). This allows statistical validity for quantitative work, but is yet another consideration which adds to the complexity of experimental design for proteomics studies. One innovation which remedies this problem is the use of fluorescent dyes to visualize spots in a method called differential in-gel electrophoresis (38: DIGE). This procedure uses one separation for the analysis of up to three protein samples simultaneously. DIGE depends upon individual labeling of each sample using Cy2, Cy3 and Cy5 fluorescent dyes before the first dimension separation. The dyes are identical in molecular weight and isoelectric point, but differ in their excitation and emission wavelengths for visualization. Separate DIGE images can be captured under the different dye emission optima and analyzed by overlapping the images. The fluorescent dyes have the exceptional qualities of nanogram sensitivity, compatibility with subsequent MS, and a linear response to protein concentrations over five orders of magnitude (38).

3.2. MALDI-TOF analysis

More recently, protein separation methods utilizing newer technological innovations have become central to proteomics. Matrix-assisted laser desorption ionization (MALDI) using supports such as cinnamic acid matrix and electrospray ionization (ESI), by which the sample is introduced into a mass spectrometer (MS) are coupled in a method which allows high throughput of experimental samples (39). Bound proteins are desorbed from the matrix using laser energy and then ionized for MS analysis. Peptides of less than 1000 Da ranging up to proteins of greater than 300 kDa can be calculated based on the time-of-flight (TOF; the duration of time between ionization and detection). A unique sample "fingerprint" will result from the mass to charge ration (m/z) of the molecules in the gas phase (TOF). The m/z patterns, rather than the actual protein characteristics, are used to identify the proteins in a sample. The coupling of MALDI-TOF technology with tandem mass spectrometers enables actual amino acid sequencing and subsequent protein identification to be performed. Limitations of the method include photo degradation by laser desorption and ionization, as well as limited ability to resolve peptides with m/z ratio of less than 500 (38).

3.3. SELDI-TOF analysis

Surface-enhanced laser desorption ionization (SELDI) time-of-flight mass spectrometry has emerged more recently as a proteomic analysis variation which is well-suited for biomarker discovery. This method introduces a specificity not present in the MALDI-TOF technique. SELDI-TOF relies upon chips made of stainless steel or aluminum, prepared with surface modifications which provide a "bait" to trap proteins of interest (38). The surface bait can be a chemical modification (immobilized anionic, cationic, hydrophilic or hydrophobic charge) or biological substrate (antibody, antigen, DNA, or other ligands) which enables differential capture of proteins. Tissues or body fluids which have been prepared by extraction of proteins using chemical solubilization and enzymatic treatment can be applied directly to the chips. Target proteins and peptides with the appropriate affinity for the bait molecules will bind to the chip. A series of wash steps with variable stringency characteristics can then be used to remove proteins which have bound weakly or non-specifically to the bait on the chip surface. The final steps in the SELDI-TOF analysis mirror the MALDI-TOF process, and the mass spectral patterns from paired control and injured specimens can be compared to identify changes associated with tendon injury (40). This method has been used for biomarker discovery in many different systems and is effective for capturing glycosylated proteins through hydrophobic capture matrices. Since glycosylated proteins are synthesized for export outside the cellular environment, this method would be ideal for selectively capturing the regulatory proteins secreted in response to tendon injury and healing (41).

3.4. Isotope labeled separation

Differential labeling of proteins or peptides with isotope labels followed by tandem mass spectroscopy (MS/MS) analysis offers a way around many of the problems of 2DE. Isotope tag-coded affinity tagging (ICAT™) compares paired protein samples by labeling each with a different stable isotope tag (42). The chemistry of the ICAT™ reagent involves a reactive group with specificity for thiol groups, a biotin affinity tag which allows the isotope-labeled peptides to be isolated, and a linker chain which connects the biotin and thiol-specific groups. The distinguishing factor in these linker chains is the side groups that are either light or heavy isotope reagent forms (early reagents used deuterium; more recently ^{13}C reagents have been developed for isotope-

tagged proteomics). The labeled peptides are analyzed using MALDI-TOF and MS/MS. The matching peaks are visualized as spectrographs from MS-separated peptides which differ by the exact size of the linker isotope, providing the ability to distinguish the control sample from the test sample. This approach produces a more reliable quantification than MS methods alone by comparing two unique but related samples within a single analysis, thereby reducing MS data variation. Similar systems include isobaric tags for quantification (iTRAQ) and stable isotope labeling with amino acids in cell culture (SILAC).

Each of these labeling methods relies on statistical methods which are prone to over-estimating the significance of differences when results are low intensity. Low intensity signals are plagued by fluctuations in the measurements which obscure signal clarity and reduce the sensitivity of an experiment (39). Conversely, differences that reach statistical significance can be underestimated if a simple fold-change cutoff criterion is used. Differential expression methods are especially problematic when experiments involve a small number of replicates. Traditional statistical methods such as the paired *t*-test are suitable for the analysis of differentially-expressed proteins in MS/MS data when a large number of replicates are tested (more than 10). The number of replicates is frequently restricted given the high cost of experiments and/or the limited nature of biological samples. Biological "averaging" where protein abundances average out when multiple individual samples are pooled may be of value in future studies of tendon proteomics (39).

3.5. Peptide mapping

Peptide mapping was used extensively in the years before 2DE became widespread. Much of what is known and understood about the structural composition of tendon collagens was elucidated using peptide mapping. Collagenous protein extracts were prepared as described in the Proteomics Methods section (3) above, and then digested using a cyanogen bromide chemical method (CNBr). CNBr cleaves the collagen molecule at methionine residues. The procedure generates a relatively small number of peptide fragments which are fractionated using selective salt precipitation (22). The peptides can be separated on SDS-PAGE gradient gels and compared with low molecular weight peptide standard markers. Densitometric scans of the bands obtained after staining with coomassie blue allow quantitative comparisons of various collagen types to be made. In one study, collagen types in rotator cuff tendons were characterized using peptide mapping. The authors found an increased presence of type III collagen at the tendon insertions using this method (22). For some proteins found in large amounts in the tendon proper, this method may continue to have value. However, the performance of such methods is not optimal. More recently, CNBr peptides of collagen samples isolated from tendon and other tissues have been separated using capillary electrophoresis coupled with mass spectrometry (43) or with MALDI-TOF mass spectrometry (39).

4. ICAT ANALYSIS IN AN ENZYME-INDUCTION INJURY MODEL

We have used isotope tagging to evaluate tendon injury in a rat collagenase injury model. Harlan Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing approximately 300g were used for this study. The right tendon of each isoflurane anesthetized rat was injured injecting ~15 µl of sterile solution to deliver 900 IU collagenase/kg (Sigma Chemical Co., St. Louis, MO). This amount was determined in preliminary experiments to produce a mild injury similar to exercise induced tendinopathy. Rats were maintained on water and rat chow *ad libidum* with a dark/ light cycle of 12/12 hours. Tendons were harvested at 24, 48 and 72 hours post injury. Non-collagenous proteins were extracted by vortex mixing each tendon in 1 mL of 0.1M TRIS solution at pH 7.5. Supernatants containing extracted proteins were removed following centrifugation to sediment insoluble materials including collagens. A BCA protein assay (Pierce Biotechnology Inc., Rockford, IL) was used to determine the protein concentration. Proteins were digested to an appropriate peptide size range (5 to 25 residues) using endoproteinase Glu-C followed by proteomics-quality bovine pancreatic trypsin (Roche Applied Science, Indianapolis, IN). Peptide fragments were dried by lyophilization and resuspended in 0.1 M TRIS buffer, pH 8.5 at 1 µg/ml concentration. Peptides were analyzed using ultralow molecular weight 16.5% TRIS-Tricine SDS PAGE and silver staining. Acetone-precipitated aqueous proteins were isotope labeled following the manufacturers protocol (Applied Biosystems, Foster City, CA). The separate paired samples of precipitated peptide fragments were solubilized in SDS buffer, and then each sample was labeled with either the light ICAT reagent (for control state) or heavy ICAT reagent (for test state). Labeled samples were then enzymatically digested with trypsin for 16 hours at room temperature. Unbound ICAT reagent was removed using a cation exchange column, and the samples were then affinity-purified using an avidin column. Samples were prepared by reverse-phase HPLC chromatography, then analyzed using MALDI-MS, quadrapole-time of flight (Q-TOF), for abundance determination with MS and sequence determination with MS/MS.

The abundance ratios for these proteins were calculated from three independent peptide quantifications. Results were compared with protein analysis worksheet (PAWS) and MassLynx database information using only *Rattus* species correlations.

In early isotope labeling experiments, we detected proteins involved in fibroblast cell proliferation: a 1.2-fold increase in DNA polymerase at 24 hours post-injury which returned to normal levels by 48 hours after injury induction and a regulator of chromatin gene transcription which dipped to 0.4-fold lower than normal at 48 hours, but then increased 1.4-fold relative to uninjured control samples by 72 hours post-injury (44). In later studies, we detected significant changes in tendon injury in proteins traditionally described as the acute-phase

Table 1. Proteins measured by isotope labeling in rat tendon injury

Protein	Confidence Score[1]	Fold-Change At 24-hours Post-Injury	Fold-Change At 48-hours Post-Injury	Fold-Change At 72-hours Post-Injury
DNA polymerase	13.4	1.2	0.9	0.9
Regulator of chromatin gene transcription	14.5	0.8	0.4	2.4
Alpha-2-macroglobulin	30	10	1.0	1.0
Alpha-1-macroglobulin	38	4.5	1.5	1.0
Serotransferrin	74	2.5	4.25	1.5

[1]A cutoff score of 15 represents a 95% confidence interval for identification of each of the listed proteins. Numbers describe the fold-changes in protein concentration for injured tendon relative to noninjured controls.

inflammatory proteins (Table 1). We found a 10-fold increase in alpha-2-macroglobulin, a 4.5-fold increase in alpha-1-macroglobulin and a 2.5-fold increase in serotransferrin at 24 hours post-injury using the ICAT™ method. By 48 hours post-injury, only the serotransferrin remained elevated in the injured tissue, at 4.25-fold higher than control uninjured tendon in contrast to the other inflammatory proteins, which had returned to near-normal levels. Serotransferrin is responsible for the transport of iron from sites of heme degradation to those of storage and utilization, as well as having a potential role in stimulating cell proliferation (45). Each of the inflammatory proteins we detected is associated with acute injury and inflammation. Our results confirm the role of inflammatory proteins in tendon injury. Macroglobulins inhibit the activity of key proteinases, limiting the degradation of damaged tissue at the site of injury. Since proteolytic enzymes modulate remodeling and tendon regeneration activities, as discussed in section 2.2, therapies which reduce local levels of the major acute phase inflammatory proteins may promote tendinopathy healing.

5. PROTEOMICS METHODS FOR WOUND HEALING ANALYSIS

Proteins and peptides important in the control of tendon healing are often secreted at the site of tissue injury. These molecules are typically present in low abundance and are difficult to characterize using traditional tissue harvest and protein extraction methods. Although proteomics using 2DE and mass spectroscopy is a useful discovery system for high abundance proteins, it is not sensitive enough for detection of regulatory proteins which may be present in low amounts (41).

Identification of secreted proteins important in the process of wound healing has been conducted using a modification of capillary ultrafiltration probes (46). Although not specific to tendon problems, this method has important implications for the study of tendinopathy. In the referenced study, a single microliter of fluid was extracted from a dorsal skin wound in a BALB/c mouse model. A semi-permeable membrane positioned within the capillary tube allowed interstitial fluid to be collected without contamination by cells and debris from the wound environment. The sensitivity of the tandem mass spectroscopy used in the analytical phase allowed two proteins to be characterized in this system (thymosin ß10 and ß4) (46). A similar method could be used to analyze control proteins in a wounded versus uninjured rat tendon model.

6. FURTHER CONSIDERATIONS

The genomics revolution of the last decade has changed the global approach to biological questions. However, it is increasingly apparent that gene sequence and protein function cannot be correlated in many systems. Post-translational modifications such as phosphorylation, glycosylation, ubiquitination, methylation, acetylation, farnesylation, sumoylation and lipidation play an important role in development, physiology and disease (47). These important variations in protein expression cannot be identified using genomic methods. The identification of protein modifications which contribute to the complexity of gene products will depend upon methods which exploit affinity purification coupled with proteomics methodologies.

Proteomics currently is in a phase of exponential growth (25). This platform of methods represents an excellent tool for the study of variations in protein expression between different states and conditions. Thousands of cancer biology and therapeutics laboratories have contributed proteomics data over the past decade, demonstrating its potency as resource in these areas. Biomarker discovery for diagnostics is another field which uses the strengths of proteomic analysis. Likewise, pharmacologic efficacy studies and other direct clinical applications are well suited for proteomics inquiry. Proteomics can provide a framework for mining many experimental systems to develop a catalogue of critical proteins and regulatory pathways.

Taking cost, availability and utility into account, 2DE is one of the most appropriate current technologies for the proteomic characterization of proteins. Advances in bioinformatics such as the increasing accumulation and widespread availability of amino acid sequence data will make this methodology of greater importance in the future. The maturation of proteomics databases with results from wound healing studies, regeneration, growth regulation, immune regulation, and modulation of inflammation should enhance the capacity for tendon proteomics, as well as the accrual of tendon-specific data (48). These factors will give investigators who apply proteomics methods in the study of tendinopathy an unprecedented ability to identify molecules previously unrecognized in tendon injury. The next decade should yield a tremendous insight

into control mechanisms involved in tendinopathy and healing.

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Abbreviations: VEGF: vascular endothelial growth factor; IGF-1: insulin-like growth factor-1; bFGF: basic fibroblast growth factor; PDGF: platelet-derived growth factor; TGF-beta: transforming growth factor-beta; BMP-2: bone morphogenic protein-2; MGF: mechano growth factor; MMPs: matrix metalloproteinases; IPG: immobilized pH gradient; p*I*: isoelectric point; 2DE: two-dimensional electrophoresis; MS: mass spectroscopy; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; DIGE: differential in-gel electrophoresis; MALDI: matrix-assisted laser desorption ionization; ESI: electrospray ionization; TOF: time-of-flight; m/z: mass to charge ratio; SELDI: surface-enhanced laser desorption ionization; DNA: deoxyribonucleic acid; MS/MS: tandem mass spectroscopy; ICAT: isotope tag-coded affinity tagging; iTRAQ: isobaric tags for quantification; SILAC:

stable isotope labeling with amino acids in cell culture; PAWS: protein analysis worksheet

Key Words: Proteomics, Mass Spectroscopy, Two-Dimensional Electrophoresis, Isotope-Labeling, Tendinopathy, Review

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