

Using Extracellular Matrix Proteomics to Understand Left Ventricular Remodeling

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Survival following myocardial infarction has improved substantially over the last 40 years; however, the incidence of subsequent congestive heart failure has increased dramatically as a consequence. Discovering plasma markers that signify adverse cardiac remodeling may allow high-risk patients to be recognized earlier and may provide an improved way to assess treatment efficacy. Alterations in extracellular matrix regulate cardiac remodeling following myocardial infarction and potentially may provide a large array of candidate indicators.

The field of cardiac proteomics has progressed rapidly over the past 20 years since publication of the first 2-dimensional electrophoretic gels of left ventricular proteins. Proteomic approaches now are used routinely to better understand how the left ventricle responds to injury.

In this review, we will discuss how methods have developed to allow comprehensive evaluation of the extracellular matrix proteome. We will explain how extracellular matrix proteomic data can be used to predict adverse remodeling for an individual patient and highlight future directions. Although this review will focus on the use of extracellular matrix proteomics to better understand post-myocardial infarction remodeling responses, these approaches have applicability to a wide-range of cardiac pathologies, including pressure overload hypertrophy, viral myocarditis, and nonischemic heart failure.

Short term (ie, 30 day) survival rates for the post-myocardial infarction (MI) patient have improved from 60% in the 1970s to >90% currently, primarily as a result of reperfusion therapy.¹⁻³ However, up to 45% of MI survivors subsequently develop congestive heart failure (CHF).⁴⁻⁶ Even with currently available therapies (reperfusion, angiotensin-converting enzyme inhibitors, and β adrenergic receptor inhibitors),^{2,7,8} novel strategies are needed to identify and treat patients who are at risk for CHF.⁹ Examining changes at the extracellular matrix (ECM) level is a promising avenue to find new mechanisms to limit adverse remodeling.¹⁰

One clinical trial that highlights the importance of ECM in post-MI remodeling is the Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival (EPHESUS) Study. In this study, the investigators evaluated the effects of

treatment with an aldosterone receptor antagonist compared with placebo on post-MI outcomes. In a substudy of 476 post-MI patients that developed CHF, serum analytes of collagen turnover were measured.¹¹ The authors combined baseline serum levels of type I collagen telopeptide and brain natriuretic peptide into 1 composite index of collagen turnover. All-cause mortality and the composite end point of cardiovascular death or CHF hospitalization both were associated with the collagen turnover composite index, with hazard ratios of 2.49 ($P=0.039$) and 3.03 ($P=0.002$), respectively. Furthermore, patients treated with eplerenone showed reduced levels of collagen turnover markers compared with those who did not receive eplerenone, indicating that collagen synthesis and degradation are active processes in post-MI patients. These results are consistent with the numerous animal studies that have shown a relationship between ECM remodeling and the development of CHF.¹²⁻¹⁴ Therefore, a better understanding of how the cardiac ECM proteome changes during disease progression may provide additional targets for therapeutic intervention.

This review will concentrate on the strengths and limitations of different proteomic approaches to glean information that is specific to ECM turnover in the post-MI setting. Proteomic strategies provide us with a means to index the proteins present, quantify levels, determine function, and explore interactions.¹⁵ This review will not concentrate on mass spectrometry technological advances that are well described in other reviews.¹⁵ Rather, we will focus on how sample preparation and labeling protocols have changed to increase the likelihood of cataloguing the cardiac ECM proteome. From our own perspective, we will describe how proteomic approaches focusing on the ECM compartment have progressed over time to current gel-free approaches using decellularized fractions. Strengths and limitations of the various proteomic approaches are summarized in Table 1.

Resolving Whole Left Ventricular Tissue Extracts by Two-Dimensional Electrophoresis

In 1992, Dunn and colleagues were the first to establish a human myocardial 2-dimensional electrophoresis database, which was updated in 1994.^{16,17} The database contained 1388

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Table 1. Strengths and Limitations of Proteomic Approaches for Evaluation of ECM

1. 2-DE gel analysis
Strengths: established technology, separates intact proteins, provides MW and pI information, can detect post-translational modifications
Limitations: no. of proteins resolved per gel is limited, multiple proteins can co-migrate in a single spot
2. MS-based methods approaches
Strengths: can be label-based or label-free, capacity for high throughput, more extensive proteome coverage than 2-DE gels by several orders of magnitude
Limitations: needs sophisticated instrumentation and software to acquire and process data
3. Protein arrays
Strengths: allows evaluation of a large no. of similar samples in high throughput manner
Limitations: can only be used for specified protein targets, ECM is not included in many of the currently available commercial arrays
4. Fractionation approaches
Strengths: focuses on cell type or organelle of interest, enrichment for ECM is possible, this approach can be coupled with any of the above approaches
Limitations: analysis dependent on purity of the preparation

2-DE indicates 2-dimensional electrophoresis; ECM, extracellular matrix; MS, mass spectrometry; MW, molecular weight; and pI, isoelectric point.

protein spots characterized by molecular weight and isoelectric point (pI). From a total of 103 protein spots analyzed, 49 were assigned by immunoblotting and 32 by *N*-terminal sequencing, for a 79% identification success rate. An additional 6 proteins tentatively were assigned by comparison with the human heart 2-dimensional electrophoresis (2-DE) protein database of Jungblut and colleagues.¹⁸ The databases from these 2 groups are the standards by which cardiac extract 2-DE gels are compared still. Annotated web-based 2-DE maps for healthy and diseased cardiac tissue, such as failing myocardium, also have been developed from these databases.¹⁹ These maps, which contain a preponderance of calcium signaling proteins and mitochondrial enzymes, highlight impairments in sarcoplasmic reticulum calcium cycling and mitochondrial signaling as markers of cardiac injury.

A major advantage of the 2-DE gel approach is that it provides apparent molecular weight and pI information. Additionally, gel analysis is broad-based and effectively unbiased from the investigator point of view (ie, you do not need to have a target already selected to examine changes). Because gel approaches are nontargeted, they can, in theory, reveal novel discoveries. Detailed image analysis of gel spots, using software such as SameSpots (Nonlinear Dynamic, Durham, NC) affords the advantage that subsequent mass spectrometry analyses only need to be conducted on spots that exhibit significant differences among comparison groups.

A major disadvantage of the 2-DE gel approach is that even though the approach is unbiased, it is limited by issues of experimental design. Another limitation is the inability to fully resolve the entire complement of left ventricular (LV) proteins on a single 2-DE gel. While using multiple immobilized pH gradient strips with overlapping pI ranges has

increased the number of proteins resolved, for each gel only approximately 10% of the total number of proteins can be visualized. Furthermore, LV proteomic profiles represent mixed cell populations that are dominated by the most abundant cell type. Therefore, comparing a normal ventricle to an infarcted ventricle merely will reflect the change from a myocyte-dominated to an inflammatory cell and fibroblast-dominated tissue. Additionally, approaches using unfractionated LV extracts will yield a predominance of cytoplasmic and mitochondrial proteins, because the myocyte is the major cell type in the LV, and mitochondria comprise 30% of the myocyte volume.^{20,21} The inability to add ECM proteins to the LV proteome catalog using this global strategy has been disappointing. In addition to being present in relatively low abundance, ECM proteins are also hydrophobic, which requires that solubility issues be addressed.

Protein Labeling Strategies

From 2-DE gels, methods progressed to stable isotopic labeling strategies that directly couple quantification and identification. A major advantage of multiplexed labeling strategies is that the experimental groups are mixed before mass spectrometry analysis, thereby minimizing issues related to analytic variability such as differences in retention time or differential suppression of ionization.^{22,23} Strategies in use over recent years have been based on a variety of approaches.²⁴ These approaches include using stable isotope incorporation (¹⁶O/¹⁸O labeling), derivatization for mass spectroscopy (eg, isotope-coded affinity tags, dimethyl labeling), derivatives that yield reporter ions in MS/MS (eg, isobaric tag for relative and absolute quantitation and tandem mass tags, and metabolic labeling (eg, stable isotope labeling with amino acids in cell culture [SILAC]).^{25–31}

A major strength of these strategies is that proteins are not first separated on 2-DE gels, which avoids issues of solubility and extremes of pI associated with the isoelectric focusing step. However, there are advantages to separating proteins by 1-dimensional electrophoresis before mass spectrometry analysis, in that molecular weight information is obtained and minimal sample cleanup is required. Labeling strategies also can be effectively coupled with multi-dimensional chromatography (combinations of ion exchange/reversed-phase [eg, MudPIT] or high-pH/low-pH reversed phase (termed high-/low-pH RP) in conjunction with tandem mass spectrometry to allow quantification and identification in one step.^{32–34}

With isobaric tag for relative and absolute quantitation and tandem mass tags, all tryptic peptides are labeled, which increases confidence in the relative quantification. A major advantage of both iTRAQ and tandem mass tags is that both approaches can be highly multiplexed. Incorporation of ¹⁶O/¹⁸O during tryptic digestion and dimethyl labeling are straightforward methods that are relatively inexpensive but do not afford a high degree of multiplexing.

SILAC involves the metabolic incorporation of stable isotope analogs of amino acids into cellular proteins. SILAC has been used to quantify protein complexes, enzyme substrates, membrane proteins, and temporal dynamics.^{35–37} Because up to 5 labels can be used, multiple comparisons can be made simultaneously. This technique is especially useful for

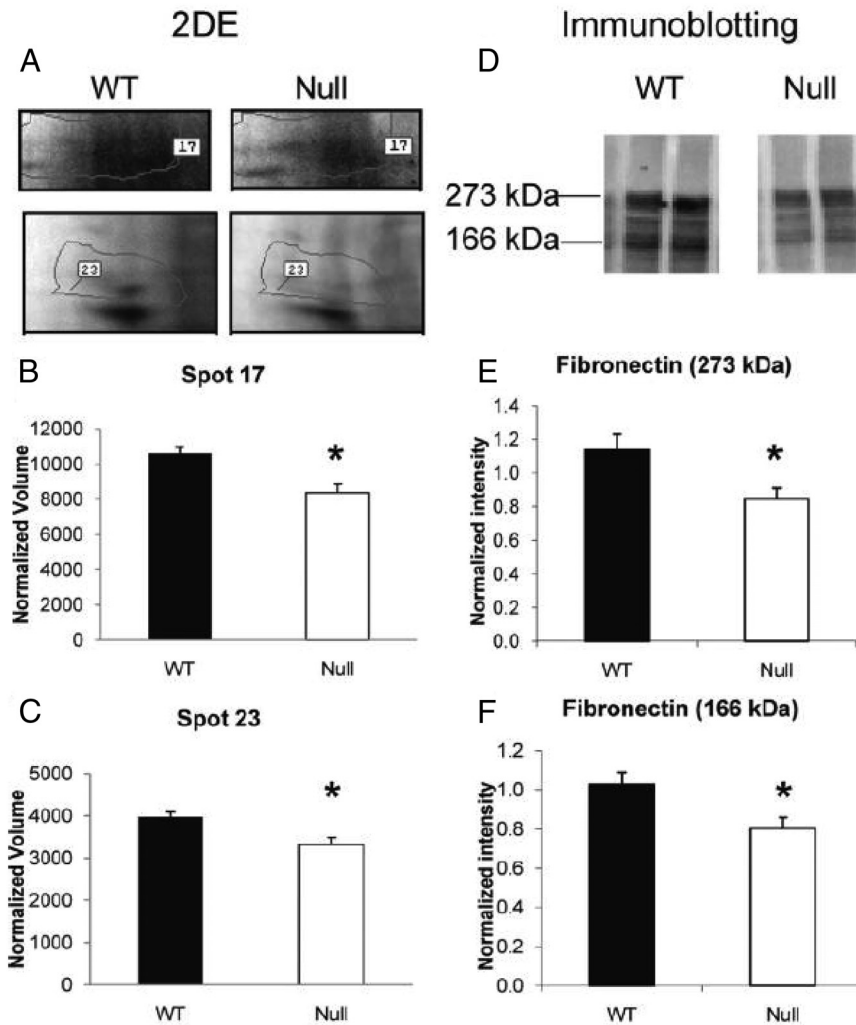


Figure 1. MMP-7 null infarcted left ventricle showed lower levels of fibronectin by 2-dimensional electrophoresis and immunoblotting. **A**, Fibronectin was identified in spot 17 and spot 23 of the 2-dimensional electrophoresis gels. Both spot 17 (**B**) and spot 23 (**C**) showed significant lower intensity in infarct left ventricle of MMP-7 null mice compared with wild type (WT). **D**, Immunoblotting of fibronectin in WT and MMP-7 null LV extracts were performed. The densitometry of the 273 kDa full-length (**E**) and the 166 kDa fragments (**F**) of fibronectin indicated both bands showed significantly lower intensity in MMP-7 null left ventricular infarct when compared with the wt ($P < 0.05$). Reproduced from Chiao YA et al⁴⁹ with permission from the American Chemical Society.

monitoring cell secretomes because newly synthesized proteins are evaluated. Pinto and colleagues demonstrated the use of SILAC for exploring sheddase activity in cultured cells to identify 2 novel substrates for snake venom metalloproteinases; a similar approach in cardiac fibroblasts may identify novel ECM substrates of matrix metalloproteinases (MMPs).^{38,39} A limitation of using SILAC to analyze a cell secretome is that the cells must be cultured in protein-free medium to allow analysis of secreted proteins in the supernatant; here, the assumption is that the proteins secreted under serum starvation conditions reflect in vivo secretion patterns.

Label-free quantification, including spectral counting (based on the number of spectra assigned to a given protein) and comparison of precursor ion intensities, can be used to determine differential protein expression levels in 2 or more experimental groups.^{40,41} The enhanced mass accuracy and resolution of high-performance mass spectrometers are particularly important for relative quantification. The availability of software for relative quantification, (eg, Progenesis LC-MS [Nonlinear], SIEVE [Thermo Fisher, Waltham, MA] based on precursor ion intensities, and Scaffold [Proteome Software, Portland, OR] and ProteoIQ [NuSep, Bogart, GA] for spectrum counting) have advanced the label-free approach by providing the computational framework necessary to analyze the data.^{42–44}

Xu and colleagues used the label-free approach to identify MMP-9 substrates in cancer cells.⁴⁵ Others are working on label-free analysis in studies of other cardiovascular diseases, such as hypertension and atherosclerotic plaque formation.^{46–48}

Focusing on Natural Tissue Enrichment Strategies

Our team used a 2-DE gel approach to identify MMP-7 and MMP-9 related changes in the left ventricle post-MI.^{49,50} Using infarct tissue only, which provided a natural enrichment for ECM, we evaluated wild-type and MMP-7 or MMP-9 null left ventricular infarcts at day 7 post-MI. In both MMP-7 and MMP-9 null mice, we identified left ventricular ECM proteins with lower expression compared with wild-type mice. Included in the list was fibronectin, a known in vitro substrate for MMP-7 and MMP-9 (Figure 1). In the wild type and MMP-7 null post-MI extract comparison, we further showed that adding exogenous recombinant MMP-7 resulted in the generation of the fibronectin fragments seen in the wild type LV samples. We also demonstrated a rescue in phenotype for tenascin C, indicating that both fibronectin and tenascin C are in vivo substrates of MMP-7. Importantly, these were the first reports of multiple ECM proteins being identified in cardiac extracts using a 2-DE gel approach,

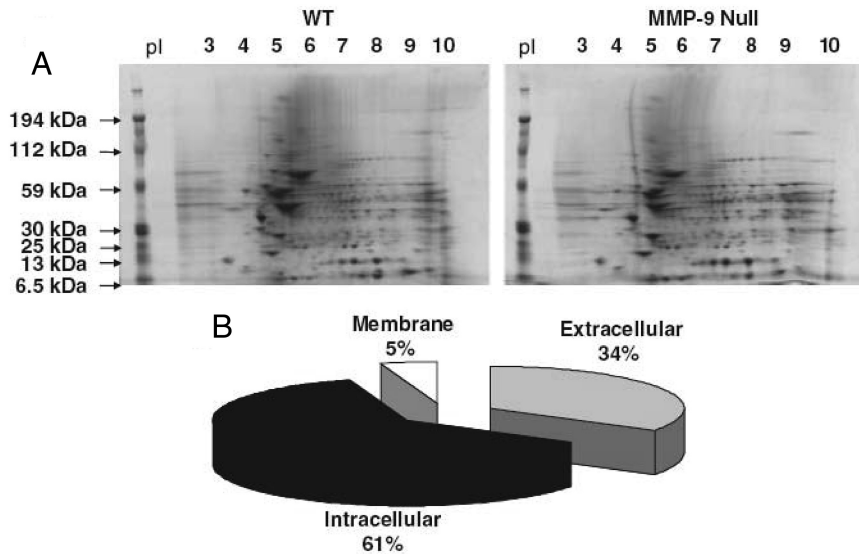


Figure 2. Representative 2-dimensional electrophoresis gels of the wild type (wt) and MMP-9 null post-MI groups (A), and of the candidate MMP-9 substrates identified (B), showing that 61% are classified as intracellular, 5% as membrane, and 34% as extracellular proteins. Reproduced from Zamilpa R et al⁵⁰ with permission from Wiley-VCH Verlag GmbH & Co.

indicating success in enriching for ECM by limiting analysis to the infarct region. In the wild type and MMP-9 null samples, 34% of the proteins present in differentially expressed spots were identified as extracellular proteins (Figure 2). A further 5% were identified as membrane proteins. However, multiple cytoplasmic and mitochondrial proteins also were present, indicating that this enrichment strategy was not optimized fully.

Proteome Simplification by Suborganelle Fractionation

Fractionation techniques include separation by organelle, which is based, to a large extent, on solubility or physicochemical properties such as size and pI. In the heart, mitochondria, caveolae, proteasome, and myofilament organelles have been examined using subfractionation coupled with proteomic approaches.^{25,51–55} Taylor and colleagues have comprehensively characterized the human heart mitochondrial proteome,⁵⁶ and several groups continue with this endeavor.⁵⁷ An excellent review on the cardiac mitochondrial proteome is provided by Gucek and colleagues.²⁵

With the subfractionation approach, the accuracy of the cardiac organelle proteome catalog generated is dependent on the purity of the isolation. Protein correlation profiling can be used to assign a protein as either a true component of an organelle or a contaminating component, thereby monitoring isolation purity and reducing false-positive identifications.⁵¹ However, the profiling strategy is complicated by the fact that proteins often shuffle among compartments and, therefore, may not be assignable to only 1 organelle. Additionally, while profiling provides information on whether the protein is present in a particular compartment, it does not elucidate function.

We have recently developed a subfractionation approach whereby the left ventricle is decellularized to remove cells while leaving the ECM intact (Figure 3).^{58,59} Our protocol was modified from work done by the Christman laboratory, which uses a decellularization protocol to prepare tissue-specific ECM coatings for cell culture and other bioengineering

applications.⁵⁸ They found that human embryonic derived stem cells grown in cardiac-derived ECM matured more fully into a cardiomyocyte (eg, formed desmosomes) compared with cells grown on the standard gelatin substrate. This technique uses sodium dodecyl sulfate to break up cell and organelle membranes. A premise to this approach is that the cytosolic proteins and intracellular organelles will be washed away in the sodium dodecyl sulfate buffer. In order for this technique to be successful, the intracellular protein content must be washed away. Additionally, residual sodium dodecyl sulfate needs to be washed away to avoid interference with downstream mass spectrometry analyses. Similar techniques have been developed by the Mayr laboratory to examine vascular ECM.⁶⁰ An advantage of this strategy is that it can be

Decellularization Protocol

1. Separate LV from atria and RV.
2. Rinse with deionized water for 30 min.
3. Incubate in 1% SDS in PBS with protease inhibitors at room temperature for 4-5 days.
4. Rinse with deionized water overnight.

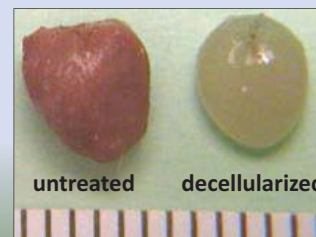


Figure 3. Decellularization strategy to enrich for relatively low abundance ECM proteins. The image shows a mouse untreated left ventricle (left) and a decellularized left ventricle (right).

Table 2. Strategies to Evaluate MMP-Mediated ECM Peptide Generation and Determine Effects of the Peptides on Post-MI Remodeling

1. Observe which ECM proteins in post-MI samples have lower than expected molecular weight as detected by mass spectrometry or immunoblotting.
2. Demonstrate that purified MMP cleaves the recombinant ECM protein in vitro.
3. Determine if the ECM proteolytic fragments generated in vitro are the same ones generated in vivo post-MI.
4. Determine whether alterations in MMP levels (e.g., using MMP-null or transgenic mice) changes the ECM peptide levels.
5. Determine if the ECM peptide has biological activity on:
 - a. Cell specific functions in vitro, relevant cells include myocytes, leukocytes (neutrophils, macrophages, and lymphocytes), fibroblasts, endothelial cells, and vascular smooth muscle cells.
 - b. LV specific functions in vivo by assessing if blocking or increasing ECM peptide levels alter left ventricular remodeling post-MI.

ECM indicates extracellular matrix; LV, left ventricular; MI, myocardial infarction; MMP, matrix metalloproteinases.

coupled with protein separation on a 1-dimensional electrophoresis gel to provide molecular weight information, which can reflect ECM proteolysis that may occur during disease progression. This approach is currently being used to examine ECM and matrix metalloproteinase-dependent ECM peptide formation during left ventricular remodeling, with a representative experimental design shown in Table 2.

Incorporating Functional Proteomics into Systems Biology Approaches to Assign Cause and Effect Relationships Between Proteases and Specific ECM Targets

Proteomics can be used to better understand protein, cell, and tissue function, for the purpose of identifying mechanistic indicators, prognostic predictors, therapeutic assessors of cardiovascular disease, and biosignatures that inform us on individual responses.^{61,62} Functional proteomic strategies also can be applied to isolated cell proteomes to give us information on cell function during disease development and progression. For example, isolating cardiac fibroblasts during different stages of post-MI remodeling can provide functional clues about how the fibroblast regulates cardiac remodeling. However, an approach that involves examination of cell-specific proteomes has the potential to overlook secreted proteins. Evaluating both the isolated cell proteome and the corresponding cell secretome is necessary to fully understand the contribution of that particular cell.⁶³ In left ventricular samples, relevant cells include the cardiac myocyte, fibroblasts, endothelial cells, smooth muscle cells, and (during injury responses) infiltrating inflammatory cells. Of these cell types, the fibroblast is the major source of cardiac ECM and the most prominent target of ECM proteomic approaches.

Future Directions and Conclusions

Several areas actively under development that have relevance to cardiac ECM proteomics include:

(1) Target ECM Using Unbiased, Informed Approaches

Protein microarrays, including currently available antibody arrays, typically ignore ECM proteins in their arrays. Protein

array data can be coupled with mRNA expression array analysis of the same samples to increase the confidence that any observed changes are accurate and true. While this approach minimizes false-positive results, it does not provide information about protein post-translational modifications, which are an abundant component of ECM complexity. This approach also assumes equal protein breakdown and synthesis rates, which may be true at equilibrium but not in the post-MI setting.

Along these same lines, profiling ECM pathways that are perturbed early in the initiation of congestive heart failure, before symptoms become irreversible, is needed. Using multiple techniques that overlap in diagnostic or prognostic capabilities and combining datasets by using pathway analysis software can increase the effect size by several orders of magnitude and allow more accurate detection of protein changes. These proteins then can be confirmed to occur in human patients using case-controlled studies.

(2) Include Post-Translational Modifications Specific to ECM

In the mitochondrial proteome, post-translational modifications notably include phosphorylation, nitrosylation, and O-GlcNAcylation, which are transient and, therefore, difficult to detect.^{25,64} In the ECM, in contrast, post-translational modifications tend to be more permanent, with the most common modifications being hydroxylation, glycosylation, and cross-linking.^{65,66} Additionally, global and site-specific glycosylation can be visualized using stains such as the Pro-Q Emerald stain.⁶⁷ Therefore, adding the posttranslational dimension to the ECM proteome catalog is quite possible.

(3) Incorporate ECM into Systems Biology Approaches

One limitation of existing pathway analysis software programs is that the ECM is largely ignored or is listed as an intracellular component. While cluster analysis is a useful method to analyze proteomic datasets, clusters are often grouped by cytosolic, microsomal, mitochondrial, and nuclear fractions, totally excluding the extracellular compartment. The same is true for computational tools, including protein correlation profiling software.²⁶ Another limitation is that currently available programs do not take into consideration the fact that left ventricular tissue is heterogeneous in terms of cell composition. Normal functioning myocytes, hypoxic myocytes, necrotic myocytes, and apoptotic myocytes all coexist in the post-MI LV, but vary in number temporally and spatially. Likewise, included in the general designation of fibroblasts are resident fibroblast cells, infiltrating fibrocytes, myofibroblasts, and protomyofibroblasts, all of which alter ECM composition in different ways.^{68–70} Therefore, consideration of the cell complexity needs to be incorporated into systems biology software tools.

(4) Extend Tissue Proteomics to the Plasma

Plasma can be a valuable source of accessible protein markers, providing distinct barcodes to reflect cardiac disease status.^{61,71,72} As recently as 35 years ago, only 40 proteins could be measured routinely in plasma.⁷³ Currently, thousands of proteins can be analyzed, after depletion of albumin and other highly abundant proteins.^{63,74,75} Analyses based on

the Luminex technology (also available commercially, for example, from Rules-Based-Medicine, Austin, TX), permit multi-analyte profiling of up to 100 cytokines, growth factors, and other analytes in a 100- μ L plasma sample. While plasma analysis serves as a promising avenue for clinical applications, there is a need for uniform blood collection and sample processing protocols to minimize handling artifacts. Consistent sampling procedures are necessary to reproducibly evaluate plasma for clinical diagnostic potential.²⁶

As in other areas of scientific investigation, the validity of the results obtained with proteomic analysis is dependent on proper study design, controls, statistical analysis, and validation. In terms of predicting which post-MI patients are likely to progress to congestive heart failure, techniques to monitor a panel of complementary markers, rather than a single marker, likely will need to be developed in order to examine ECM patterns and obtain a more complete prognostic picture. Additionally, the most informative markers likely will be found in multiple screens and demonstrate a functional role in the initiation or progression of CHF. In conclusion, the progress in ECM proteomics seen in the last 10 years undoubtedly will continue into the future as we develop this list of markers.

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Disclosures

None.

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