Published in final edited form as:
Circulation. 2012 February 14; 125(6): 746–748. doi:10.1161/CIRCULATIONAHA.111.086835.

Extracellular Matrix Proteomics in Cardiac Ischemia/ Reperfusion: The Search is On

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Keywords

Editorial; Extracellular Matrix Proteomics; Extracellular Matrix; Ischemia-Reperfusion; Proteomics

Prolonged myocardial ischemia leads to myocytenecrosis, which initiates a localized inflammatory response that catabolizes the cellular debris and initiates deposition of extracellular matrix (ECM) proteins.[1] In addition to providing a vital structural framework for the three-dimensional organization of cells, the ECM determines the physical properties of biological tissues and acts as a dynamic microenvironment for cellular signaling. A number of cardiac diseases, including myocardial ischemia and reperfusion (I/R), are associated with qualitative and quantitative alterations in ECM proteins.[2] Understanding ECM changes is important for identifying factors that tip the balance between favorable reparative remodeling following myonecrosis and adverse remodeling that leads to progressive ventricular dilatation and congestive heart failure.

Extracellular matrix proteomics is the comprehensive description of the ECM expressed in a tissue at the time of evaluation. This approach generates a global, integrated view of extracellular processes and networks at the protein level. The goal of extracellular matrix proteomics is to reconcile gene activation to a particular extracellular phenotype. The ability to examine multiple proteins simultaneously is a substantial improvement over previous ECM studies that simply evaluated the total concentration of single proteins (i.e., collagen) without consideration of type (e.g., type I vs III vs IV) or quality (full-length collagen vs partially degraded).

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Conflict of Interest Disclosures: None

In this issue of *Circulation*, Barallobre-Barreiro and colleagues present an innovative extracellular matridomic approach for the analysis of ECM protein changes in a porcine I/R model.[3] Using a novel proteomic method to enrich for ECM proteins, tissue from infarcted and border zone regions of the left ventricle (LV) were analyzed separately by liquid chromatography tandem mass spectrometry (LC-MS) for peptide identification and compared to control animals without I/R. Using these techniques, they identified 139 ECM proteins in porcine hearts subjected to 2 hours of ischemia followed by 15 or 60 days of reperfusion. Of the 139 proteins, 15 were identified for the first time in cardiac ECM (Table 1). Whereas many of the ECM proteins catalogued are known to have roles in wound healing, fibrosis, tissue remodeling, ECM polymerization, collagen fibril organization and cell-ECM interaction, many of the newly identified cardiac ECM proteins have heretofore been associated with cartilage homeostasis, which involves pathways not known to be relevant to cardiac remodeling. For example, cartilage intermediate layer protein 1 has previously been associated with chondrocytes and adipocytes, but not with cardiac myocytes or fibroblasts.

In addition to identifying ECM proteins, the investigators performed a principal component analysis, which provides a visual display of regional and temporal differences in ECM post I/R. This statistical method is useful in analyzing complex data sets to reveal hidden data trends by summarizing (but not clustering) the data. Using this analysis, the investigators show that for both soluble and insoluble protein fractions the ECM proteins identified in the I/R groups are distinct from the controls.

Examining ECM at the proteomic level has several advantages over examining it at the genomic level. In contrast to gene array studies that only reveal which genes are upregulated at the time of evaluation, ECM extracellular matrix proteomics assesses proteins that have accumulated in the tissue over time. Proteomics also provides information regarding post-translational protein modifications -- which are highly abundant in ECM -- and plasma and extra-cardiac cell derived ECM proteins that are not regulated by cardiac genes and, therefore, not identified by gene array analysis. Hence, to fully understand cardiac remodeling following I/R, an assessment of extracellular matrix proteomics is essential.

A major challenge faced by investigators who study cardiac ECM is detecting and identifying low abundance proteins at their physiologic concentrations. For example, analysis of the entire LV in animal models of myocardial infarction (MI)via two-dimensional electrophoresis gels results in the identification of a preponderance of mitochondrial proteins and no ECM proteins, as cardiomyocytes in the non-infarcted region contribute to 90% of the tissue volume and mitochondria make up 30% of the cardiomyocyte cell volume. Thus, the contribution of the ECM, while prominent in the infarct scar, is minimized when the entire LV is analyzed.[4] A similar obstacle is encountered when human myocardial samples are analyzed.[5] To enhance detection of ECM, the infarct-which is devoid of cardiomyocytes -- and non-infarcted regions can be analyzed separately. Using this approach, we previously demonstrated matrix metalloproteinase (MMP)-9 dependent changes in infarct ECM composition post-MI, with fibronectin being an *in vivo* substrate of MMP-9.[6] Similarly, Barallobre-Barreirot *et al* demonstrate robust expression of fibronectin at day 15 post-I/R.

The study in this issue of *Circulation* is notable for several reasons. First, by using a sequential extraction approach Barallobre-Barreiro and colleagues identified soluble, newly synthesized ECM as well as insoluble integral ECM components (e.g., cross-linked collagen, proteoglycans, and glycoproteins). In cataloging the ECM matridome at 15 and 60 days post-I/R, they also provide candidate soluble ECM components that may be detected in the plasma for monitoring the post-MI repair process to determine if optimal cardiac

remodeling is occurring. The catalogue also provides targets to explore for therapeutic intervention. Since repeated tissue sampling (i.e., cardiac biopsy) to determine cardiac ECM composition is not feasible in the clinical setting, identification of novel plasma biomarkers and bio-signatures for prognostication in the post-MI setting is essential. Examining the soluble fractions also adds an extra dimension to the ECM catalogue by providing information on proteins that may undergo proteolysis in the I/R setting. ECM fragments (designated asmatricryptins) serve as potent stimulators of cell signaling, but information regarding their formation in I/R is limited.[7]

Second, by performing real-time PCR on the same tissue specimens used for proteomic analysis, the investigators revealed a discordant regulation of protein and messenger RNA levels in the injury and border-zone regions: although gene expression for selected ECM proteins was similar in both regions, the corresponding protein levels were much higher in the infarct area. This underscores the varying, but complimentary, information obtained from genomic and proteomic analyses. That there was little overlap between the gene and protein levels also underscores the fact that the best way to validate proteomic data is to use immunoblotting or ELISA methods to confirm protein level changes.

Third, by analyzing border and infarct regions at 15 and 60 days post-MI, the investigators provide spatial and temporal data on ECM scar composition changes in response to I/R. At 15 days post-MI, few ECM proteins changed significantly at the border zone while more pronounced changes were observed within the infarct area. Of special interest, cartilage and bone-related proteins such as aggrecan and chondroadher in were present at day 60 but not 15 days post-I/R. By combining the protein signature of early and late-stage remodeling with an analysis of protein network interactions, the investigators propose that transforming growth factor beta 1 ($TGF\beta_1$) plays a pivotal role in regulating ECM post-MI. However, it is interesting to note that even though several members of the TGF signaling pathway were identified in their study, $TGF\beta_1$ itself was not. Further studies are needed to establish the causal and effect relationships of the proteins interactions identified.

Fourth, several of the novel cardiac ECM proteins identified in the porcine I/R model were validated in LV tissue from patients with ischemic cardiomyopathy obtained at the time of cardiac transplantation, which demonstrates the potential clinical application of their findings. Although their I/R model and end stage ischemic cardiomyopathy represent different time points along the post-ischemia time continuum, the persistent increase in these particular ECM proteins may provide mechanistic clues on factors that influence the progression to adverse remodeling.

The study has several limitations. Despite reperfusion, the prolonged (i.e., 2 hour) episode of ischemia caused irreversible myocardial damage. Therefore, this study examined the ECM changes following reperfusion of infarcted, rather than ischemic, myocardium. Whether similar ECM changes occur in myocardium that is (a) ischemic but viable or (b) infarcted without reperfusion remains to be tested. Because of the cost and complexity of the study, the analysis was limited to 2 time points (15 and 60 days post-I/R) analyzed in a relatively small number of animals. A more thorough evaluation of early ECM changes is needed to understand the interplay between the inflammatory and fibrotic responses and to identify appropriate prognostic and therapeutic targets. For example, MMP-2 was the only MMP identified in the ECM matridome, whereas other MMPs known to be involved in early post-MI remodeling, including MMP-3, -7, and -9 [8], were not identified.

One caveat of the procedure utilized by the investigators for ECM enrichment is that this protocol does not likely solubilize the entire ECM, thereby providing an incomplete representation of ECM composition. Future studies using a solubilization buffer that

completely dissolves the ECM into constituent components may better inform how cross linked ECM is modified during the post-I/R response. Along these same lines, the investigators did not analyze cardiac tissue in the non-infarcted remote region of hearts subjected to I/R. Hence, it is unknown if the ECM changes (a) are the result of local signals and hencerestricted to the infarct and border zones or (b) occur diffusely throughout the myocardium and result from hemodynamic (i.e., LV wall stress) or systemic neuro hormonal activation (i.e., the renin-angiotensin and sympathetic nervous system). Finally, the animals studied did not receive drugs that are routinely administered to patients post-MI (i.e., angiotensin-converting enzyme inhibitors, beta-adrenergic blockers, statins, antiplatelet agents, and aldosterone inhibitors), many of which are known to affect cardiac remodeling and may alter the ECM composition.

In conclusion, the in-depth analysis of the cardiac ECM response to I/R by Barallobre-Barreiro *et al* provides candidate biomarkers of remodeling that extend our list beyond collagen. Future animal studies using experimental conditions, such as genetic deletions in one (or more) components of pathways thought to be important in ECM regulation may provide a more comprehensive understanding of ECM involvement in cardiac remodeling. The information gained from ECM matridomic studies should continue to advance our understanding on how to prevent, or at least diagnose, progressive cardiac remodeling that will transition to heart failure.

Acknowledgments

Sources of Funding: We acknowledge support from NIH NHLBI T32 HL07446 to KYD; from the Rapoport Foundation for Cardiovascular Research to RAL; and from NHLBIHHSN 268201000036C (N01-HV-00244) for the UTHSCSA Cardiovascular Proteomics Centerand R01 HL075360, the Max and Minnie Tomerlin Voelcker Fund, and the Veteran's Administration (Merit) to MLL.

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Table 1

Newly identified proteins in the post-I/R cardiac ECM.

Accession number	Name	Currently known functions
AGRIN_HUMAN	Agrin	Aggregation of acetylcholine receptors
ASPN_HUMAN	Asporin	Negative regulator of chondrogenesis
CILP1_HUMAN	Cartilage intermediate layer protein 1	Cartilage scaffolding
CO4A2_HUMAN	Collagen alpha-2 (IV)	Structural integrity of basement membrane
COCA1_HUMAN	Collagen alpha-1 (XII)	Mediates interaction between collagen fibrils and other extracellular matrix proteins
COEA1_HUMAN	Collagen alpha-1 (XIV)	Integrating collagen bundles
EMIL1_HUMAN	EMILIN-1	Elastogenesis and maintenance of vascular cell morphology
MATN4_HUMAN	Matrilin-4	Formation of filamentous networks
NID1_HUMAN	Nidogen-1	Connects network of collagens and laminins
PODN_HUMAN	Podocan	Growth and migration of cells in cardiovascular tissues
PRELP_HUMAN	Prolargin	Anchoring basement membranes to underlying connective tissue
SPON1_HUMAN	Spondin-1	Axon outgrowth and cell migration during development
TENX_HUMAN	Tenascin-X	Matrix maturation during wound healing
TETN_HUMAN	Tetranectin	Fibrinolysys and proteolysis during tissue remodeling
TINAL_HUMAN	Tubulointerstitial nephritis antigen-like	Promotes cell adhesion and spreading