Extracellular Matrix Remodeling: The Common Denominator in Connective Tissue Diseases

Possibilities for Evaluation and Current Understanding of the Matrix as More Than a Passive Architecture, But a Key Player in Tissue Failure

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ABSTRACT

Increased attention is paid to the structural components of tissues. These components are mostly collagens and various proteoglycans. Emerging evidence suggests that altered components and noncoded modifications of the matrix may be both initiators and drivers of disease, exemplified by excessive tissue remodeling leading to tissue stiffness, as well as by changes in the signaling potential of both intact matrix and fragments thereof. Although tissue structure until recently was viewed as a simple architecture anchoring cells and proteins, this complex grid may contain essential information enabling the maintenance of the structure and normal functioning of tissue. The aims of this review are to (1) discuss the structural components of the matrix and the relevance of their mutations to the pathology of diseases such as fibrosis and cancer, (2) introduce the possibility that post-translational modifications (PTMs), such as protease cleavage, citrullination, cross-linking, nitrosylation, glycosylation, and isomerization, generated during pathology, may be unique, disease-specific biochemical markers, (3) list and review the range of simple enzyme-linked immunosorbent assays (ELISAs) that have been developed for assessing the extracellular matrix (ECM) and detecting abnormal ECM remodeling, and (4) discuss whether some PTMs are the cause or consequence of disease. New evidence clearly suggests that the ECM at some point in the pathogenesis becomes a driver of disease. These pathological modified ECM proteins may allow insights into complicated pathologies in which the end stage is excessive tissue remodeling, and provide unique and more pathology-specific biochemical markers.

INTRODUCTION

The extracellular matrix (ECM) is of paramount importance for tissue function, and controls cell phenotype and function. That was initially illustrated by Mintz and colleagues who showed that the normal mouse embryonic tissue microenvironment could repress expression of the tumor phenotype;[1,2] thus, the ECM was able to control genotype/phenotype relationships. These interactions between cells and the ECM components are mediated through receptors, such as integrins and the discoidin receptors.[3] To maintain healthy tissue, the ECM must regenerate itself by normal remodeling, in which old or damaged proteins are broken down in a specific sequence of proteolytic events and replaced by new proteins. However, during pathological conditions, such as cancer, fibrosis, and inflammation, the delicate repair–response balance is disturbed.[4,5] The original proteins of the ECM are replaced by different matrix constituents, and consequently, the composition and quality of the matrix are altered. During cancer and fibrosis propagation, the ECM may be stiffened, and this can actually enhance tumor cell migration, myofibroblast activation, and collagen deposition,[6–14] thereby linking the actual matrix quality to disease progression.

ABBREVIATIONS: 2-D, two-dimensional; 3-D, three-dimensional; AASLD, American Association for the Study of Liver Disease; AGE, advanced glycation/glycosylation end product; BM, basement membrane; BSAP, bone-specific alkaline phosphatase; CIA, collagen-induced arthritis; CL, cutis laxa; COMP, cartilage oligomeric matrix protein; CRP, C-reactive protein; CTX-I, cross-linked C-terminal telopeptide of type I collagen; CTX-II, cross-linked C-terminal telopeptide of type II collagen; DDR, discoidin domain receptors; EAE, experimental autoimmune encephalomyelitis; ECM, extracellular matrix; ECMR, ECM remodeling; ELISA, enzyme-linked immunosorbent assay; EMT, epithelial-to-mesenchymal transition; GAG, glycosaminoglycan; hMFB, hepatic myofibroblast-like cells; HDAC1, histone deacetylase 1; HSCs, hepatic stellate cells; ICTP, C-terminal telopeptide of type I collagen; LN, laminin N-terminal; LOX, lysyl oxidase; MED, multiple epiphyseal dysplasia; MHC, major histocompatibility complex; MMP, matrix metalloproteinase; MS, multiple sclerosis; NO, nitrogen oxide; NTX, N-telopeptide of type I collagen; PADs, peptidylarginine deiminases; PDGF, platelet-derived growth factor; PIIANP, type IIA procollagen N-terminal peptide; PRELP, proline arginine-rich end leucine-rich repeat protein; PSACH, pseudoachondroplasia; PTM, post-translational modifications; RA, rheumatoid arthritis; RAGE, receptor for AGEs; RNS, reactive nitrogen species; ROS, reactive oxygen species; SLE, systemic lupus erythematosus; SLRPs, small leucine-rich proteoglycans; SMCD, Schmid type metaphyseal chondrodysplasia; SMD, spondylometaphyseal dysplasia; SVAS, supravalvular aortic stenosis; TG, transglutaminase; TGF, transforming growth factor; TNF, tumor necrosis factor; WBS, William-Beuren syndrome.

DOI: 10.1089/adt.2012.474

During this pathological remodeling of the ECM, excessive levels of tissue- and pathology-specific turnover products are released into the circulation. Turnover products holding post-translational modifications (PTMs) are defined as modifications made secondary to translation of the protein into the peptide sequence from mRNA. Thus, most PTMs are not directly DNA coded, and are a consequence of tissue physiology and pathophysiology.[15,16] PTMs may be derived from processes, such as aging (in which amino acid isomerization occurs), citrullination (during inflammation), protease degradation (fibrosis and inflammation), and glycosylation (diabetes),[15,16] as will be carefully discussed. Protease-generated neoepitopes have, to date, received more attention than other PTMs. However, potentially important PTMs that are believed to be specific for cancer as well as fibrotic and other pathological conditions have recently been identified.[15,17–19] The PTMs made to proteins result in unique protein fingerprints.[20] These modified structures are prime candidates for biochemical marker development, as they may be more related to the pathogenesis than unmodified proteins. Several lines of independent evidence suggest that PTMs to specific proteins contribute to abnormal cellular proliferation, adhesion characteristics, and morphology,[21] and may cause many of the differences in cancer tissue compared to normal tissue.[21–26] Furthermore, the generation of PTMs of key structural proteins, generated by protease cleavage, citrullination, nitrosylation, glycosylation, and isomerization, is emerging as a critical factor in tissue homeostasis and remodeling. Thus, PTM profiles may be used as biochemical fingerprints for detecting and verifying the function and activity of key cellular signaling pathways[21–26] involved in tissue homeostasis and integrity. Additional lines of evidence highlight that the structural components of the matrix, after PTM, are central part of the pathogenesis itself,[15] thus highlighting the matrix structural proteins as central and active participants rather than passive bystanders in disease pathogenesis.

The aims of this review are to discuss the structural components of the matrix, the potential applicability to pathology, and the measurement of structural molecules in serum. We review the PTMs, which may be both a consequence of disease and a part of the pathogenesis, as exemplified by the role of tissue stiffness in cancer and fibrosis. Lastly, we list the current methods for measuring post-translational modified matrix proteins in serum. These PTMs may serve as disease-specific biochemical markers and assist in the identification of key molecular pathways leading to enhanced connective tissue remodeling.

FUNCTION OF THE ECM

The ECM is a three-dimensional (3-D) structure that encapsulates cells and defines their microenvironment.[27] It consists of a meshwork of proteins to which soluble factors, such as growth factors and cytokines, can bind. There are two main types of ECM. The first is the basement membrane (BM), which interacts directly with the epithelium and endothelium, and it is composed of primarily of type IV collagen, laminins, entactin/nidogen, and heparan sulfate proteoglycans (*e.g.*, perlecan) (*Fig. 1*).[28]

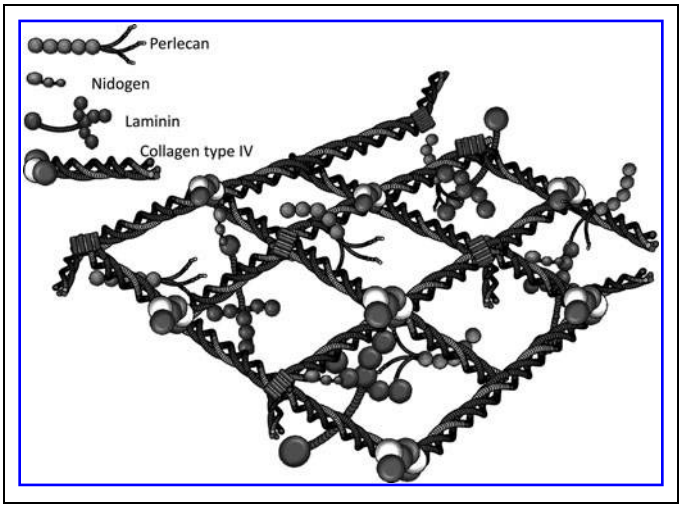

Fig. 1. The molecular structure of a typical basal lamina. The basal lamina is formed by specific interactions between the proteins type IV collagen, laminin, and entactin plus the proteoglycan Perlecan. Adapted by S.H. Madsen, from Yurchenco and Schittny.[28]

The second type is the interstitial matrix, which makes up the bulk of the ECM in the body. The interstitial matrix consists of many types of collagens, including types I and III, together with fibronectin. The interstitial matrix additionally consists of tenascin and proteoglycans that provide tissue hydration, enable binding of growth factors and cytokines to the tissue, and cross-link the matrix to enhance its integrity.[29]

Although originally considered as merely a support system for the cells within the tissue, the ECM is now recognized as a central regulator of cell and tissue behavior via transmembrane signaling.[1,30–33] While the basic characteristics and composition of the BM and interstitial matrix are constant across tissues, variations in ECM components, such as protein isoform expression, ratio between individual matrix components, and PTMs, contribute to differences in ECM organization and structure and ensure tissue specificity.[15] PTMs, such as glycosylation and cross-linking, significantly affect the mechanical properties of the ECM, including its viscoelasticity or stiffness. Both the stiffness and topology (3-D appearance) of the ECM regulate the growth, remodeling, differentiation, migration, and phenotype of a wide variety of cell and tissue types.[8–14,34]

MATRIX COMPOSITION AFFECTS CELL PHENOTYPE

The importance of matrix stiffness in tissue-specific differentiation is exemplified by the fact that cells grown as monolayers (two-dimensional: 2-D) on top of either a plastic substrate or a glass coverslip, with or without ECM ligand, fail to assemble the same tissue-like structures as those growing in the normal ECM (3-D). Cells growing on plastic or glass are less likely to express differentiated proteins upon stimulation,[34] or respond to growth factors or protease inhibitors in the same way as cells growing in a 3-D setting.[35] These phenotypic disparities can be explained, in part, by the fact that

living tissues in 3-D emit biological signals that may be read by specific integrins, but this signaling is nonexistent in 2-D substrata such as tissue culture on plastic. The role of cell polarity versus non-polarity in cultures is receiving increased attention. Another illustration of this phenomenon is that when epithelial cells and melanocytes are grown in a 3-D ECM microenvironment, they assemble into tissue-like structures and express differentiated proteins when given the correct soluble stimuli.[36] Neither behavior is seen when the same cells are cultured on 2-D plastic substrata.

The architecture of the interstitial matrix *in vivo* also differs substantially from that found typically in tissues cultured on plastic, and this too can have dramatic effects on cell behavior.[35] For instance, osteoblasts grown on plastic in 2-D do not rely on matrix metalloproteinases (MMPs) for survival, whereas osteoblasts embedded in an interstitial matrix, such as 3-D type I collagen, are critically dependent for their survival on MMP activation of latent transforming growth factor (TGF)-β.[35] Thus, the matrix architecture is crucial to the phenotype and survival of cells. Interestingly, the orientation of collagen fibers can critically regulate cell and tissue behavior.[37–39] This 3-D contextual information is lost when cells are grown in 2-D.

Varying components of the ECM also influence the ability of the matrix to regulate cell and tissue behavior. The ECM transmits signals through various specialized cell membrane receptors, including integrins, discoidin domain receptors (DDRs), and syndecans.[40–44] Integrins provide an excellent model of how an altered ECM could promote tumor progression. Integrins consist of 24 distinct transmembrane heterodimers that relay cues from the surrounding ECM to regulate cell growth, survival, motility, invasion, and differentiation.[40–44] They are able to interact with the ECM externally, and with cytoplasmic adhesion plaque proteins and the cytoskeleton intracellularly to influence cell behavior. Integrin–ECM interactions regulate the cell fate by activating multiple biochemical signaling circuits and altering the cell shape.[45,46] This occurs either through direct interactions between ECM receptors and actin-linked proteins or cytoskeletal reorganization induced by activating cytoskeletal-remodeling enzymes, such as RhoGTPases.[45,46]

This section highlights that the composition of the ECM affects the phenotype of cells through specific receptor-mediated interactions. Certain ECM compositions and structures result in a context-dependent response to a given stimulus, which is absent in other experimental settings.

ECM PROTEINS

The ECM mainly consists of collagens and proteoglycans, each with their unique function. In the following section, the most important and well-investigated collagens and proteoglycans are discussed, together with other important structural components of the ECM.

Collagens

Collagens are a family of proteins made up of three α-chains supercoiled around each other completely or partially in a triple helix with a characteristic Gly-X-Y repeat. Intra- and intermolecular cross-links bring stability to the collagen molecules, contributing to the characteristically high tensile strength and minimal extensibility of collagen. Type I, II, III, and V collagens belong to the group of fibrillar collagens, which are the most abundant collagen group in the body. In addition to the triple-helical domain, they also contain N- and C-terminal propeptide domains that are cleaved off by N- and C-procollagenases, respectively, before fibril assembly.[47] Type I–VI collagens are the most well described at present and are the focus of this section.

Type I collagen. Type I collagen is composed of the heterotrimer $\alpha_1\alpha_1\alpha_2$(I) and is the most abundant type of collagen that is ubiquitously expressed. It provides tensile stiffness in bone and has important load-bearing, tensile strength, and stress-carrying properties in other tissues as well. In tendons, type I collagen fibrils are arranged in parallel to form bundles, whereas in skin, the arrangement is more random, forming a complex network of interlaced fibrils. These different arrangements contribute to the different properties of the tissues. Type I collagen is often incorporated into fibrils with either type III[48] or type V collagen.[49] The synthesis, concentration, and circulating levels (serum concentration) of degradation products of type I collagen have been proven to be increased during breast, bone, lung, ovarian, prostate, and skin malignancy.[50–55]

Type II collagen. Type II collagen is the major component of hyaline cartilage, but is also found in the vitreous body of the eye, the corneal epithelium, the notochord, the nucleus pulposus of invertebral discs, and embryonic epithelial-to-mesenchymal transitions (EMTs).[47] Type II collagen is a homotrimer consisting of three α_1(II) chains, and the primary sequence has a high content of hydroxylysine and glycosyl residues, which mediate interactions with proteoglycans, another important component of hyaline cartilage. Type II collagen degradation is mainly associated with rheulatological diseases such as osteoarthritis and rheumatoid artiritis.[56]

Type III collagen. Type III collagen is mainly present in association with type I collagen and is an important component of the interstitial tissues of the lung, liver, dermis, spleen, and vessels. Type III collagen is a homotrimer consisting of three α_1(III) chains. A characteristic feature of type III collagen is that it is correlated to extensibility of tissues, and that it may contribute to elasticity, a property that is uniquely connected to this type of collagen.[57] Type III collagen has been mostly assiociated with various fibrotic diseases.[58–61]

Type IV collagen. Type IV collagen is the main component of the BM, a specialized type of ECM that separates the epithelium from the stroma in all tissues in the body. It consists of three domains: N-terminal 7S domain, a central triple helix, and a large C-terminal NC1 globular domain. Its triple helix is ∼25% longer than those seen in the fibrillar collagens, and the Gly-X-Y repeat is frequently interrupted, accounting for the relatively high flexibility of this type of collagen.[28] Instead of fibrils, type IV collagen molecules assemble into a flexible 3-D network. The most abundant isoform of type IV collagen is $\alpha_1\alpha_1\alpha_2$(IV), but tissue-specific isoforms also exist:

$\alpha_3\alpha_4\alpha_5$(IV) heterotrimers are found in the lung, glomeruli of the kidney, cochlea, eyes, and testis, whereas $\alpha_5\alpha_5\alpha_6$(IV) is found in skin, Bowman's capsule of the kidney, the esophagus, and the knee joint.[62] Turnover of the basement membrane is associated with a range of diseases.

Type V collagen. The most common structure of type V collagen is $\alpha_1\alpha_1\alpha_2$(V), although homotrimers of three α_1(V) chains and heterotrimers of the $\alpha_1\alpha_2\alpha_3$(V) isoforms have also been detected.[63] Type V collagen is expressed in tissues containing type I collagen, but is a quantitatively minor component.[64] It typically forms heterofibrils with type I collagen,[49,64] where it makes up the core structure of these heterotypic fibrils. Type V collagen is of special importance for the structure of tissues. It has been shown to be essential for the correct assembly of collagen fibrils and to regulate their size and organization.[65] This characteristic makes type V collagen especially unique and interesting to study. The N-terminal domain contains a high level of tyrosine sulfated residues that contribute to the strong interactions that type V collagen has with triple-helical domains of other collagen types. This enhances the stability of fibrils.[66]

Type VI collagen. Type VI collagen is a heterotrimeric molecule with the isoform $\alpha_1\alpha_2\alpha_3$(VI) and consists of a short triple helix flanked by two extended globular domains, and it is expressed, albeit variable, in virtually all tissues. The primary fibrils are arranged in overlapping dimers in an antiparallel manner and form parallel tetramers that are stabilized by intermolecular disulfide bonds. They aggregate to form filaments and an independent microfibrillar network. Type VI collagen molecules have a uniquely beaded appearance and interact with several ECM components such as type I collagen and fibronectin.[67]

Proteoglycans

Proteoglycans are ECM macromolecules formed by a protein core with one or more glycosaminoglycans (GAGs) bound covalently. Due to the negative charge and structural conformation of GAGs, proteoglycans can interact with a large variety of macromolecules.[68] Proteoglycans can be divided into five families according to the structural properties of their core protein.[69,70]

The small leucine-rich proteoglycan (SLRP) family is formed by proteoglycans that bind specifically to other ECM constituents and contribute to the structural framework of connective tissues. SLRPs are small molecules, with core proteins of 40 kDa, and possess characteristic 6–10 leucine residues at conserved locations between the flanking cystein-rich disulfide-bonded domains at the N- and C-terminus that participate in protein–protein interactions with collagens, matrix glycoproteins, and cell membrane components.[70,71] Based on several parameters, including gene organization and amino acid homologies, SLRPs are further divided into five classes: class I includes decorin, biglycan, and asporin; class II includes fibromodulin, lumican, keratocan, proline arginine-rich end leucine-rich repeat protein (PRELP), and osteoadherin; class III includes epiphycan, mimecan, and opticin; class IV includes chondroadherin and nyctalopin; and class V includes podocan.[69,72]

Decorin, fibromodulin, asporin, lumican, PRELP, and chondroadherin can interact with collagen and influence collagen fibril formation and interaction.[69] In addition to their ECM functions in tissue hydration and collagen fibrillogenesis, proteoglycans are able to influence tissue repair and tumor growth, to facilitate cellular adhesion, proliferation, and migration, and to modulate growth factors and cytokine activities. For this reason, they are referred to as matricellular protein, with the ability to modulate cell–matrix interactions and cell functions.[72] In particular, decorin, biglycan, and lumican exert many modulation roles in different biological processes. These functions highlight the important effect of ECM components in the cellular phenotype by influencing cell communication through, that is, signal transduction, cytokine modulation, adhesion, and migration.[72] All the different matricellular functions exerted by these three important SLRPs are detailed in *Table 1*.[3,73–118]

Some of the most important proteoglycans expressed in the ECM are briefly described in the following paragraphs.

Aggrecan. Aggrecan is the major proteoglycan of the cartilage, and it is the most highly glycosylated, with 150 chondroitin sulfate and keratan sulfate GAGs bound to a large central core protein. Through its specific binding with hyaluronan and link protein, it forms a supramolecular structure whose characteristics make it able to retain water molecules in the cartilage, providing the tissue with the property of resisting compressional forces with minimal deformation.[69]

Versican. Versican is a large interstitial chondroitin sulfate proteoglycan. It is present in many tissues, and it is one of the principal ECM components of normal blood vessels where it influences the assembly of ECM and controls elastic fiber fibrillogenesis.[119] It is present in the intima and adventitia of most arteries and veins, and it is synthesized by vascular smooth muscle cells as well as endothelial cells, myofibroblasts, and macrophages.[120] Versican interacts with hyaluronan and link protein to form high molecular weight stable aggregates. These complexes create a reversibly compressive compartment and provide a swelling pressure within the ECM that is compensated by collagen and elastic fibers. Dramatically increased levels of versican have been observed in atherosclerosis and restenosis, implying that this proteoglycan is a specific component of developing lesions and contributes to their progression in atherosclerosis and restenosis.[119]

Perlecan. Perlecan is a heparan sulfate proteoglycan widely distributed in BMs, and it has the largest core protein found in proteoglycans. It is able to self-associate or interact with several other BM macromolecules, including laminin and type IV collagen.[68]

Decorin. Decorin is the most abundant SLRP in cartilage. It contains one GAG chain, often dermatan sulfate, which can adopt complex secondary structures and form specific interactions with matrix

Table 1. The Matricellular Effects of Extracellular Matrix Components

Protein or PTM	Cellular phenotype	Responsible receptor	Reference
Elastin-derived peptides	Chemotaxis of monocytes, fibroblasts, and endothelial cells	Elastin-binding protein in complex with protective protein/cathepsin A and neuraminidase-1	73,74
	Proliferation of fibroblasts and smooth muscle cells		
	Protease release from fibroblasts and leukocytes		
Thrombospondin	Inhibition of angiogenesis	CD36 and CD47	75–77
Type I collagen	Fibroblast migration	DDR2; integrins α_1, α_2, α_{10}, α_{11}, and β_1	3,78
Acetylated Proline–Glycine–Proline (acPGP; fragment of type I collagen)	Neutrophil chemotaxis	CXCR1 and CXCR2	79,80
Arresten, canstatin, and tumstatin (fragments of type IV collagen)	Inhibition of angiogenesis, tumor growth, and endothelial cell proliferation and migration.	Various integrins	81,82
	Induction of apoptosis		
Endostatin (fragment of collagen type XVIII)	Inhibition of endothelial proliferation, angiogenesis, and tumor growth	Glypicans, nucleolin	83–86
	Induction of endothelial cell apoptosis		
RGD motif (present in collagens, laminin, and fibronectin)	Cell adhesion, angiogenesis, and apoptosis	Various integrins	87,88
Fibromodulin	Proliferation, migration, and chemotaxis of HSCs	Unknown	89
Laminin-332 (elastase-generated fragment of γ_2)	Neutrophil chemotaxis	Unknown	90
SIKVAV and ASKVKV (sequences in linker regions between coiled-coil and globular domains of laminin α_1 and α_5 chains)	Neutrophil and macrophage chemotaxis	Unknown receptors; SIKVAV interacts with integrins α_1, α_6, and β_1 in the salivary gland carcinoma cell line	91,92
Laminin	Chemotactic migration of malignant cells toward laminin	67LR (LamR)	93,94
Lumican	Regulation of inflammation and innate immunity	CD14, FasL, CXCL1	95–98
	Apoptosis induction	Fas	
Biglycan	Regulation of inflammation and innate immunity	TLR2, TLR4, P2X4/P2X7, selectin L/CD44, C1q	99–110
	Cytokine modulation (PDGF, TGF-β, TNF-α, WISP-1, BMP-4)		
	Adhesion and migration	RhoA, Rac1	
Decorin	Signal transduction	LRP-1, c-MET	102–106, 110–118
	Cytokine modulation (PDGF, TGF-β, TNF-α, VWF, and WISP-1)		
	Regulation of inflammation and innate immunity	TGB-β, C1q	
	Antiapoptotic effect.	IGF-IR	
	Antioncogenic effect.	EGF-R, VEGF-R2,	
	Adhesion and migration	IGF-IR, integrin $\alpha_2\beta_1$, RhoA, Rac1	

PTMs, post-translational modifications; HSC, hepatic stellate cell; PDGF, platelet-derived growth factor; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

molecules. Its level increases with age. Its main function is to regulate collagen fibrillogenesis and to maintain tissue integrity by its binding with fibronectin and thrombospondin.[69] However, decorin also exerts important matricellular functions, favoring the cell–matrix interactions and influencing cell phenotype (see *Table 1*). Decorin is an important antifibrotic agent: it influences fibrogenesis in different organs by inhibiting TGF-β; it regulates ECM synthesis and turnover, and it is involved in regulation of cell death, adhesion, and migration.[72]

Biglycan. Biglycan is a small SLRP. It is found in many connective tissues, such as skin, bones, and blood vessels. Within the hyaline cartilage tissue, biglycan is localized mainly pericellularly.[70] Together with decorin, biglycan is a key regulator of the lateral assembly of collagen fibres, and it interacts primarily with type VI collagen.[69] Biglycan is thought to have a role also in fibrogenesis and in assembly of elastin fibers.[121] Moreover, this proteoglycan is able to bind to the membrane-bound proteoglycan, dystroglycan, and to a wide variety of proteins. It is involved, for instance, in cell signal transduction during cell growth and differentiation and in regulating cytokine activity through its capacity to bind TGF-β and tumor necrosis factor (TNF) α (see *Table 1*).[99]

Mimecan. Mimecan is a keratan sulfate proteoglycan belonging to the SLRP family, and it is the product of the gene that encodes for osteoglycin.[122] Its main role consists in regulating the collagen fibril diameter.[123] Apart from corneal tissue, where it has been first identified, mimecan is expressed also in other tissues, such as medullary bone,[124] amniotic membrane,[125] cartilage[126] and pituitary.[127] In the lung, mimecan mRNA expression is correlated to nonsmall-cell lung cancers,[128] and in arteries, there is an indication that it can be involved in arterial remodeling during atherosclerosis.[129]

Fibromodulin. Fibromodulin is one predominant SLRP in cartilage. It contains up to four keratan sulfate chains, and it is able to influence collagen fibril formation and maintain a sustained interaction with the formed fibrils.[69,130]

Lumican. Lumican is a highly biologically active SLRP. It can exist as proteoglycan (with GAG chains) and as glycoprotein (with mono- or polysaccharide chains). In the human adult cornea, it is present in the first form, whereas it is in a glycoprotein form in embryonic cornea and in skin. Lumican expression in cornea has been widely studied. In this tissue, it exerts its main role in controlling the polymerization of collagen into small-diameter fibrils.[131] Lumican is also highly present in skeletal muscle, kidneys, placenta, heart, intervertebral discs, blood vessels, intestine, uterus, and pancreas,[71] and it has a widespread distribution in connective tissues, including cartilage, where it modulates collagen fibrillogenesis and regulates the assembly and diameter of collagen fibers and interfibrillar spacing, enhancing collagen fibril stability.[132] Together with decorin and biglycan, it is an important component of the ECM exerting matricellular functions (see *Table 1*).

Other proteins. The glycoproteins fibronectin and tenascin C modulate the integrin-mediated adhesion of cells to other ECM proteins, such as collagens, and as such play a key role in cancer invasion. A single gene encodes fibronectin, but alternative splicing allows formation of multiple isoforms from which some are tumor specific.[133] The fibulins, Galectin-1 and Fibulin-1, function as intramolecular bridges in the organization of ECM supramolecular structures, such as elastic fibres and BMs.[134] Galectin-1 and Fibulin-1 can bind ECM components, that is, laminin and fibronectin, and therefore modifies the adhesive properties of cancer cells.[134–136]

Effect of Structural Proteins on Cellular Phenotype: Selected Examples

There is growing evidence that ECM molecules have functions other than structural roles, but as integrated players in the structure and functional homeostasis of tissue. A nonexhaustive list of these proteins is given in *Table 1*. This highlights that ECM proteins are beginning to be recognized as paracrine-signaling molecules, with profound effects on cellular phenotypes that until recently was restricted to cytokines, growth factors, and hormones.[137] Of particular relevance, which will be discussed later in this review, some proteins do not change cellular phenotypes in their native conformation, whereas subsequent to a specific PTM, a highly potent and novel function of that protein is revealed. A well-thought example of such cryptic sites is RGD sequences that are either exposed by protease digestion in most collagen species[87] or even more scholarly exemplified by endostatin, which is a fragment of collagen type XVIII that by cleavage becomes possibly the most powerful anti-angiogenic molecule to date.[83]

Table 1 lists examples from outstanding research groups which serve to highlight that the matrix encompasses strong signaling motifs that may be revealed during the pathological process. Consequently, the matrix molecules themselves, in addition to cytokines, growth factors, and hormones, become essential players in tissue homeostasis. As the ECM molecules both anchor cells in the right spatial distribution and cell orientation, these structural components may have a dual effect due to their emerging signaling roles.

ECM REMODELLING IN CANCER AND FIBROSIS

Cancer and fibrosis share a number of abnormal characteristics of the ECM structure and function, including constitutively high matrix degradation, formation, and turnover. Interestingly, both diseases involve aspects of inflammation and matrix assembly, destruction, and disorganization.[24,138,139] As illustrated in *Figure 2*, cancer cell metastasis results in extensive ECM remodeling (ECMR), resulting in the release of matrix components, including neoepitopes, into the circulation. ECM components and remodeling enzymes are known to be elevated in the circulation of cancer patients.[140,141]

The architecture of the tumor-associated ECM is fundamentally different from that of the normal tissue stroma.[142] As an example, type I collagen is situated parallel to the epithelial cells in healthy tissue, but is less organized in the stroma surrounding metastases.[143] These stromal changes to the ECM promote transformation, tumor

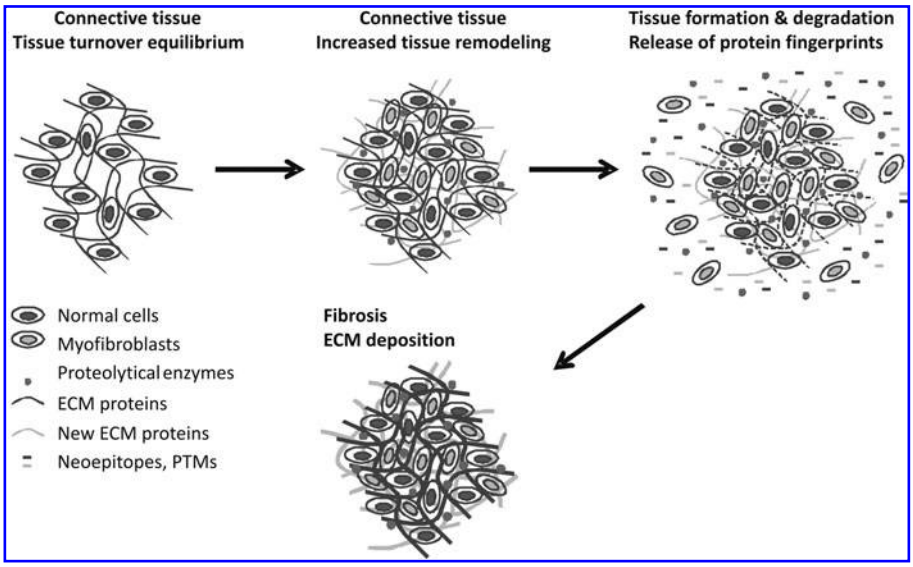

Fig. 2. Schematic representation of the high extracellular matrix remodeling in fibrosis. All steps involve extracellular matrix (ECM) remodeling that generates unique protein degradation fingerprints. These enzymes degrade the ECM, releasing smaller fragments of protein from the ECM into the circulation. Interestingly, many of the same processes occur in both fibrosis and cancer.

growth, motility, and invasion; enhance cancer cell survival; enable metastatic dissemination; and facilitate the establishment of tumor cells at distant sites.[143] Cancer is caused when the essential rules governing how cells should be organized in a stable manner within all living tissue are disregarded. Uncontrolled cell growth is necessary for cancer formation. Such growth becomes self-directed, leading to a disorganization of the normal tissue architecture, which is known as neoplastic transformation. More than 90% of malignant tumors are epithelial tumors,[4] occurring where there is a collapse in the boundary between the epithelial and connective tissues that encompass a given organ. Interruption of these tissue boundaries promotes cancer cell migration to nearby blood vessels or the lymph node system, enabling the cells to metastasize to remote organs resulting in multiorgan failure and death.

Fibrosis is an end-stage representation of a repair–response process after an injury. Like cancer, it may lead to serious organ damage. The development of liver fibrosis resembles the process of wound healing, including the three essential phases after tissue injury: inflammation, synthesis of collagenous and noncollagenous ECM components, and tissue remodeling. Fibrosis may begin in response to various acute or chronic stimuli, including infections, autoimmune reactions, toxins, radiation, and mechanical injury.[144] The pathogenic process driving fibrogenesis is believed to be a dynamic series of events involving complex cellular and molecular mechanisms evolving from the acute or chronic activation of tissue repair that follows repeated tissue injury,[5] In the case of liver fibrosis, these stimuli give rise to a series of events that involve several cell types working in synergy toward irreversible damage of the liver.[145]

Identification and characterization of the cell types and the different mediators involved in liver fibrogenesis have expanded significantly during recent years.[146–148] Hepatic stellate cells (HSCs) have been identified as the driving force of liver fibrosis. When HSCs are activated by inflammatory mediators,[149] they differentiate into hepatic myofibroblast-like cells (hMFB) capable of expression and secretion of several connective tissue components (for example, collagens, elastin, proteoglycans, and hyaluronan).[149,150] HSCs are believed to be the main source of ECM proteins accumulated in the liver during chronic liver disease. Recent research has clearly demonstrated that other cell types contribute to the hMFB-pool.[151–153] These cells can come from local sources such as portal myofibroblasts[154] or, may be, newly formed HSCs that originate from a process called EMT, in which biliary epithelial cells or hepatocytes transform into fibroblasts.[155] In addition, contributions to the hMFB-pool come from outside the liver from cells like bone marrow[156] and circulating fibrocytes.[157] The bone marrow-derived myofibroblasts have been shown to be of a surprisingly large importance, as they can transdifferentiate into epithelial cells.[158–161] The accumulation of fibrous tissue and myofibroblast contraction in the liver leads to mechanical increase of hepatic vascular resistance to portal vein blood.[159,162] This in turn leads to loss of oxygen to the surrounding tissue, facilitating neoangiogenesis as HSCs and Kupffer cells begin overexpressing proangiogenic growth factors and cytokines.[163]

The activation of HSCs involves multiple intracellular pathways and gene regulation. Regulation of growth factors plays an important role in HSC activation, with platelet-derived growth factor (PDGF) signaling is the best-characterized pathway leading to HSC activation. Binding of PDGF results in dimerization and phosphorylation of the tyrosine residues in the intracellular domain of the receptor. This activates the Ras/MAPK and the PI3K-AKT/PKB pathways, leading to cellular proliferation.[164] The increased matrix production by HSCs is controlled by TGF-β, which is the most potent fibrogenic cytokine in the liver, by signaling via Smad proteins.[165] Chemokines induce the NFκB signaling pathway, leading to further migration and proliferation of HSCs. Continued deposition of matrix proteins is controlled by a positive feedback loop that sustains the inflammatory response and proliferation and migration of HSCs as chemokines interact with immune cells.[166,167]

Disregulation of ECM homeostasis is also central in the development of fibrosis of the lung, although the origin of fibrogenic precursors remains a subject of debate, and is potentially multifactorial in nature. Activation of resident fibroblasts, recruitment of circulating progenitors such as fibrocytes or other candidate progenitors,

and EMT of alveolar epithelia have all been implicated in the formation of activated myofibroblasts[168–172] in the lung. Consistent with findings in fibrotic liver disease, these activated myofibroblasts produce fibrillar collagens such as type I collagen and other matrix proteins, which apart from promoting remodeling and ultimately scarring the lung parenchyma, drive a sustained cycle of ongoing fibrogenesis, even in the absence of ongoing inflammatory insult. As with liver fibrosis, studies in disease models indicate that TGF-β is a key fibrogenic cytokine. Together with other cytokines, signaling pathways, and matrix proteins, TGF-β contributes to the ongoing disease cascade and destructive remodeling of the lung.[172–178]

As a biomechanically sensitive organ, the lung could be considered as particularly dependent on the composition and architectural organization of ECM components, including BM collagens such as type IV collagen, structural fibrillar collagens (type I and III), and elastin.[179] The importance of collagen remodeling in resolution of fibrosis has been demonstrated in models in which inhibition of the lysyl oxidase (LOX) family member, LOXL2, which catalyzes the cross-linking of fibrillar collagen, and thereby increases tissue tension, was sufficient to reverse established fibroblast activation and reduce TGF-β signaling, cytokine production, inflammation, and other markers of profibrogenic imbalance.[14] These findings are consistent with previous data showing that ongoing myofibroblast activation and TGF-β signaling from the latency-associated complex can be driven by altered mechanical tension in a feed-forward loop.[180,181] Selective inhibition of LOXL2, which is overexpressed in both human fibrotic disease and disease models, may also constitute a therapeutic target. Inhibition of aberrant fibrogenesis, while avoiding inhibition of other LOX family members, such as LOX and LOXL1, may play a critical role in elastin homeostasis in the lung.[182,183]

Studies in humans and in animal models have suggested that some elements of fibrosis are reversible, and in specific circumstances, restoration to near-normal organ architecture can be achieved.[184–189] Consequent to these findings is an emerging interest in the fibrosis field with focus on the ECM components. Measurement of the individual molecules gives a deeper understanding of fibrosis and attenuates pathological processes.

Noninvasive biomarkers of liver fibrosis have been sought for decades, and the FibroTest multimarker panel is approved for clinical usage in Europe. However, all of the current markers and panels have limitations,[190] and none have been recommended by the American Association for the Study of Liver Disease (AASLD) to replace liver biopsy.[191] Clearly, novel markers are still needed, and measuring neoepitopes of ECM proteins composes a snapshot of matrix dynamics that may be of diagnostic and prognostic value.[192] Examples of well-studied liver ECM markers include collagen propeptides, notably PIIINP,[193] and caspase fragments of cytokeratin 18.[194] A recent mass spectrometry study of the ECM in two rodent models identified 16 different collagens in the liver, and profiled changes in the abundance of collagens and integrins in tumors compared with healthy livers and precancerous fibrotic livers.[195] Neoepitopes of these proteins may serve as valuable markers of liver ECMR. Promising candidates have been reported, including those derived from type IV collagen,[196,197] type I collagen,[198] type V collagen,[199] and type VI collagen.[200] Systemic approaches, such as global profiling of serum glycoproteins, have also been utilized,[201] and this technique is now being validated in rodent models (*e.g.*, Fang *et al.*,[202] Blomme *et al.*,[203]) and in additional cohorts of liver disease patients.[204,205]

A range of diseases involve excessive matrix remodeling in specific matrices. For example, in rheumatoid arthritis the turnover of type I, II, and III collagens are highly upregulated in the cartilage and synovium.[206] The high turnover of ECM proteins are also found in other diseases, such as:

- osteoarthritis affecting the articular cartilage (type II collagen and aggrecan)[56]
- metabolic bone diseases (type I collagen)[138,207–210]
- sarcopenia (type VI collagen)[211–213]
- cancer (basement membrane and desmoplasia)[4,33,214–216]
- atherosclerosis (type I and III collagens, titin and versican)[217]
- various fibrotic diseases including liver (type I, III, IV, V, VI collagens and biglycan),[59–61,144,196,197,199,200,218] lung (elastin, type I, III, and V collagen),[74,220–232] and kidney.[233]

Key lessons on the importance of the structural components of the matrix may be harvested from the genetic mutations that lead to pathologies. *Table 2* contains a summary of key structural proteins and their known mutations leading to matrix and tissue failure.[224,233–271] These disease phenotypes provide pivotal information on proteins important for tissue function, and thus how they are involved in some pathologies of nongenomic disorders, and subsequently how treatments that affect these proteins may counter disease progression.

PTMS IN THE ECM

PTMs are non-DNA-coded modifications to the composition or structure of proteins, which generate unique parts of a molecule known as neoepitopes.[17] Pathologically relevant protein modifications are not restricted to protease activity, although the subpopulation of neoepitopes generated through this mechanism may be of paramount importance. *Figure 3* depicts a handful of different types of PTMs. Some have been identified and used as biochemical markers as a measure of the disease activity,[272] but also as contributions to disease process,[17] as they change the functionality of the proteins.

One Gene, 1,000 Protein Subtypes

The importance of PTMs is best described by the fact that one gene may result in 1,000 different and unique proteins with different functional implications. This is illustrated in *Figure 3*. Here, modifications to amino acids by specific PTMs or degradation of the protein result in both immunologically different as well as functionally different proteins. Measurement of the same protein may provide highly different information, such as either protein formation or protein degradation, which obviously entails opposite information. Some pathologies may further modify the protein specifically, and thus give a specific protein fingerprint of pathology

Table 2. Genetic Mutations in Structural Proteins Leading to Distinct Pathologies

Protein	Disease	Reference
Type I collagen	Osteogenesis imperfecta, Ehlers-Danlos syndrome type VII	234,235
Type II collagen	Several chondrodysplasias, osteoarthritis	236–239
Type III collagen	Ehlers-Danlos syndrome type IV, aortic aneurysms	240,241
Type IV collagen	Kidney fibrosis, Alport syndrome	233,242–244
Type V collagen	Ehlers-Danlos syndrome type I and II	245,246
Type VI collagen	Bethlem myopathy, Ullrich congenital muscular dystrophy	247
Type VII collagen	Epidermolysis bullosa dystrophica	248
Type IX collagen	MED	249
Type X collagen	SMCD and Japanese-type SMD	250,251
Type XV collagen	Cardiac and muscle phenotypes	243
Ypye XVII collagen	Growth retardation	243
Type XVIII collagen	Renal filtration defects	243
Elastin	Lung, skin and arterial defects, SVAS, WBS, CL	224,252,253
Laminin	Alport syndrome	233
Biglycan	Cardiovascular disease, osteoporosis	254–256
Biglycan/decorin	Osteopenia and skin fragility	257
Biglycan/fibromodulin	Osteoarthritis	258
Perlecan	Multiple developmental defects and myotonia. Schwartz-Jampel syndrome	243
Nidogen 1 and 2	Lung and kidney development	243
Fibromodulin	Osteoarthritis	259
Lumican/fibromodulin	Joint laxity and impaired tendon integrity	260,261
Lumican	Reduced corneal transparency and skin fragility	262
Decorin	Intestinal tumor; skin fragility; Ehlers-Danlos syndrome-like.	263–265
Mimecan	Colorectal cancer early formation	266
Fibrillin	Marfan syndrome	267
COMP	PSACH and MED	268–270
Matrillin-3	MED	271

MED, multiple epiphyseal dysplasia; SMCD, Schmid-type metaphyseal chondrodysplasia; SMD, spondylometaphyseal dysplasia; SVAS, supravalvular aortic stenosis; WBS, William-Beuren syndrome; CL, cutis laxa; COMP, Cartilage oligomeric matrix protein; PSACH, pseudoachondroplasia.

such as glycosylation of hemoglobin resulting in HbA1c type I diabetes.[273] A long line of evidence suggests that measurement of the intact protein provides some information, and measurement of one of these subforms of a protein provides different and more pathologically-relevant information. These have been carefully described for C-reactive protein (CRP),[274] type I and XVI collagens,[275,276] osteocalcin,[277] and a selection of other analytes. Thus, it is becoming increasingly clear that measurement of a given pathologically-modified protein enables refinement of clinical chemistry and diagnostic procedures. Most likely the best example is hemoglobin, in which the modification of glycosylation is the gold standard marker of diabetes; thus, the intact protein is a necessity of life, whereas the PTM-modified protein is a pathology-specific marker.

As will later be described in larger molecular detail, many amino acids are amenable to specific modifications (citrullinations, phosphorylations, acetylations, methylations, nitrosylations, and glycosylations[17]). These modifications can have both positive and negative impacts on the function of the protein, and even target the protein for degradation. In addition, many proteins are born with a propeptide that needs to be cleaved before the protein is in the active configuration that being enzymatic activity or structural enablement. Both N- and C-terminal propeptides are present, which may be further modified, and thus is a waste underestimation of the complexity of these peptides. The degradation products of proteins may be the specific action of pathological-specific enzymes, and there is an accumulating amount of evidence suggesting that different fragments of the same protein may have different physiological and pathophysiological meanings.[278] Lastly, polymerization may both be understood as aggregates of the same protein such as hyperphosphorylated Tau or cross-linked collagens, but also pentameric CRP. Each of these subpools obviously holds unique information.

Cross-Linking

Cross-linking plays an important role in the ECM meshwork and thereby in tissue integrity. Cross-linking between different ECM components or between different protein chains can result from enzymatic and nonenzymatic pathways. Enzymatic cross-linking is often processed by members of the LOX enzyme family, whose members have been shown to promote the linearization of interstitial collagens which stiffen the tissues, thus leading to neoplastic progression of tumor cells.[280–283] Interestingly, this matrix stiffness was associated with different phenotypes and enhanced mechanoresponsiveness of the epithelium.[280,281] Therefore, cross-linking plays an important part in both the initiation and

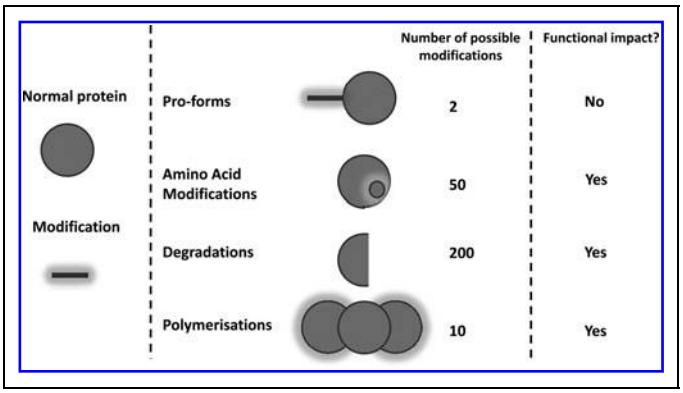

Fig. 3. Schematic figure of the modifications made to a protein that causes unique subpools to be generated, which each may entail specific pathological or physiological information.

progression of metastasis. Similarly, in fibrotic disease, increases in tissue tension mediated by cross-linking can lead to activation of TGF-β signaling from the latency-associated complex and other signaling changes, driving a fibrogenic feed-forward loop.

Valuable assays for evaluation of bone- and cartilage-related diseases have been developed using antibodies highly specific for protease-cleaved sites in type I collagen[276] and type II collagen,[284] respectively. The antibodies in these assays also assess the cross-linking between the lysines in the epitopes. C-terminal telopeptide of type I collagen (CTX-I) is an 8-amino acid fragment from the C-telopeptide of type I collagen generated by cathepsin K activity, and the rate of its release from bone is a useful reflection of the resorbing activity of osteoclasts.[285] Measuring this fragment is useful for the evaluation of treatment efficacy in bone diseases such as osteoporosis.[286] The CTX epitope contains an aspartylglycine motif (DG) that is prone to spontaneous isomerization. In other words, EKAH-D(α)GGR epitopes are released during degradation of newly synthesized type I collagen, whereas EKAHD(β)GGR epitopes are released from matured type I collagen. It has been established that the α/β ratio is a useful measure of the age of bone tissue;[287–289] the lower the ratio, the older the bone tissue.[290] Further, the lysine residue of the CTX residue is cross-linked. *Figure 4* outlines schematically how assessment of both a cross-linked and cathepsin K-degraded epitope may be undertaken through the use of sandwich enzyme-linked immunosorbent assay (ELISA) technology. Additional ECM assays may be constructed by a similar approach, to include as much possible information of protein subtype as possible. Resorption rates of newly synthesized collagen type I can be assessed by specific immunoassays targeting the detection of αCTX in urine samples.[291] Degradation rate of matured, isomerized collagen can be estimated by another specific assay targeting βCTX in both urine and serum samples.[276]

Another way of cross-linking is through the actions of tissue transglutaminases (TGs). They play a fundamental role in tissue stabilization by transamidation of glutamine residues of one protein chain to the amino group of a lysine residue in a second protein

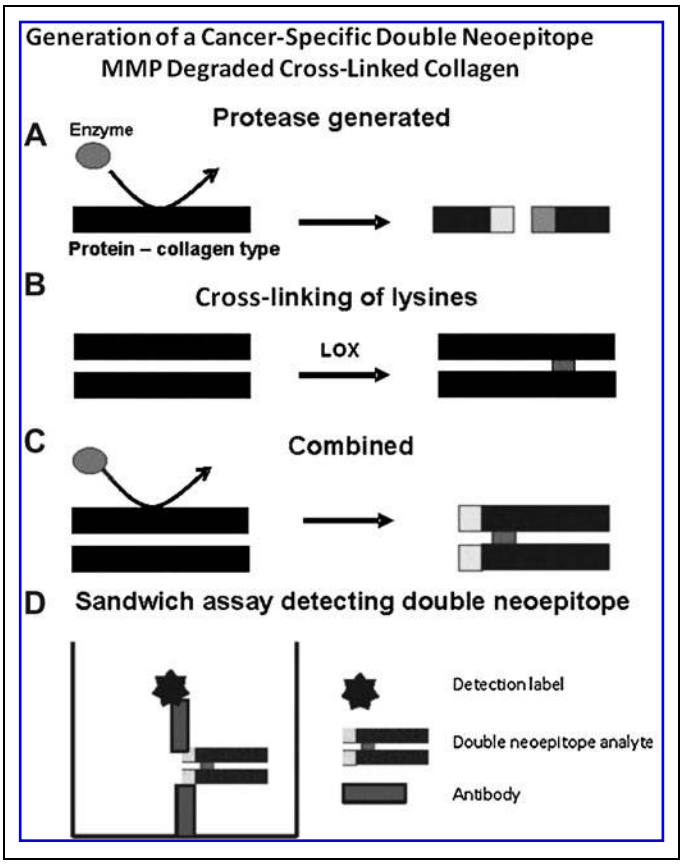

Fig. 4. Development of an assay to detect a cancer-specific double neoepitope. **(A)** An enzyme, most likely an MMP, cleaves collagen molecules. This produces a cut in the peptide sequence, exposing an N- and C-terminal-truncated molecule. **(B)** Lysil oxidase family members are highly upregulated in many cancers. This family of enzymes enzymatically cross-links the lysines in the collagen chains, resulting in stiffened tissue. In the local area of cancer metastasis and growth, these processes are occurring at a more rapid pace than in other parts of the body, resulting in increased expression of a range of collagen, proteases, and other enzymes. **(C)** The processes of protease generation and lysine cross-linking are combined. **(D)** Design and generation of a sandwich assay to detect both the lysine cross-link and the protease-generated degradation product. Thus, this type of ELISA contains more information than traditional assays (*i.e.,* both degradation and cross-link information).

chain. This results in the formation of the covalent N-γ-glutaminyl-ε-lysyl-isopeptide bond, which is resistant to proteolytic degradation.[292] As several ECM proteins, such as collagens, fibronectin, laminin, and vimentin, act as substrates for TGs, they are involved in physiologic tissue integrity while being associated with various pathologies, including neurodegenerative diseases, cancer, inflammation, and fibrosis. In fibrosis, TGF-β promotes activation of TG cross-linking, thereby reducing the ECM turnover, leading to deposition and accumulation of ECM proteins, and thus stabilizing the ECM network and facilitating proteolytic resistance. In cancer, intracellular cross-

linking by TG2 has been shown to be both pro- and antiapoptotic, and favoring cell survival, invasion, and motility by the close association with surface integrins.[293,294]

Oxidations and Hydroxylations

Oxidative damage to proteins is often caused by the action of free radicals, reactive oxygen species (ROS) and reactive nitrogen species such as hydrogen peroxide and nitric oxide, generated in cells by the mitochondrial respiratory chain.[295] Oxidizing PTMs have been implicated in several pathological and healthy tissue turnover processes. Although many amino acids can be attacked by ROS, some seem more likely to undergo oxidation than others. For example, lysine and proline are readily oxidized to aldehydes; methionine is sulfoxidized; and tyrosines are nitrosylated.[296] Under normal conditions, these ROS are strictly regulated by antioxidants, such as peroxidases and dimutases among others.[297] However, under pathological conditions, oxidation may be implicated in tissue destruction. The role of ROS in almost all aspects of cancer initiation and development[139,295,298–303] is still debated. Measurement of specific components of the ECM that hold these oxidized PTMs may be useful for both early diagnosis and prognosis of cancer.

Protease-Generated Neoepitopes

Matrix remodeling at specific disease stages results in both elevated levels of, and uniquely modified, proteins. Endopeptidases, such as MMPs and cysteine proteases, play major roles in the degradation of extracellular macromolecules such as collagens and proteoglycans. Specific proteolytic activities are a prerequisite for a range of cellular functions and interactions with the ECM, resulting in the generation of specific cleavage fragments. Even though many components of the ECM, as well as enzymes responsible for remodeling, are present in different tissues, the combination of a specific peptidase and specific ECM protein may provide a unique combination that elucidates activity in a particular tissue or a specific disease mechanism.

One often-taught example of protease degradation of a given tissue is that of joint degenerative diseases. Joint degenerative diseases lead to alterations in the metabolism of the articular cartilage and subchondral bone.[278,304–309] Cartilage is for the most part composed of collagen type II, which accounts for 60%–70% of the dry weight of cartilage, and proteoglycans accounting for 10% of the dry weight, of which aggrecan is the most abundant.[310] Since type II collagen is the most abundant protein in cartilage, several different degradation fragments of collagen type II have been indicated as useful for monitoring degenerative diseases of the cartilage.[272,311] C-terminal telopeptide of type II collagen (CTX-II) is an MMP-generated neoepitope derived from the C-terminal part of type II collagen,[310] and measurement of CTX-II is highly useful for monitoring degradation of type II collagen in experimental setups assessing cartilage degradation.[278,312] Examples of a range of protease-generated neoepitopes have already been described in the literature, but they have not been utilized by applied science to produce quantifiable methods of disease assessment. Assays detecting a few neoepitopes that have been developed and that are used in both clinical and preclinical studies were reviewed recently.[313]

To some extent, C-terminal telopeptide of type I collagen (ICTP) and MMP-derived fragments of type I collagen assays[53,54,314–316] as an indicator of cancer progression have been developed and used in prognosis of lung and ovarian cancers. A range of biochemical markers based on degradation products of the ECM, particularly collagen, may be identified and used in cancer. The collagen composition of the BM and interstitial matrix may be relevant for the development of the given marker for the ECMR associated with soft tissue metastasis.

Isomerization: Advanced Glycation End Product of ECM Proteins

Proteins containing aspartate (D), asparagine (N), glutamate (E), or glutamine (Q) residues linked to a low–molecular-weight amino acid, such as glycine (G), can undergo spontaneous nonenzymatic isomerization.[15] This isomerization introduces a kink in the conformation of the molecule, as the peptide backbone is redirected from the γ-carboxyl group in the native newly synthesized form to the side chain γ-carboxyl.[290] Peptides that contain amino acid isomerizations are often resistant to proteolysis.[317,318] This feature affects the processing of antigens for presentation on the major histocompatibility complex II during the immune response signaling for the production of T-cells and antibodies.[15] In preclinical studies, it has been shown that various known autoantigens contain sites prone to deamidation and isomerization. These autoantigens are involved in type I diabetes, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and experimental autoimmune encephalomyelitis.[317,319–322]

The C-telopeptide of type I collagen marker CTX-I is a marker of bone resorption. It has been shown that assessment of the nonisomerized epitope (αCTX-I) is more sensitive as a marker for bone metastases secondary to breast and prostate cancer than the isomerized epitope (βCTX-I).[207] This is due to the high ECMR of type I collagen in the bone area invaded by cancer cells, and thus a high amount of newly formed nonisomerized collagen type I undergoes resorption by osteoclasts.

Nonenzymatic Glycosylation

Nonenzymatic glycosylation is also called the Maillard reaction, and leads to PTMs of proteins, nucleic acids, and lipids.[273] A common cause of nonenzymatic glycosylation is increased blood glucose levels, and accordingly, most knowledge about nonenzymatic glycosylation arises from studies performed in diabetics.[273] The marker HbA1c is an established PTM marker in type II diabetes. Recently, advanced glycation end products (AGEs) have been implicated in cancers. The nicotine-induced accumulation of AGEs is a cause of cancer.[323] The receptor for AGEs, called RAGE, is currently under intense investigation as both a marker and an inducer of cancer[324] and to assess whether there is a link between chronic inflammation and cancer, since inflammatory mediators can both be pro- and antitumorigenic.[139,216,324,325]

Citrullination

Citrullination or deimination is the term used for the PTM of the amino acid arginine, which can transform into the amino acid citrulline. The change is facilitated by peptidylarginine deiminases (PADs).[326,327] The conversion of arginine into citrulline can have important consequences for the structure and function of proteins, since arginine is positively charged at a neutral pH, whereas citrulline is uncharged. The positive charge increases the hydrophobicity of the protein, leading to changes in protein folding.

Histone deacetylase 1 (HDAC1) inhibitors are currently under development for the treatment of certain cancers, particularly breast cancer.[21] Histone lysine and arginine residues contain a wide array of PTM-producing processes, including methylation, citrullination, acetylation, ubquitination, and sumoylation. The combined action of these modifications regulates critical DNA processes, including replication, repair, and transcription. In addition, enzymes that modify histone lysine and arginine residues have been correlated with not only cancer but also arthritis, heart disease, diabetes, and neurodegenerative disorders.[328,329]

Histone methylation plays a key role in regulating the chromatin structure and function. The recent identification of enzymes that antagonize or remove histone methylation offers new insights into histone methylation plasticity in the regulation of epigenetic pathways. Peptidylarginine deiminase 4 (PADI4; also known as PAD4) was the first enzyme shown to antagonize histone methylation. PADI4 functions as a histone deiminase, converting a methylarginine residue to citrulline at specific sites on the tails of histones H3 and H4. PADI4 associates with HDAC1.[328–330]

NOVEL TECHNIQUES CURRENTLY AVAILABLE FOR ASSESSING THE STRUCTURE OF THE ECM

Clinical biochemistry provides a battery of assessments for profiling tissue turnover profiles. A range of serological assessments have been developed to investigate some of the key structural proteins of the ECM (*Table 3*).[56,60,129,144,196–200,217,274,276,278,311,314,331–347] Measurement of these proteins may provide key information in clinical settings on the tissue turnover profile, and thereby assists in patient diagnosis, in identification of those patients in most need of treatment, and finally, in monitoring of clinical efficacy of interventions. These technologies may

Table 3. Currently Available Serological Markers Assessing the Structure of the Extracellular Matrix

Name of protein fragment	ECM component	Reference
C1M	MMP-mediated type I collagen degradation	198
C2M	MMP-mediated type II collagen degradation	56
C3M	MMP-mediated type III collagen degradation	60
C4M	MMP-mediated type IV collagen degradation	197
C5M	MMP-mediated type V collagen degradation	331
C6M	MMP-mediated type VI collagen degradation	200
P1NP	Type I collagen formation in tissues other than bone	144
P4NP 7S	Type IV collagen formation	196
P5CP	Type V collagen formation	199
PIIANP	Type II collagen formation	332,333
PIIINP	Type III collagen formation	334
VICM	MMP-mediated citrullinated vimentin degradation	335
CRPM	MMP-mediated CRP degradation	274
ELM	MMP-mediated elastin degradation	217
BGM	MMP-mediated biglycan degradation	336
MIM	MMP-mediated mimican degradation	129
VCANM	MMP-mediated versican degradation	337
TIM	MMP-mediated titin degradation	338
Aggrecan	MMP- and aggrenase-cleaved aggrecan	278
COMP	Intact COMP	339
Osteocalcin	Intact osteocalcin bone formation	340
HA	Hyalonic acid	334
ICTP	MMP-mediated type I collagen destruction	314
CTX-I	Cathepsin K degraded type I collagen	276,311,341,342
CTX-II	MMP-degraded type II collagen	343,344
C2C	MMP-degraded type II collagen	345
TELO-I	Citrullinated carboxyterminal telopeptides of type I collagen	346
TELO-II	Citrullinated carboxyterminal telopeptides of type II collagen	346
MCV	Mutated citrullinated vimentin	347

ECM, extracellular matrix; MMP, matrix metalloproteinase; CRP, C-reactive protein.

also be used in preclinical settings, in *ex vivo* and *in vitro* cultures, to determine the molecular mode of action in the assembly and maintenance of the matrix.[311]

An Example of a Combined Aged, Cross-Linked, and Cleaved Neoepitope for the Evaluation of Bone Metastases

The relationship between skeletal tumor load and elevations in serum or urine levels of αCTX and seven other biomarkers related to bone turnover has been investigated in a pooled group of breast and prostate cancer patients.[207] Patients were stratified according to the Soloway score:

- Score 0: 0 bone metastases
- Score 1: <6 bone metastases
- Score 2: 6–20 bone metastases
- Score 3: >20 bone metastases
- Score 4: superscan showing that >75% ribs, vertebrae, and pelvic bone are affected.

In breast cancer patients, a strong linear association was observed between bone metastases and all biomarkers, except osteoprotegerin and receptor activator of nuclear factor κB ligand (*Figure 5*). All six remaining markers were significantly elevated in patients with a Soloway score of 1. The relative percent increases in biomarker levels in the presence of bone metastases were most pronounced for αCTX-I, which was elevated by more than 600% in patients with Soloway score of 3. The next highest increases were in bone-specific alkaline phosphatase and N-telopeptide of type I collagen (NTX), which were elevated by 470% and 440% at Soloway score 3, respectively. These findings were supported by observations in prostate cancer patients, which showed that of the seven biomarkers, αCTX-I was the most sensitive for bone metastases.[348] The higher sensitivity of αCTX-I could be explained by the fact that this epitope is released from sites of high bone remodeling, where collagen fibrils do not have time to mature and undergo β-isomerization. The αCTX-1 epitope was located by immunostaining adjacent sections of bones invaded by breast cancer or prostate cancer,[208] and at the sites of high bone remodeling.

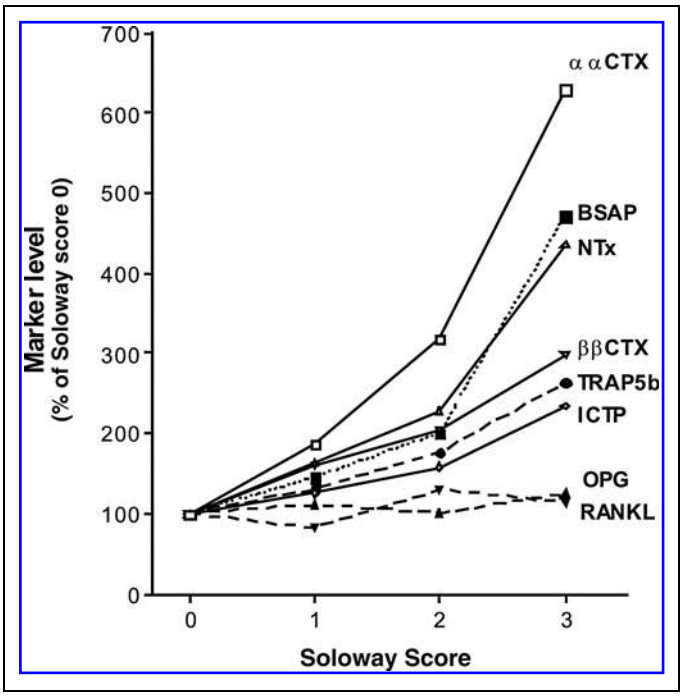

Fig. 5. Relative increases in bone resorption, bone formation, and osteoclastogenesis marker levels as a function of the extent of skeletal metastasis, assessed in 132 patients with breast or prostate cancer. Relative increases are expressed as a percentage of levels in patients with a Soloway score 0.[207]

These data support that careful selection of matrix constituents and, in particular, those that carry one or more PTMs such as isomerization in a type I collagen fragment generated by cathepsin K as described in this example may be superior markers reflecting pathological, including malignant, events in the ECM.

An Example of MMP-Degraded Type III Collagen for the Assessment of Liver Fibrosis

The central pathological change in fibrosis is uncontrolled ECMR.[349,350] During fibrogenesis, the quantity and composition of matrix proteins in the liver change, resulting in excessive accumulation of fibrous (scar) tissue and an overall increase in the ECM density.[351] ECM matrix proteins in a normal liver are distributed mainly in the portal tracts, whereas a BM-like matrix is located in the perisinusoidal space of Disse. The most abundant collagens in the liver are type I and III collagens, which by immunohistochemistry are found predominantly in the perisinusoidal spaces, in portal tracts, and in subcapsular areas.[5,352] The ECM of the cirrhotic liver contains approximately six times as much matrix as the normal liver,[353] which is a result of increased levels of type I, III, and IV collagens.[354] However, levels of MMPs such as MMP-9 also increase in cirrhosis.[349,355] The combination of active and overexpressed MMP-9 with the accumulation of type III collagen poses the interesting hypothesis that an MMP-9-generated fragment of type III collagen could be used as a biochemical marker of liver fibrosis.

Type III collagen degradation by MMPs, and even MMP-9 exclusively, may result in many unique fragments, such as those derived from type II collagen and previously published.[81] The CO3-610 (C3M) fragment (KNGETGPQGP) is one of those, and is exclusively derived from MMP-9. When this C3M fragment was assessed in two separate animal models of liver fibrosis, the BDL and CCl4 animal models, a >200% fold upregulation was observed, as well as a highly significant correlation to portal pressure.[60,356,357] These data strongly suggest that liver fibrosis is not merely an accumulation of ECM proteins, but a dynamic condition with accelerated ECM turnover, in which both tissue formation and tissue degradation are highly upregulated. In the case of liver fibrosis, ECM tissue formation outstrips tissue degradation, leading to a net accumulation of scar tissue over time. This example also suggests that PTMs released by protease degradation of proteins may in some cases be more sensitive markers for pathology than intact proteins. This idea is receiving increased

attention.[17] This approach has been recently been described as the protein fingerprint technology, in which the different subpools of the total pool of information about one protein during formation or degradation provide distinct and important data.[20]

PTMs: THE CAUSE OR CONSEQUENCE OF THE DISEASE?

Proteins are complex molecules susceptible to numerous PTMs occurring spontaneously during aging or as a consequence of physiologic or pathologic processes. Today, it is well established that PTMs can uncover cryptic epitopes and/or create novel epitopes, to which no tolerance exists.[15] Antigenicity and interactions of proteins with components of the immune system may be profoundly affected by PTMs. Thus, modified self-antigens may be absent (indicating they are not tolerated) during early T-cell selection, and trigger reactions by the immune system as they arise later in life. In turn, this may play a role in the initiation and pathogenesis of autoimmune diseases.[15] Several studies have shown that various types of PTMs are hallmarks of aging and are associated with autoimmune diseases, such as RA, SLE, and type I diabetes.[15,16,358–375]

The presence of PTMs in several known autoantigens has been reported. Many of these modifications have been implicated in the antigenicity of the proteins, as outlined in *Table 4* (modified from Cloos and Christgau[15]).[296,311,319–322,346,347,376–389] These observations have sparked a growing interest in the role and assessment of PTMs in autoimmune diseases as well as other pathological conditions associated with aging. Whether the presence of PTMs is merely a secondary phenomenon accompanying the disease or a primary event in disease initiation remains to be resolved.

It is noteworthy that T-cell responses to modified antigens in general are very specific.[390,391] In contrast, autoantibodies recognizing modified proteins tend to be more nonspecific and often cross-react with the native antigen. This B-cell promiscuity may play an important role in the phenomenon of epitope-spreading characteristics of many autoimmune diseases,[392] which in part may be the disease driver in illnesses such as RA. These examples serve to highlight that in the immune system, PTMs, in various ways, may initiate, play parts in the pathogenesis, or even constitute the central events in some diseases. Regardless of whether PTMs are the chicken or the egg, these examples further emphasize that PTMs are relevant markers of diseases. Tools developed to measure specific monoclonal antibodies may aid the understanding of the temporal events leading to PTMs, and their role in disease mechanisms.

FUTURE DIRECTIONS

In this review, we have described the key components of the ECM and highlighted recent developments in the identification and measurement of PTMs. There is a growing body of evidence that modifications made to the structural proteins of the matrix may both be a consequence of the disease as well as drivers of disease progression. Thus, PTMs to specific ECM proteins may be more integrated in pathogenesis than previously thought. Indeed, the matrix serves as much more than just a structural framework for tissues.

Table 4. List of Post-Translational Modifications Involved in Immune Responses in Different Autoimmune Diseases

Autoantigen	Relevant disease/ animal model	Modification	Reference
MBP	MS/EAE	Acetylation	376
		Citrullination	377
		Isomerization	296
		Phosphorylation	378
aB-crystallin	MS/EAE	Citrullination	379,380
		Isomerization	321
		Phosphorylation	381
Type I collagen	RA	Citrullination	346
Type II collagen	RA/CIA	Glycosylation	382
		Protease degradation	311
		Hydroxylation	382
		Citrullination	346
Fibrin	RA	Citrullination	383
Fillagrin	RA	Citrullination	384
Vimentin	RA	Citrullination	347,385
IgG	RA	Isomerization	296
		Glycation	386
Insulin	Type I diabetes	Deamidation	319
		Isomerization	319
GAD	Type I diabetes	Oxidative damage	387
Histone H2B	SLE	Isomerization	322
		Deamidation	388
Transglutamination			388
SnRNP D	SLE	Isomerization	320
SnRNP 70k	SLE	Phosphorylation	389

MS, multiple sclerosis; EAE, experimental autoimmune ecephalomyelitis; RA, rheumatoid arthritis; CIA, collagen-induced arthritis; SLE, systemic lupus erythematosus.

Fibrosis and cancer involve signature proteins and enzymes. These enzymes degrade the ECM and create a range of other PTMs, releasing smaller fragments of ECM proteins into the circulation. An optimal biochemical marker may be designed by identifying the common denominator of specific pathophysiological processes to determine

the marker's tissue specificity and sensitivity. Different cells of a particular tissue predominately express given proteases that in combination with different signature proteins from different host tissues, which may provide optimal selective markers for connective tissue diseases. Biochemical markers based on the advanced disease/tissue neoepitope approach could become an important tool to be used in combination with others for diagnosing and staging disease as well as assessing efficacy and safety of new therapeutic interventions.

ACKNOWLEDGMENTS

We acknowledge funding from the Danish Ministry of Science, Technology, and Science, and the Danish Science Foundation (Den Danske Forskningsfond).

DISCLOSURE STATEMENT

No competing financial interests exist.

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