Extracellular Matrix Markers and Methods for Their Study (Review)

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The extracellular matrix (ECM) is a complex meshwork consisting mainly of proteins and carbohydrates; it is currently viewed as a key factor of tissue organization and homeostasis. In each organ, the composition of ECM is different: it includes a variety of fibrillar components, such as collagens, fibronectin, and elastin, as well as non-fibrillar molecules: proteoglycans, hyaluronan, glycoproteins, and matrix proteins. ECM is an active tissue, where the *de novo* syntheses of structural components are constantly taking place. In parallel, ECM components undergo degradation catalyzed by a number of enzymes including matrix metalloproteinases. The synthesis and degradation of ECM components are controlled by mediators and cytokines, metabolic, epigenetic, and environmental factors. Currently, a large amount of evidence indicates that modifications (remodeling) of ECM play an important role in the pathogenesis of clinical conditions. This may explain the increasing interest in the markers of ECM remodeling both in health and disease. In recent years, many of the ECM markers were considered targets for diagnosing, predicting, and treating diseases. In this review, we discuss some of the currently known ECM markers and methods used for their determination.

Key words: extracellular matrix; matrix remodeling; biomarkers; collagen; metalloproteinases.

Introduction

Extracellular matrix (ECM) is the basis of connective tissue and a partial component of liquid connective tissues (blood, lymph); it provides mechanical support for cells and intercellular interactions as well as transport of chemicals and migration of cells [1–3]. Earlier, ECM was thought to be a stable structure, changing mainly in accordance with the needs of growth and repair. However, recent studies have shown that ECM is a metabolically active and constantly reconstructing tissue [4].

In the body, two processes occur simultaneously: continuous enzymatic degradation of ECM and — in parallel — the synthesis of ECM components and their restructuring (remodeling). These changes involve ECM cells by regulating their proliferation, migration, and differentiation [5–7]. To ensure the physiological migration and proliferation of cells in the connective tissue, local degradation of ECM is a necessary condition; when this process is blocked, for example, in connective tissue dysplasia, it can lead to neoplasms [8]. Normally, ECM homeostasis is characterized by an optimal balance between the formation, secretion, alteration, and degradation of the matrix [9].

The increasing interest in the ECM structural changes is due to their importance for human physiology and

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pathophysiology. In recent years, new technologies for studying the ECM became available; that included advanced methods of visualizing the ECM structure, e.g., optical coherence tomography (OCT) and labeled monoclonal antibodies to individual ECM elements [10–13]. Also, genetic engineering approaches to the therapeutic correction of ECM metabolic disorders [14, 15] are under development [16].

ECM reconstruction, i.e. changes in its structure and geometry, is termed "remodeling". Remodeling plays an important physiological role in a healthy body; abnormal remodeling is part of the pathogenesis of various diseases. In health, ECM remodeling can be observed from birth to maturity that makes it an element of growth processes. Abnormal remodeling of ECM is characteristic of chronic obstructive pulmonary disease (COPD) and bronchial asthma [5]. In our recent studies [17–22], we found that a sizable proportion of children with atopic asthma had intranasal structure abnormalities, hypertrophic changes in the pharyngeal tonsil, and hypertrophy of the nasal mucosa. On the one hand, it can be viewed as a manifestation of multimorbidity typical for respiratory allergy in children, which may indicate a systemic involvement of ECM in the pathological process. On the other hand, the identified changes may be indicative of pathological remodeling, including the potentiation of hypertrophic changes in the upper respiratory tract in children with atopic bronchial asthma.

The proven involvement of ECM remodeling in the pathogenesis of various diseases underscores the importance of continuous monitoring of ECM changes. For such monitoring, a study of ECM in individual locations is not sufficient; rather detecting the molecules resulted from the systemic ECM remodeling seems the right approach to determine the clinically relevant ECM biomarkers. Therefore, below we discuss the most informative biomarkers associated with a specific disease as well as their changes during therapy. The general overview of the ECM that is present in all organs and tissues is important to understand the origin of multimorbid diseases, including multisystem manifestations in patients with atopic diseases, connective tissue dysplasia and other disorders [19, 23–25].

In this review, we describe major biomarkers that can be used to monitor the current status and changes in ECM. We also address the clinical significance of these markers as well as the methods for their determination.

Markers of the state of the extracellular matrix

Because of the complexity of the ECM, it is currently considered insufficient to use any single marker to characterize the status of the matrix. Other difficulties are due to age-related and pathophysiological features of the ECM metabolism. It has been demonstrated that the somatic development of the human body and, therefore, the formation of the ECM may depend on the existing chronic pathology [3, 18, 26–28].

Currently, the following markers reflecting the state of the ECM are being actively studied:

1. Markers characterizing the ECM components:

markers of the synthesis and degradation of various collagens — the main structure-forming proteins of the ECM;

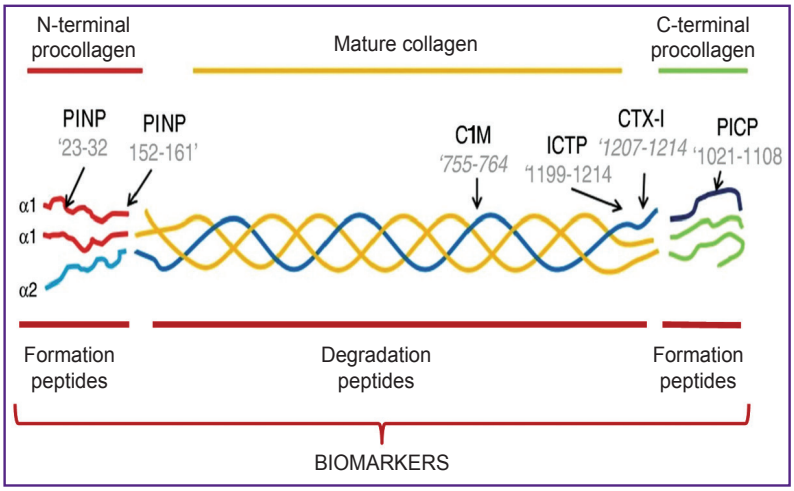
elastin degradation products;

metabolites of other components of ECM.

2. Markers characterizing the level and activity of proteinases (matrix metalloproteinases — MMP, caspases, chymases, etc.) and their inhibitors.

3. Mediators and cytokines involved in ECM metabolism.

The difficulty of determining the most informative biomarkers of ECM is also due to the fact that several processes can develop simultaneously in opposite directions [29]. In the initial phase, following damage to the basement membrane, endothelial or epithelial cells, degraded proteins of the basement membrane are released; for example, C4M — fragment of type IV mature collagen degraded by the action of MMP (see the Figure). Then the process continues in deeper tissues with the formation of such markers as MMP-generated fragments of type I and III collagens — C1M and C3M, respectively, which can be considered markers of chronic inflammation. In the opposite direction — in the ECM recovery — fibroblasts generate new proteins for



Markers of synthesis and degradation of type I collagen (modified from Genovese and Karsdal, 2016 [29])

Here: PINP — amino-terminal propeptides of type I collagen, fragments 152–161 and 23–32; ICTP — marker of metalloproteinase-mediated collagen degradation; CTX-I — C-terminal telopeptide of type I collagen released by cathepsin K and originated from bone tissue only; PICP — collagen carboxy-terminal propeptide. For collagens of other types, fragments of amino-terminal (Pro-Cx/PxNP) and mature collagen (CxM) are also measured (x denotes the type of collagen)

the basement membrane and the extracellular matrix to replace the degraded ones. Markers of collagen synthesis include, for example, cleaved amino-terminal propeptide of type III procollagen (Pro-C3, PIIINP).

In accordance with this mechanism, the life cycle of collagen can be traced by measuring the serum levels of the carboxy-terminal telopeptide of type I collagen (PICP, ICTP) and type III procollagen (PIIINP) [30]. ICTP is a small molecule resulting from the MMP-catalyzed degradation of type I collagen. The PIIINP levels reflect the synthesis of collagen [31]. Together, the ICTP and PIIINP characterize the intensity of the collagen synthesis/degradation cycle and serve as biomarkers of collagen metabolism.

With the persistence of the inflammatory process and the induction of fibrosis, proteases destroy the newly formed matrix and, at the same time, activated fibroblasts produce new matrix proteins at an accelerated rate. The new matrix can be formed by both normal and altered proteins, such as immature collagen, which can be incorporated into the matrix. This collagen will undergo post-translational modifications, such as cross-linking mediated by specific enzymes that are activated in the fibrous microenvironment [29].

Markers characterizing the composition of the extracellular matrix

Markers of collagen synthesis and breakdown. Currently, the presence of collagen degradation products is most often determined in various biological fluids (blood, urine, gastric juice, synovial fluid). Of these, the most accessible and well-studied marker is hydroxyproline, an amino acid that differs from proline by the presence of a hydroxyl group on one of the carbon atoms. Free hydroxyproline is a marker of collagen destruction, whereas its peptide-bound form reflects both the degradation and synthesis of collagen. Since sizable amounts of hydroxyproline are formed as a result of degradation of newly synthesized collagen simultaneously in different tissues, the concentration of hydroxyproline in the urine is a non-specific indicator, which has recently been replaced by more specific markers [32]. These specific markers include:

1. Collagen breakdown markers:

deoxypyridinoline or pyrilinks-D in 24 h urine (the main product of cross-linking of mature bone tissue collagen);

type I collagen degradation products known by various names: C-telopeptide, beta-cross-linked, carboxy-terminal cross-linking telopeptide of bone collagen, collagen cross-linked C-telopeptide, collagen CTX, crosslaps, type 1 collagen, beta-cross laps, CT, b-CTx;

fragments of degraded mature collagen: C1M, C3M, C4M, C5M, C6M [33].

The advantage of using these markers is that they reflect the metabolism of the existing matrix, in contrast to, for example, hydroxyproline that partially reflects the intake of collagen and other substances from food.

2. Collagen synthesis markers:

osteocalcin — a non-collagen protein synthesized by osteoblasts; it promotes the binding of calcium and hydroxyapatites;

amino-terminal propeptides of types I, II, III, IV, V, VI procollagen: Pro-C1/PINP, Pro-C2/PIINP, Pro-C3/PIIINP, Pro-C4, Pro-C5, Pro-C6, respectively [34, 35];

carboxy-terminal propeptide type I procollagen [36].

These metabolites are tissue-specific that enables using them as markers of the ECM metabolism in different organs and tissues.

Collagen breakdown products can be biologically active. The main biological effects of the peptide derivatives of types I and IV collagens are summarized in Table 1; they include:

1) chemoattraction of neutrophils and endothelial cells;

2) regulation of cell migration, including fibroblasts and tumor cells;

Table 1

Collagen-derived bioactive peptides and their biological activities (modified from Kisling et al., 2019 [10])

Substrate	Matrix metalloproteinase	Source	Bioactive peptide	Biological activity	References
Type I collagen	MMP-8 MMP-9	Collagens and elastins from various tissues	Proline-glycin-proline	Regulatory peptide of inflammation; neutrophil chemoattractant	[37–39]
	MMP-2 MMP-9	Heart tissues	C-1158/59	Heal improvement; cell migration	[40, 41]
		Bone tissue	C-propeptide	Tissue vascularization; endothelial chemoattractant	[42]
Type IV collagen	MMP-2 MMP-9	Epithelial and endothelial tissues	Arrestin	Multifunction proteins; angiogenesis inhibition; nesenchymal-endothelial transition	[43–46]
	MMP-2	Epithelial and endothelial tissues	Canstatin	Epithelial cell proliferation; fibroblast migration; angiogenesis inhibition	[47]

The end of the Table 1

Substrate	Matrix metalloproteinase	Source	Bioactive peptide	Biological activity	References
	MMP-9	Epithelial and endothelial tissues	Tumstatin	Antiangiogenic and anti-inflammatory effect; fibroblast proliferation and migration; reverse remodeling in experimental asthma	[48–50]
	MMP-2 MMP-9	Epithelial and endothelial tissues	Tetrastatin	Antitumor migration effect; anti-migration effect on tumor cells	[51, 52]
	MMP-2 MMP-9	Lung and breast tissues	Pentostatin	Antiangiogenic effect	[53]
	MMP-2 MMP-9	Eye tissue, vascular endothelium	Hexastatin	Antiangiogenic effect; antimigration effect	[54]

Here: ***MMP*** is metalloproteinase, matrikin-bioactive ECM fragment that modulates various physiological processes by binding to cell surface receptors for chemokines, cytokines, and growth factors; ***proline-glycine-proline*** — collagen cleavage product, regulatory peptide; ***C-1158/59*** — fragment of collagen C-1158/59; it was identified in patient's blood plasma after myocardial infarction; ***C-propeptide*** — C-propeptide of type I procollagen — terminal fragment of type I procollagen, a marker of type I collagen biosynthesis; ***arrestin*** — family of multifunctional proteins that originate from the non-collagen domain of the type IV collagen α1 chain; ***canstatin*** — non-collagen canstatin — C-terminal fragment of the type IV collagen α2 chain; ***tumstatin*** — type IV collagen derivative, α3 chain derivative — matricriptin; ***tetrastatin*** — type IV collagen derivative, α4 chain derivative — matricriptin; ***pentostatin-1*** — type IV collagen derivative, α5 chain derivative — matricriptin; ***hexastatin*** — type IV collagen derivative, α6 chain derivative — matricriptin; ***matricryptin*** is a ligand with "hidden" or functionally "inactive" domains that become open or "active" after fragmentation of the parent molecule; ***mesenchymal-endothelial transition*** — the process of changing the mesenchymal phenotype for the epithelial one in mesenchymal cells.

3) regulation of tissue vascularization including inhibition of angiogenesis;

4) regulation of proliferation of epithelial cells and fibroblasts.

It should also be noted that the type VI collagen propeptide is also regarded as hormone called endotrophin [55, 56]. It stimulates profibrogenesis and neoangiogenesis, attracts macrophages, monocytes, and tumor cells, and increases tissue resistance to insulin [57–60].

Elastin degradation products. Elastin is a structural protein that provides for the elasticity of tissues, including the respiratory system [61, 62]. Under inflammation, elastin is destroyed by neutrophil elastase; as a result, the elastin fragment EL-NE is generated and released into the circulation [63]. Of particular importance is that a specific antibody to this fragment binds only to EL-NE without binding to intact elastin or other elastin fragments produced by MMR or cathepsin G [63].

Elastin is an important structural protein of the lung [64]. Ronnow et al. [65] recently demonstrated that specific elastin fragments resulted from its degradation by proteinase 3, cathepsin G, neutrophilic elastase, MMP-7 or MMP-9, MMP-12 could be used as biomarkers typical for patients with COPD with a poor prognosis.

Among biomarkers reflecting the abnormal metabolism of ECM associated with degradation of elastin, a special role is played by the amino acids desmosine and isodesmosine, which are found in the elastin matrix only. The presence of these amino acids can be accurately determined in the plasma, urine, and sputum. These amino acids reflect changes in the systemic balance between the activation and deactivation of elastase that may be caused by systemic inflammation. The levels of these biomarkers in the sputum reflect the degradation of elastin in the lungs. Clinical data accumulated over several decades indicate a correlation between the levels of desmosine and isodesmosine, the course of COPD and responses to therapy [66–68].

Metabolites of other ECM components. To characterize the biochemical processes that occur in the ECM, it is also important to note other non-collagen structural proteins, such as proteoglycans and adhesive proteins (laminins, fibronectin). Currently, improved enzyme immunoassay spectrometric methods for the determination of sulfated glycosaminoglycans (carbohydrate parts of proteoglycans) — heparan, dermatan, hyaluronan, and keratane are available [69].

Among non-sulfated glycosaminoglycans, it is hyaluronan (hyaluronic acid, HA) that generates is of particular interest as a potential marker of ECM. As a systemic marker of liver fibrosis, HA determination does not require a biopsy [70, 71]. The high molecular mass HA (molecular mass of more than 1000 kDa) is a biologically active form of HA with anti-inflammatory and anti-angiogenic properties promoting cell survival. In contrast, the low molecular weight HA (150–350 kDa) produced during inflammation, has pro-inflammatory and pro-angiogenic properties and promotes cell migration to the site of inflammation [72, 73]. Under the action of hyaluronidase, HA is degraded to derivatives.

They actively participate in stimulating the expression of inflammation-associated genes in various immune cells at the site of injury [74]. Observations in patients with asthma indicate the direct involvement of HA in the pathophysiology of allergic diseases of the respiratory tract and demonstrate a change in the HA levels in persistent and severe bronchial asthma [75]. The initiation and maintenance of allergic asthma depend on the balance between the low and high molecular weight HAs [76]. The expression of HA in the apical layer of the mucosal epithelium maintains the activity of the beating cilia and regulates the mucociliary clearance; therapy with exogenous HA has a beneficial effect on the upper respiratory tract [77].

The level and activity of proteinases

Various proteases involved in the degradation of ECM components are commonly used as biomarkers for remodeling of structural proteins; particular attention is paid to matrix metalloproteinases. For example, MMP-2, MMP-9, and MMP-13 cause indirect destruction of types I, III, V, and VI collagens [78–80]. In fact, the combination of a specific protease, which is activated in a particular disease, and an ECM protein, which is expressed in a specific organ, can generate a very specific biomarker (Table 2).

In recent years, close attention has been paid to the role of matrix proteinases in the physiological

Table 2

ECM fragments and their potential use as biomarkers of clinical conditions (modified from Genovese and Karsdal, 2016 [29])

ECM fragment	Bio-sample	Protein/protease	Condition	References
C1M	Blood serum	Type I collagen/metalloproteinases	Idiopathic pulmonary fibrosis and COPD, ankylosing spondylitis, breast and ovarian cancer	[81–85]
C2M	Blood serum	Type II collagen/metalloproteinases	Ankylosing spondylitis, osteoarthritis	[84, 86, 87]
CTX-II	Blood serum, synovial fluid	Type II collagen/metalloproteinases	Osteoarthritis	[88, 89]
C2C	Blood serum	Type II collagen/metalloproteinases	Osteoarthritis	[90]
TIINE	Urine, synovial fluid	Type II collagen/metalloproteinases	Osteoarthritis	[91]
C3M	Blood serum, urine	Type III collagen/metalloproteinases	Idiopathic pulmonary fibrosis and COPD, ankylosing spondylitis, chronic kidney disease, breast and ovarian cancer	[81–84, 92–94]
C3A	Blood serum	Type III collagen/ADAMTs	Idiopathic pulmonary fibrosis and COPD	[82]
C4M	Blood serum	Type IV collagen/metalloproteinases	Ankylosing spondylitis, COPD, breast and ovarian cancer	[81, 82, 84, 92]
C4M12a3	Blood serum	Type IV collagen/metalloproteinase-12	Idiopathic pulmonary fibrosis and COPD, breast and ovarian cancer	[50, 81, 85, 92, 95]
C5M	Blood serum	Type V collagen/metalloproteinases	Idiopathic pulmonary fibrosis and COPD, ankylosing spondylitis	[80, 82]
C6M	Blood serum	Type VI collagen/metalloproteinases	Idiopathic pulmonary fibrosis, ankylosing spondylitis, COPD	[83, 84, 92]
ELM7	Blood serum	Elastin/metalloproteinase-7	Idiopathic pulmonary fibrosis and COPD, lung cancer	[61, 62, 65]
EL-NE	Blood serum	Elastin/HNE	COPD	[62, 65]
ELM	Blood serum	Elastin/metalloproteinase-9 or -12	COPD, acute myocardial infarction	[65, 96]
ELM2	Blood serum	Elastin/metalloproteinase-9 or -12	Acute myocardial infarction	[96]
VCANM	Blood plasma	Versican/metalloproteinases	Chronic kidney disease	[97]
PIIANP	Blood serum	Type II collagen — amino-terminal propeptide	Rheumatoid arthritis, osteoarthritis	[98]
PRO-C2 (PIIBNP)	Blood serum	Type II collagen/metalloproteinase	Osteoarthritis	[34]
Pro-C3	Urine, blood serum	Type III collagen/aminoproteinase	Chronic kidney disease, liver fibrosis	[93, 99]
Pro-C5	Blood serum	Type V collagen/carboxyproteinase	Liver fibrosis, COPD	[85]
TEGE373-374ARGS	Blood serum, synovial fluid	Aggrecan/aggrecanase	Degenerative joint diseases	[100]

The end of the Table 2

ECM fragment	Bio-sample	Protein/protease	Condition	References
IPEN341-342FFGV	Blood serum, synovial fluid	Aggrecan/metalloproteinases	Degenerative joint diseases	[100]
32mer	Cartilage	Aggrecan (fragment between 341–373)	Degenerative joint diseases	[101]

and pathological remodeling of the lung tissue. There is evidence [102] that the expression, regulation, and function of MMP are highly significant for the development and maintenance of adequate lung oxygenation in both health and disease. In particular, in stressful conditions such as acute respiratory infection or allergic inflammation, MMP-2 and MMP-9 play a protective role, providing bacterial clearance and facilitating chemotaxis. Therefore, understanding the expression and function of MMPs, both in normal and diseased lungs may contribute to the development of new therapeutic technologies.

Matrix metalloproteinases destroy both collagens and proteoglycans [102–106]. It is important to analyze the balance between ECM proteases and their inhibitors. For example, in COPD, an imbalance between proteases and anti-proteases is evidenced by the excess of MMP and the relative deficiency of the tissue inhibitor of metalloproteinases (TIMP) [104].

Some of the metalloproteinases have been proposed to serve biomarkers of the ECM in special cases, for example, for measuring the TIMP/MMP-9 ratio in sputum supernatants or fibroblasts in patients with COPD [107]. According to Mulyadi et al. [108], MMP-9 activity in the saliva did not correlate with lung function in patients with COPD, whereas it did correlate (p=0.002) with the patients' age in the older age group.

There are reports on the determination of individual metalloproteinases in bronchial asthma [109–112] and eosinophilic fasciitis [113]. Naveed et al. [114] showed that in patients with asthma, the level of MMP-1 in the respiratory tract was 5.4 times of that in control; the number of mast cells correlated with the proliferation of bronchial smooth muscle (BSM), whereas the MMP-1 protein was associated with airway hyper-reactivity. The authors conclude that MMP-1 is activated by the mast cell tryptase (and this leads to ECM proliferation) and that the interactions of BSM with mast cells aggravate the disease by briefly activating the MMP, the BSM growth, and the airway hyper-reactivity.

To determine the activity of MMPs, substrate gel electrophoresis can be used. Interpretation of the results is rather difficult and must take into account additional aspects. MMPs are produced in the form of propeptides; they transform to active proteolytic enzymes only after the removal of part of their molecule. As a propeptide MMPs show some proteolytic activity when in gel but not in solution. In addition, an overestimation of proteinase activity can occur in samples containing non-covalent enzyme-inhibitor complexes, such as MMP or TIMP [115].

Another histological method for the determination of proteinase activity in tissues is the electrophoretic technique — *in situ* zymography. Frozen tissue sections are incubated with a fluorescently labeled substrate to determine the location and the quantity of proteins [116].

Mediators and cytokines involved in the metabolism of extracellular matrix

The ECM metabolism is regulated by a number of mediators and cytokines; their structures are reviewed in detail by Richards, Tsuda, Schönherr and Hausser [117–119]. Special attention is given to cytokines synthesized by macrophages and innate lymphoid cells (ILC). The cytokines, transforming growth factor β (TGF-β) and interleukin 13 (IL-13), activate myofibroblasts, which produce ECM components. At the same time, other cytokines including interleukin 1 (IL-1), tumor necrosis factor α (TNF-α), and interferon γ (IFN-γ) can stimulate metalloproteinases and/or reduce collagen synthesis by fibroblasts. Metabolic changes, age, and epigenetic mechanisms influence the function of fibroblasts; this should be taken into account when the ECM metabolism in normal and pathological conditions is discussed [117].

One of the most well studied mediators of ECM is periostin, a protein expressed in epithelial and matrix cells. It is activated by cytokines, including IL-4 and IL-13, and involved in the processes of fibrosis of the basement membrane. Hoshino et al. [120] investigated the relationship between the wall thickness of the respiratory tract and serum periostin in asthmatic patients who did not use steroids. They found that the level of periostin correlated with the wall thickness in the respiratory tract under inflammation but decreased with inhaled glucocorticoids in parallel with a drop in the number of sputum eosinophils. In fact, periostin levels can be used to predict clinical responses to treatments in bronchial asthma and other allergic diseases, including the treatment based on the inhibition of IL-13 [121–123].

TGF-β is also considered a key cytokine regulating the remodeling of ECM [124, 125]. TGF-β1 was proven to inhibit myoblast differentiation and ECM degradation [126]. TGF-β1 is also a strong fibrogenic agent: it induces the production of collagen and other extracellular matrix

components, reduces the expression of MMPs, and activates the synthesis of protease inhibitors [127]. Structural deficiency of fibrillin-1 leads to dysregulation of TGF-β activity [128, 129].

In addition to the measurements of ECM components, their derivatives, mediators and cytokines in tissues and body fluids, the ECM-relevant genes are also analyzed [130, 131]. The currently available sequencing techniques provide significant support to this kind of research.

Methods for studying the extracellular matrix

The existing information on ECM has been obtained mainly by using general research methods. Below, we address those methods and also mention specific assays for biomarkers. In addition, we discuss methods that can be used for assaying highly specific ECM biomarkers.

Methods that do not involve or partially involve biomarkers

Ultrastructural analysis of the extracellular matrix. Evaluation of the ECM ultrastructure is carried out mainly by light and electron microscopy. To perform these studies, preliminary preparation of biological samples is needed; that may include negative staining, immunological labeling, and rotational shading [132].

Nonlinear optical imaging of extracellular matrix proteins. Nonlinear imaging techniques such as multiharmonic imaging microscopy have been used to visualize nonlabeled biological structures. Multiharmonic signals are generated when an intense electromagnetic field propagates through a sample that either has a specific molecular orientation or exhibits other specific properties. The method provides additional morphological information when combined with other methods of nonlinear optical imaging, such as two-photon excitation (TPE). The ability to visualize biological samples without additional genome labeling makes this method well applicable to tissues enriched with collagen and elastin [133].

Visualization, structure analysis, and degradation chemistry of extracellular matrix proteins. Methods for visualization of complex matrices *in vivo* and biochemical analysis of individual ECM proteins are available. General laboratory methods for visualizing ECM components include the second harmonic protocols, scanning electron microscopy as well as histological methods for detecting and analyzing the degradation products of ECM components — immunohistochemistry, trichrome staining and *in situ* zymography.

In addition, a method for determining cell migration and cell invasion (a common transwell invasion assay) has been developed. It allows for real-time visualization of cells with a specific focus on collagen and other ECM proteins.

Classical biochemical methods based on protein electrophoresis remain relevant for the analysis of ECM proteins [134].

Histochemical methods to study the extracellular matrix. The thickness of the collagen fibers, as well as the packaging of collagen, can be determined histochemically by staining with picrosirius red and subsequent polarization microscopy. The study of collagen fibers by this method helps to distinguish between normally packed thick bundles of collagen and pathological collagen (thin and poorly packaged unorganized collagen) [135].

Microscopy of the second harmonic. Studying the extracellular matrix is possible using the modern high-resolution method — nonlinear microscopy based on recording of the second-harmonic generation signal [136]. Second-harmonic microscopy is a nonlinear imaging method based on polarization microscopy. In this case, the optical or infrared laser radiation propagates through a sample, in which, due to the optical nonlinearity of the material, radiation of the double frequency is generated. Although the fraction of the transformed radiation is small, it is possible to record the second harmonic due to the high intensity of the original laser radiation and the absence of interference in the double frequency region. The method is very sensitive to changes in surface properties, mechanical stresses and the state of biological objects. It allows one to characterize the molecular nonlinear-optical properties of collagen and determine the three-dimensional structure of collagen fibers inside the tendon. Its main advantage is the ability to visualize living, unstained, and unfixed tissues, as well as fixed ones [137]. An additional advantage of this method is the possibility of its use simultaneously with other visualization methods in one sample [138].

Tomographic and ultrasound methods. Gorska et al. [139] used high-resolution tomography and endobronchial ultrasound in patients with asthma and COPD to assess the respiratory tract wall thickness. They concluded that ultrasound was more suitable for this purpose, although there was a good comparability between the results of both methods. Hartley et al. [140] compared the thoracic tomographic parameters and the lung function in patients with asthma and COPD; in both diseases, the distal bronchioles were found overstretched and air traps formed.

The method of endoscopic OCT was used by Ding et al. [141] to assess the stage of COPD and the morphology of the respiratory tract in these patients; the authors also analyzed the architecture of the respiratory tract in heavy smokers with normal FEV1 and subjects who have never smoked. A high correlation was found between the histological and OCT data in respect to the bronchial wall remodeling [142, 143]. Low amplitude OCT scattering compatible with bronchial and peribronchial edema (i.e., excessive ECM hydrophilicity) and powerful OCT scattering during epithelial delamination [144] were found.

Methods for biomarker analysis

Immunoenzyme, radioimmune, immunological and immunohistochemical methods are used to detect ECM-associated biomarkers [134]. For clinical monitoring, samples obtained from body fluids are the most convenient (urine or blood plasma). In these biosamples, soluble derivatives of ECM components (elastin, collagen, fibronectin, or hyaluronan) can be determined. Tissue biopsies or intraoperative material can be used to examine almost all components of the matrix, but they are not suitable for continuous monitoring in the clinic.

The below biochemical methods are used to detect ECM biomarkers.

Determination of total protein content. Although the total protein content in tissue or cell samples does not apply to ECM biomarkers, it is often a necessary characteristic, since it allows to establish the relative number of ECM components as well as products of their synthesis and degradation. The total protein content can be quickly assessed using a simple colorimetric analysis of the amino acid mixture formed during acid hydrolysis of the sample. The reagent is ninhydrin (2,2-dihydroxyindan-1,3-dione) that gives an intense blue staining (575 nm) when combined with the primary amino groups of free amino acids. Calibration requires standard samples of protein hydrolyzate that are commercially available or can be prepared from a soluble protein. Acid hydrolysis of tissue is carried out with concentrated HCl. A full description of the technique can be found in Stoilov et al. [145].

Determination of elastin by immunoassay. Elastin is an insoluble protein that cannot be extracted from tissue by surfactants, chaotropic agents, reducing agents or proteases; yet, it can be converted to a soluble form by special methods of solubilization. After that, determination of elastin can be performed on the basis of desmosine or isodesmosine with high-performance liquid chromatography, radioimmunological analysis (RIA), enzymatic immunosorbent analysis (ELISA), capillary electrophoresis or mass spectrometry. However, only RIA and ELISA provide highly efficient analysis necessary for serial tests. For detailed protocols and discussion on these methods, see Stoilov et al. [145].

Determination of hydroxyproline. Hydroxyproline is a specific amino acid of fibrillar collagen, constituting about 13.5% of this protein. It is easily determined in hydrolyzates of proteinaceous tissue using the Ehrlich's reagent and colorimetry at 550 nm. This method allows one to determine hydroxyproline with good accuracy even in microgram samples. It should be noted that hydroxyproline is contained not only in collagen but also in elastin. Giving that its concentration in elastin does not exceed 1%, the presence of elastin is not expected to introduce a significant error in the measurement of collagen based on hydroxyproline [146].

Determination of lysyl oxidase activity. Lysyl oxidase and related mammalian enzymes catalyze the extracellular oxidative deamination of lysine in elastin, as well as lysine and hydroxylysine in collagen. The resulting aldehyde forms of proteins then spontaneously form lysine bridges necessary for the normal functioning of tissues. Any abnormal course of this process may be a sign of fibrosis or other pathological conditions; therefore, the lysine oxidase activity is a very important marker of ECM remodeling. The activity of lysine oxidase can be measured by the RIA method; however, tritium labeled substrates needed for this undertaking makes this approach hardly suitable for continuous monitoring of ECM.

A simpler way of analysis is the determination of hydrogen peroxide released during oxidative deamination of lysine (hydroxylysine) based on enzymatic oxidation of homovanilic acid or amplex red in the presence of horseradish peroxidase with the measurement of oxidation products by the fluorescence method (homovanilic acid — 315/425 nm, amplex red — 563/587 nm). Although this method is about twice less sensitive than the RIA, it allows for the analysis of series of samples, including the use of robotic analytical systems. Protocols for tissue extraction, sample preparation and analysis are presented in Trackman et al. [147].

Plasma fibronectin determination. Fibronectin is a glycoprotein that is present in the body of most mammals in two forms — the insoluble fibrillar mesh on the cell surface and extracellular matrix and the soluble form in the blood. Fibronectin is synthesized by many types of cells with the exception of some nerve cells. The soluble fibronectin is synthesized predominantly by hepatocytes and circulates at a concentration close to micromolar. Fibronectin accumulates in fibrils of the extracellular matrix on the cell surface together with locally produced cellular fibronectin. Commercial enzyme immunoassays (EIA) are most commonly used for analyzing the plasma content of fibronectin [148].

Determination of hyaluronan (hyaluronic acid). This is glycosaminoglycan, a high molecular weight polysaccharide with an unbranched main chain consisting of alternating β-(1-4)-glucuronic acid residues and β-(1-3)-N-acetylglucosamine. Each dimer is considered one structural unit with a molecular weight of about 450 Da. Hyaluronan is produced mainly by fibroblasts and other specialized connective tissue cells. It plays a structural role in the connective tissue matrix and participates in various intercellular interactions. Hyaluronan is widely distributed in the body and can be found free in plasma, serum and synovial fluid [149]. It is measured by electrophoresis or EIA. In test systems for the determination of hyaluronan by the EIA produced by Corgenix Medical Corp. the material derived from bull cartilage is used.

Immunohistochemical methods. Immunohistochemical microscopic examination provides the most

specific identification of a tissue component of interest. In this method, tissue slices are labeled with specific antibodies to a protein of interest, which serves an antigen. For the first time, a method of staining cellular components by using specific antibodies was proposed by Coons et al. as early as in 1941. Later, antibodies labeled with enzymes have been developed.

There are many ways of using immunohistochemistry for detecting ECM proteins and their degradation products. Immunohistochemical methods allow for the localization of various extracellular matrix proteins in tissues with the help of antibodies to various ECM proteins of interest. For example, antibodies to type I and type IV collagen, laminin and fibronectin were used to demonstrate the presence and localization of these ECM components in the human submesothelial zone of the peritoneum [150].

To assess the matrix degradation, more sophisticated versions of immunohistochemistry have been developed. Antibodies to neo-epitopes emerged after metalloproteinase-driven collagen degradation are available. For example, an antibody against a type II collagen neoepitope was used to study the role of MMP-13 in the development of the bone tissue [151].

Physico-chemical methods of isolation and preparation of ECM components. In most cases, the isolation and concentration of ECM components or products of their synthesis/degradation are performed using electrophoresis and highly sensitive liquid chromatography. However, these methods are mainly used to determine markers of ECM metabolism in liquid samples. Recently, methods for non-liquid tissue samples preparation and measurement have been developed. For example, the levels of elastin and collagen in tissues are often difficult to measure due to the limited solubility of these proteins. To approach this problem, the biomaterial is subjected to acid hydrolysis, which cleaves tissue-associated proteins to their constituent amino acids. Then, the amino acids that are unique for each protein (desmosine for elastin and hydroxyproline for collagen) are quantified. The total protein content can also be assayed with part of the hydrolyzate using the reagent for free amino groups [145].

Conclusion

Today, determining the ECM markers is important for monitoring the ECM remodeling. Further studies on the known and novel ECM markers are needed for understanding the mechanisms of multimorbidity and, accordingly, for the development of new treatment strategies. Despite significant progress in this area, many unresolved issues about the implementation of the above methods remain. Among them, the limited access to ECM in organs and tissues; that necessitates using non-invasive or minimally invasive methods. Secondly, the existing methods for the isolation and analysis of ECM markers are technically difficult and/or costly (sequencing, immunohistochemistry methods). Further research should focus on new, more accessible markers of the ECM, a comprehensive assessment of their role in physiology and pathogenesis, and improvement of methods for their accurate and rapid determination.

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