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REVIEW ARTICLE

Connective tissue and diseases: from morphology to proteomics towards the development of new therapeutic approaches

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ABSTRACT

Connective tissue consists of cells separated by the extracellular matrix, whose composition and amount vary according to age, to functional requirements, and to the presence of pathologic conditions. Within this non-random macromolecular assembly, collagens, elastin, proteoglycans and structural glycoproteins are mutually interdependent and modifications of one component, by extrinsic (environmental) and/or intrinsic (systemic, genetic, age-related) factors, may have consequences on the tissue as a whole. Since decades, different microscopical techniques have been applied mainly for diagnostic purposes and for detailed descriptions of changes occurring in cells and in matrix components. More recently, in order to dissect the molecular complexity of the matrix network, to analyse the interactions between cells and matrix and to look for modulators of cell phenotype, histomorphologic investigations have been implemented with proteomic studies that allow to identify possible diagnostic markers, and to better understand patho-mechanisms enabling the design of novel therapeutic strategies. Therefore, the progressively expanding, although incomplete, knowledge on connective tissue biology, sheds new light on the pathogenesis of diseases affecting single molecules (i.e. collagenopathies, mucopolysaccharidoses, elastinopathies) and discloses the importance of matrix components as fundamental regulators of cell phenotype, in relation, for instance, to the aging process and/or to cancer development and progression. Few examples will be presented demonstrating the promises of proteomics as a technique leading to the discovery of new therapies and possibly to the development of individualized treatments for a better patient care.

Keywords: pathology, proteomics, fibrosis, rheumatology, cancer

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INTRODUCTION

The composition of the extracellular matrix (ECM) can be schematically represented as a non-random macromolecular assembly of constituents belonging to four major families of molecules: collagens, elastin, proteoglycans and structural glycoproteins. Since decades, histopathological investigations on connective tissues lead to the discovery of an ever-growing ECM components, which rapidly increased from the originally recognized "collagen, elastin and mucopolysaccharides" to more than 29 different collagen types, a large number of proteoglycans, an increasing amount of glycoproteins, besides the microheterogeneity created by alternative splicings of primary transcripts. This complexity was accompanied by the discovery of membrane

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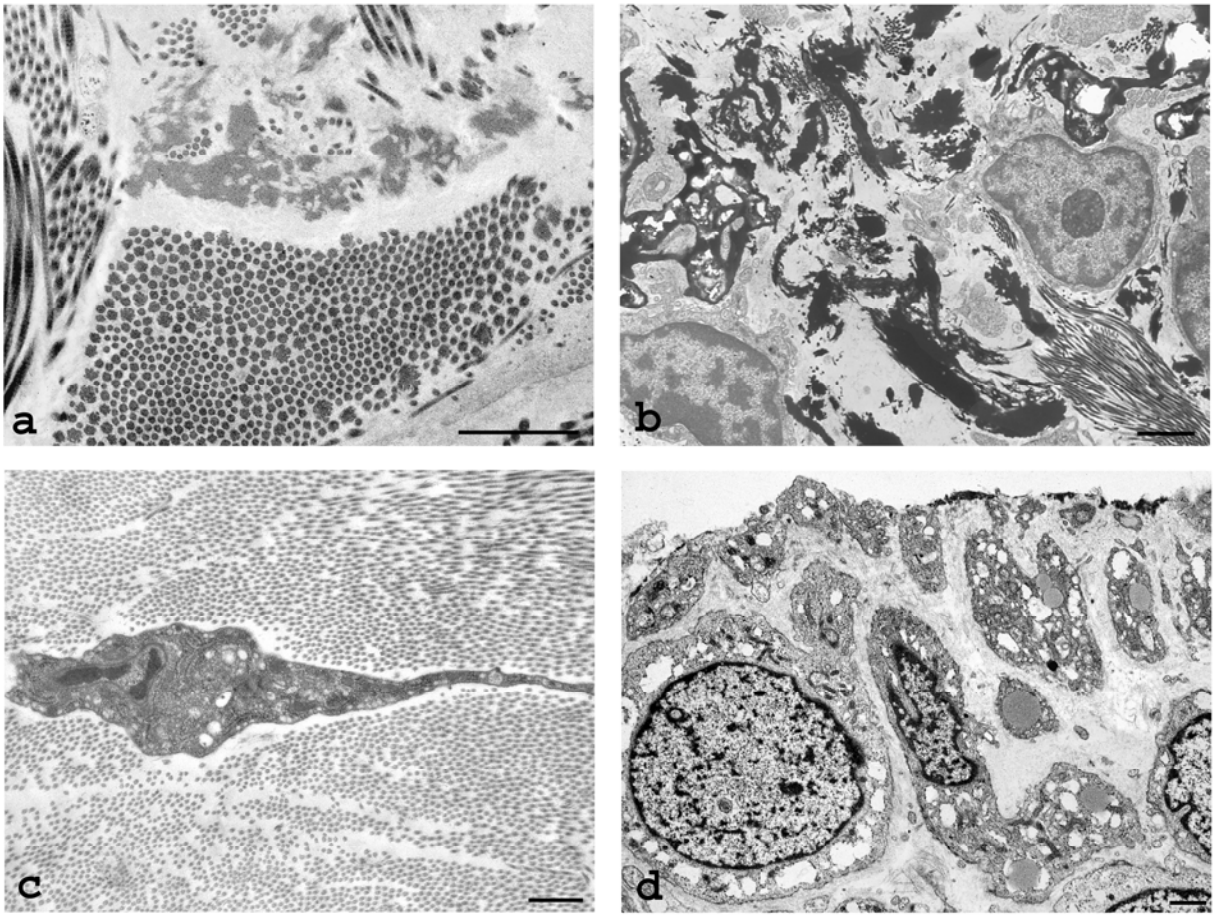

Figure 1. Transmission electron microscopy of : a) skin from a patient affected by a collagenopathy showing collagen flowers indicative of abnormal fibrillogenesis, and small fragments of amorphous elstin forming functionally inefficient elastic fibers; b) skin from a patient affected by pseudoxanthoma elasticum showing numerous irregular and polymorphous elastic fibers, in some areas the fibers are deformed by mineral precipitates; c) aponeurotic cell immersed in a collagenous matrix from a fibrotic fascia of a Dupuytren's affected patient, d) a multilayer of synoviocytes in a patient suffering from osteoarthritis. Bar: 1 μm

receptors mediating cell–matrix interactions as well as signaling pathways (1). The microenvironment is capable to greatly influence gene expression and the behaviour of cells is largely determined by interactions with extracellular matrix, with neighbouring cells and with soluble local and systemic factors. Since decades, different microscopical techniques (from light to transmission electron microscopy, from histochemistry to immunoelectronmicroscopy) have been applied mainly for diagnostic purposes and for detailed descriptions of changes occurring on cells and on matrix components (2-4) (Figure 1). More recently, the development of new structural approaches and the possibility to observe samples in a physiological environment improved the knowledge of connective tissue biology and disclosed new pathways in the pathogenesis of genetic and/or acquired diseases (5-7). Therefore, the importance of connective tissue components, cellular as well as extracellular, has been highlighted in genetic diseases mainly affecting single molecules (i.e. collagenopathies, mucopolysaccharidoses, elastinopathies), in acquired disorders, mostly related to the aging process (i.e. localized or

systemic fibroses, emphysema, rheumatologic diseases, osteoporosis, vascular complications) and in the modulation of neoplastic phenotype by interactions between cancer cells and stroma. In order to dissect the molecular complexity of the matrix network, to analyse the interactions between cells and matrix and to look for modulators of cell phenotype, histomorphologic investigations have been implemented by proteomic studies that, through a broad characterization of cellular protein profiles (Figure 2) and/or of biological fluids, allow to identify possible diagnostic markers and to better understand patho-mechanisms enabling the design of novel therapeutic strategies (8).

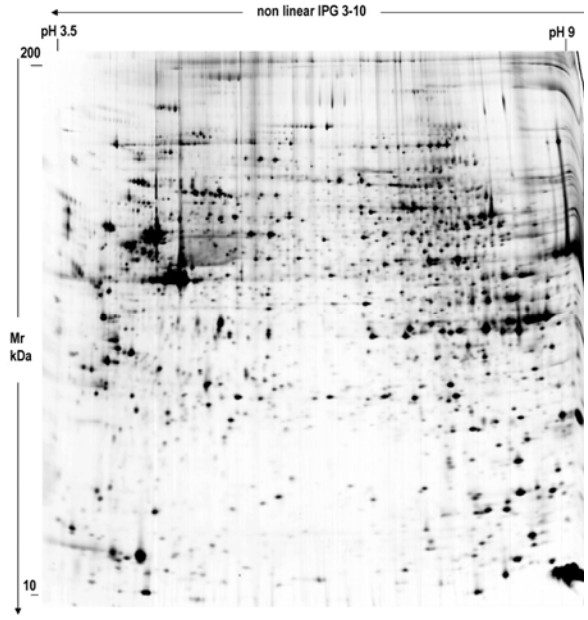

Figure 2. Silver stained 2D electrophoretogram of human dermal fibroblasts. Proteins have been separated by molecular weight and isoelectric point.

A few examples will be presented in order to provide the reader of the "flavour" of the complexity and importance of connective tissues as well as the potentiality of proteomics applications in disclosing molecular pathways towards the discovery of new therapies, possibly leading to the development of individualized treatments for a better patient care.

ELASTINOPATHIES

Elastin and elastic fibers, among all connective tissue macromolecules, represent a unique model for investigating the influence of endogenous and exogenous noxae in matrix homeostasis. They are produced only in the perinatal period, but are designed to maintain elastic function for lifetime and their importance is due to the fact that they endow critical mechanical properties on elastic tissues and regulate cell fate in several organs.

Loss of elasticity due to degradative/degenerative changes is one of the major contributing factors to connective tissue ageing, to development of aortic aneurysms and lung emphysema, and to changes in sun-damaged skin. The dramatic consequences of the loss of elastin and/or of elastic fiber elasticity are clearly visible in numerous diseases among which the most important are pseudoxanthoma elasticum, elastosis perforans serpiginosa, Menkes, cutis laxa, Marfan, Buschke-Ollendorff syndrome and Hutchinson-Gilford progeria, although some of these alterations have been noticed also in collagenopathies as prolidase deficiency, Elhers Danlos and osteogenesis imperfecta (9). Connective tissue is, in fact, a complex and integrated network of several macromolecules that may be modified as a result of mutations in genes coding for matrix constituents, as a consequence of altered cellular homeostasis, and in response to exogenous factors. One of the most striking alterations of elastin elasticity, which may take place with aging, as well as in genetic and acquired diseases, is the occurrence of ectopic calcifications. As a disease model, we have investigated, since years, the pathogenesis of pseudoxanthoma elasticum (PXE), a genetic disorder that was unpredictably demonstrated to be due to mutations in the ABCC6 gene belonging to the ABC transporter

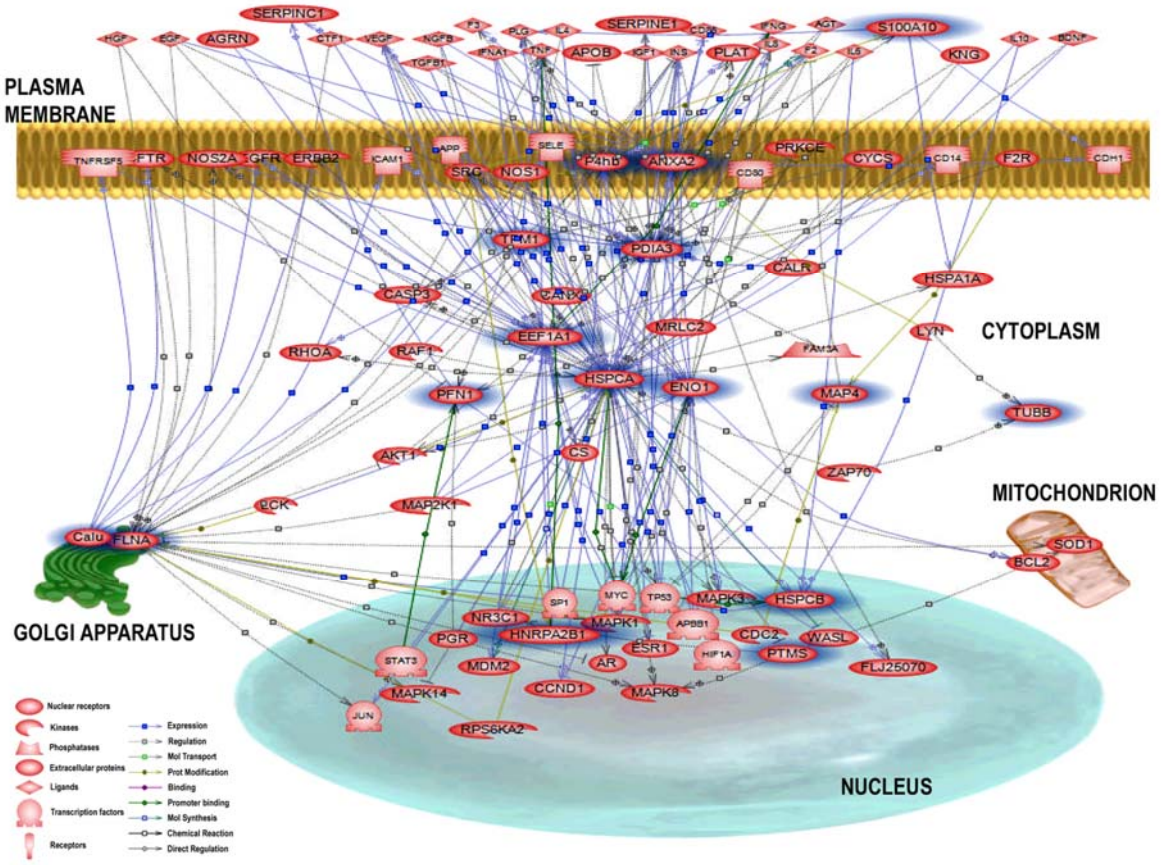

Figure 3. Interaction map obtained after the analysis of the protein profile of PXE compared to control fibroblasts. The map was generated with the ResNetCore database from Ariadne Genomics at www.ariadnegenomics.com. Proteins, that were differentially expressed and identified by MS/MS, appear highlighted.

family (10). PXE is characterized by progressive mineralization of elastic fibers, with major clinical consequences on skin, eyes and cardiovascular system. By light microscopy, elastic fibers in the mid-dermis appear polymorphous, fragmented and mineralized, whereas collagen bundles are sometimes poorly organized. By electron microscopy, elastic fibers exhibit two different types of mineral precipitates: fine deposits in the center of the fiber and bulky precipitates deforming and breaking the fibers (11). In the mineralized areas of the dermis, deposits of thread-like material and collagen fibrils in form of "collagen flowers" are always present, supporting the hypothesis that even tough the most dramatic alterations involve the elastic component; the whole matrix homeostasis is affected due to abnormal mesenchymal cell phenotype (12). Dermal fibroblasts, for instance, are numerous, exhibit a hypertrophic endoplasmic reticulum with numerous cisternal dilatations (11) and are characterized by abnormal cell-matrix interactions and increased proteolytic potential (12,13). The characterization of the protein profile of fibroblasts isolated from the dermis of PXE patients, lead us to the identification of several differentially expressed proteins mainly related to cytoskeleton, redox balance, endoplasmic reticulum stress response, metabolism, membrane stability (Figure 3) and to the demonstration that oxidative stress has profound and endurable consequences on PXE fibroblast phenotype being

responsible for reduced levels of global DNA methylation, increased amount of carbonylated proteins and of lipid peroxidation products. In particular, by identifying a network of proteins, i.e. matrix gla protein, calumenin and protein disulfide isomerase, affecting elastic fibre calcification through inefficient vitamin K recycling, new light was shed on the pathogenetic pathways in PXE, and on the role of proteins that could be regarded as druggable targets aiming to delay and/or revert the pathologic phenotype of PXE fibroblasts and in particular elastic fiber mineralization (14).

FIBROTIC DISEASES

Fibrosis is defined by the overgrowth, hardening and/or scarring of tissues and organs including lung, liver, kidney, intestine, heart, skin and bone marrow. It is attributed to excess deposition of extracellular matrix components as collagen, possibly due to chronic inflammatory reactions induced by a variety of stimuli (i.e. persistent infections, autoimmune reactions, allergic responses, chemical insults, radiation, and tissue injury). Although current treatments for fibrotic diseases such as idiopathic pulmonary fibrosis, liver cirrhosis, systemic sclerosis, progressive kidney disease, and cardiovascular fibrosis typically target the inflammatory response, there is accumulating evidence that the mechanisms leading to fibrosis are different from those typical of inflammation (15) and that in several circumstances the key cellular mediator is the myofibroblast, a primary collagen-producing cell that can be found in granulation tissue, in fibrotic conditions, but also in tumour stroma, thus promoting cancer progression. These data highlight the role of myofibroblast as a promising target for anti-fibrotic and anti-cancer therapy (16).

Myofibroblasts may derive from resident mesenchymal cells, from circulating fibroblast-like cells originating from bone-marrow stem cells, and from epithelial and endothelial cells through a process called epithelial/endothelial-mesenchymal (EMT/EndMT) transition. Moreover, they are activated by a variety of mechanisms, including paracrine signals derived from lymphocytes and macrophages, autocrine factors secreted by myofibroblasts, and pathogen-associated molecules (15). The term myofibroblast was introduced by Majno and co-workers (17) with the aim to identify cells observed in experimental wound-healing. During the next decade, due to the development of immunohistochemical techniques, myofibroblasts were found to contain α-smooth-muscle actin (SMA), however, the unambiguous characterization of myofibroblasts was given by electron microscopy with the detailed visualization of cell structural features as the spindle cell morphology, the prominent endoplasmic reticulum, the peripheral smooth-muscle myofilaments and fibronexus junctions in association with a positivity for SMA. A more precise characterization of myofibroblasts and of their role in fibrotic disorders, as interstitial lung fibrosis, Dupytren's and Peyronie's diseases, has been recently revealed by proteome analysis.

Idiopathic pulmonary fibrosis (IPF) is an interstitial lung disease morphologically characterized by alternating regions of normal lung parenchyma, interstitial inflammation, fibrosis, and "honeycombing", as the result of failed alveolar reepithelialization, fibroblast persistence, and excessive deposition of collagen and other ECM components. Long-term survival of IPF patients is poor, with a 5-year survival rate of only 20%. Given the poor efficacy of treatments with corticosteroids and/or cytotoxic agents such as prednisone, novel therapeutic strategies are required to improve IPF management. This goal can be achieved through a better understanding of the molecular mechanisms underlying the pathogenesis and progression of

this disease (18). A comparison of the protein profile of primary human lung fibroblasts derived from patients with lung fibrosis with that of control fibroblasts of unaffected lung tissues, revealed a significant modulation in the expression of proteins related to the cytoskeleton, lower levels of components of the antioxidative system, as well as a reduced expression of major histocompatibility complex class I C (19). Within this context, the widespread use of high-throughput approaches, already started to disclose, in pulmonary fibrosis, molecular pathways and consequently key molecular targets that involve epithelial and endothelial injury, hypercoagulation, fibroblast activation and differentiation, EMT, fibrocyte recruitment, ECM deposition, angiogenesis, and aberrant repair mechanisms, thus offering a new spectrum of theraputic intervention, some of which are already tested in clinical trials, as anti-transforming growth factor b1, 2 and 3, anti-connective tissue growth factor (FG-3019), interferon-g1, anticoagulants, pirfenidone, imatinib, rapamycin (everolimus), bosentan (18).

Among other fibrotic diseases that have been recently investigated by proteome analysis, it could be mentioned Dupuytren's disease (DD), that is characterized by cell proliferation and progressive collagen deposition causing the contracture of the aponeurotic fascia and consequently the invalidity of the fingers involved. Genetic, metabolic and environmental factors are involved in the pathogenesis of the disease, but their role is not clearly established. Although Dupuytren's contracture might resemble the phases occurring during the repair processes and could therefore involve myofibroblasts in its pathogenesis, ultrastructural evaluations performed on aponeurotic tissue from patients affected by Dupuytren's contracture, revealed the presence of cell and matrix alterations in different parts of the diseased aponeuroses (20). Surprisingly, myofibroblasts or myofibroblast-like cells were only occasionally visible in the diseased aponeuroses. Morphological data sustain the hypothesis that aponeurotic cells in DD have abnormal cell-matrix interactions and modified integrin expression and that altered cell phenotype might have functional consequences (21).

Different therapies have been proposed for DD treatment, but their real effectiveness has not been shown and surgical therapy (fasciectomy) is the most used treatment to correct finger deformity and to avoid joint ankylosis (22).

Proteomic profiling of pathologic versus unaffected patient-matched palmar fasciae tissues from DD patients revealed the involvement of several different molecular processes related to disease progression, including extra- and intra-cellular signalling, oxidative stress, cytoskeletal changes, and alterations in cellular metabolism. In particular, autocrine regulation through ERBB-2 and IGF-1R receptors and the Akt signalling pathway have emerged as novel components of pro-survival cell signalling, thus providing a basis for a new therapeutic strategy in DD (23).

DEGENERATIVE DISORDERS

This broad group of diseases, such as Parkinson's, Alzheimer, diabetes, atherosclerosis, osteoporosis, arthritis, is characterized by a progressive deterioration of the function or structure of affected tissues or organs. Patients can often live for years with their diseases, although symptoms usually become more severe and disabling with time and death may occur as a result of complications of these disorders.

For instance, in inflammatory rheumatic diseases, e.g., rheumatoid arthritis (RA) or spondyloarthropathy (SpA), one of the primary targets of inflammation is the synovial tissue, that lines the joint cavity and is responsible for joint homeostasis (24). In pathologic conditions as Ra and SpA, the phenotype of the synovium changes into an activated, proliferative tissue, leading to

narrowing of the joint space, increased efflux of synovial fluid and subsequent swollen joints. Moreover, synovial hyperplasia is accompanied by infiltration of lymphocytes and monocytes and by proteolytic enzyme production (mainly matrix metalloproteinases), that play a major role in cartilage and bone loss (25).

By contrast, is non-inflammatory rheumatic disease as Osteoarthritis (OA), neutrophils are absent and the primary target is the articular cartilage that degenerates due to imbalanced anabolic and catabolic processes within chondrocytes.

Despite these well known histopathological features (26), rheumatologic disorders are relatively poorly understood and not easily managed from the clinical point of view. In addition to traditional disease-modifying anti-rheumatic drugs as injectable gold, sulfasalazine, methotrexate, corticosteroids, cyclosporine, an increasing number of other therapeutic agents became available in the last decade, namely anti-TNF agents (infliximab, etanercept, and adalimumab), as well as treatments directed against interleukin (IL)-1 (anakinra), IL-6, IL-15, T cells (abatacept), and B cells (rituximab), never the less, most patients show large inter-individual variability in drug response and toxicity, due to genetic, physiological, pathophysiological, or environmental factors (27).

Therefore, proteomic-based approaches, mainly focusing on protein target identification by differential screening of biological fluids (serum and synovial fluid) or rheumatic tissues (synovial tissue, cartilage and chondrocytes) and on identification and characterization of autoantigens, provide an increasing panel of molecules that may have the potential to serve as early biomarkers as well as potential druggable targets (28). Using 2D gel electrophoresis, Tillerman and co-workers, by hierarchical clustering based on the protein expression profiles, were able to discriminate between various forms of rheumatic diseases (29). As an example, evaluation of calgranulin-A expression differentiates OA from RA, whereas S100A8, that is specific for cells of myeloid origin such as granulocytes, monocytes and macrophages and is released during the interaction of monocytes with the inflammatory activated endothelium, allows to discriminate between diseases of inflammatory and non-inflammatory origin. Furthermore, 2D gel electrophoresis, followed by western blotting and incubation of patients' sera, contributes to reveal a variety of potential autoantibodies in patients with RA. Antibodies against citrulline-containing proteins, for instance, have shown to be highly specific for RA (specificity: 96–98%) (30), and citrullinated α-enolase has been identified as an abundantly expressed autoantigen in synovial tissue of RA patients, Therefore, the discovery of new autoantigens and/or the evaluation of specific post-translational modifications of these autoantigens by proteome analysis could open new doors for drug targets.

CONNECTIVE TISSUE AND CANCER

Fibroblasts play a key role in modulating connective tissue homeostasis. Several humoral and cellular factors may contribute to activate or to inhibit fibroblast activity or, after being released from fibroblasts, may reciprocally act on other mesenchymal and epithelial cells. Tumor progression, for instance, is influenced by extracellular matrices and by soluble factors or cytokines that are locally produced by host tissue cells and that may sustain one of the most insidious aspects of tumors, namely their propensity to invade normal tissues and to form secondary foci (metastases) in organs at distant sites from the primary tumor. During this process, invasive cells interact with cells of the host tissue (i.e. fibroblasts, endothelial cells, macrophages, lymphocytes) (31). Neoplastic and normal cells may be able to mutually modulate their activities

through soluble messages (cytokines) and through insoluble molecules of the extracellular matrix. Cancer cells, for instance, may regulate the biosynthetic activities of fibroblasts, thus altering the scaffold of the tumor. Reciprocally, host cells secrete extracellular matrix proteins and cytokines, which may influence growth and activities of tumor cells. In addition, host cells may produce proteolytic enzymes contributing to host tissue destruction and cancerous cells migration (32). Although, there is no evidence that stromal cells are directly involved in carcinogenesis, several studies have shown that stroma is a key determinant of epithelial proliferation, cell death, motility and differentiation (33) and that normal fibroblasts are able to convert or modulate malignant tumor cell lines into morphological benign or biologically less aggressive cell populations (34). Therefore, modulation of tumor stroma may result in more efficient tumor regression and this aspect could be very important in cancer treatment by depriving tumor cells of an essential structural and functional support system (33). Despite the large number of studies performed so far, the complex interactions between tumor and mesenchymal cells are still elusive and studies aiming to understand these interactions may hence open new perspectives for therapeutic intervention. For instance, a comparison of the proteome profiles of normal human skin fibroblasts and of melanoma-associated fibroblasts (35) revealed possible candidate biomarkers. Beside glutathione peroxidase GPX5, that is secreted by melanoma cells, two proteins are specifically produced by melanoma-associated fibroblasts, namely periostin and stanniocalcin-1, thus contributing to the identification of molecular markers, which may also represent possible new targets for therapeutic intervention.

CONCLUSIONS

Within connective tissues, the extracellular matrix provides cells with a mechanical scaffold for adhesion and migration, but represents also a reservoir of cytokines/growth factors and a functionally active substrate for cellular crosstalk. This heterogeneous and composite network plays a crucial role in numerous diseases, as already clearly demonstrated by different morphological approaches. The progressively expanding use of proteomic analyses, by dissecting the molecular pathogenetic pathways, provides a fundamental contribution to the identification of effective molecular targets, allowing the discovery and the development of novel therapeutics. In addition, it is worth mentioning that proteomics may also represent a precious tool for understanding the efficacy and/or the toxicity of specific components within herbal medicine preparations that have been used with promising results, for instance, in the traditional Chinese medicine. Since, an increased attention has been recently paid to natural products isolated from plants, marine flora and microorganisms, the identification of selected active components by screening with high throughput technologies, as proteomics, will offer a great advantage to pick up compounds and eventually to improve their therapeutic potential by molecular modifications (36-38).

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