Proteases and Cancer

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METHODS IN MOLECULAR BIOLOGY™

Proteases and Cancer

Methods and Protocols

Edited by

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Preface

The recent availability of the complete genomic sequences of several mammalian organisms has led to an explosion in the knowledge of proteolytic enzymes. The realization that proteases constitute more than 2% of the total genes in the human genome underscores the importance of proteolysis to human biology and to human diseases such as cancer. Cancer development is characterized by genetic alterations in critical genes, including oncogenes and tumor suppressor genes, that provide proliferative or survival advantages to the developing tumor. Tumor cells further epigenetically subvert normal physiological processes in order to promote their own growth and survival. Proteases decisively contribute to cancer development and promotion by regulating the activities of growth factors/cytokines and signaling receptors, as well as the composition of the extracellular matrix, thereby suppressing cell death pathways and activating cell survival pathways. In addition, proteases are recognized to contribute to malignant potential in several tissue contexts by stimulating tumor cell migration and invasion. The tumor microenvironment, which is increasingly recognized to play a critical role in tumor development and progression, is critically modulated by protease activities, through activation of tumor-promoting inflammatory, angiogenic, and immune pathways. Thus, effective targeting of specific protease activities associated with tumor development holds considerable promise for improving the diagnosis and treatment of cancers.

Proteases are enzymes that catalyze the cleavage of proteins through the hydrolysis of peptide bonds. The known array of proteases and protease homologues comprises five protease families known as metalloproteinases, serine proteases, cysteine proteases, aspartic acid proteases, and threonine proteases. Protease cleavage is usually irreversible; therefore, the activities of proteases must be tightly regulated in order to prevent inappropriate and frequently pathogenic proteolysis. Most proteases are either synthesized as inactive zymogens or they are sequestered in latent forms that are specifically activated to unleash cellular responses critical for normal physiology. The sequelae of biological responses that may be generated by such protease zymogen cascades are frequently underestimated. Proteases are further controlled by regulated access of proteases to their appropriate substrates and spatial compartmentalization of proteases in cells and tissues. The regulated termination of proteolytic activities is also critical and may be accomplished by specific endogenous inhibitors and clearance receptors. Because of the complexity and intricate regulation of protease systems, delineating the activities and specific biological impact of protease pathways will require refinement of current technologies and development of unique molecular tools.

The goal of this volume is to bring together a wide range of complimentary techniques that have been developed for the specific detection and analysis of proteases and their activities in cancer. While a variety of techniques are now available for detection and imaging of proteins and receptors in many cellular contexts, the technologies for determining specific protease expression, activity, and functionally important downstream targets remain relatively poorly developed when compared to other classes of enzymes. As a prelude to understanding the approaches taken by investigators toward understanding

the broad biological functions of proteases in the pathogenesis of cancer, we first present an overview of major protease classes implicated in cancer development and the issues involved in delineating protease functions during cancer development. The complexity of the roles played by secreted, pericellular, and intracellular proteases is highlighted. Examples of mechanistic studies using organ-specific mouse models of cancer development combined with transgenic targeting of proteases highlight the diverse and complex roles that proteases may play in tumor initiation and progression.

The spectrum of proteases that are expressed at a specific moment or circumstance by a cell, tissue, or organism has been termed the "degradome." New insights from genomic and computational analyses of degradomes and the challenges faced in the analysis of the large amount of information and techniques for simplifying the complexity of protease cascades are discussed next. This analysis has provided unique perspectives into the evolution of mammalian organisms. The spectrum of proteases, protease inhibitors, and protease interactors associated with cancer development can be experimentally identified at the transcript level by the profiling of gene expression using microarray technologies. The study of the expression of protease genes involved in cancer development presents additional unique challenges, however, since proteases are expressed not only by the tumor cells, but also by stromal cells that are located within the tumor microenvironment surrounding the tumor (e.g., fibroblasts, endothelial cells). Determination of which cells are expressing proteases and the molecules that regulate them is critical to understanding the functional roles of the proteases and designing effective therapeutic applications. An elegant approach to this challenge is presented using a microarray strategy that has the advantage of distinguishing the cellular origin of the genes detected through differential species specificity. The approach involves xenograft models in which human tumors are implanted into mice. Tissues are analyzed using a dual species protease/inhibitor microarray chip, validated for its ability to distinguish between human and mouse transcripts.

Proteases are associated with specific unique recognition sequences where the substrate peptide bond is cleaved, designated …P1-P1′…, where cleavage occurs between P1-P1′ amino acid residues. Biochemical and proteomics methods have proved invaluable in the identification of preferred cleavage sequences, which can then in turn be used in the design of experimental tools for detection or inhibition of protease activity and to assist in predicting endogenous substrates and signaling pathways that involve the protease of interest. Ideally, these approaches must be rapid yet comprehensive with respect to the inclusion of all potential target recognition sequences. Presented in this volume are several complementary high-throughput approaches aimed at identifying preferred cleavage sequences for proteases. In the first, positional scanning synthetic combinatorial libraries of fluorogenic peptides have been developed to identify the preferred amino acid residues at P1 through P4 positions for a given protease. This strategy has proven extremely useful for determining preferred cleavage sequences specific to a protease; however, possible interdependence between positions may not be identified by this method, since every position other than the position of interest is randomized. In an alternative strategy, protease cleavage site preferences are determined using mixture-based peptide libraries where random amino-terminally capped peptide mixtures are digested with the protease of interest and the cleavage products analyzed by automated Edman sequencing. Based on initial determinations, the process is reiterated until the full cleavage motif preference of the protease is known. In the final strategy, a substrate phage display technique is presented where randomized peptide substrates are displayed as fusion proteins on the outside of a bacteriophage. Here, the technique has been adapted to a semiautomatic

platform in order to obtain a rapid but comprehensive substrate recognition profile for a given protease.

Knowledge of the preferred cleavage sequence of the given protease is the first step toward enabling detection of protease activity. Proteolysis within the pericellular environment occurs predominantly on the cell surface, where inactive zymogens, protease inhibitors, and other protease interactors assemble into multiprotein complexes that mediate the sequential and spatially restricted activation of protease zymogens and their cleavage of target substrates. Therefore, a critical aspect of our understanding of proteases in cancer biology is the ability to detect expression of specific protease activities on cells. A novel method for revealing protease activity on single living cells is presented, which employs a simple noninvasive assay for detecting the presence of specific cell surface proteolytic activity that is based on the use of modified bacterial cytotoxins.

Organ and tumor-type specific regulation of protease activities and substrates is an important consideration in our understanding of protease functions in cancer biology. Preferred cleavage sequences reveal only what substrates can be cleaved, not necessarily what substrates are cleaved in vivo. Identifying and verifying biologically relevant substrates is a major challenge for the protease field. An approach to this problem is presented where differential isotopic labeling is applied to selectively identify potential protein substrates in complex protein mixtures, followed by identification and quantification by tandem mass spectrometry proteomics and database searching.

There is an urgent need to develop more effective strategies for earlier diagnosis of cancer, earlier detection of tumor recurrence, and therapeutic targeting of tumors. Ideally, new diagnostic technologies should be directed toward noninvasive in vivo imaging, which would enable more efficient patient screening and better patient management, thus leading to improved outcomes. Several approaches for catalytic targeting of protease activities associated with cancers that have potential for both the diagnosis and treatment of tumors are presented. In the first, a noninvasive molecular imaging approach is employed for detection of tumor-associated proteolytic activity in living animals. This optical imaging approach utilizes visible and near-infared fluorescence resonance energy transfer (FRET) fluorophore pairs and has been successfully applied to detect and measure matrix metalloproteinase proteolytic activity in tumors in mouse models of cancer. Antiprotease therapy for cancer is a potentially powerful strategy to specifically target rate-limiting steps or proteolytic pathways within tumor cells. An elegant strategy involving the reengineering of protease-activated anthrax toxins to eliminate tumor cells, which has shown promise, is presented. This approach has been demonstrated to successfully target tumor cells that exploit the uPA and MMP protease pathways on the tumor cell surface is next presented, whereby the anthrax toxin PrAg-furin cleavage site is reengineered to generate a PrAg cleaved by the protease of interest. Finally, the development of protease-cleavable linkers that may enable rapid clearance from the circulation of radioisotopes "on demand" is presented, with clinical potential for radioimmunotherapeutic approaches to the treatment of cancer.

The editors are grateful to the contributing authors for providing their expertise in order to bring this volume to fruition. We are also grateful to our all of our colleagues in the protease community who have contributed thoughtful insights into the biological and biochemical approaches discussed within these pages.

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