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Autoantigens

The Critical Partner in Initiating and Propagating Systemic Autoimmunity

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ABSTRACT: The increasing recognition that cancer is frequently associated with an autoantibody response, and observations that systemic autoimmunity is sometimes associated with the diagnosis of a variety of malignancies (many detected near the onset of autoimmune disease), strongly underscore a potential mechanistic connection between cancer immunity and systemic autoimmunity. Accumulating data suggest that autoantigens are critical partners in driving the autoimmune response. Furthermore, unique changes in antigen expression and conformation in the immunizing tumor and the target tissue may play a role in antigen selection and ongoing damage. This construct has important implications for diagnosis, monitoring, and treatment of autoimmunity and, potentially, cancer immunotherapy.

KEYWORDS: autoimmunity; autoantigens; cancer; nucleophosmin; myositis; B23

INTRODUCTION

Systemic autoimmune diseases are complex phenomena in which the immune system recognizes and destroys the body's own tissues. The causes and mechanisms of systemic autoimmune diseases remain unclear. These disorders deserve attention not only because they are often devastating clinically but also because of their immense potential to teach us about the fundamentals of immunologic perception. In this review, we focus on autoantigens in systemic autoimmunity and the powerful insights they can provide into disease initiation and propagation, particularly when studied in the relevant target tissues.

AUTOANTIBODIES

Historically, studies of disease mechanism in systemic autoimmunity have focused on characterization of autoantibodies and identification of their antigenic targets.¹ Although the pathogenic role of autoantibodies remains unclear except in a

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few rare cases,² this body of work has revealed a profound conundrum in human autoimmunity—while many autoantigens can be widely expressed, autoantibodies recognizing specific molecules correlate strongly with certain clinical patterns of organ-specific damage.^{3,4} For example, antibodies to topoisomerase I are found exclusively in patients with the diffuse form of scleroderma, where they are frequently associated with interstitial lung disease.^{5–7} Similarly, antibodies recognizing several aminoacyl tRNA synthetases are found in autoimmune myositis, where they are associated with a distinct phenotype called the synthetase syndrome.^{8–11} Antibodies to the Sm ribonucleoprotein (part of the mRNA splicing complex) are specific for systemic lupus erythematosus (SLE).^{12,13} Interestingly, in some cases, similar targets of autoantibodies are found in a systemic autoimmune disease as well as in cancer. For example, B23/nucleophosmin (B23/NPM) is recognized by antibodies in scleroderma patients with pulmonary hypertension,¹⁴ as well as in patients with hepatocellular¹⁵ or breast¹⁶ cancer. In hepatocellular cancer, autoantibodies seem to appear first around the time of diagnosis.¹⁷ The mechanisms underlying this striking association of specific immune response and phenotype remain unclear.

There are at least three components that interact to produce systemic autoimmune disease: (i) The T-cell and B-cell repertoires capable of immunological specificity and memory, (ii) the physiological context (e.g., infection, Toll-like receptor ligation) integrated by antigen-presenting cells, and (iii) the antigen that initiates and drives the ongoing autoimmune response. Autoantibodies and antigen-specific T cells provide invaluable information about which antigens (or antigenic complexes) are relevant to autoimmune disease. However, they are but enigmatic clues to the complex chronology of autoimmunity.

Most investigations into human autoimmune disease deal with a more mature phase of autoimmunity, when autoimmune processes have generated observable clinical phenotypes. Thus, the specific patterns of autoantibodies and self-reactive T cells seen at these moments result from immunologic integration and filtering of critical events that have already occurred, some of which may have been recent but many of which were probably distant in time.¹⁸ Recent investigations indicate that studying autoantigens in the affected tissue itself will constitute an additional, critically important resource in the analysis of autoimmune initiation and propagation.

Antigen Structure and Conformation Affect Processing and Epitope Selection: Implications for Proteolysis of Autoantigens during Apoptosis

Significant data have demonstrated that antigen-specific T cells are central orchestrators of autoimmunity, making it particularly vital to understand the mechanisms by which T cells become activated and respond to self-antigens. One theory of immunologic perception is especially powerful in explaining how potentially self-reactive T cells may escape central and peripheral tolerance (reviewed in Ref. 19). In the Sercarz construct of immunodominance, T lymphocytes are exposed to a limited number of all the possible peptide fragments that could be derived from protein antigens. Those peptides that can stimulate T cells, having been effectively loaded into the groove of major histocompatibility complex (MHC) molecules during normal pathways of antigen processing, are termed dominant. Those peptides that fail to compete effectively for MHC loading are termed cryptic. The structure of the antigen itself, the presence of interacting proteins, and the nature of processing and

presentation pathways all determine which peptides are efficiently presented and therefore seen by T cells. Any given set of circumstances consistently produces a certain small set of such dominant epitopes in the steady state. T cells undergoing negative selection in the thymus or periphery will be deleted only if they recognize dominant epitopes of autoantigens, namely those peptides that are preferentially generated and presented in that particular microenvironment. Therefore, the T-cell repertoire retains reactivity to cryptic autoantigen epitopes that are not usually presented (or presented only at subthreshold levels) *in vivo*. If changes occur in antigen structure and/or the processing and presentation pathways, cryptic epitopes may be revealed in suprathreshold amounts. Under proinflammatory circumstances, an immune response may ensue and spread to other epitopes of the same molecule.^{20–22}

The hierarchy of processing and the critical role of certain proteases in the selection of epitopes have been shown experimentally in several model systems. Colin Watts' group demonstrated that an asparaginyl endopeptidase (AEP) activity was required in B cells to process tetanus toxin and reveal the dominant epitopes recognized by reactive T cells.^{23,24} The cleavage site for this protease was distant in primary sequence from the T-cell epitopes generated, occurring instead in a hinge region between two domains of the antigen. Thus, an early proteolytic event can determine the accessibility of certain regions to subsequent processing and presentation, thereby influencing the final antigenic image perceived by the immune system. For other antigens, proteases may also cleave and destroy certain epitopes, rendering these sequences cryptic.^{25,26}

Additionally, posttranslational modifications of antigens can affect proteolytic processing, epitope selection, and epitope structure itself. Manoury *et al.*²⁴ found that *N*-glycosylation of the cleavage site asparagine inhibited AEP activity, suggesting that epitopes generated downstream of AEP would be sensitive to this modification. In a mouse model of autoimmunity, Mamula *et al.*²⁷ showed that a minor posttranslational change in protein structure can break tolerance. The conversion of one aspartate to an isoaspartyl linkage in a 25-amino-acid cytochrome *c* peptide produced a potent immunogen, generating robust T-cell responses in their mouse model. T lymphocytes isolated from mice immunized with the isoaspartyl peptide responded only to this form of the antigen in *ex vivo* proliferation assays and not to the aspartyl form of the peptide. Interestingly, autoantibodies from similarly immunized mice recognized both the aspartyl and the isoaspartyl cytochrome *c* peptides by enzyme-linked immunosorbent assay.

Collectively, the above studies demonstrate that the generation and selection of epitopes for presentation to T cells, although generally operating by predictable rules for a specific antigen–APC pair, may be profoundly affected by even subtle modifications of the antigen itself. As noted previously, there is a paucity of data examining autoantigen structural changes in disease-relevant tissues and the role such alterations may play in autoimmune pathogenesis.

Autoantigen Structure Is Modified during Cell Death

In an initial series of studies examining autoantigen structure in a variety of physiological states, we observed that a striking, near-unifying feature of autoantigens is their susceptibility to undergo proteolytic modification during cell death.^{28–31} Of particular relevance in this regard is the cytotoxic T lymphocyte (CTL)–mediated

destruction of virally infected or cancerous cells, in which unique autoantigen fragments are generated by granzyme B.³⁰ Granzyme B, a serine protease found in CTL granules, is essential for initiation of apoptosis in target cells.^{32,33} Also, granzyme B displays great specificity in substrate and site selection, with clear primary sequence requirements surrounding the cleavage site of its substrate,^{34–36} as well as probable macromolecular preferences.³⁷ The autoantigen fragments produced by granzyme B were not generated during other forms of cell death and were especially prominent when caspase activity was inhibited during granule-mediated death.³⁰ There is currently little information available about the function of the CTL granule pathway and its effects on autoantigens in the actual tissues targeted by autoimmune processes during human disease.

To understand autoimmune initiation and propagation, the expression, structure, and cleavage of autoantigens in the most biologically and immunologically relevant microenvironments must be defined. Clinically affected tissues and organs offer appropriate sites in which to examine the forms of autoantigens that propagate autoimmune diseases. In contrast, we know much less about the circumstances of disease initiation, making studies of the status of specific autoantigens in the immunizing tissue difficult. Recent work indicates that a variety of autoimmunity-associated cancers may be appropriate in this regard.

Autoimmunity and Cancer

Previous groups have noted a striking association between various autoimmune disorders (e.g., myositis, scleroderma, SLE, vasculitis) and cancer, with cancer diagnosis and autoimmune detection frequently clustering in time.^{38–40} The association of distinct autoantibodies with unique phenotypes in systemic autoimmune disease is reminiscent of a variety of paraneoplastic autoimmune syndromes where autoantibody and T-cell responses target a set of autoantigens expressed only in the inciting tumor and the tissue under immune attack.^{41–43} For example, expression of neurooncological ventral antigen (NOVA) proteins is increased in tumor tissue from patients with the clinically distinct paraneoplastic opsoclonus–myoclonus ataxia,⁴⁴ and expression of NOVA in healthy adults is restricted to neuronal tissue.^{45,46} Similarly, a high-titer autoantibody response to the Hu antigen is observed in patients with paraneoplastic encephalomyelitis and sensory neuropathy, and elevated levels of Hu antigens are present in small-cell lung cancers and various neuronal tissues.^{47–49} These studies indicate that cancerous tissues may present an altered and proimmune microenvironment where self-proteins with abnormal expression and/or structure are first registered by the immune system. The autoimmune responses originally directed against tumor antigens then spreads to nontumor tissues with shared antigen profiles, leading to tissue destruction manifesting as a paraneoplastic syndrome.

The above findings have focused our attention on whether analogous connections exist in systemic autoimmune diseases, namely that cancer tissues associated with certain syndromes are the appropriate sites of autoimmune initiation in those disorders. We have therefore examined autoantigen expression and structure in relevant tumor tissues to determine if immunogenic properties may be altered in these biological settings. Furthermore, we have investigated the possible pathogenic role of a cross-reactive antitumor response by studying autoantigen expression in the specific

tissues targeted in a particular human disease, as well as in the corresponding tumors.

***Hepatocellular Carcinoma Antigen B23/NPM Has
a Unique Conformation in Liver Tumor Tissues***

The initial model chosen for study was that of hepatocellular carcinoma (HCC), in which patient autoantibodies recognize a variety of nucleolar antigens including B23/NPM. B23/NPM is an abundant and ubiquitously expressed nucleolar protein targeted by autoantibodies in scleroderma¹⁴ and HCC.^{15,17} Imai *et al.*¹⁷ found that several patients with chronic hepatitis or cirrhosis showed increases in antinucleolar antibody titers before diagnoses of liver cancer, indicating that the immune response to nucleolar antigens may reflect changes in the structure and function of these molecules.

When B23/NPM expression levels in HCC liver or nontumor liver were analyzed by immunoblotting, liver cancer expressed increased amounts of B23/NPM compared with nontumor liver. Furthermore, HCC B23/NPM ran slightly faster by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) when compared with nontumor liver, both from healthy individuals and from the surrounding cirrhotic tissue of HCC patients. We also examined the susceptibility of B23/NPM to cleavage by granzyme B in these different lysates: whereas B23/NPM in nontumor liver was extremely resistant to proteolysis by granzyme B, the form in HCC samples was efficiently cleaved. This enhanced susceptibility to cleavage, along with the formation of an SDS-stable oligomeric complex not seen in normal and cirrhotic samples, indicated that B23/NPM had an altered conformation distinct from that of normal liver protein.

We defined the granzyme B cleavage sites in B23/NPM and generated an antibody specific for the amino-terminal fragment resulting from proteolysis at aspartate 161. Using this antibody for immunohistochemistry, we found a robust signal in tumor tissue alone, although we could not detect granzyme B fragments by immunoblot analysis of tumor lysates. These data strongly suggested that HCC B23/NPM displays a novel structure, thus exposing a normally hidden granzyme B cleavage site. A mutant protein lacking only the first six amino acids had similar biochemical properties to HCC B23/NPM, as well as an increased susceptibility to granzyme B cleavage. Hence, truncation (or other posttranslational modifications) at the amino terminus may be responsible for the conformational differences seen in tumor samples.

That small changes in the amino terminus of B23/NPM may significantly affect its three-dimensional structure has interesting implications for the role of altered B23/NPM function in liver tumorigenesis. B23/NPM was found to interact with critical tumor suppressors^{50–54} p14^{ARF} and p53 and is overexpressed in several cancer tissues and cell lines.^{55–57} Several groups have also described phosphorylation of B23/NPM by various cell cycle-regulated kinases, including CDK2/cyclin E,⁵⁸ casein kinase II,⁵⁹ and Polo-like kinase 1 (Plk1).⁶⁰ Of particular note, Plk1, which has critical functions in proper spindle maturation and chromosomal segregation during mitosis,⁶¹ phosphorylates B23/NPM on serine 4.⁶⁰ Thus, reversible modification of the amino terminus likely occurs during normal physiological processes, with subsequent changes in B23/NPM activity and structure. A tantalizing model is

that a more permanent change in the amino terminus is favored and propagated in cancer tissues because of some growth advantage conferred by altered B23/NPM function, and the resulting conformational changes then reveal key cryptic epitopes for autoimmune initiation.

More studies are required to confirm the proposed changes in HCC B23/NPM and to demonstrate structural consequences. Furthermore, alterations in processing and presentation of the tumor form of B23/NPM need to be investigated. Defining the T-cell response to B23/NPM in HCC will be invaluable in these efforts.

***Autoantigens in the Target Tissue in Systemic Autoimmunity:
Insights from Autoimmune Inflammatory Myopathies***

Although B23/NPM provided an excellent view into autoantigen expression and structure in tumors, there was not a natural extension to an associated autoimmune syndrome that had corresponding autoantibodies to B23/NPM. We therefore proceeded to study autoimmune myositis, since a correlation between autoimmune myositis and cancer has been demonstrated repeatedly.^{39,62,63} Interestingly, the most frequent cancers associated with both dermatomyositis (DM) and polymyositis (PM) are adenocarcinomas, being approximately 80% of cancers in patients with cancer and DM and 70% of patients with cancer and PM.³⁹ Also, the muscle tissue targeted in these diseases is readily available for biochemical and histological analysis of antigen expression.

When we examined skeletal muscle tissues from patients with myositis, we observed several striking features of myositis-specific autoantigen expression: (i) myositis autoantigens were expressed at low levels in control muscle but at high levels in myositis muscle and (ii) increased autoantigen expression correlated with differentiation state, such that myositis autoantigens were particularly abundant in cells demonstrating features of muscle cell regeneration. For example, histidyl tRNA synthetase (HRS) is a myositis autoantigen with 10%–20% of patients showing this autoantibody specificity.¹¹ Myositis muscle lysates contained at least fourfold the amount of HRS found in normal control samples. We also detected abundant signal for HRS by immunocytochemistry in diseased muscle sections, whereas few cells showed positive staining in normal tissues. Analysis of serial sections for markers of muscle regeneration, such as neural cell adhesion molecule (NCAM) or neonatal myosin heavy chain, revealed that regions expressing HRS contained differentiating muscle cells. Furthermore, HRS and several other myositis autoantigens were plentifully expressed early on during *ex vivo* differentiation of cultured myoblasts but decreased substantially in myotubes. Finally, we found increased autoantigen expression in several cancers associated with autoimmune myositis, but not in the related normal tissues, demonstrating that tumor cells and undifferentiated myoblasts are antigenically similar.

These data strongly implicate regenerating muscle cells rather than mature myotubes as (i) targets of cytolytic attack in autoimmune myositis and (ii) the source of ongoing antigen supply in this disease. Taking into consideration the additional resemblance of differentiating myoblasts to cancer cells in autoantigen profile, we proposed that cancerous tissue is the relevant microenvironment for disease initiation in autoimmune myositis. A variety of inducers of change (e.g., trauma, exercise, infection) could stimulate muscle cells to regenerate and express suprathreshold levels of

autoantigens recognized by the antitumor response. This would then enable a feed-forward loop with further cycles of damage and regeneration in muscle tissues. Our model provides a plausible explanation for the self-sustaining nature of the autoimmune response and the exacerbations that are general features of autoimmune diseases.

CONCLUSION

Human autoimmune rheumatic diseases are highly complex processes, exhibiting marked genetic, environmental, and kinetic diversity. Despite this, there is a striking correlation between the targets of the immune response in each disease and the clinical phenotype manifested, indicating that the specificity of the autoimmune response marks a centrally important pathogenic principle. The increasing recognition that cancer is frequently associated with an autoantibody response, and observations that systemic autoimmunity is sometimes associated with the diagnosis of a variety of malignancies (many detected near the onset of autoimmune disease), strongly underscore a potential mechanistic connection between cancer immunity and systemic autoimmunity. Accumulating data suggest that autoantigens are critical partners in driving the autoimmune response. Furthermore, unique changes in antigen expression and conformation in the immunizing tumor and the target tissue may play a role in antigen selection and ongoing damage. This construct has important implications for diagnosis, monitoring, and treatment of autoimmunity and, potentially, cancer immunotherapy.

[*Competing interests:* The authors declare that they have no competing financial interests.]

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