Enhanced autoantigen expression in regenerating muscle cells in idiopathic inflammatory myopathy

Livia Casciola-Rosen,1,2 Kanneboyina Nagaraju,1 Paul Plotz,6 Kondi Wang,7 Stuart Levine,1 Edward Gabrielson,5 Andrea Corse,4 and Antony Rosen1,3,5

1Department of Medicine, Division of Rheumatology, 2Department of Dermatology, 3Department Cell Biology and Anatomy, 4Department of Neurology, and 5Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD 21224
5Arthritis and Rheumatism Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD 20892
7Department of Neuropathology, Armed Forces Institute of Pathology, Washington DC 20306

Unique autoantibody specificities are strongly associated with distinct clinical phenotypes, making autoantibodies useful for diagnosis and prognosis. To investigate the mechanisms underlying this striking association, we examined autoantigen expression in normal muscle and in muscle from patients with autoimmune myositis. Although myositis autoantigens are expressed at very low levels in control muscle, they are found at high levels in myositis muscle. Furthermore, increased autoantigen expression correlates with differentiation state, such that myositis autoantigen expression is increased in cells that have features of regenerating muscle cells. Consistent with this, we found that cultured myoblasts express high levels of autoantigens, which are strikingly down-regulated as cells differentiate into myotubes in vitro. These data strongly implicate regenerating muscle cells rather than mature myotubes as the source of ongoing antigen supply in autoimmune myositis. Myositis autoantigen expression is also markedly increased in several cancers known to be associated with autoimmune myositis, but not in their related normal tissues, demonstrating that tumor cells and undifferentiated myoblasts are antigenically similar. We propose that in cancer-associated myositis, an autoimmune response directed against cancer cross-reacts with regenerating muscle cells, enabling a feed-forward loop of tissue damage and antigen selection. Regulating pathways of antigen expression may provide unrecognized therapeutic opportunities in autoimmune diseases.

In systemic autoimmune diseases, there is a strong association of autoantibodies against specific autoantigens with unique clinical phenotypes, making autoantibodies useful diagnostically and prognostically (1, 2). For example, Mi-2 autoantibodies are found in patients with dermatomyositis (DM; reference 3); antibodies recognizing several of the aminoacyltrNA synthetases are found in patients with myositis frequently associated with interstitial lung disease (4, 5); autoantibodies recognizing topoisomerase-1 are associated with diffuse scleroderma and lung disease (6, 7); and autoantibodies recognizing dsDNA are diagnostic of lupus (8, 9). In spite of such a strong association with phenotype, these autoantigens are ubiquitously expressed. Understanding the mechanisms underlying this striking association of targets of the immune response with disease phenotype may identify pathways of pathogenic and therapeutic relevance.

The antibody–phenotype association is reminiscent of a variety of paraneoplastic autoimmune syndromes, where autoantibody and T cell responses target autoantigens that are limited in expression to the inciting tumor and the target organ (10, 11). For example, expression of the (neuro-oncological ventral antigen) NOVA antigens is increased in tumor tissue from patients with the clinically distinct paraneoplastic opsoclonus-myoclonus ataxia (12), and expression of NOVA in adults is restricted to neuronal tissue (13, 14). 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 target antigens are expressed in small cell lung cancers and various
neuronal tissues (15–17). It remains unknown whether a similar principle may underlie antigen selection and target tissue specificity in systemic autoimmune rheumatic diseases, in which autoantibodies are directed at proteins that are widely expressed. In this regard, it is of interest that a variety of tumor-associated autoantigens (not related to paraneoplastic syndromes) may be oncogenic proteins up-regulated in tumors (18–20).

Human idiopathic inflammatory myopathy (hereafter referred to as autoimmune myositis) provides an excellent model with which to probe such autoantigen expression for several reasons: (a) several myositis-specific autoantigens have been well-defined (21); (b) unlike systemic lupus erythematosus (SLE) and scleroderma (in which the target tissues are inadequately defined or poorly accessible), muscle is routinely obtained during diagnostic workup of autoimmune myositis and provides sufficient material for biochemical and histological analysis; and (c) an association between autoimmune myositis and cancer has been demonstrated repeatedly (22–24). Although present for both DM and polymyositis (PM), the risk of cancer is substantially greater for DM. Interestingly, the most frequent cancers associated with both DM and PM are adenocarcinomas, being ~80% of cancers in patients with cancer and DM and 70% of patients with cancer and PM (24). Therefore, we examined autoantigen expression in diseased tissue in human autoimmune myositis and in several adenocarcinomas that are associated with this autoimmune phenotype.

The studies yielded several striking insights that were not predicted. Myositis autoantigens are expressed at low levels in control muscle, but at high levels in myositis muscle. Furthermore, increased autoantigen expression correlates with differentiation state, such that myositis autoantigen expression is increased particularly in cells that have features of regenerating muscle cells. Consistent with this in vivo observation, we found that cultured myoblasts express high levels of autoantigens, which are strikingly down-regulated as cells are differentiated into myotubes in vitro. These data strongly implicate regenerating muscle cells rather than mature myotubes as (a) targets of cytolytic attack in autoimmune myositis and (b) the source of ongoing antigen supply in this disease. By focusing damage onto those cells accomplishing repair, a feed-forward loop is enabled, likely accounting for the self-sustaining nature of the autoimmune response and

Figure 1. Myositis-specific autoantigens are expressed at low levels in control muscle and at high levels in myositis muscle. (A) Equal protein amounts of lysed human muscle biopsies, obtained from normal, DM, and PM patients, were immunoblotted with antibodies against the indicated antigens. The myositis-specific autoantigens (Mi-2, U1-70 kD, HRS, and DNA-PKcs) were expressed at high levels in myositis muscle. In contrast, levels of nonmyositis autoantigens and nonautoantigens were not increased in the myositis tissue. Of note, immunoblotted U1-70 kD was detected in two different forms in the tissue lysates (70 and 68 kD). These represent different phosphorylation states of U1-70 kD. Such altered forms can be detected in vitro; U1-70 kD in unincubated cell lysates migrates at 70 kD, but after incubating the lysates at 37°C for 30 min with λ-phosphatase, only the 68-kD form is blotted (not depicted). Black lines indicate that intervening lanes have been spliced out. (B) The immunoblots were scanned, and the data were quantitated by normalizing the immunoblotted level of each autoantigen relative to that of vinculin in the same lysate. In the set of immunoblots shown (representative of two to six blots performed with each different lysed muscle biopsy), the vinculin data shown in the fifth panel was used to normalize all of the immunoblotted antigens except fodrin. The α-fodrin data were obtained using a different set of lysates, with its own matched vinculin blot (the latter is not depicted), and these vinculin values were used to normalize the α-fodrin data.
injury that is a general feature of autoimmune diseases. Interestingly, autoantigen expression is also markedly increased in several cancers associated with autoimmune myositis, but not in the related normal tissues, demonstrating that tumor cells and undifferentiated myoblasts are antigenically similar, and suggesting a mechanism for disease initiation and propagation in autoimmune myositis.

**RESULTS**

Myositis-specific autoantigens are expressed at low levels in control muscle, but at high levels in myositis muscle

The autoantigens frequently targeted in human autoimmune myositis have been well defined. These include histidyl tRNA synthetase (HRS/Jo-1; 10–20% of patients), Mi-2 (15–30% of DM patients), U1-70kD (5–20% of patients), and Ku/the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs; 5–20% of patients). It is noteworthy that even the most common individual established autoantibodies are found in less than one third of myositis patients (25–28).

Several of these antigens (e.g., HRS and Mi-2) are myositis specific (29), whereas others may be targeted in myositis as well as other systemic autoimmune syndromes (e.g., U1-70kD and DNA-PKcs are targeted in lupus, myositis, and overlap syndromes; references 30, 31). To define whether autoantigen expression in myositis muscle is altered compared with control muscle, biochemical levels of autoantigens in equal protein amounts of detergent lysates of human muscle biopsy specimens were determined by immunoblotting (Fig. 1 A). In preliminary studies, we showed that vinculin expression was constant in equal protein amounts of the different muscle biopsies (Fig. 1 A). To facilitate quantitative biochemical analysis of human biopsies in which there can be significant interindividual variation, we analyzed lysates made from eight different normal biopsies, six patients with DM, and nine patients with PM, and normalized protein expression to vinculin as a loading control. Expression levels of Mi-2, U1-70kD, HRS, and DNA-PKcs were low in control muscle biopsy lysates (Fig. 1 A, lanes 1–4), but were strikingly increased in patients with autoimmune myositis (U1-70 kD: 34-fold increase, P < 0.002; HRS: 4.3-fold increase, P < 0.045; and DNA-PKcs: 6.9-fold increase, P < 0.006). Mi-2 levels were increased exclusively in DM biopsies (mean of 10-fold increase, P < 0.045), but not in PM biopsies (1.2-fold, P = 0.9; Fig. 1 B), a finding of particular interest because Mi-2 autoantibodies are found selectively in DM (3, 4). Of note, levels of control nonautoantigens (vinculins, elongation factor 1α, Bid, and p115) and nonmyositis autoantigen molecules (α-fodrin, golgin 160, topoisomerase I, and B23) were not significantly different in muscle biopsy lysates made from normals or patients with myositis (Fig. 1 B and not depicted).

Autoantigen expression is increased in muscle cells in myositis muscle

Because immunoblotting detects antigen levels in lysates comprising both muscle cells and immigrant inflammatory cell populations, we examined autoantigen expression in control and inflamed muscle by immunocytochemistry when appropriate reagents were available. Staining of HRS with affinity-purified polyclonal antipeptide antibodies in control muscle showed diffuse low-level staining in all cells (Fig. 2, A); staining was completely competed away with the cognate peptide, but not with an irrelevant peptide (not depicted). In PM and DM, increased staining of HRS was evident; this staining was restricted to the cytoplasm of muscle cells and was completely competed by specific peptide (Fig. 2, B and C, and not depicted). Similarly, low levels of DNA-PKcs and Ku70 were demonstrable in control muscle biopsies (Fig. 2, D and G). Levels of DNA-PKcs and Ku70 were increased in the nuclei of both PM and DM biopsies (Fig. 2, E, F, H, and I, respectively). At higher magnifications, it was clear that these increased levels of DNA-PKcs were associated with muscle cell nuclei as well as inflammatory cells (see Fig. 4).

Muscle cell regeneration is a prominent feature in myositis

Several different markers of muscle cell regeneration were used to interrogate the amount of regeneration in muscle biopsies. Both immunohistochemical analysis and immunoblotting were used to quantitate the regeneration activity in
these biopsies. Regenerating cells (positive for neural cell adhesion molecule [NCAM; reference 32], neonatal myosin heavy chain [nMHC] [33, 34]) were rare in control biopsies, but were frequent in biopsies from patients with either DM or PM (Fig. 3, A–F, and not depicted). NCAM-positive fibers were significantly increased in DM (35.5 ± 10.1 vs. 5.2 ± 1.1, mean ± SE; DM vs. normal: P < 0.05) and PM (26.9 ± 6.9 vs. 5.2 ± 1.1, mean ± SE; PM vs. normal, P < 0.05) (Fig. 3, G). The difference between PM and DM was not significant. Normal biopsies showed small numbers of NCAM-positive fibers, which were only present in a few fields. NCAM staining was undetectable by immunoblotting in control biopsies (n = 4), but was positive (in some cases markedly so) in 75% (n = 8) of myositis biopsies (n = 4, P = 0.013; Fisher’s exact test). The pattern of staining of all regeneration markers was similar to the primary histological patterns noted in each phenotype. Thus, regenerating cells were particularly prominent with a perifascicular pattern in DM and an intrafascicular pattern in PM.

Interestingly, autoantigen levels were highest in cells with the morphologic features of regeneration; staining of serial DM or PM muscle sections with antibodies to DNA-PKcs and neonatal myosin heavy chain showed strong staining of DNA-PKcs in regenerating cells (Fig. 4 and not depicted). Strikingly, in DM biopsies (where regeneration has a predominantly perifascicular distribution), areas devoid of regenerating muscle cells (neonatal myosin heavy chain negative) also did not stain with the anti–DNA-PKcs antibody. Similar findings were made for HRS in which highest levels of expression were observed in regenerating cells (Fig. 4 B, data for NCAM and HRS staining). We were unable to examine Mi-2 expression in normal and myositis muscle by immunohistochemistry due to the lack of availability of appropriate antibodies. This pattern of absent expression in control muscle with marked increases in expression in myositis muscle is strikingly reminiscent of MHC class I (35–37). When we examined MHC class I expression in normal and myositis muscle, cells expressing regeneration markers (neonatal myosin heavy chain) also ex-
pressed high levels of MHC class I as well as DNA-PKcs (unpublished data). Together, these data demonstrate that autoantigen expression is particularly enhanced in regenerating muscle cells.

Autoantigen expression changes significantly during in vitro differentiation of myoblasts

Because (a) the expression level of several myositis autoantigens was increased in myositis tissue compared with controls, (b) the number of muscle cells expressing markers of regeneration was increased in myositis, and (c) there was evidence supporting the preferential expression of increased antigen levels in these less-differentiated cells, we sought to examine the effect of differentiation on autoantigen expression in primary human muscle cells differentiating in vitro. Equal protein amounts of muscle cell lysates were studied at increasing times after initiation of differentiation, and differentiation state was confirmed by examining levels of NCAM and adult heavy chain myosin expression. Equal protein amounts of muscle cell lysates were studied at increasing times after initiation of differentiation, and differentiation state was confirmed by examining levels of NCAM and adult heavy chain myosin expression. Thus, high levels of NCAM and undetectable levels of adult myosin heavy chain were found in the cultured myoblasts, whereas day-14 myotubes had undetectable levels of NCAM and high levels of adult myosin heavy chain (Fig. 5 A). These findings were consistent with the predicted differentiation status of the cells at both points.

Levels of Mi-2, poly(ADP ribose polymerase) (PARP), U1-70kD, DNA-PKcs, and HRS were high in undifferentiated cells, and diminished as myotubes formed over time. In contrast, levels of vinculin and the vesicle marker p115 remained unchanged (Fig. 5 B and C). The expression levels of nonmyositis autoantigens were variably affected in this in vitro differentiation assay. Although some antigen levels remained unchanged (B23 and α-fodrin), others had a similar pattern to myositis antigens (i.e., high in myoblasts and low in myotubes). Examples of this pattern include topoisomerase I, GM 130, and RNA polymerase II (unpublished data).

We also examined the expression of myositis autoantigens in proliferating and quiescent vascular smooth muscle cells (Fig. 6). We initially confirmed the proliferative/differentiation state of the cell populations using proliferating cell nuclear antigen (PCNA) expression as a marker of cell proliferation, and smooth muscle actin as a marker of the differentiated phenotype. PCNA expression was robust in proliferating cells, but absent in quiescent cells (ratio of 84.5:1). In contrast, α-smooth muscle actin was expressed at low levels in proliferating cells, but was increased in quiescent cells (ratio of 1:25). Interestingly, the relative expression of Mi-2 (normalized to vinculin) in proliferating vascular smooth muscle cells was decreased 5.2-fold compared with that in skeletal muscle myoblasts (unpublished data). Furthermore, unlike skeletal muscle cells, Mi-2 and DNA-PKcs levels were not increased in proliferating over quiescent cells when normalized for protein loading (Fig. 6). Together, these data strongly indicate that myositis autoantigens are differentially expressed during muscle cell differentiation and that this enhanced expression is not a general property of proliferating cells. Therefore, the data focus attention on the regenerating skeletal muscle cell as the source of antigen that feeds the ongoing immune response in myositis.

Figure 4. High levels of DNA–PKcs and HRS staining are found in regenerating myocytes in DM muscle. (A) Serial DM muscle biopsy sections were stained with antibodies against neonatal myosin heavy chain or DNA–PKcs. (left and center) Both antibodies stained an overlapping subset of cells in serial sections with a striking perifascicular pattern characteristic of DM. Note that areas within the fascicle are not stained with either antibody. (right) Higher magnification showed increased DNA–PKcs staining in many muscle cell nuclei. (B) A separate set of serial DM muscle biopsy sections was stained with antibodies against NCAM (monoclonal) or HRS (affinity-purified anti-HRS polyclonal antibody). These antibodies stained an overlapping subset of cells on the serial sections. (A and B) Unless otherwise indicated, all panels have a magnification of 5.
Myositis autoantigens are frequently expressed at low levels in a variety of normal tissues, but at increased levels in tumors

Because the association of myositis with a wide variety of malignancies has been repeatedly demonstrated (22–24), we evaluated Mi-2, DNA-PKcs, HRS, and vinculin (a loading control) expression by immunoblotting detergent lysates of several tumors known to be associated with myositis, and their corresponding normal tissues. Initial studies demonstrated that the very low levels of Mi-2 expression in normal muscle were representative of all other normal primary tissues tested; thus, Mi-2 was not detectable in normal adult human breast, liver, thymus, and lung (Figs. 1 A and 7 A). In contrast, Mi-2 was expressed at high levels in breast and lung adenocarcinomas (Fig. 7 B), as well as in hepatocellular carcinoma (not depicted). Mean Mi-2 levels (normalized to vinculin) were elevated 53-fold and 11-fold in breast and lung adenocarcinomas, respectively, relative to the corresponding normal tissues. Initial studies demonstrated that the very low levels of Mi-2 expression in normal muscle were representative of all other normal primary tissues tested; thus, Mi-2 was not detectable in normal adult human breast, liver, thymus, and lung (Figs. 1 A and 7 A). In contrast, Mi-2 was expressed at high levels in breast and lung adenocarcinomas (Fig. 7 B), as well as in hepatocellular carcinoma (not depicted). Mean Mi-2 levels (normalized to vinculin) were elevated 53-fold and 11-fold in breast and lung adenocarcinomas, respectively, relative to the corresponding normal tissues. Initial studies demonstrated that the very low levels of Mi-2 expression in normal muscle were representative of all other normal primary tissues tested; thus, Mi-2 was not detectable in normal adult human breast, liver, thymus, and lung (Figs. 1 A and 7 A). In contrast, Mi-2 was expressed at high levels in breast and lung adenocarcinomas (Fig. 7 B), as well as in hepatocellular carcinoma (not depicted). Mean Mi-2 levels (normalized to vinculin) were elevated 53-fold and 11-fold in breast and lung adenocarcinomas, respectively, relative to the corresponding normal tissues. Initial studies demonstrated that the very low levels of Mi-2 expression in normal muscle were representative of all other normal primary tissues tested; thus, Mi-2 was not detectable in normal adult human breast, liver, thymus, and lung (Figs. 1 A and 7 A).
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was a variable and modest increase in HRS expression in lung cancers (Fig. 7 B).

**DISCUSSION**

Although autoantigens in systemic autoimmune diseases are ubiquitously expressed molecules that function in conserved, nontissue-specific pathways, the expression of such molecules in the relevant target tissues in humans has not been defined previously. In these studies, we have examined autoantigen expression in normal and diseased target tissue in human autoimmune myositis, and have made the unexpected finding that myositis autoantigens are expressed at very low levels in control muscle. Therefore, it is unlikely that initiation and propagation of the immune response to myositis antigens occurs in normal, unperturbed muscle. In contrast, myositis autoantigens are expressed at high levels in myositis muscle, where the enhanced autoantigen expression is found in muscle cells that express markers of an immature phenotype. Furthermore, such changes in autoantigen expression can be recapitulated during in vitro differentiation of human myoblasts into myotubes. Studies on proliferating and quiescent smooth muscle cells indicate that the enhanced expression of myositis autoantigens is not a general marker of proliferating cells. This suggests a new paradigm for disease propagation in myositis that focuses, for the first time, on regenerating cells as the source of ongoing antigen drive.

Muscle cell damage and regeneration occur in both PM and DM, but the pathologic phenotypes are generally distinguishable, based in part on the pattern of muscle involvement (perifascicular vs. intrafascicular), and the relationship to blood vessel involvement (38). Interestingly, the expression of Mi-2, an autoantigen targeted specifically in DM, is significantly increased (~10-fold) only in muscle biopsies from patients with DM, and not PM. This phenotype-specific expression of Mi-2 is of particular interest because it demonstrates that a DM-specific pattern of autoantigen expression correlates with the DM-specific autoantibody response. Because both DM and PM have similar numbers of regenerating cells, this enhanced expression of Mi-2 cannot be due to expression in all dividing cells. In contrast with Mi-2, the expression of U1-70kD (an autoantigen targeted in patients with DM and PM as well as overlap syndromes) is increased equally in muscle from patients with PM and DM. Expression of α-fodrin (specifically targeted in Sjogren’s syndrome; references 39–41), golgin-160 (targeted in lupus/Sjogren’s syndrome; reference 42) and B23 (targeted in scleroderma and SLE; references 43, 44) was not significantly different in normal and myositis muscle. The augmented expression in myositis muscle of several autoantigens targeted specifically in myositis, but not other systemic autoimmune syndromes, further supports a role for distinct states of the target tissue in shaping the specificity of the phenotype-specific autoantibody response.

The preferential expression of myositis autoantigens in regenerating muscle cells may also account for the well-defined, patchy histologic changes in myositis, where areas of muscle damage, inflammation, and regeneration can be found adjacent to areas that are apparently histologically normal (45). Similar patchy pathologic changes are found in several other autoimmune diseases, raising the possibility that generation of disease-specific autoantibodies in systemic autoimmune syndromes reflects distinct antigen expression patterns in each unique propagating microenvironment. It will be important to define whether regenerating cells in different tissues (e.g., lung, skin, synovium, blood vessel) exhibit unique patterns of autoantigen expression and to elucidate the mechanisms that underlie these differences in expression.

The studies reported here do not identify the site of initiation of the immune response in myositis. It is possible that

**Figure 7.** Myositis autoantigens are frequently expressed at low levels in normal tissue, but at increased levels in tumors. (A and B) Lysates were made from the indicated normal and tumorous human tissues. Equal protein amounts were electrophoresed in each gel lane on 10% SDS-polyacrylamide gels, and immunoblotted with a human serum monospecific for Mi-2, a polyclonal anti-HRS antibody, or monoclonal antibodies against DNA-PKcs, and vinculin. The data shown are representative of those obtained from 8–10 different cancer biopsies of each type in two to three separate experiments. Black lines indicate that intervening lanes have been spliced out. (B) Mi-2 levels were elevated in 10/10 (breast) and 9/10 (lung) cancer tissue lysates; DNA-PKcs expression was increased in 9/10 (breast) and 8/8 (lung) cancer tissue lysates, and HRS levels were elevated in 3/3 (breast) cancer tissue lysates. HRS was expressed fairly robustly in normal lung with only small increases (if any) detected in cancerous lung.
unique forms of proinflammatory muscle injury inducing significant muscle regeneration may initiate autoimmunity directly in muscle. In particular, it is possible that autoantigen overexpression in diseased tissue in DM or PM might be associated with increased DNA damage, leading to up-regulation of repair proteins. This would be consistent with increased expression of DNA-PKcs, PARP, and Mi-2 in regenerating cells, and with previous observations that myositis is associated with autoantibodies to proteins involved in DNA repair (46).

Our studies also demonstrate that the antigenic fingerprint of tumors associated with myositis strongly resembles that of regenerating muscle cells in myositis, raising the interesting possibility that the immune response in myositis is initiated as an antitumor response. The high level expression of Mi-2, DNA-PKcs, and HRS in adenocarcinomas of the lung and breast, in contrast with absent expression in normal liver, muscle, and breast (all three antigens), and normal lung (for Mi-2 and DNA-PKcs only) is reminiscent of the restricted expression of Ri/NOVA autoantigens to tumors and specific areas of the central nervous system that become targets for immune-mediated damage (for review see reference 10). The major difference is that autoantigen expression in the target tissue in myositis is not constitutive, but is induced in regenerating muscle cells. The observation that levels of HRS may be increased in normal lung compared with other tissues is of potential interest because this autoantibody response is associated with interstitial lung disease in myositis (47, 48).

Model of cancer-associated myositis
A variety of tumors, but not normal healthy tissue, express high levels of myositis autoantigens. As with the PND model (10, 11), in which autoantigen expression in tumors is almost universal but immune responses occur in only a minority of patients (<15%), we propose that an effective, adaptive cytokytic antitumor immune response develops infrequently and is directed against a group of antigens shared with immature or regenerating muscle cells. Because normal muscle contains very low levels of myositis autoantigens at baseline and does not express MHC class I (49), healthy muscle is not targeted by the antitumor immune response. In the setting of nonspecific muscle injury (e.g., trauma or overuse, viral infection, drugs, or DNA damage), increased numbers of regenerating cells that express myositis autoantigens at high levels are induced, providing the target to focus antigen-specific immune responses onto muscle. Of particular relevance here is the observation that MHC class I expression is strikingly elevated in the context of muscle damage (50), particularly on regenerating cells (unpublished data). Once muscle injury has occurred in the setting of a cytokolytic immune response that cross-reacts with regenerating muscle cells, a feed-forward cycle of injury is induced that is focused at regions of muscle regeneration. The uniform susceptibility of myositis autoantigens to cleavage by granzyme B (51), and the marked enrichment of perforin- and granzyme-expressing activated CD8+ T cells in DM and PM muscle (52) is of great interest in this regard. Direct demonstration of myositis antigen-reactive CD8+ T cells is a major priority. In this construct, muscle regeneration could be viewed as a cause, as well as a consequence of, muscle damage. Adjacent noninjured muscle (not expressing significant levels of autoantigens or MHC class I) would not be susceptible to damage, potentially explaining the striking observation that nonaffected areas of muscle exist in myositis immediately adjacent to areas of damage and regeneration (45).

The kinetic relationship of an antitumor immune response and development of myositis remain unknown. Recent studies in lupus have demonstrated that initiation of an autoimmune response and development of the diagnostic phenotype are frequently separated in time, sometimes for a period of years (53). In these studies, development of an antinuclear antibody and antiphospholipid antibodies generally predated the development of symptoms of SLE, which correlated with the onset of an autoantibody response directed against the spliceosome (53). It is possible that a similar separation of initiation of the immune response and development of the diagnostic phenotype occurs in myositis, the latter event being stimulated by nonspecific muscle damage occurring in the setting of a prior antitumor immune response.

In summary, the studies reported here identify several previously unrecognized principles relevant to the initiation and propagation of autoimmune myositis and potentially other autoimmune rheumatic diseases. First, mature, healthy tissue may not be the primary target of autoimmunity, but rather injured and repairing tissue in which stem cells or differentiating cells are replacing injured cells. Second, the phenotype-specific antigen fingerprint and phenotype-specific autoantibody response are related. Third, immature muscle cells and tumor cells share similarities in their antigenic composition, which differs from that present in mature, differentiated tissues. Although available therapies frequently target the immune effector components of autoimmune diseases, inhibiting antigen expression might be of even greater therapeutic relevance. We propose that regulating cell differentiation and/or pathways responsible for unique antigen expression patterns in target tissue will provide unique therapeutic opportunities in autoimmune diseases.

MATERIALS AND METHODS
Human tissue and disease status. All studies on human materials were performed on samples provided in compliance with Institutional Review Board and Health Insurance Portability And Accountability Act regulations. Muscle biopsies were obtained from patients seen at the Neuromuscular Clinic at Johns Hopkins Hospital and at the Armed Forces Institute of Pathology. All other human tissues were obtained from the Departments of Surgery or Pathology at the Johns Hopkins Medical Institutions. Research tissue samples were obtained within 1 h of surgical resection. The surgical procedures were performed for patient management, and the research tissue samples were in excess of tissue required for routine diagnostic purposes. Muscle biopsies were obtained from six patients with DM, nine patients with PM, and eight controls whose biopsies were histologically normal. Histologic criteria for the muscle biopsies identified as consistent with DM included perifascicular atrophy; myofiber degeneration, regeneration, and necrosis; and perivascular inflammation with or without endomyosial inflam-
tion. Histologic criteria for the muscle biopsies consistent with PM included demonstration of primary endomysial inflammation defined as chronic mononuclear inflammatory cells surrounding and/or invading normal-appearing myofibres, as well as evidence of myofiber degeneration, regeneration, and necrosis. The breast cancer biopsies represented nine cases of ductal adenocarcinoma (all grade 2 or 3) and one case of lobular adenocarcinoma. Normal human mammary tissue was obtained as discarded material from reduction mammoplasty, and was treated as described previously (54). The 10 lung cancer biopsies were all cases of pulmonary adenocarcinoma.

Cell culture and differentiation. Normal human skeletal muscle cells from a single donor (Clonetics) were cultured as described previously (55). When the cultures were ~80% confluent, the cells were induced to differentiate into myotubes by replacing the growth medium with medium A containing DMEM, 2% horse serum, and 1-galactose, and growing the cells for a further 2 wk without subculturing. Human aortic smooth muscle cells were cultured and differentiated exactly as described previously (56).

Immunoblotting of antigens in cultured cells. Biochemical levels of antigens expressed in myoblasts and myotubes were assessed by harvesting myoblasts immediately after changing into medium A (day 0) and at days 2, 5, 9, and 14 (for HRS/Jo-1) or days 1, 3, 7, 11, and 14 (all other antigens are shown). Culture dishes were placed on ice, and the cells were washed twice in PBS and lysed in lysis buffer containing 1% Nonidet P-40, 20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, and protease inhibitors (57). 70 µg of each sample was electrophoresed on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membrane and immunoblotted with patient sera monospecific for Mi-2, PARP, U1-70 kD, or HRS, or monoclonal antibodies against vinculin (Sigma-Aldrich), DNA-PKcs (Oncogene Research Products), p115 (Transduction Labs), NCAM (Soretce), and myosin (Sigma-Aldrich) as described previously (58, 59). Time course experiments were performed at least three times with similar results for each antigen on every occasion. Biochemical levels of antigens expressed in aortic smooth muscle cells were assessed by harvesting the cells before or after differentiation, when the cells were proliferating or quiescent, respectively. 3 × 10^5 cells were loaded in each gel lane. Immunoblots were performed as described before. In addition, immunoblots were performed with monoclonal antibodies against α-smooth muscle actin (Sigma-Aldrich) and PCNA (BD Biosciences).

Immunoblotting and quantitation of antigens in lysates made from biopsies of human muscle, as well as lung and breast adenocarcinomas. Biopsy pieces were weighed, placed in ice-cold Petri dishes (with all subsequent steps being performed at 4°C), cut into small pieces, and transferred to a glass Kontes homogenizer containing lysis buffer (~10–20 µl/mg tissue) and protease inhibitors. The tissue was dounced with 20 strokes, the homogenate was centrifuged (16,000 g, 4°C, 10 min), and the supernatant was used for protein assay and to make gel samples. Equal amounts of lysate protein were electrophoresed in each gel lane, transferred to nitrocellulose, and immunoblotted as described before. Antibodies used to immunoblot Mi-2, U1-70 kD, DNA-PKcs, and vinculin were as described before. HRS and topoisomerase I were immunoblotted with polyclonal antibodies raised against purified full-length HRS or an NH2-terminal topoisomerase I peptide, respectively. Fodrin and elongation factor 1 were detected using a monoclonal antibody purchased from Upstate Biotechnology and Chemicon International, respectively. In those cases where patient sera were used to immunoblot human muscle biopsy lysates (Mi-2 and U1-70 kD), great care was taken to ensure that those parts of the transfers containing human immunoglobulins were discarded to eliminate any cross-reactivity with the horseradish peroxidase–labeled anti–human IgG used as a secondary antibody. Representative examples of immunoblots are shown in Fig. 1 A. The immunoblots were quantified by densitometry, and the data were normalized for each antigen relative to vinculin blotted in the same lysate. The NCSS/PASS statistical analysis program (NCSS statistical software) was used for appropriate statistical analyses and to create box plots.

Immunohistochemistry. Frozen muscle biopsy sections (5–8 microns) were fixed in acetone and endogenous peroxidase activity was blocked by applying hydrogen peroxide at room temperature for 10 min as described previously (55). Sections were incubated overnight at 4°C in a humidified chamber with the following primary antibodies: mouse anti–rabbit neonatal myosin heavy chain (cross reacts with human; Novo castra); mouse anti–human DNA-PKcs, mouse anti–human Ku70 (Abcam Inc.), mouse anti–human NCAM (Becton Dickinson), and rabbit anti–human HRS (raised against a peptide at the COOH terminus of HRS and affinity-purified using the coupled immunizing peptide). The sections were washed in Tris-buffered saline and incubated with appropriate secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature. Staining was visualized after incubating with 3,3-diaminobenzidine and all sections were counterstained with Mayers’ hematoxylin. Negative controls were performed either by incubating with isotype matched antibody, by omitting the primary antibody, or (in the case of HRS) by preincubating the affinity-purified primary antibody with the HRS peptide used for immunization.

Quantitation of regenerating muscle fibers in muscle biopsies. Several markers of regenerating muscle cells (NCAM, neonatal myosin heavy chain, developmental myosin heavy chain) were used in immunohistochemistry as described before. We quantified NCAM-positive muscle fibers in PM (n = 10), DM (n = 7), and normal (n = 5) muscle biopsies. Frozen muscle sections were stained with anti-NCAM antibody as described before. The intensity and pattern of NCAM reactivity (Figs. 3 and 4, visualized as a brown color) varied among positive fibers, with staining being observed in small and large fibers. Some fibers showed both cell surface and intense cytoplasmic staining, whereas others stained strongly at the cell surface but diffusely in the cytoplasm. The number of NCAM-positive fibers in five nonoverlapping, randomly selected areas of each biopsy was counted using a 10X objective on an Axioscope microscope (Carl Zeiss MicroImaging, Inc.). The average number of positive fibers per field was determined for each group, and statistical significance was calculated using the Student’s t test. Similar data was obtained with neonatal myosin heavy chain and developmental myosin heavy chain (unpublished data).

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