Nitric Oxide Signaling Triggered by the Rheumatoid Arthritis–Shared Epitope

A New Paradigm for MHC–Disease Association?

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ABSTRACT: Many immune-mediated diseases are associated with particular MHC class I or class II alleles. In rheumatoid arthritis (RA-shared), the vast majority of patients possess *HLA-DRB1* alleles encoding a shared epitope, which is a five–amino acid sequence motif in positions 70–74 of the HLA-DRβ chain. The mechanistic basis for this association is unknown. Here we discuss recent evidence suggesting that the shared epitope may act as an allele-specific ligand that triggers increased nitric oxide (NO) production in opposite cells with resultant immune dysregulation. We propose that by doing that, the RA-shared shared epitope may form an unintended bridge between the innate and adaptive immune systems, thereby allowing aberrant signaling events that could trigger disease.

KEYWORDS: autoimmunity; MHC–disease association; signal transduction

INTRODUCTION

Genetic associations with particular class I and class II major histocompatibility complex (MHC) alleles have been observed in many immune-mediated diseases.[1] On the basis of the known role of MHC gene products in antigen presentation, it has been postulated that the mechanism of these associations involves T cell repertoire selection and/or recognition of specific antigens. Evidence to support this paradigm exists in some diseases, but in other cases the basis of MHC–disease association does not appear to involve antigen presentation. For example, approximately 90% of patients with narcolepsy express the *DQB1*0602* allele,[2] yet to this date, no conclusive evidence for an immune-based mechanism has been established in this sleep disorder. Instead, recent

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studies suggest that signaling aberrations in the orexin-activated pathway may be involved.[3] Similarly, in hereditary hemochromatosis, an association with HLA-A3 has been known for decades.[4] More recently, however, the association was found to be due to linkage disequilibrium with a point mutation in *HFE*, an adjacent class I HLA-related gene. The mutant HFE protein does not allow proper transferrin receptor-mediated signaling, with resultant iron overload.[5] Thus, in at least two HLA-associated diseases, the etiopathogenesis involves signaling aberrations rather than antigen presentation. Below, we discuss evidence suggesting that HLA-disease association in rheumatoid arthritis (RA) may also be attributed to aberrant signaling.

THE RA SHARED EPITOPE: UNANSWERED QUESTIONS

Rheumatoid arthritis (RA) is one of the most prevalent HLA-associated diseases. Its association with HLA-DR4 was noticed three decades ago.[6] More recently, it has been found that the vast majority of RA patients possess *HLA-DRB1* alleles encoding a "shared epitope" (SE), consisted of QKRAA, QRRAA, or RRRAA sequences in residues 70–74 of the DRβ chain.[7] Immunogenetic analyses[8] have demonstrated that among the five SE amino acids, residues 70, 71, and 74 are critically important, while the identities of residues 72 and 73 do not seem to matter. Thus, a (Q/R)-(K/R)-x-x-A consensus motif (glutamine or arginine in position 70, lysine or arginine in position 71, and alanine in position 74) has been proposed as essential and sufficient to confer RA susceptibility. Further analyses have demonstrated that the nature of residue 70 is critical for both susceptibility to and protection against RA. While *HLA-DRB1* alleles encoding glutamine or arginine in position 70 are associated with increased RA susceptibility, alleles encoding aspartic acid in that position confer protection against the disease.[9–11]

The mechanism by which the SE affects disease susceptibility is unknown. Several hypotheses have been proposed, including presentation of arthritogenic self-peptides,[1] molecular mimicry with foreign antigens,[12] or T cell repertoire selection.[13] While these hypotheses are all plausible, they are difficult to reconcile with the fact that data supporting antigen-specific immune responses as the primary event in RA are equivocal. Furthermore, SE–disease association is not unique to RA, as several other human diseases have also been shown to be associated with SE-encoding *DRB1* alleles.[14–17] Additionally, the SE has also been shown to confer disease susceptibility in other species. For example, an association with SE-expressing MHC class II alleles has been reported in spontaneous RA-like disease in dogs,[18] and transgenic mice carrying SE-encoding *HLA-DRB1* alleles have been found to have more severe collagen-induced arthritis[19] and experimental encephalomyelitis.[20] Thus, the promiscuous association of the SE with pathogenically diverse diseases, with no apparent antigen- or species-specificity, suggests that in

addition to its role in antigen presentation, the SE may have antigen nonspecific effects.

Another feature of RA–SE association, which is difficult to attribute to antigen presentation, is the fact that SE influence in RA is not limited to disease susceptibility. Several groups have demonstrated correlations between the SE and disease severity.[21] According to those studies, RA in SE-positive individuals, in particular those possessing two SE-encoding *HLA-DRB1* alleles, is more severe, involves more erosions, and starts at an earlier age than RA in individuals who are SE-negative.[21] Furthermore, the SE appears to affect penetrance as well. As discussed below, the concordance rate of RA in monozygotic (MZ) twins is very low.[22,23] Quite interestingly, however, the concordance rate directly correlates with the SE gene dose. While in SE-negative MZ twins the concordance rate is only 5%, in twins with one SE-encoding *HLA-DRB1* allele it is 13%, and in MZ with two alleles, RA concordance rate reaches 27%.[24] Additionally, life table analyses revealed that the probability of RA discordant twin pairs to become concordant over time correlates with the number of SE-encoding *HLA-DRB1* alleles, with the shortest time to concordance in twins possessing two such alleles.[24]

Thus, the lack of evidence to support an antigen-specific immune response, the promiscuous association of the SE with other diseases, and SE effect on RA severity and penetrance, all argue against antigen presentation function as the sole basis of RA–SE association. An alternative hypothesis has been therefore proposed that, rather than emanating from the antigen presentation function of the HLA-DR molecule, the RA–SE association is a result of linkage disequilibrium with another gene. According to this hypothesis, the SE is a surrogate marker for another gene, such as TNF-α,[25] which is the actual culprit. Linkage disequilibrium has been convincingly implicated in another MHC-associated disease, hereditary hemochromatosis.[5] In RA, however, this mechanism appears to be less likely, since it fails to explain the seemingly random occurrence of RA among genetically susceptible individuals, best illustrated by the very low concordance rate of RA in MZ twins.

It has long been observed that despite the strong influence of genetic factors in RA, the concordance rate of the disease in MZ twins is only 12–15%.[22,23] This surprisingly low penetrance contrasts with concordance rates reported in other autoimmune diseases, such as systemic lupus erythematosus or ankylosing spondylitis,[26] and suggests that environmentally driven stochastic (random) events may be responsible for triggering disease onset in RA. Consistent with this hypothesis, there are many indications of increased incidence of stochastically occurring molecular events in RA, including protein and lipid damage, accelerated telomere shortening, DNA damage, and somatic mutations.[27–30]

The impact of aging on RA incidence and evidence of accelerated immune senescence in this disease[31] lend further support to the theory that stochastically occurring events may be involved in RA. Epidemiological surveys indicate that advancing age is a strong risk factor for developing RA. The incidence of the

disease continuously increases with age and peaks in the eighth decade of life. Such age-associated increasing susceptibility argues against presentation of specific antigens, since adaptive immune responses are expected to decline with age.[31]

A common denominator to cellular aging and stochastic events is that they are both associated with oxidative stress, a factor that has long been implicated in RA pathogenesis.[32] Thus, a conceivable unifying explanation could be that the SE may lead to disease onset by conducing to a pro-oxidative milieu, which in turn increases the risk of deleterious stochastic events. Consistent with this model, a significant interaction between the SE and smoking, a well-known pro-oxidant stressor,[33] has been recently reported.[34] Padyukov *et al*. have found that in SE-positive smokers the relative risk for RA was 7, while in SE-negative smokers it was 2.4, and a relative risk of 2.8 was found in SE-positive individuals without a history of smoking. Importantly, smokers carrying two copies of the SE were found to have a relative risk of 15.7.[34]

NITRIC OXIDE (NO) SIGNALING TRIGGERED BY THE SE

As discussed above, there is no convincing evidence to support an adaptive immunity role for the SE in RA. Could the SE possess as yet unknown innate immunity functions? As a first step to address this question, we have undertaken to examine the effect of the SE on NO signaling.[35]

NO is a ubiquitous signaling molecule with versatile roles in the immune system. Its proinflammatory effects have been noted in RA,[36] where increased NO levels correlate significantly with inflammatory markers of the disease[37] and antirheumatic agents have been shown to suppress NO production.[36,38] In addition, excessive NO levels, either alone, or in conjunction with intercurrent oxidative challenges, can cause mutations,[39] increase the risk of lymphoma,[40] and accelerate telomere shortening[41]—events that have all been noted to associate with RA.[27–30] Thus, several lines of evidence suggest that NO may be an important factor in RA and in its association with the SE.

Our recent data indicate that the SE acts as a ligand, which triggers NO signaling in opposite cells.[35] For example, SE-positive B lymphocyte lines exhibited much higher rates of spontaneous NO production compared to SE-negative lines. There were no significant differences between SE-positive healthy individuals and SE-positive RA patients or between SE-positive RA MZ twins and their healthy co-twins, suggesting that spontaneous NO overproduction is a SE- rather than RA-associated aberration.

Studies involving L cell transfectants indicate that NO overproduction in SE-positive cells is contributed by the *DRB1* gene itself, rather than by another gene secondary to linkage disequilibrium. Transfectants expressing on their surface a SE-positive HLA-DRβ chain encoded by allele *DRB1*0401* showed a much higher spontaneous NO production rate compared to transfectants

expressing on their surface a SE-negative HLA-DRβ chain encoded by allele *DRB1*0402*.[35]

The HLA-DR molecule is directly involved in NO signaling, as indicated by experiments using tetrameric HLA-DR molecules and multimeric protein particles engineered to express multiple copies of the SE. Class II MHC-negative fibroblasts stimulated with the SE-positive T-DRB1*0401 tetramer generated much higher NO levels, compared to cells stimulated with the control, SE-negative T-DRB1*1501 tetramer. The SE-positive tetramer was found to be a highly specific and potent stimulator of NO production, with an EC_{50} of $\sim 3.0 \times 10^{-8}$ M.[35] To further map the active site on SE-expressing proteins, we genetically engineered the third allelic hypervariable region (HV3, residues 65–79) encoded by SE-positive or SE-negative *DRB1* alleles into the spikes of a recombinant hepatitis B core (HBc) protein capsid, which form α helical structures, mimicking the native conformation of the HV3. Stimulation of class II-negative cells with SE-positive HBc chimeric capsids, but not with the SE-negative capsids, triggered rapid NO production.[35] Since the HV3 encoded by the two alleles differs by only three amino acid residues within the HV3 region, these data map the active site to that region. These results also indicate that NO signaling can be triggered by "naked" SE-expressing α-helical loops, independent of any antigenic groove peptide.

Activation of NO signals could also be triggered by soluble 15mer synthetic peptides corresponding to the HV3 of the SE-positive *DRB1* allele and when immobilized on a solid phase could trigger NO signaling within 15–30 min. SE-triggered NO signaling could be seen in a wide variety of human and murine cell lineages and involved rapid activation of NO synthase (NOS), followed by increased levels of cGMP.[35]

NO is a pleiotropic signaling molecule. Among many other effects, it has been previously shown to inhibit apoptotic target killing by cytotoxic T cells.[42,43] Since impaired apoptotic elimination of autoreactive cells has been proposed as a triggering event in RA,[44,45] we investigated whether the SE might be a contributing factor. Our data indicate that SE-positive target cells are much more resistant to apoptotic killing by cytolytic γδ T cells. The resistance could be reversed by preincubation with a NOS inhibitor, indicating that the resistance was due to NO overproduction.[35]

A PROPOSED MODEL

Our data indicate that the SE acts as an allele-specific ligand that triggers NO-mediated signaling events. This pathway is constitutively active in B cells carrying SE-positive *DRB1* alleles and in class II-negative cells transfected with SE-encoding *DRB1* cDNA. The pathway can also be activated by recombinant proteins engineered to express the SE ligand in its natural conformation and by short synthetic peptides expressing the SE motif.

In its native conformation, the SE occupies approximately 1.5 α-helical loops in the DRβ1 domain and builds the lateral wall of the fourth pocket of the peptide-binding groove. It is therefore legitimate to wonder whether interaction between the SE and a putative receptor on opposite cells is spatially possible. It should be remembered, however, that the SE is part of the "sidewall" of the peptide groove, but it is not playing a major role in peptide anchoring, a function that is mediated primarily by groove "floor" residues. Among the five amino acid residues of the SE, only one (K/R71) has been shown to interact with some groove peptides. The other residues interact with outside molecules, such as the T cell receptor, and/or merely help maintaining the SE α-helical conformation. Additionally, X-ray crystallography studies[46] have shown that the 70–74 region forms a free protrusion, which allows a sizable gap between the collective electron densities of antigenic peptides and the van der Waals surface at the region 70–74 of the DRβ chain. Thus, the SE represents a relatively free subdomain, which, depending on the level of antigenic peptide occupancy and the nature of that peptide, could allow sufficient room for interaction with other molecules.

We propose that the SE may act as a receptor-binding ligand that triggers innate signaling events in opposite cells, analogous to signaling effects mediated by particular α-helical loops in the class I MHC α2 domain (which closely resemble spatially the DRβ1 domain). It is well established that distinct loops in the class I MHC α2 domain bind to cell-surface receptors, such as killer immunoglobulin-like receptors (KIR), NKG2/CD94 receptors, the transferrin receptor, or the pheromone receptor V2R, and activate signal transduction events through these receptors.[47–50] Thus, there is ample evidence that MHC α-helical loops can function as receptor-binding ligands.

FIGURE 1 depicts a proposed model of SE-triggered signaling. According to this model, the SE ligand interacts with a putative cell-surface receptor and activates NOS, which in turn activates a cGMP-dependent kinase. We propose that the consequences of SE-triggered signaling could be dual: (1) Overproduction of NO could contribute to RA pathogenesis through its proinflammatory and immune modulation effects. Increased NO levels could also lead to T cell hyporesponsiveness and resistance to apoptosis,[51] two aberrations previously noted in RA.[44,52] (2) Chronic overproduction of NO, perhaps in conjunction with recurrent daily oxidative stresses, could increase the likelihood of stochastic, disease-initiating events.

We believe this model provides plausible explanations for many of the unanswered questions surrounding the role of the SE in RA. For example, SE-triggered, antigen-independent NO signaling could better explain the lack of evidence for an antigen-specific immune response in RA on the one hand and the promiscuous association of SE-expressing alleles with other diseases on the other. A signaling-based mechanism could also provide a better explanation for the association of the SE with disease severity and its gene–dose effect.

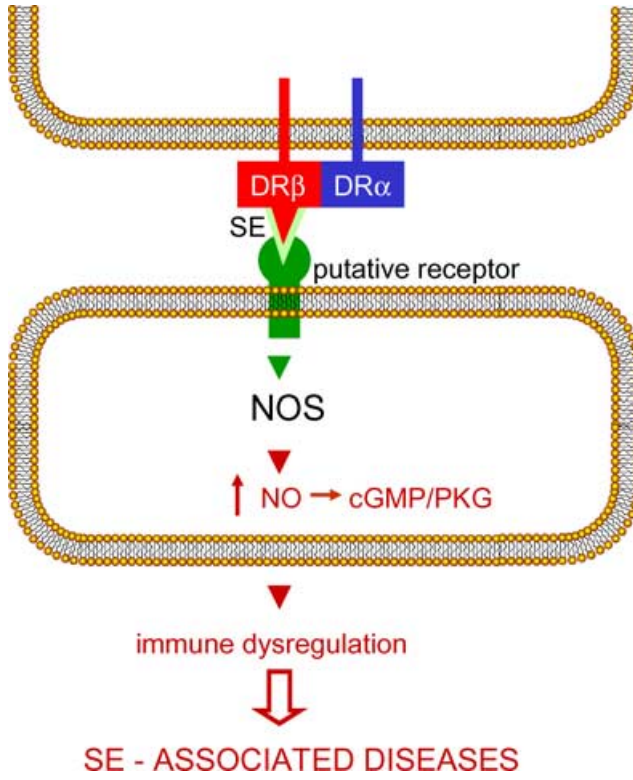

FIGURE 1. A proposed model of SE-triggered signaling aberration. SE-positive HLA-DR surface molecules interact *in trans* with a putative receptor on opposite cells and activate NOS with resultant activation of a NO-cGMP-PKG signaling cascade. The aberrantly activated pathway can lead to several functional consequences, including delayed apoptotic elimination of lymphocytes, immune dysregulation, and increased susceptibility to oxidative stresses. These aberrations, in turn, could allow the emergence of autoreactive immune cells, with resultant RA or other SE-associated diseases, depending on the genetic background of the individual.

Additionally, as a result of the pro-oxidative effects of NO[53] the SE could conceivably increase the likelihood of deleterious stochastic events, which could explain the seemingly random occurrence of RA in genetically susceptible individuals, the SE effect on penetrance, the association of the disease with advancing age, and the increased incidence of somatic mutations and premature telomere attrition observed in RA.

Obviously, many questions remain unanswered. Prominent among them is the fact that up to 10% of all RA patients do not carry any SE-encoding *DRB1* allele. Therefore, the possibility that disease-inducing NO signaling could also be triggered by SE-independent mechanisms deserves consideration. It should

be also clarified that the paradigm proposed here and the prevailing adaptive immunity-based theories are not mutually exclusive. It is conceivable that while presentation of a putative arthritogenic antigen by the SE is required to initiate an immune response, the pathogenic consequences of that response are determined by the unique ability of the SE to co-trigger NO-mediated innate immunity signals.

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