



# Understanding the pathogenesis of ANCA: Where are we today?

GLORIA A. PRESTON, PhD; JIA JIN YANG, MD; HONG XIAO, MD; AND RONALD J. FALK, MD

In the intervening years between the ANCA workshop in Groningen, The Netherlands, and the current proceedings in Cleveland, Ohio, there have been substantial advances in understanding the pathogenesis of ANCA small-vessel vasculitis (SVV). There have been and still are several hypotheses generated on the basis of both *in vivo* and *in vitro* observations. These include the possibilities that: 1) ANCA induce the activation and degranulation neutrophils and monocytes in the circulation or, after adherence, to the endothelium; 2) ANCA bind to target antigens on the surface of endothelial or epithelial cells that synthesize and express these antigens or to target antigens that are deposited on the surfaces; 3) ANCA antigens have direct effects on the vascular endothelium; 4) there is no relationship between ANCA, their target antigens, and SVV.

It is most likely that some or all of these mechanisms are involved. So too, it is likely that more than a “single hit” is required for disease generation and proliferation. Thus, in any given patient it is plausible that the antibody forms in susceptible patients, and that there are genetic factors and environmental pressures (ie, especially silica or infectious diseases with superantigen formation) that conspire to cause an inflammatory reaction.

## ■ ANIMAL MODELS

Some of the most dramatic progress has been made in elucidating an animal model of ANCA SVV. There are currently several models of SVV in animals. Some of these models rely upon the spontaneous induction of vasculitis associated with a polyclonal autoantibody response. One such model is in a rat strain susceptible to autoimmune syndrome in response to mercuric chloride.<sup>1</sup> These animals develop autoantibodies to myeloperoxidase (MPO), but also to a host of other proteins and nuclear antigens. The model provides some intriguing clues about the potential role of infection in antibody-induced vasculitis.

For instance, treatment with antibiotics in these animals diminished mercuric chloride-induced vasculitis. Unfortunately, these animals never developed necrotizing glomerulonephritis. Furthermore, the range of autoantibodies that occurs precludes any possibility of establishing a prominent role for any particular antibody as a driving force in vasculitis. Similarly, the SCG/Kj mice in which anti-MPO antibodies are found develop a crescentic glomerulonephritis and necrotizing vasculitis.<sup>2</sup> However, this anti-MPO response is only part of a polyclonal immune response.

There have been two examples of anti-MPO-induced crescentic glomerulonephritis requiring pretreatment with anti-glomerular basement membrane antibodies. First, Kobayashi and then Heeringa treated rats with subnephritogenic anti-glomerular basement membrane disease and then either administered rabbit anti-rat MPO antibodies or induced the development of an anti-MPO response in rats. In the Heeringa model, rats developed lesions characterized by fibrinoid necrosis and crescentic formation.<sup>3</sup>

Recently, Hong Xiao made use of a model in which MPO knockout mice are immunized with murine MPO. As reported in these proceedings, these mice developed a brisk anti-MPO antibody response. When splenocytes from these animals are transferred to RAG2 mice, SVV and a necrotizing and crescentic glomerulonephritis develops. When mice are immunized with control antigen such as bovine serum albumin and splenocytes transferred, no lesion occurs. Interestingly, both experimental and control mice have a baseline immune complex deposition in their glomeruli. In preliminary observations, transfer of anti-MPO antibodies alone, derived from immunized MPO knockout mice, into RAG2 mice results in a necrotizing glomerulonephritis and crescentic glomerulonephritis.

These observations would suggest that the anti-MPO antibody alone is capable of creating a necrotizing glomerulonephritis. It is most likely that the transfer of the splenocytes results in more aggressive crescentic glomerulonephritis, providing insights into the stimulatory roles of T cells in this process. Much work remains to be done with respect to the relative roles of T and B cell constituent stimuli as a granulomatous angiitis and to the contributing components of the antibodies and their receptors used for anti-MPO-induced damage.

From the Department of Medicine, Division of Nephrology and Hypertension (G.A.P., J.J.Y., and R.J.F.), and the Department of Pathology and Laboratory Medicine (H.X. and R.J.F.), University of North Carolina at Chapel Hill.

Address correspondence to R.J.F., Department of Medicine, Division of Nephrology and Hypertension, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599.

**TABLE 1**  
BIOLOGICAL ACTIVITIES OF PR3

Regulation of differentiation
<ul style="list-style-type: none"> <li>• Truncation of NF-<math>\kappa</math>B<sup>24</sup></li> <li>• Hydrolyzation of Hsp 28<sup>25</sup></li> <li>• Truncation of Sp1<sup>26</sup></li> <li>• Component of leukemia-associated inhibitor<sup>27</sup></li> </ul>
Impact on cytokine network
<ul style="list-style-type: none"> <li>• Conversion of IL-8 to active form<sup>28</sup></li> <li>• Conversion of TNF-<math>\alpha</math> to active form<sup>29</sup></li> <li>• Conversion of IL-1<math>\beta</math> to active form<sup>29</sup></li> <li>• Activator of latent TGF<math>\beta</math>1<sup>30</sup></li> <li>• Enhances IL-8 production by endothelial cells<sup>31</sup></li> </ul>
Other substrates and physiological functions
<ul style="list-style-type: none"> <li>• Cleavage of C1 inhibitor<sup>32</sup></li> <li>• Cleavage and inactivation of the thrombin receptor<sup>33</sup></li> <li>• Cleavage of matrix macromolecules (elastin, fibronectin, laminin, vitronectin, type IV collagen)<sup>34</sup></li> <li>• Activator of MMP-2<sup>35</sup></li> </ul>
Effect on endothelial cells
<ul style="list-style-type: none"> <li>• Internalization into cells<sup>4</sup></li> <li>• Induction of apoptosis<sup>4</sup></li> <li>• Activates signaling molecules<sup>36</sup></li> <li>• Stimulates tissue factor production<sup>37</sup></li> </ul>

### ■ ANCA ANTIGENS ARE MORE DESTRUCTIVE

Another area of substantial research in this field pertains to the roles of the target antigens MPO and proteinase 3 in the development of endothelial injury. It has been demonstrated that both MPO and proteinase 3 were capable of entering endothelial cells of many types.<sup>4</sup> This process of antigen entry into cells most likely is a consequence of receptor-mediated endocytosis. The exact nature of the receptor remains controversial, although Dr. Daha suggested previously that 111 kb protein was an important ligand.<sup>5</sup> Whether there are multiple receptors including the soluble protein C receptor for proteinase 3 is not clear at this time.<sup>6</sup> However, once proteinase 3 or MPO enters a cell, there are many potential consequences. It is already known that proteinase 3 has numerous effects other than that of just a destructive enzyme (Table 1). Similarly, once inside the cell, MPO has a number of effects as well (Table 2). We now know that entry of proteinase 3 into endothelial cells pushes the balance of cellular signaling pathways toward a proapoptotic event through JNK and p38MAPK pathways. Although these signals result in a pro-apoptotic pathway under some circumstances, they may have proliferative effects under other conditions. Once inside the cell, proteinase 3 cleaves p65 NF- $\kappa$ B at a site in the vicinity of a caspase 3 site, rendering it dysfunctional. These results have implications not just for vasculitis, but for inflammation in general, as it would suggest that proteinase 3 may function in a manner analogous to granzyme B released from lymphocytes.<sup>7,8</sup> While the substrates are different, the killing effect of these proteases is quite similar.

Myeloperoxidase and proteinase 3 are cationic in nature. These enzymes bind ionic proteins on endothelial cell surface. As such, these antigens may be the source of

**TABLE 2**  
BIOLOGICAL ACTIVITIES OF MPO

<ul style="list-style-type: none"> <li>• Bactericidal through enzymatic production of hypochlorous acid<sup>38</sup></li> <li>• Functions as a peroxidase to produce free radicals causing lipid peroxidation of low-density lipoproteins<sup>39</sup></li> <li>• Produces oxidants that activate cell-signaling pathways<sup>40</sup></li> <li>• Produces hypochlorous acid activates NF-<math>\kappa</math>B transcription factor<sup>41</sup></li> <li>• Produces advanced glycation end products at sites of inflammation<sup>42</sup></li> <li>• Internalized by endothelial cells causing increased free radical production<sup>4</sup></li> <li>• Tyrosine nitration of vascular ECM proteins<sup>38</sup></li> </ul>
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ANCA binding that would result in local cell injury. Whether the antibody antigen interaction on the surface of the endothelial cell is a prime effector arm of endothelial cell injury remains controversial.<sup>9</sup> There is really no good evidence for linear or granular staining of immune reactivities along the endothelial cells in vivo.

There still remains substantial controversy as to whether proteinase 3 and MPO are expressed by cells other than those of myeloid lineage. Most evidence suggests that proteinase 3 is not made by endothelial cells,<sup>10,11</sup> although there are data on both sides of this controversy.<sup>12</sup> Of note, proteinase 3 message may be expressed by glomerular epithelial cells and may be associated with crescent formation.<sup>13</sup> Proteinase 3 protein and message were found in distal tubular epithelial cells and glomerular epithelial cells in normal kidney and in patients with crescentic glomerulonephritis. In fact, glomerular proteinase 3 RNA expression was associated with the percentage of cellular crescents.

Over the years, ANCA have been shown to activate leukocytes in an ever-expanding number of ways, including induction of reactive oxygen production, degranulation, activators of 5-lipoxygenase pathway, and stimulation of cytokine message and protein, including IL-8 and IL-1 $\beta$ . Each of these effects may be involved in direct tissue damage as well as the recruitment of new leukocytes to areas of acute then chronic inflammation.

### ■ ANCA ACTIVATE LEUKOCYTES

There is controversy as to the mechanism by which ANCA induce neutrophil and monocyte activation. What is the relative contribution of the Fc $\gamma$  receptor engagement versus F(ab')<sub>2</sub> antigen binding in activation? Part of the controversy likely stems from the "readout" or outcome variable studies. Several studies have used superoxide anion production or release of a granule protein. We have recently studied the effect of the whole antibody versus F(ab')<sub>2</sub> fragment on the stimulation of transcription of a distinct set of genes in leukocytes from healthy

donors. Interestingly, some changes in gene expression were unique to whole IgG, some unique to F(ab')<sub>2</sub> fragments, and some to both. We concentrated on a gene called "differentiation-dependent gene 2 (DIF2)," also known as "IEX-1," investigating both message and protein levels. Levels are increased in leukocytes activated by ANCA F(ab')<sub>2</sub> and by the whole immunoglobulin. Similarly, we have looked at the transcription of IL-8 and COX-2, and we found that there are differences between ANCA IgG and their respective F(ab')<sub>2</sub>. This would suggest that in some circumstances one gene may be more responsive to particular signals than another. Whether these in vitro phenomena have any in vivo correlate has recently been studied by looking at RNA and protein levels in circulating leukocytes of ANCA patients with active disease when compared to ANCA patients in remission and disease controls (systemic lupus and IgA nephropathy). The corresponding increase of genes in vivo found in active disease that are stimulated in vitro gives credence to these in vitro observations.

The mechanisms by which ANCA alter neutrophils and monocytes seem to require ANCA binding to the antigen. It is likely that the F(ab')<sub>2</sub> signal is different than the combined signals stimulated by the whole endeavor. Signals to activate transcription of certain genes may originate from a different portion of the antibody, while binding to the Fc receptor may predominantly signal leukocyte activation. Neutrophils respond to the physical cues of ANCA by upregulating transcription of IL-1β and IL-8.<sup>14-18</sup> Some evidence has linked ANCA with protein kinase C activation and IP<sub>3</sub> generation.<sup>19</sup> ANCA-induced signaling can synergize with arachidonic acid path-

ways,<sup>20</sup> and with TNF-α signaling pathways.<sup>21</sup> A major function of signaling networks is to place a value on a signal such that it is either dissipated or converted into further biochemical events. Consequently, in the hierarchical framework of signaling networks, the strongest signal prevails.<sup>22</sup> It is possible that there are multiple signals that are "integrated" through several different pathways that result in leukocyte activation or leukocyte production of phlogistic effectors or in the development of an apoptotic signal. The point that F(ab')<sub>2</sub> binding engages signaling components was further confirmed by Harper et al,<sup>23</sup> who reported that neutrophils from ANCA patients had a greater degree of apoptosis that correlated with higher concentrations of surface proteinase 3. Moreover, once the cells became apoptotic, they became unresponsive to ANCA binding and signaling, indicating that ANCA require an intact signaling network to mediate a response.

ANCA signaling is most likely a consolidation of signals produced by both ANCA-F(ab')<sub>2</sub> and ANCA-Fc engagement. These signals are probably not mutually exclusive, and the complexity of outputs results in a variety of neutrophil and monocyte functions. We still have much to learn about the consequences of ANCA on the clinical and pathologic phenotype of ANCA vasculitis.

## ■ SUMMARY

The role of ANCA, ANCA antigens, endothelial cell damage, genetic and environmental pressures, and the "activatability" of leukocytes will probably prove to be important variables in human ANCA vasculitis. The advent of a reliable animal model may open new areas of investigation and treatment of these vasculitic conditions.

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