Reactive Nitrogen Intermediates in Giant Cell Arteritis

Selective Nitration of Neocapillaries

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Arterial wall damage in giant cell arteritis (GCA) is mediated by several different macrophage effector functions, including the production of metalloproteinases and lipid peroxidation. Tissue-invading macrophages also express nitric oxide synthase (NOS)-2, but it is not known whether nitric oxide-related mechanisms contribute to the disease process. Nitric oxide can form nitrating agents, including peroxynitrite, a nitric oxide congener formed in the presence of reactive oxygen intermediates. Protein nitration selectively targets tyrosine residues and can result in a gain, as well as a loss, of protein function. Nitrated tyrosine residues in GCA arteries were detected almost exclusively on endothelial cells of newly formed microcapillaries in the media, whereas microvessels in the adventitia and the intima were spared. Nitration correlated with endothelial NOS-3 expression and not with NOS-2-producing macrophages, which preferentially homed to the hyperplastic intima. The restriction of nitration to the media coincided with the production of reactive oxygen intermediates as demonstrated by the presence of the toxic aldehyde, 4-hydroxynonenal. Depletion of tissue-infiltrating macrophages in human temporal artery-SCID mouse chimeras disrupted nitrotyrosine generation, demonstrating a critical role of macrophages in the nitration process that targeted medial microvessels. Thus, protein nitration in GCA is highly compartmentalized, reflecting the production of reactive oxygen and reactive nitrogen intermediates in the inflamed arterial wall. Heterogeneity of microvessels in NOS-3 regulation may be an additional determinant contributing to this compartmentalization and could explain the preferential targeting of newly generated capillary beds. (*Am J Pathol* 2002, 161:115–123)

Giant cell arteritis (GCA) is the most frequent form of vasculitis in the western world. The diagnosis of GCA is established by biopsy of the temporal artery, a preferred site of involvement in this arteritis. Typically, the temporal artery lesion shows a panarteritis with infiltrates composed of T cells and macrophages that have accumulated in all layers of the vessel wall.^{1,2} Granuloma formation has a tendency to focus on the media and the media-intima border with multinucleated giant cells in close proximity to the fragmented internal elastic lamina. The inflammation primarily targets defined vascular beds, in particular the extracranial and upper extremity branches of the aorta. Consequently, ischemic complications of the disease center on the head and the central nervous system, with blindness being the most feared manifestation.^{3,4}

Progress has been made in delineating the pathogenic mechanisms involved in GCA.^{3,2} It is now understood that GCA is a T-cell-driven syndrome with T-cell activation occurring in the adventitia of the vessel.^{5,6} T cells regulate the function and activity of effector macrophages, which mediate tissue damage, including the production of tissue-destructive metalloproteinases and reactive oxygen intermediates (ROIs).^{7,8} A major component of arterial damage in GCA is related to a maladaptive injury-response program of the arterial wall in reaction to the immunological insult. This injury-response program encompasses a rapid and concentric proliferation of the intimal layer, causing luminal obstruction and tissue ischemia.⁹ Intimal hyperplasia is accompanied by neoangiogenesis with neocapillaries being formed in the normally avascular medial and intimal layers of the arterial wall.¹⁰

One of the emerging principles in GCA pathogenesis is the strict compartmentalization of immunological events in the vessel wall.¹¹ Tissue-infiltrating lymphocytes and macrophages have been subdivided into distinct subsets based on their topographic arrangement in the artery.¹² T cells and macrophages in the adventitia

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are specialized to produce interferon- γ , interleukin-1, and interleukin-6. Medial macrophages, especially multinucleated giant cells, are committed to the production of metalloproteinases and growth factors, such as platelet-derived growth factor and vascular endothelial growth factor, which are instrumental in controlling the process of intimal proliferation. Macrophages infiltrating the medial smooth muscle cell layer have also been implicated in lipid peroxidation, a process of oxidative damage leading to cell dysfunction and death. Macrophages located in the hyperplastic intima characteristically express nitric oxide synthase (NOS)-2, giving them the ability to contribute to nitrosative stress. Although it is not understood how the close correlation between tissue localization and function is regulated, it suggests that inflammatory cells have information about their whereabouts in the microenvironment and are involved in a highly regulated crosstalk with the arterial tissue.

This study addressed whether the production of reactive nitrogen intermediates (RNIs) contributed to the disease process. Given that RNIs and ROIs can act synergistically and that lipid peroxidation is focused on the media,^{7,13} it is also important to understand whether the pathways leading to ROI and RNI formation are independently and spatially differentially regulated. Because of their extremely short half-lives, ROIs and RNIs cannot be directly demonstrated in the tissue lesion, but their biological effects can be detected as a fingerprint of metabolites generated as downstream products. Nitric oxide (NO) can react with superoxide to form a nitrating agent, peroxynitrite.^{14,15} Peroxynitrite reacts avidly with tyrosine residues in proteins to form nitrotyrosine (NT).¹⁶ This pathway of nitration has received particular attention because this protein modification can have profound impact on protein function and turnover.^{14,17} NT can be detected by immunohistological techniques, which also allow for detailed localization of cellular structures affected by the nitration process.¹⁸ Our studies show that NT formation is a regular event in GCA that, intriguingly, affects only selected cell populations in a restricted area of the vascular wall. Specifically, NOS-3⁺ endothelial cells lining neocapillaries in the media of the artery exclusively undergo nitration. Nitration was dependent on macrophages, as shown in depletion experiments in temporal artery-SCID mouse chimeras, and it correlated with ROI production but not NOS-2-expressing macrophages.

Materials and Methods

Temporal Artery Specimens

Temporal artery specimens were obtained from routine diagnostic biopsies. All tissues were obtained from patients who gave informed consent, and all experiments were performed in accordance with an Institutional Review Board-approved protocol. Thirty-three biopsies showed typical signs of GCA with characteristic inflammatory infiltrates. Eight of these patients were on pred-

nisone at the time of biopsy. All inflamed specimens were graded on serial sections as to whether they had marked (>50% lumen obstruction, defined as the area of the lumen as a percentage of the area internal of the internal elastic lamina) or minimal to moderate intimal hyperplasia (<50% stenosis). Tissue specimens from control patients had no infiltrates in multiple serial sections. Specimens were embedded in OCT compound (Sakura Finetek, Torrance, CA), immediately frozen, and stored at -80°C for immunohistochemical analysis.

Treatment of Temporal Artery-SCID Mouse Chimeras

All animal experiments were performed in accordance with an Institutional Animal Care and Use Committee-approved protocol. Segments of temporal arteries from patients with GCA were implanted subcutaneously into NOD-SCID mice (NOD.CB17-*Prkdc*^{scid}/J; Jackson Laboratories, Bar Harbor, ME) as described previously.^{13,19} Mice were anesthetized with 50 mg/kg of pentobarbital before the surgery. Starting on day 8 after implantation, mice were injected intraperitoneally for 3 consecutive days with 200 μg or 400 μg of anti-CD11b monoclonal antibody (mAb) (clone OKM-1, CRL-8026; American Type Culture Collection, Manassas, VA) diluted in RPMI 1640 medium/phosphate-buffered saline. The control group was sham-injected with medium only. Sixteen days after implantation, the mice were euthanized by CO_2 inhalation, and the temporal arteries were explanted and embedded in OCT compound for immunohistochemical analysis.

Immunohistochemical Analysis

Single- and two-color immunohistochemistry and multi-color immunofluorescence were performed on frozen sections of temporal arteries as previously described.^{7,10} The following reagents were purchased: rabbit anti-NOS III (BD-Transduction Laboratories, Lexington, KY), rabbit anti-NT (Upstate Biotechnology, Lake Placid, NY), goat anti-CD14 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Factor VIII (DAKO, Carpinteria, CA), biotinylated goat anti-rabbit (Affinity Bioreagents, Golden, CO), biotinylated rabbit anti-goat (DAKO), Alexa-488-conjugated goat anti-rabbit (Molecular Probes, Eugene, OR), Vector ABC kit, Vector Red substrate, and Vector Blue substrate (all Vector Laboratories, Burlingame, CA). Rabbit antibodies to the toxic aldehyde, 4-hydroxynonenal (HNE), were a gift from Luke Szveda (Case Western Reserve, Cleveland, OH).

Blocking of nonspecific binding was performed with 5% goat or rabbit serum, depending on the secondary antibody (Ab) being used. Nuclear fast red (Vector Laboratories) or DAPI (4,6-diamidino-2-phenylindole; Sigma, St Louis, MO) were used for counterstaining as indicated.

Sections were stained with rabbit anti-NT (1:250), followed by biotinylated goat anti-rabbit Ig, developed with the Vector ABC kit and Vector Blue, and counterstained with nuclear fast red. In the two-color stains,

slides were stained with the anti-NT Ab as described and developed with the Vector Red substrate. For the second staining, rabbit anti-Factor VIII (1:200), rabbit anti-HNE (1:500), rabbit anti-NOS-2 (1:200), or rabbit anti-NOS-3 (1:200) were used followed by Alexa 488-labeled secondary goat anti-rabbit Ab (1:300) and counterstaining with DAPI.

Temporal artery sections explanted from NOD-SCID mice were stained with anti-human CD14, anti-NT, or anti-HNE Ab, incubated with the appropriate secondary antibody, and developed with the Vector ABC kit with Vector blue substrate and counterstained with nuclear fast red (all Vector Laboratories).

Image Analysis

To semiquantify the extent of NT formation or lipid peroxidation in the arterial wall and to estimate the density of Factor VIII⁺ vessels, tissue sections were stained with anti-NT, anti-HNE, and anti-Factor VIII Ab and developed with Vector red (Vector Laboratories). Slides were imaged with the Kontron Imaging System (Kontron Elektronik, Eiching, Germany) for light microscopy or scanned with a confocal laser microscope (LSM-510; Zeiss, Thornwood, NY) for immunofluorescence. The percentage of stained areas was calculated in each of the arterial wall layers separately using KS-400 software (Kontron Elektronik).

Statistical Analysis

All statistical analyses were performed using SigmaStat Software (SPSS, Chicago, IL). Comparisons of measurements from the immunohistochemical analysis were done with the nonparametric rank sum test.

Results

Expression of NT in Vasculitic Lesions

Biological consequences of NO release on the tissue-invading inflammatory cells and resident vessel wall structures were examined by detecting nitrated tyrosine residues in tissue sections of temporal arteries. Arterial sections from 15 normal temporal arteries lacking inflammatory infiltrates did not stain for NT. In tissue sections from 33 temporal arteries with arteritis, NT-positive cells were consistently detected (Figure 1). The extent of NT expression in GCA arteries was variable, with some patients having only a few cell clusters positive for nitrated tyrosine and other patients having extensive and widespread NT formation. There was no correlation between nitration and corticosteroid treatment. However, only the minority of GCA patients was on corticosteroids and in most of these patients, treatment had been started only a few days earlier. A good predictor for the presence of NT⁺ cells was the degree of intimal hyperplasia and luminal occlusion. A quantitative analysis for NT expression in relationship to the extent of intimal hyperplasia is

Figure 1. Protein nitration in GCA. Nitrated tyrosine residues were detected immunohistochemically in temporal arteries from patients with GCA. Tissue sections were stained with Abs specific for NT, developed with Vector blue substrate, and counterstained with nuclear fast red. NT could not be detected in temporal arteries lacking inflammation (**A**), but it was expressed in arteries with typical vasculitic lesions (**B** and **C**). Original magnifications: $\times 100$ (**A** and **B**); $\times 400$ (**C**).

shown in Figure 2. In arteries with lumen-occlusive hyperplastic intima ($n = 10$), NT was much more abundant than in vessels with minimal intimal thickening ($n = 5$). Previous studies have shown that the profile of inflammatory cytokines and growth factors produced in the vasculitic lesions and not necessarily the density of the infil-

Figure 2. Correlation of NT expression with neointima formation. Tissue sections from temporal arteries that had inflammatory infiltrates consistent with GCA were stained with anti-NT Ab as described in Figure 1. Arteries were categorized as having extensive or minimal to moderate intimal hyperplasia as defined above. The stained area was quantified by laser confocal microscopy and the percentage of stained tissue in relation to the entire arterial circumference minus the lumen was calculated. Results are displayed as box plots with median, 25th, and 75th percentiles as boxes, and the 10th and 90th percentiles as whiskers. Median NT expression was at least 10-fold higher in inflamed temporal arteries with lumen-stenosing intimal hyperplasia.

trate correlate with intimal hyperplasia in temporal arteries,^{10,20} suggesting a possible relationship between the process of NT formation and the nature of the inflammatory insult to the arterial wall.

Tyrosine Nitration Selectively Affects Endothelial Cells of Microcapillaries

The distribution of NT-specific staining suggested that endothelial cells were the targets of NO-related species. NT-positive cells were arranged in isolated clusters, often surrounding a small lumen. Two-color immunofluorescence was used to identify the cells with NT residues. As shown in Figure 3, NT-specific staining was limited to Factor VIII⁺ endothelial cells. Endothelial cells lining the macrolumen were negative for NT-specific staining, and not all capillaries in the inflamed arterial wall reacted with anti-NT Ab. The nitration process did not affect extracellular matrix proteins. Also, NT was explicitly rare on non-endothelial cells. High-power microscopic imaging demonstrated that cells in close proximity to the endothelial cell layer were spared from NT formation, raising the possibility that the processes involving NO-related species were limited to the intracellular space.

The nonrandomness of NT staining throughout the wall raised the question whether capillary beds in distinct regions were differentially susceptible to nitration. In Figure 4, the expression of Factor VIII and NT was semi-quantified in the medial and intimal layers of 20 GCA arteries. Capillaries appeared with similar density in the media and the intima, whereas nitrated proteins were strongly biased to the media ($P = 0.01$).

ROI Availability Is Restricted to the Media, and NO-Related Species Derive from NOS-3⁺ Cells

The generation of peroxynitrite requires the interaction between NO and ROI. The selectivity of NT formation in

Figure 3. Expression of nitrated proteins by endothelial cells lining microcapillaries. Frozen sections of temporal arteries affected by GCA were stained with Abs specific for NT and for Factor VIII. Anti-NT binding was detected with Vector red (red) and anti-Factor VIII was visualized with Alexa 488-labeled secondary Abs (green). Co-occurrence of NT and Factor VIII generates a yellow signal. Blue identifies nuclei stained with DAPI. NT is found almost exclusively on endothelial cells lining microvessels in the arterial media (**left**), and NT staining accumulated in the cytoplasm of the cell (**right**). Original magnifications: $\times 100$ (**left**); $\times 400$ (**right**).

Figure 4. Nitration of endothelial proteins is typical of neocapillaries in the media. Frozen sections of temporal arteries with GCA were stained and analyzed by confocal laser microscopy as described in Figure 2. Nitrated proteins were detected with anti-NT Ab; Factor VIII was used as a specific marker of endothelial cells. The toxic aldehyde, 4-HNE, was identified with a specific Ab. The area of staining was determined independently for each layer of the artery wall and results are shown for the intima (macrolumen to internal elastic lamina) and the media (internal to external elastic laminae). Results are expressed as box plots as described in Figure 2. Similar numbers of endothelial cells lining newly formed microcapillaries were detected in the intima and the media (**left**). Generation of NT ($P = 0.01$) and 4-HNE ($P = 0.003$) as markers of nitration and oxidative stress, respectively, occurred preferentially in the media with relative sparing of the intima.

medial microvessels could be caused by restricted availability of superoxide. Because of their short half-life, ROIs cannot be directly measured in the tissue. We have previously reported that lipid peroxidation is an important mechanism of tissue damage in GCA.^{7,21} Specifically, the toxic aldehyde, HNE, is formed in GCA lesions with compartmentalization of the lipid peroxidation process to the medial layer. HNE has been seen on the surface of smooth muscle cells and also on the surface of macrophages and T cells that have invaded the smooth muscle cell layer.¹³ Generation of HNE has been correlated with up-regulation of mitochondrial activity in CD68⁺ macrophages, a feature that is seen exclusively in macrophages homing to the media. To examine the precise relationship between the tissue compartments in which NO and ROI were generated, staining of tissue with anti-HNE Ab was quantified by digital surface analysis and compared with the distribution of NT. The two markers of oxidative stress and nitration were closely correlated; both were abundant in the media but were relatively sparse in the intima. Figure 5 demonstrates the close co-localization of HNE and NT, both of which affect structures in the medial wall layer. The co-occurrence of HNE and NT in a selected area of the vessel wall suggested that the ROIs necessary for HNE and NT generation might be similarly regulated.

We have previously reported that NOS-2 is selectively detected in macrophages recruited to the intima¹² and is absent in macrophages accumulating in the media and around the internal elastic lamina. The relative sparing of the intimal tissue from NT generation suggested that NO produced by NOS-2⁺ cells was unlikely to contribute to protein nitration. Staining of tissue sections with anti-NOS-2 and anti-NT Ab confirmed that both markers were nonoverlapping. Instead, NOS-3 was found in essentially all endothelial cells, including those lining the newly formed microcapillaries in the media and intima (Figure 6). Two-color immunofluorescence correlated NT production with NOS-3⁺ endothelial cells in the media (Figure 6C). Positive staining for NT around NOS-2⁺ macro-

phages in the intima was infrequently seen (data not shown).

Macrophages Are Essential in Nitrosative Stress of Endothelial Cells Lining Medial Neovessels

Initial studies of lipid peroxidation in GCA have implicated macrophages in oxidative stress.⁷ To examine whether the restricted nitration occurring in neocapillaries of the media was dependent on macrophages, macrophages were depleted from inflamed temporal arteries and NT expression was compared in tissues with and without macrophages. For these depletion experiments, human temporal artery-SCID mouse chimeras were used. After engrafting human temporal arteries containing GCA lesions into NOD-SCID mice, the chimeras were treated for 3 days with anti-CD11b mAb. Seven days after antibody injections, the arterial grafts were retrieved. Histomorphology demonstrated that three daily injections of 200 μ g of anti-CD11b Ab were sufficient to eradicate CD14⁺ cells from the inflammatory lesions while the lymphocytic infiltrates were maintained. As demonstrated in Figure 7, depletion of CD14⁺ macrophages completely disrupted NT formation. NT⁺ endothelial cells persisted in the control grafts. These data established that macrophages are critically involved in protein nitration in GCA. Because macrophages in the inflamed media do not express NOS-2, their contribution is most likely that of supplying oxygen-derived intermediates for the nitration of tyrosine.

Discussion

Mechanisms of arterial wall injury in GCA are diverse and include oxidative damage. Therapeutic interventions for the treatment of this vasculitis are currently limited to nonspecific immunosuppression.²² Better understanding of the inflammatory pathways and mediators of tissue damage involved in this disease is necessary to broaden the spectrum of therapeutic targets. In this study, we investigated whether NO produced in the vascular lesions has cytotoxic effects. Among the specific cellular constituents, multiple targets for RNI have been identified, including DNA, lipids, and proteins.^{23,24} We focused on tyrosine nitration, a protein modification that can be attributed to peroxynitrite. Tyrosine nitration can distort tyrosine phosphorylation and, thus, have a major impact on intracellular signaling pathways.^{25,26}

We found that nitration of tyrosine residues is an ongoing process in the vascular lesions. Intriguingly, NT formation was highly restricted. Nitrated tyrosine was almost exclusively detected on endothelial cells lining newly formed microcapillaries in the media; microvessels in the intima and the adventitia were spared. It remains possible that other pathways of NO-related stress are operational in GCA that could possibly affect other microcompartments and other cellular targets.²⁷

The lack of NT formation in the intima cannot be explained by the absence of NO-related species because

Figure 5. Topographic relationship of lipid peroxidation and NT formation. Temporal artery sections were double-stained with Abs specific for NT and 4-HNE adducts. NT-specific binding was detected with Vector red substrate (**A**, red), and reactivity to HNE was visualized with a secondary Ab labeled with Alexa 488 (**B**, green). Concomitant expression of NT and 4-HNE generates a yellow signal (**C**). Blue identifies nuclei stained with DAPI. An area from the arterial media is shown. Cellular structures affected by NT formation were also targeted by lipid peroxidation. Original magnifications, $\times 400$.

NOS-3⁺ endothelial cells and NOS-2⁺ macrophages are abundant in this compartment of the artery. Also, there was no evidence that protein nitration was relevant in the adventitia, in particular, not in the vasa vasorum supplying the arterial wall. NOS-3 expression was a universal feature of vasa vasorum, suggesting that additional factors besides NO are necessary to initiate nitration. ROIs could be the limiting factor sparing intimal and adventitial structures from NO-related damage, re-emphasizing the strict compartmentalization of tissue-damaging mechanisms in GCA.

The surprising finding of this study was that NT was almost exclusively found in endothelial cells of the media. NT has been reported in vascular disease, particularly in atherosclerotic lesions. In all reports, NT in the tissue has been correlated with NOS-2 expression^{28–30} and has not been localized to NOS-3-producing microendothelial cells. NOS-2 has also been found to be the critical source of RNIs and peroxynitrite in brain ischemia. NOS-2 knockout mice were found to be resistant to NT formation in the vascular endothelium of the peri-infarct region.³¹ In the current study, NOS-2 expressed by macrophages could

not be implicated in tyrosine nitration, leaving the question unanswered of what the precise role of NO-releasing macrophages in GCA arteries is.¹² NO, acting as a vasodilator, is an important modulator of vascular function. Whether regulation of vascular tone is altered in GCA is unknown, but this may not be hemodynamically relevant, given that extensive inflammatory changes and lumen-occlusive intimal hyperplasia are the leading causes of tissue ischemia.

High-power immunohistochemistry and immunofluorescence indicated NT accumulation in the cytoplasm and not on the cell surface (Figures 1 and 3), suggesting that peroxynitrite may be formed within the endothelial cell itself. Endothelial cells can provide superoxide via NADPH oxidases and may, therefore, provide superoxide as well as NO to form peroxynitrite. Depletion experiments, however, demonstrated that CD14⁺ macrophages are critical in the process leading to NT generation in microendothelial cells. One possibility is that ROIs and RNIs participating in the nitration process originate from different cell populations. This model would predict that the coordinated regulation of two pathways is a

Figure 6. Expression of NT by NOS-3⁺ endothelial cells. To identify the cellular source of NO required for NT formation, two-color immunostaining of temporal artery sections from patients with GCA was performed. NT was detected with specific Abs and developed with Vector red (**A**, red). NOS-3 production in endothelial cells lining microcapillaries was demonstrated with anti-NOS-3 Ab, visualized after developing with Alexa 488-labeled secondary Ab (**B**, green). Double-positive cells expressing NT and NOS-3 (shown as yellow) were mainly detected in the newly formed capillary network of the media (**C**). Counterstain with DAPI (blue); original magnifications, $\times 400$.

Figure 7. Tissue-resident macrophages are required for NT formation in capillary endothelial cells. Temporal artery-SCID mouse chimeras were generated by implanting pieces of inflamed temporal arteries into SCID mice. After engraftment of the human tissue, the chimeras were either sham treated or injected with anti-CD11b mAb. Arterial grafts were harvested and tissue sections were immunostained with anti-CD14 (**top**) or anti-NT Ab (**bottom**). Binding of anti-CD14 Ab was detected with Vector red (red, **top**) and NT was visualized after development with Vector blue (blue, **bottom**). Arteries from anti-CD11b-treated chimeras were depleted of tissue-invading macrophages (**A**), which were maintained in sham-treated grafts (**B**). Macrophage-depleted arteries were negative for NT (**C**). Protein nitration continued in sham-treated temporal arteries (**D**). These results are suggestive that nitration was dependent on macrophages. Original magnifications: $\times 100$ (**A** and **B**); $\times 200$ (**C** and **D**); $\times 400$ (**inset**).

prerequisite of NT generation in GCA. NOS-3 is constitutively expressed in endothelial cells. ROS production by selected medial macrophages could be the limiting step.

Under physiological circumstances, the media and intima of medium-size arteries are free of capillaries. The process of neocapillarization in GCA is highly organized and typically leads to microvessels appearing in the media and the hyperplastic intima.¹⁰ Intimal neovessels were spared from NT formation, although they expressed NOS-3 like all other vessels in the wall. Nitrated tyrosine residues were also not detected on adventitial vasa vasorum, a vascular bed consistently positive for NOS-3. NOS activity and, consequently, NO production are post-transcriptionally regulated. NOS-3 activity may be different in distinct vascular beds, even if NOS-3 is equally expressed. Alternatively, NO production by itself is insufficient to create nitrative stress. We considered the possibility that endothelial beds in the wall of the artery were heterogeneous. Studies examining the expression of adhesion molecules, necessary for migration of inflammatory cells into the vascular lesions, demonstrated that

there were no differences between adventitial, medial, and intimal capillaries.³³

To test the functional consequences of tyrosine nitration of microcapillaries *in vivo*, we have used a series of markers to examine microendothelial function in medial neovessels in GCA arteries (data not shown). Studies of apoptotic cells in GCA arteries have demonstrated that cellular apoptosis is seen in the adventitia, yet it is infrequent in the media and the hyperproliferative intima. Medial cells undergoing apoptosis have been described to be smooth muscle cells,^{13,34} in line with the observation that the smooth muscle cell layer loses thickness in inflamed temporal arteries. The expression of adhesion molecules on medial capillaries indicated that the microvessels remained functionally intact.

Beneficial effects of tyrosine nitration in endothelial cells have also been described. Lefer and colleagues³⁵ studied the direct action of peroxynitrite on endothelium *in vivo* and *in vitro* and reported inhibition of neutrophil adhesion as well as attenuation of neutrophil accumulation after ischemia reperfusion injury. This cytoprotective

effect of peroxynitrite was associated with inhibition of P-selectin expression. These authors used gene-targeted animals to demonstrate that NO derived from NOS-3 is critical in regulating leukocyte/endothelial cell interaction.³⁶ Peroxynitrite has also been implicated in the mechanoactivation of endothelial cells. In studies by Go and colleagues,³⁷ peroxynitrite acted as a signaling molecule by activating c-Jun NH(2) terminal kinase, an enzyme involved in determining cell survival in response to environmental stresses. In essence, nitration of tyrosine residues is not necessarily a sign of deleterious effects, but it is possible that peroxynitrite participates in controlling the function of endothelial cells lining the newly emerged capillaries in the inflamed arterial media. The functional competence of endothelial cells in the media is obviously critical, because they can control cell migration through the vessel wall, perpetuating the vasculitic reaction.

Although the precise impact of NT formation specifically targeting the endothelial lining of neocapillaries in the media is not known, our study provides important clues on the role of RNs in GCA. Expression of NOS-2 in macrophages does not seem to have any tissue-injurious function; at least, nitration products are only infrequently found in the vicinity of NOS-2-positive macrophages. Such macrophages are preferentially found in the hyperplastic intima, suggesting that NO release may have vasoregulatory function. Nitration is restricted to the media of the inflamed blood vessel and is dependent on ROI-producing macrophages. ROI production is strictly compartmentalized. Although cells in the adventitia seem to hold the key to ultimate control, effector functions of macrophages in the media become central in determining the pathogenic events. The mechanism by which lymphocytes in the adventitia communicate to macrophages in the media is not understood, but it likely involves the action of interferon- γ produced by adventitial T cells.³⁸

Interfering with oxidative stress in GCA has not been explored as a therapeutic option. Patients with GCA are currently treated with high doses of corticosteroids,²² which have suppressive effects on some but not all mediators of disease. Our studies define ROI formation by macrophages as a critical mechanism not only for lipid peroxidation, but also for tyrosine nitration, suggesting that they may be suitable targets for therapy.

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