Varicella-zoster virus vasculopathy

Immune characteristics of virus-infected arteries

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ABSTRACT

Objective: Pathologic changes in varicella-zoster virus (VZV)-infected arteries include inflammation, thickened intima, and paucity of smooth muscle cells. Since no criteria have been established for early vs late VZV vasculopathy, we examined inflammatory cells and their distribution in 6 normal arteries, and 2 VZV-infected arteries 3 days after onset of disease (early) and 10 months after protracted neurologic disease (late).

Methods: VZV-infected temporal artery obtained 3 days after onset of ischemic optic neuropathy from an 80-year-old man, VZV-infected middle cerebral artery (MCA) obtained 10 months after protracted disease from a 73-year-old man, and 5 MCAs and 1 temporal artery from normal subjects, age 22–60 years, were examined histologically and immunohistochemically using antibodies against VZV and inflammatory cell subsets.

Results: In both early and late VZV vasculopathy, T cells, activated macrophages, and rare B cells were found in adventitia and intima. In adventitia of early VZV vasculopathy, neutrophils and VZV antigen were abundant and a thickened intima was associated with inflammatory cells in vaso vasorum vessels. In media of late VZV vasculopathy, viral antigen, but not leukocytes, was found. VZV was not seen in inflammatory cells. Inflammatory cells were absent in control arteries.

Conclusions: Both VZV and neutrophils exclusively in adventitia in early VZV vasculopathy indicate that disease begins there. Late VZV vasculopathy is distinguished by viral antigen without inflammation in media, revealing a human virus in an immunoprivileged arterial media. Association of thickened intima and inflammation in vaso vasorum vessels in early VZV vasculopathy support the role of virus-induced inflammation in vessel wall remodeling. *Neurology*® 2013;80:62–68

GLOSSARY

DAB = diaminobenzidine; **MCA** = middle cerebral artery; **PAH** = pulmonary arterial hypertension; **PBS** = phosphate-buffered saline; **VSMC** = vascular smooth muscle cell; **VZV** = varicella-zoster virus.

In varicella-zoster virus (VZV) vasculopathy, virus-infected regions are associated with a thickened intima composed of myofibroblasts, a paucity of smooth muscle cells in media, and disruption of internal elastic lamina.¹ Similar morphologic changes have been found in other vascular diseases, such as pulmonary arterial hypertension (PAH) and atherosclerosis,² in which inflammation has emerged as a pathogenic component. For example, in PAH, inflammatory cells surrounding pulmonary artery lesions secrete CX3CL1, which induces vascular smooth muscle cell (VSMC) proliferation.³ In atherosclerosis, activated macrophages, in addition to other cells, secrete platelet-derived growth factor–BB and insulin-like growth factor–I, which promote VSMC migration⁴; interleukin-1B also promotes VSMC proliferation.^{5,6} We hypothesize that 1) after reactivation from trigeminal ganglia, virus spreads transaxonally to infect the adventitia, followed by transmural migration to the media^{1,7}; 2) inflammatory cells are recruited to virus-infected sites; and 3) inflammatory cells secrete factors that contribute to vessel wall changes seen in VZV vasculopathy. Characterization of immune cells involved in VZV vasculopathy could elucidate mechanisms of VZV-induced vascular remodeling and identify potential targets for therapy.

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Although no criteria have been established for early vs late VZV vasculopathy, we describe inflammatory cell subtypes and their distribution in a VZV-infected temporal artery 3 days after onset of disease (early) and a VZV-infected middle cerebral artery (MCA) 10 months after protracted neurologic disease (late) compared to their presence and distribution in 5 normal MCAs and 1 normal temporal artery.

METHODS Clinical features and arteries examined. Temporal and middle cerebral arteries from 2 subjects with VZV vasculopathy were studied; pathologic and virologic analyses of these arteries have been described.^{1,7-9} The VZV-infected temporal artery was obtained 3 days after ischemic optic neuropathy (early VZV vasculopathy) in an 80-year-old man who developed left ophthalmic-distribution zoster 4 weeks earlier⁷; the temporal artery did not exhibit pathologic changes of giant-cell arteritis, but did contain VZV antigen and CSF contained anti-VZV immunoglobulin G and M consistent with the diagnosis of VZV vasculopathy. The patient improved after antiviral treatment. The VZV-infected MCA was obtained at autopsy 10 months after protracted VZV vasculopathy (late VZV vasculopathy) in a 73-year-old man.^{8,9} Control arteries included 5 MCAs obtained postmortem from 5 subjects (3 men and 2 women), age 22–60 years, and 1 temporal artery obtained during biopsy from a 43-year-old man with chronic headaches that was normal histologically. All subjects from whom control arteries were obtained had no history of zoster, immunosuppression, TIAs, or stroke.

Classification of early and late VZV vasculopathy. There are no established criteria that define early and late VZV vasculopathy. A VZV-infected temporal artery was studied 3 days after onset of disease (designated early VZV vasculopathy), and a VZV-infected MCA 10 months after protracted neurologic disease (designated late VZV vasculopathy).

Standard protocol approvals, registrations, and patient consents. The MCA from a subject with late VZV vasculopathy was archival autopsy material obtained in 1995 and published as a clinicopathologic conference in the *New England Journal of Medicine*.^{8,9} The 5 control MCAs obtained at autopsy were de-identified and deemed exempt from review by the Colorado Multiple Institutional Review Board. The normal temporal artery and VZV-infected temporal artery were biopsy specimens sent to the neurovirology laboratory at the University of Colorado School of Medicine for virologic diagnostic evaluation.

Histopathology. Formalin-fixed, paraffin-embedded 5- μ m sections were incubated at 72°C for 30 minutes and stained with hematoxylin & eosin.

Immunohistochemistry. Primary antibodies used were a 1:5,000 dilution of polyclonal rabbit anti-VZV 63¹⁰; undiluted rabbit monoclonal anti-CD3 (Ventana, Tucson, AZ); undiluted rabbit monoclonal anti-CD4 (Ventana); a 1:100 dilution of mouse monoclonal anti-CD8 (Dako, Carpinteria, CA); undiluted mouse monoclonal anti-CD15 (Ventana); undiluted mouse monoclonal anti-CD20 (Ventana); undiluted mouse monoclonal anti-CD45 for automated slide staining (Ventana); a 1:50 dilution of mouse monoclonal anti-CD45 for immunofluorescence (Dako); a 1:80 dilution of mouse monoclonal anti-CD57 (Becton Dickinson, San Jose, CA); undiluted mouse monoclonal anti-CD68 (Ventana); and a 1:100 dilution of rabbit monoclonal anti-CD117 (Cell Marque, Rocklin, CA). Except when indicated, all incubations were at room temperature.

To detect VZV gene 63 protein, 5- μ m sections were deparaffinized in 100% xylene (3 times for 5 minutes each) followed by 100% ethanol (3 times for 5 minutes each). After sequential dipping in 95%, 70%, and 50% ethanol, sections were placed in distilled water, blocked in phosphate-buffered saline (PBS) containing 5% normal goat serum for 1 hour, washed 3 times with PBS, and incubated with anti-VZV antibody or normal rabbit serum overnight at 4°C. After warming to room temperature, sections were rinsed 3 times with PBS, incubated with a 1:1,000 dilution of biotinylated goat antirabbit antibody (Dako) for 1 hour, rinsed 3 times in PBS, and incubated with prediluted alkaline phosphatase-conjugated streptavidin (Becton-Dickinson) for 1 hour. The color reaction was developed for 2 minutes using the fresh fuchsin substrate system (Dako) in the presence of levamisole (Dako), final concentration 24 μ g/mL.

For CD3, CD4, CD8, CD15, CD20, CD45, CD57, and CD68 immunostainings, sections were deparaffinized, and heated for 30 minutes for epitope retrieval in Tris-EDTA, pH 9. Slides were incubated with primary antibody at 37°C for 32 minutes, except for CD4 at 20 minutes, CD15 at 24 minutes, and CD68 at 16 minutes. Slides were then processed with biotinylated secondary antibody followed by horseradish peroxidase and diaminobenzidine (DAB) using an automated slide stainer according to the manufacturer's instructions (reagents/protocol in iVIEW DAB Detection Kit, Ventana).

For CD117 immunostaining, slides were deparaffinized, heated in 10 mM sodium citrate buffer, pH 6, for 20 minutes for epitope retrieval, followed by blocking for 15 minutes at room temperature with Ultra V Block (Thermo Scientific, Kalamazoo, MI), and incubated with anti-CD117 antibody for 1 hour at room temperature; slides were processed with biotinylated secondary antibody followed by horseradish peroxidase and DAB using an automated slide stainer according to the manufacturer's instructions (reagents/protocol in Bond Polymer Refine Detection kit; Leica, Buffalo Grove, IL).

For dual immunofluorescence (VZV 63 and CD45), slides were deparaffinized, heated in 10 mM sodium citrate buffer, pH 6, for 20 minutes for epitope retrieval, blocked for 1 hour at room temperature with normal donkey serum and incubated with anti-VZV 63 and anti-CD45 antibody, washed with PBS 3 times for 3 minutes each, and further incubated with a 1:1,000 dilution of donkey antirabbit 488 (Invitrogen, Grand Island, NY) and a 1:1,000 dilution of donkey antimouse 594 (Invitrogen) for 1 hour at room temperature. After washing with PBS 3 times for 3 minutes each, slides were mounted with Vectashield Mounting Media (Vector Labs, Burlingame, CA).

Cells expressing specific leukocyte markers were identified by the presence of brown color or fluorescence compared to their presence or absence in control arteries. Positive control cells expressing leukocyte markers were provided by sections of human tonsil and lymph node. All slides were viewed using a Nikon Eclipse E800 microscope with Axiovision digital imaging software.

RESULTS Distribution of VZV antigen and leukocytes in cerebral and temporal arteries. No pathologic changes, VZV antigen, or CD45-expressing cells were detected in normal cerebral or temporal arteries (figure 1, A–C). A thickened intima was seen in both the temporal artery (figure 1D) in early VZV vasculopathy and in the MCA (figure 1G) in late VZV vasculopathy. In early VZV vasculopathy, the adventitia contained both VZV antigen (figure 1E) and leukocytes expressing CD45 (figure 1F), while in late VZV vasculopathy, VZV antigen was found only in the media, which was devoid of leukocytes (figure 1H), although some leukocytes were seen in adventitia and luminal surface of the intima (figure 1I).

Figure 1 Distribution of varicella-zoster virus (VZV) antigen and leukocytes expressing CD45 in cerebral arteries from normal subjects and from patients with VZV vasculopathy

(A–I) Hematoxylin & eosin (H&E) staining shows normal artery morphology in a normal artery (A) with a single layer of endothelial cells in the intima (I), smooth muscle cells in the media (M), and fibroblasts and connective tissue in the outer adventitia (Adv). A thickened intima (I) was seen in both early (D) and late (G) VZV vasculopathy. VZV antigen was not seen in the normal artery (B), but was present in the adventitia (E) in early VZV vasculopathy and in the media (H) in late VZV vasculopathy (black arrows, pink color). Leukocytes expressing CD45 were not present in the normal artery (C), but were seen in the adventitia (F, black arrows, brown color), where VZV antigen was found in early VZV vasculopathy. In late VZV vasculopathy, leukocytes expressing CD45 were seen predominantly in the luminal surface of the thickened intima and to a lesser degree in the adventitia (I, black arrows, brown color). In contrast to the presence of both VZV antigen and leukocytes in the adventitia in early VZV vasculopathy, the media in late VZV vasculopathy contained only VZV antigen. Magnification = $\times 200$ in panels A–C and G–I; $\times 100$ in panels D–F.

To determine whether VZV antigen was present in leukocytes, arteries from subjects with early and late VZV vasculopathy were dual-stained with antibodies directed against VZV antigen and CD45 and examined by immunofluorescence. Cells containing VZV antigen but not expressing CD45 were identified in the adventitia of early VZV vasculopathy (figure 2, A–C) and in the media of late VZV vasculopathy (figure 2, D–F).

Distribution of immune cell subsets in VZV vasculopathy.

Since none of the 5 normal cerebral arteries contained leukocytes expressing CD45, they were not examined further for leukocyte subsets. In both early and late VZV vasculopathy, T cells expressing CD3 (figure 3, A and D, respectively), CD4 (figure 3, B and E, respectively), and CD8 (figure 3, C and F, respectively) were present primarily in the adventitia and thickened intima. Rare B cells expressing CD20 were detected in the adventitia and thickened intima in early (figure 4A) and late (figure 4D) VZV vasculopathy. Activated macrophages expressing CD68 were seen in the adventitia and intima in early (figure 4B) and late (figure 4E) VZV vasculopathy. In early VZV vasculopathy, activated macrophages expressing CD68 were detected in the

lumen of the vaso vasorum that extended into the media (figure 4B). Abundant neutrophils expressing CD15 were present predominantly in the adventitia and rarely in the intima in early VZV vasculopathy (figure 4C), whereas neutrophils were rarely seen in the intima in late VZV vasculopathy (figure 4F). No or only rare natural killer cells expressing CD57 or mast cells expressing CD117 were found in either early or late VZV vasculopathy (not shown).

Association of inflammatory cells with thickened intima in early VZV vasculopathy.

T cells expressing CD3 (figure 5) as well as cells expressing CD4, CD8, CD20, and CD68 were present in the adventitia associated with a thickened intima in early VZV vasculopathy. Natural killer cells expressing CD57 and mast cells expressing CD117 were not seen (not shown). Neutrophils expressing CD15 were seen on the luminal surface of the artery that contained an asymmetric thickened intima; their distribution was random and not associated with intimal thickening (not shown). A thickened intima was not seen in areas devoid of cells expressing CD3, CD4, CD8, CD20, and CD68.

Figure 2 Cells in cerebral arteries containing varicella-zoster virus (VZV) antigen do not express CD45

Cerebral arteries from subjects with early and late VZV vasculopathy were dual-stained with antibodies directed against VZV antigen and CD45. In the adventitia of early VZV vasculopathy, immunofluorescent staining identified cells containing VZV antigen (A, arrows, green color) but not expressing CD45. Leukocytes that expressed CD45 were devoid of VZV antigen (B, arrows, red color). The merged image also shows VZV-infected cells that did not express CD45 (C, arrows, green color). In the media of late VZV vasculopathy, cells expressing VZV antigen (D, arrow, green color) did not express CD45 (E, arrows, red color). The merged image in late VZV vasculopathy also shows VZV-infected cells (F). Nonspecific green and red autofluorescence is seen in the arterial folds (A-C, long thin arrows) and in the internal elastic lamina (D-F, long thin arrows). Magnification = $\times 200$.

Figure 3 T-cell subsets in varicella-zoster virus (VZV) vasculopathy

Cells expressing CD3 were seen primarily in the adventitia (A, black line, thin black arrows, brown color) as well as in the thickened intima (D, white line, thick black arrows, brown color) in early VZV vasculopathy, whereas cells expressing CD3 were found primarily in the thickened intima (D, white line, thick black arrows, brown color) and much less in the adventitia (D, black line, thin black arrows, brown color) in late VZV vasculopathy. Cells expressing CD4 were present in the adventitia and intima in early and late VZV vasculopathy (B and E, thin black arrows in adventitia; thick black arrows in intima, brown color). Similarly, cells expressing CD8 were present in the adventitia and intima in early and late VZV vasculopathy (C and F, thin black arrows in adventitia, thick black arrows in intima; brown color). Magnification = $\times 100$ in panels A-C and $\times 200$ in panels D-F.

In early (A) and late (D) VZV vasculopathy, rare or no B cells expressing CD20 were seen in the adventitia (black line, thin black arrows, brown color) and intima (white line, thick black arrows, brown color). Activated macrophages expressing CD68 were seen in the adventitia and intima in early (B) and late (E) VZV vasculopathy (thin black arrows in adventitia; thick black arrows in intima, brown color). In early VZV vasculopathy, activated macrophages expressing CD68 were found within the lumen of the vaso vasorum that extended into the media (B, thick white arrows). Abundant neutrophils expressing CD15 were present predominantly in the adventitia and rarely in the intima in early VZV vasculopathy (C, thin black arrows in adventitia; thick black arrow in intima, brown color), whereas only rare or no neutrophils were seen in the intima in late VZV vasculopathy (F, thick black arrow, brown color). Magnification = $\times 100$ in panels A-C and $\times 200$ in panels D-F.

Overall, during both early disease and late disease, T cells expressing CD4 and CD8, macrophages expressing CD68, and B cells expressing CD20 are present in both intima and adventitia, with rare to no natural killer cells expressing CD57 or mast cells expressing CD117. Unique to early disease was the presence of VZV antigen and neutrophils in adventitia, while in late disease, VZV was present in the media in the absence of leukocytes.

DISCUSSION Since inflammation has emerged as a major contributor to vessel wall remodeling, we characterized inflammatory cell subsets and their distribution in early and late VZV vasculopathy. Earlier limited immunologic analyses of VZV-infected arteries detected T cells expressing CD3 and CD8, and macrophages expressing CD68.^{11,12} Herein, we describe multiple T cell, B cell, macrophage, neutrophil, natural killer cell, and mast cell subsets in early and late VZV vasculopathy.

Our detection of viral antigen and neutrophils in adventitia in early VZV vasculopathy supports the notion that after reactivation from ganglia, virus travels transaxonally to arterial adventitia where disease begins.^{1,7} The presence of neutrophils in early VZV vasculopathy is supported by their abundance in vesicular fluid of

zoster patients¹³ and in CSF throughout the protracted course of VZV vasculopathy, meningoencephalitis, encephalomyelitis, myelitis, and inflammatory brainstem disease.^{9,14–17} Although mechanisms of vascular remodeling triggered by VZV are unknown, neutrophils may play a role. When exposed to VZV, they produce reactive oxygen species which mediate smooth muscle cell proliferation and migration^{18,19} and induce apoptosis and loss of vascular smooth muscle cells.^{20,21} Neutrophils also secrete elastase, which can lead to extracellular matrix breakdown and weakening of the vessel wall.^{22–24} Finally, the association of a thickened intima with inflammation in the adventitia of vaso vasorum vessels in early VZV vasculopathy supports the notion that inflammatory cells produce factors involved in vascular modeling.

Furthermore, detection of viral antigen and absence of leukocytes in the immunoprivileged media²⁵ in late disease confirms earlier reports supporting intramural spread of virus to media. Eidelberg et al.²⁶ described 2 fatal cases of VZV vasculopathy of 3 and 10 months duration, respectively. In both cases, VZV antigen was found in the media of MCA without inflammation. Two other case reports found VZV in media of late VZV vasculopathy, although without comment regarding

Figure 5 In early varicella-zoster virus (VZV) vasculopathy, the thickened intima is associated with inflammatory cells in the vaso vasorum

Within the adventitia of the early VZV vasculopathy artery, 2 vaso vasorum vessels contained T cells expressing CD3 (long black arrows, brown color) adjacent to the thickened intima (black bars). Short black arrows in areas where the intima is not thickened were devoid of cells expressing CD3. A thickened intima was also associated with cells expressing CD4, CD8, CD20, and CD68 (not shown). Magnification = $\times 100$.

location of inflammation. The first subject was a 69-year-old woman who died 3 months after the onset of VZV vasculopathy; herpesvirus particles were seen by electron microscopy in smooth muscle cells of the MCA in addition to necrotizing arteritis.²⁷ The second subject was a 4-year-old girl who developed VZV vasculopathy after varicella and died 13 months later; viral antigen and multinucleated giant cells were seen in media of the MCA, along with lymphocytes, predominantly T cells expressing CD3 in an unclear distribution.¹¹

Finally, the unique findings described herein require confirmation by immunologic analysis of more arteries from subjects with early and late VZV vasculopathy.

AUTHOR CONTRIBUTIONS

M.A. Nagel: drafting/revising the manuscript for content, including medical writing for content; study concept or design; analysis or interpretation of data; contribution of vital reagents/tools/patents; acquisition of data; statistical analysis; study supervision or coordination; obtaining funding. I. Traktinskiy: analysis or interpretation of data; acquisition of data. K.R. Stenmark: drafting/revising the manuscript for content, including medical writing for content; analysis or interpretation of data. M.G. Frid: drafting/revising the manuscript for content, including medical writing for content; analysis or interpretation of data. A. Choe: analysis or interpretation of data; acquisition of data. D. Gilden: drafting/revising the manuscript for content, including medical writing for content; study concept or design; analysis or interpretation of data; contribution of vital reagents/tools/patents; acquisition of data; statistical analysis; study supervision or coordination; obtaining funding.

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