Selective Expansion of Influenza A Virus–Specific T Cells in Symptomatic Human Carotid Artery Atherosclerotic Plaques

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Background and Purpose—Evidence is accumulating that infection with influenza A virus contributes to atherothrombosis. Vaccination against influenza decreases the risk of atherosclerotic syndromes, indicating that inflammatory mechanisms may be involved. We tested the hypothesis that influenza A virus–specific T cells contribute to atherosclerotic plaque inflammation, which mediates the onset of plaque rupture.

Methods—T-cell cultures were generated from atherosclerotic segments and peripheral blood of 30 patients with symptomatic carotid artery disease. The response of plaque and peripheral blood T cells to influenza A virus was analyzed and expressed as a stimulation index (SI). Selective outgrowth of intraplaque influenza A–specific T cells was calculated by the ratio of plaque T cell SI and peripheral blood T cell SI for each patient. Accordingly, the patients were categorized as high- (SI ratio >5), intermediate- (5 < SI ratio ≤2), and non- (SI ratio <2) responders. The presence of influenza A virus in the vessel fragments was evaluated by reverse transcription–polymerase chain reaction.

Results—High proliferative responses of plaque-derived T cells to influenza A virus were frequently observed. Among the 30 patients, 5 were categorized as high responders, 10 were intermediate responders, and 15 were nonresponders. Live influenza A virus could not be detected in the atherosclerotic plaques by polymerase chain reaction.

Conclusions—Selective outgrowth of influenza A virus–specific T cells occurs within the microenvironment of human atherosclerotic plaques. Influenza virus–derived antigens or alternatively, mimicry antigens, appear to be potential candidates for triggering or sustaining plaque inflammation, which eventually leads to symptomatic plaque complications. (Stroke. 2008;39:174-179.)

Key Words: atherosclerosis ■ carotid artery ■ infectious disease ■ influenza ■ T lymphocytes
many studies have shown that influenza increases the risk of acute coronary syndromes and stroke,11–13 and interestingly, vaccination against influenza contributes to a decreased risk of myocardial infarction and stroke in the subsequent influenza season.14–17 In experimental studies, a link between influenza A virus infection and atherosclerotic plaque inflammation has been proposed, based on experiments showing that infection of apoE-deficient mice with influenza virus resulted in an acute arterial wall inflammatory response,18 composed predominantly of macrophages and T lymphocytes. These studies suggest that influenza virus may directly trigger plaque inflammation.

Still, the pathophysiologic mechanisms that underlie the relation between influenza virus infection and atherosclerotic syndromes are unknown. The observations alluded to raise the possibility that locally activated, influenza A–specific T cells could contribute to atherosclerotic plaque inflammation. To investigate this concept, we tested the antigenic specificity of T cells for influenza A virus that were isolated from culprit carotid lesions of patients with cerebrovascular disease. Results were compared with the rate of influenza A virus–specific responsiveness of peripheral blood–derived T cells from the same patients. Furthermore, the presence of influenza A virus in these lesions was investigated by sensitive reverse transcription–polymerase chain reaction (RT-PCR).

Subjects and Methods

Patients

Human carotid endarterectomy specimens were collected at surgery from 30 consecutive patients with symptomatic carotid artery disease and a carotid artery stenosis of at least 70% (NASCET method). A portion of the plaque tissue was fixed in RNA later (Ambion, Huntingdon, UK) for conservation of RNA for RT-PCR. The remaining tissue was used for isolation of plaque T cells. Blood (50 mL) was also collected from all patients for the isolation of peripheral blood mononuclear cells (PBMCs), which served as a source of antigen-presenting cells (APCs) and peripheral blood T-cell lines. A small portion of the blood was collected in a Vacutainer serum tube (BD, Breda, Netherlands) for serologic analysis. The study was approved by the local ethics committee, and all patients gave informed consent.

Antigen-Presenting Cells

PBMCs were isolated from freshly drawn blood of each patient by gradient centrifugation with Ficoll-Paque (Pharmacia Biotech, Uppsal, Sweden). The obtained cells, to be used as APCs, were frozen and stored in LN2 until further use.

Culture of Plaque and Peripheral Blood–Derived T Cells

Primary T-cell cultures were generated as described previously.9,19 In brief, endarterectomy specimens were washed in RPMI to remove adherent blood cells and thrombi. Atherosclerotic tissue was minced into small fragments (~1 mm³), washed, and placed in 24-well plates (Costar, Cambridge, Mass). The fragments were cultured in T-cell medium consisting of Iscove’s modification of Dulbecco’s medium (Life Technologies, Breda, Netherlands) supplemented with 10% heat-inactivated pooled human serum (Bio-Whittaker, Walkersville, Md), antibiotics (penicillin/streptomycin, Life Technologies), and 10 U/mL recombinant interleukin-2 (IL-2; Eurocetus, Amsterdam, Netherlands) for 7 to 10 days, resulting in spontaneous migration of T cells from the plaque tissue into the culture medium. T cells were harvested and further expanded by 1 stimulation with 10 µg/mL phytohemagglutinin (DIFCO Laboratories, Detroit, Mich) and irradiated feeder cells (a mixture of PBMCs from 2 buffy coats [Sanquin, Amsterdam, Netherlands] and 10% JY cells) in T-cell medium. Cultures were refreshed every 2 to 3 days with T-cell medium. This protocol yielded 50 to 80 million T cells per culture. With the same protocol as described earlier, PBMCs from the peripheral blood of the same patients were cultured to generate peripheral blood T-cell lines.

Influenza Virus

Sucrose gradient–purified influenza A virus (H3N2) Resvir-9, a reassortant between A/Puerto Rico/8/34 (H1N1) and A/Nanchang/933/95 (H3N2), containing the hemagglutinin, neuraminidase, and nucleoprotein of A/Nanchang/933/95, served as an antigenic stimu-

lus. In addition to influenza A virus, sucrose gradient–purified influenza B virus (B/Harbin/7/94) was also used as an antigenic stimulus. The infectious virus titer were determined in cell culture with Madin-Darby canine kidney cells as indicator cells.20

T-Cell Proliferation Assays

Ten days after stimulation with phytohemagglutinin, IL-2, and feeder cells, the specificity of the T-cell cultures (plaque as well as peripheral blood derived) was analyzed by incubating 5×10⁵ T cells and 5×10⁴ autologous, irradiated PBMCs as APCs for 4 days in T-cell medium either with or without influenza virus (10 tissue culture infectious dose [TCID₅₀] per cell) in 96-well, round-bottom culture plates (Costar). All combinations were analyzed in quadruplicate. During the last 16 hours of culture, 0.3 µCi [³H]-thymidine (Amersham Biosciences Europe, Rosendaal, Netherlands) was present per well. Cultures were harvested, and incorporated radioactivity was measured by liquid scintillation counting. Positive controls (10 µg/mL phytohemagglutinin) and negative controls (irradiated APCs only, APCs with virus only, and T cells with virus only) were also included. Furthermore, the major histocompatibility complex (MHC) restriction of the response was tested by performing blocking experiments with monoclonal antibodies against HLA-DR and HLA-ABC (clones L243 and W6/32, respectively; both from American Type Culture Collection, Manassas, Va). Additional controls were performed with paraformaldehyde–fixed influenza A virus. For the latter, influenza A virus was treated with paraformaldehyde (final concentration, 1% for 20 minutes), followed by glycine treatment (final concentration, 0.2 mol/L) and dialysis against phosphate-buffered saline.

The stimulation index (SI) was calculated as the mean counts per minute of cultures in the presence of the virus divided by the mean cpm of parallel cultures without virus. Differences between plaque T cells and peripheral blood T cells of each individual patient were also determined by calculating the ratio between plaque and peripheral SIs (SI ratio).

Immunohistochemistry

Cytospins were prepared from plaque and peripheral blood T cells of all cases. Immunohistochemistry for CD4 and CD8 was performed with a monoclonal rabbit anti-CD8 (clone sp16, Labvision, Fremont, Calif) and a monoclonal mouse anti-CD4 (clone 4b12, Labvision). Anti-mouse horseradish peroxidase polymer– and anti-rabbit alkaline phosphatase (AP) polymer–conjugated secondary antibodies (Labvision) were used, and antibody-labeled cells were visualized with Vector Blue and Vector Nova Red as chromogens (Vector, Burlingame, Calif).

Detection of Influenza A Virus in Atherosclerotic Plaque Tissue by Real-Time RT-PCR

Frozen cryostat sections of plaque tissue were cut and collected (~25 to 50 mg) in sterile Eppendorf tubes and treated with Trizol reagent. cDNA synthesis was performed with 1 µg RNA and a random-hexamer cDNA synthesis kit (Applera, Foster City, Calif). Five of 25 µL cDNA was used for amplification in a quantitative real-time PCR23 (ABI PRISM 7700 sequence detector system). The following primers were used: 5'-GGACTCGAGGAGAGACGCT (sense), 5'-ATCTGTGTTGATATGAGCCCAT (antisense), and 5'-
CTCAGTTATTCTGCTGTGCACCTTGCC (5'-FAM--labeled probe) that recognize a shared sequence in matrix proteins M1 and M2. Serial dilutions of a positive control (strain A/PR8/34, 70 000 viral RNA copies, electron microscopy [EM] counted) were also included. The detection limit with this protocol was 50 copies of viral genome. The integrity of the cDNA preparations was checked by conventional PCR with primers specific for human viral genome. The specificity of the influenza A virus–induced T-cell proliferation response to influenza A virus in plaque- and peripheral blood–derived T-cell cultures from all patients, sorted by SI ratio. Five patients were classified as high responders (SI ratio > 5), and 10 patients were classified as intermediate responders (2 ≤ SI ratio < 5). In these 15 patients, the response of plaque-derived T cells was statistically significantly increased compared with that of peripheral blood T cells. Fifteen patients were categorized as nonresponders (SI ratio < 2). In 3 of these patients, the response of plaque-derived T cells was significantly lower compared with that of peripheral blood T cells (P < 0.05).

To investigate whether our culture protocol influenced the observed responses against influenza, the responses of peripheral blood T-cell lines and freshly isolated (not cultured) PBMCs from the same patient against influenza were also compared. We did not observe any differences in the responses against influenza between cultured peripheral blood T cells and freshly isolated PBMCs (data not shown), indicating that the culture procedure did not bias the proliferative response to influenza A.

The specificities of the influenza A virus–induced T-cell response was further analyzed with different control experiments. To test whether the influenza A virus–induced proliferation was restricted by MHC, blocking experiments with antibodies against HLA-ABC and HLA-DR were performed. In Figure 2, the results obtained for the high responders are illustrated. The strongest inhibition was observed with antibodies against HLA-DR except in patient No. 27, whose levels of cultured T-cell inhibition with HLA-DR and HLA-ABC were equal. Similar results were observed for the intermediate responders; in all cases the response could be inhibited with antibodies against HLA-ABC and HLA-DR, with the strongest effect with anti–HLA-DR.

Next we tested whether antigen processing and presentation by the APCs of virus proteins are required for eliciting a T-cell response. Therefore, the responses of plaque-derived T cells from 3 patients with a high SI were tested with formalin-fixed influenza A virus and compared with the responses of live virus. We did not observe any differences in terms of proliferation between live and inactivated virus.
apoE−/− mice with influenza A virus resulted in aggravated atherosclerotic plaque inflammation. This effect was limited to atherosclerotic lesions, and no effects were observed on the normal vessel wall; moreover, these authors could not demonstrate the presence of virus in the affected lesions. Basically, we observed a similar situation: the atherosclerotic lesions contained influenza A virus–specific T cells, but the virus itself could not be detected by RT-PCR in the lesions, at least at the time of our analysis.

It is not clear whether influenza A virus is capable of infecting atherosclerotic plaques, because it is generally considered to infect cells in the pulmonary tree only, although viral genes have been detected in the peripheral blood of influenza-infected patients. These observations raise the possibility that antigenic cross-reactivity between influenza-specific epitopes and resident plaque epitopes can develop within the microenvironment of an inflamed plaque. Such a mechanism of antigenic cross-reactivity is not uncommon in the course of longstanding chronic inflammatory processes, but apart from epitope mimicry, it cannot be ruled out that influenza A virus had been present in the plaques but later disappeared, eg, by eradication through the elicited inflammatory response.

In a subset of patients, we found higher frequencies of influenza A–specific T cells in the atherosclerotic plaque compared with peripheral blood, most likely the result of antigen-driven proliferation of specific T-cell subsets. Indeed, proliferating T cells have been detected in atherosclerotic lesions, and recently it was shown that unstable atherosclerotic lesions contain clonotypic expansions of specific T-cell subsets, a phenomenon that was not observed in stable lesions despite the fact that these lesions do contain T lymphocytes. In support of this, we found that the frequency of activated T lymphocytes was higher in unstable atherosclerotic lesions than in stable lesions. Selective recruitment of influenza A–specific T-cell subsets without a subsequent antigen-driven proliferation would result in comparable frequencies among patients of antigen-specific T cells in both compartments, which was not the case.

In similar studies with EBV, we found that only 3 of 19 patients showed increased proliferation of atherosclerotic plaque T cells compared with peripheral blood T cells. In the present study, 15 of 30 patients showed an increased prolif-
mediated by both CD4- as well as CD8-positive T cells. Moreover, influenza-induced T-cell responses in vivo are plaque T-cell lines consisted of both CD4 and CD8 T cells. T-cell responses. This is not unlikely, as we found that our other infectious pathogens have been reported. However, plaque-derived T cells to lipids, heat shock proteins, and genic stimuli are also likely involved. In vitro, responses of triggers atherosclerotic plaque inflammation, and other anti-murine T cells, suggesting that the stimulation was independent of the endogenous route of antigen presentation. This indicates that at least CD4+ T cells must have been involved, and indeed, the virus-specific response of the polyclonal T-cell populations could be inhibited with antibodies directed to MHC class II antigens. In this context, it is interesting to note that activated T cells in atherosclerotic lesions are virtually all of the CD4 subtype. However, an inhibitory effect was also observed with antibodies directed to MHC class I antigens. Therefore, CD8+ T cells also may have contributed to the virus-specific T-cell responses. This is not unlikely, as we found that our plaque T-cell lines consisted of both CD4 and CD8 T cells. Moreover, influenza-induced T-cell responses in vivo are mediated by both CD4- as well as CD8-positive T cells.

Influenza infection is associated with type 1 immune responses, leading to the production of interferon-γ by CD4- and CD8-positive T cells. Production of interferon-γ in the microenvironment of the atherosclerotic lesion leads to macrophage activation, a well-known phenomenon in plaque inflammation. Indeed, interferon-γ has been shown to be the most dominant T cell–derived cytokine in atherosclerotic lesions.

Influenza virus is certainly not the only pathogen that triggers atherosclerotic plaque inflammation, and other antigenic stimuli are also likely involved. In vitro, responses of plaque-derived T cells to lipids, heat shock proteins, and other infectious pathogens have been reported. However, given the widespread occurrence of influenza within the total population, the contribution of influenza A–induced plaque inflammation to the onset of acute cerebral and vascular disease may be relevant in a substantial proportion of atherosclerotic patients (in the group of patients enrolled in our study, potentially 50%).

In conclusion, the findings of this study suggest that influenza A virus may act as an important contributor to inflammation in plaques. Future research should focus on the influenza virus (mimicking) antigens in atherosclerotic plaques responsible for T-cell activation.

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Disclosures
None.

References


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