Bioelectric Features (Inflammation and Neoangiogenesis) and Atherosclerotic Risk Factors in Carotid Plaques and Calcified Aortic Valve Stenosis

Two Different Sites of the Same Disease?

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Abstract

Neoangiogenesis and inflammation have a pivotal role in atherosclerosis. Observations support the hypothesis that calcified aortic valve stenosis is an inflammatory process, similar to atherosclerosis in tissue features and risk factors. We studied 2 groups of cases: 47 were affected by hemodynamic atherosclerotic carotid plaque (group 1) and 35 by severe calcified aortic valve stenosis (group 2). We compared the groups for atherosclerosis risk factors, morphologic features, and immunohistochemical phenotypes.

In both groups, men, smokers, and hypertensive subjects prevailed, and histologic analysis showed an elevated score for T-lymphocyte infiltrates, neoangiogenesis, calcium, and sclerosis. Adhesion molecule expression was present in both lesions. Expression of intercellular adhesion molecule 1 correlated with inflammatory infiltrates (group 1, P = .0007; group 2, P = .06). Neoangiogenesis also correlated with inflammatory infiltrates (group 1, P = .035; group 2, P = .045). In valves, neoangiogenesis correlated with calcium (P = .048). Carotid plaque and calcified valve stenosis showed common risk factors and biologic hallmarks of a chronic inflammatory process. Inflammation and neoangiogenesis have a crucial role in plaque evolution and in the progression of aortic valve stenosis.

Atherosclerosis is considered a complex cardiovascular inflammatory disease.1-2 Recent advances in basic science have established a fundamental role for inflammation in mediating all stages of atherosclerosis, from initiation through progression and in latter complications such as thrombosis or calcification. Clinical studies have confirmed that these inflammatory activities apply directly to humans. Through a series of molecular pathways, oxidative stress, toxic metabolites produced by smoking and increased dietary fat intake, mechanical stress injury, and infection are all involved in promoting an inflammatory response in sites of endothelial lesion formation, mainly in medium and large arteries.3

“Degenerative” aortic valve stenosis (AS) is a valve disease that increases in prevalence with advancing age in comparison with rheumatic diseases, which prevail in younger populations. AS is the most common valve disease and reason for aortic valve replacement in Europe and North America.4 Many clinical studies on the risk factors leading to AS have identified similar factors involved in atherosclerosis and progression of valve lesions.4-9 The histologic analysis of valve lesions in different stages of the disease showed similarities to atherosclerotic plaque evolution, such as the presence of lipoproteins, foam cells, macrophages, T lymphocytes, extracellular matrix proteins, heterotopic calcification, and bone tissue.10-13 These tissue factors may be the result of “active” biologic processes of chronic inflammation and tissue repair, sustained by neoangiogenesis similar to the processes involved in atherosclerosis.14-18

We compared 2 groups of patients: the first was affected by hemodynamic carotid plaque (CP; group 1), and the second was affected by severe calcified AS and was, on average, a few years older (group 2). To verify correlation for risk factors and
biologic tissue features, a relevant question may be whether atherosclerotic processes are specific to arterial vessels or whether endothelium damage may be more diffuse in the cardiovascular system and evolve as a progressive inflammatory disease with different clinical manifestations.

We aimed to verify whether the different sites of the arterial tree can show clinical and biologic characteristics that could indicate the same disease processes in different sites. A possible confirmation of this hypothesis could lead to a common, effective strategy to modify the course of the disease and prevent or slow progression of the chronic inflammatory process.

Materials and Methods

Clinical Findings and Atherosclerosis Risk Factors

We examined 2 clinically different groups with cardiovascular diseases. Group 1 patients (n = 47; mean ± SD age, 68.6 ± 9.1 years) were asymptomatic for hemodynamic atherosclerotic CPs according to the criteria of the North American Symptomatic Carotid Endarterectomy Trial and underwent endarterectomy. Group 2 patients (n = 35; mean ± SD age, 73.3 ± 5.7 years) underwent surgical aortic valve replacement for severe and symptomatic calcified non-rheumatic AS. The exclusion criteria were any evidence of postrheumatic endocarditis and significant aortic regurgitation or other coexistent valvular disease, with the exception of calcification of the mitral ring.

The cardiovascular risk factors considered in the study were age, sex, family history of cardiovascular disease, diabetes mellitus (defined as hyperglycemia requiring pharmacologic therapy), hypercholesterolemia (defined as a total cholesterol level >200 mg/dL [5.17 mmol/L]), hypertriglyceridemia (defined as a triglyceride level >160 mg/dL [1.81 mmol/L]), hypertension (defined as systolic or diastolic increase of blood pressure >140/90 mm Hg), and smoking (current smokers and those who had quit within the previous 12 months).

Some clinical data (high-density lipoprotein cholesterol and human serum C-reactive protein) were lacking because the 2 sets of cases were recruited in different clinical structures.

Instrument Diagnostic Evaluation

Both groups were evaluated by cardiac and vascular color Doppler ultrasound echocardiography to document “multidirectional” atherosclerosis and by coronary angiography to detect associated coronary artery disease.

The degree of CP stenosis was evaluated by means of high-resolution vascular color Doppler ultrasound echocardiography and confirmed by carotid angiography (North American Symptomatic Carotid Endarterectomy Trial and European Carotid Surgery Trialists criteria). Endarterectomy was carried out when CP stenosis was 70% or more. AS was defined as thickened leaflets with reduced systolic opening in 2-dimensional imaging and an increased anterograde velocity (>2.5 m/s) measured by a continuity equation.

Aortic valve “sclerosis” was defined as a focal area of increased echogenicity and thickening of the aortic valve leaflets without restriction of leaflet motion and a transaortic flow velocity of less than 2.5 m/s, measured by transthoracic echocardiography using the criteria of Stewart et al and Otto et al.

Diagnosis was confirmed by inspection of the plaque and valve during surgery. The research protocol was approved by the University of Siena Human Ethics Review Committee.

Histologic Analysis of CP and Aortic Valve Surgical Samples

CP and AS surgical samples were fixed in 10% neutral buffered formalin for 24 hours and decalcified overnight with formic acid. The whole carotid endarterectomy sample was removed and sectioned transversely to the longitudinal axis of the vessel; the valve samples used for histologic analysis were taken vertically through the valve cusp near the center of the leaflet. The samples sectioned were processed for routine paraffin embedding; 5-µm-thick histologic sections were stained with H&E and Weigert–van Gieson.

The histologic sections from groups 1 and 2 were analyzed semiquantitatively according to a scoring system for inflammatory cells, sclerosis, calcium, and neoangiogenesis. (The latter is characterized by the presence of small vessels of irregular shape; the vessel wall is constituted by endothelium and a thin connective or muscular layer, with scant smooth muscle cells.) We also evaluated the morphologic characteristics of neovessels (thin-walled vessel thickness, <10 µm, vs thick-walled vessel thickness, >10 µm). In group 1, we also studied sections for the presence of atheroma using semiquantitative analysis (0, absence; 1, small atheroma; 2, large atheroma; 3, extensive atheroma) and thrombus or intraplaque hemorrhage (present or absent). All histomorphometric parameters analyzed were considered of null or minimum value (0 and 1 scores) or medium or high value (2 and 3 scores).

Immunohistochemical Analysis

Immunohistochemical analysis was carried out in both groups to detect B (CD20, dilution 1:200; DAKO, Glostrup, Denmark) and T lymphocytes (UCHL1, dilution 1:200; DAKO), macrophages (CD68, dilution 1:1,000; DAKO), endothelial cells (CD34, dilution 1:100; DAKO), vascular cell adhesion molecule 1 (VCAM-1; CD106, dilution 1:75; Novocastra Laboratories, Newcastle upon Tyne, England), and...
intercellular adhesion molecule 1 (ICAM-1; CD54, dilution 1:25; Novocastra). For detection, ABC Elite (Vector Laboratories, Burlingame, CA) was used with a standard peroxidase enzyme substrate (3,3'-diaminobenzidine), that yields a black reaction product. VCAM-1 and ICAM-1 expression was graded on a semiquantitative scale, with 0-1 indicating minimal or no expression and 2-3 indicating medium or high expression.

Statistical Analysis
For comparisons within and between groups, the non-parametric Kruskal-Wallis test was used for quantitative parameters, and the \( \chi^2 \) test in contingency tables was used for qualitative parameters. Comparisons within each group also were performed by using the \( t \) test for continuous variables. Statistical significance was set at a \( P \) value of less than .05. Data were analyzed with the aid of the Statview statistical package (Abacus Concepts, Berkeley, CA).

Results

Clinical Findings and Atherosclerosis Risk Factors
Patients in group 1 were younger than those in group 2 (\( P = .009 \)), and group 1 included more men than group 2. Patients in both groups were prevalently smokers or hypertensive, and the percentage in each group with hypercholesterolemia was similar (group 1, 55%; group 2, 54%). Coronary artery disease and peripheral artery disease were present in both groups at variable percentages. Both groups showed carotid atherosclerosis, but the plaque stenosis was not hemodynamic in group 2. In group 1, 49% of patients showed echocardiographic parameters of “aortic valve sclerosis,” and 4% showed moderate AS.

In group 1, 22 asymptomatic patients (47%) had no history of cerebral ischemic symptoms; the other 25 patients (53%) reported remote symptoms (>120 days before surgery) of major or minor clinical cerebrovascular ischemia. In group 2, surgical patients were symptomatic for angina (3%), dyspnea (89%), and syncope (29%).

Tissue Features

Macroscopic Findings
Almost all CP lesions showed eccentric morphologic features (32/47), with sclerosis (40/47), calcium deposits (38/47), and large atheromas (35/47). Of the 47 samples, 25 showed intraplaque hemorrhagic areas, and 15 showed minimal surface thrombosis.
All aortic valves examined were tricuspid. Macroscopically, surgical samples appeared thickened and irregular with large calcium nodules extending from the base toward the middle portion of the valves and had preserved commissures.

**Histomorphometric Analysis**

Data are given in Table 3. In the 47 group 1 cases, 44 (94%) showed prevalence of atheroma, 42 (89%) of calcium, and 47 (100%) of fibrosis. Hemorrhage was found in 26 (55%), thrombosis in 16 (34%), and neoangiogenesis in 45 (96%).

Inflammatory cells were present in 100% of group 1 cases in variable amounts, with a high prevalence of T lymphocytes and monocytes/macrophages and scant granulocytes. The inflammatory infiltrates were most dense in the areas where neovessels were more numerous (P < .025). The thin-walled neovessels (<10 µm thick) were surrounded by widespread areas of inflammatory cells, whereas the thick-walled neovessels (>10 µm thick) were surrounded predominantly by areas of fibrosis. Group 1 samples showed greatly increased inflammatory infiltration around thin neovessels (P = .03).

Group 2 samples showed large amounts of calcification in nodular form, and dense fibrosis was observed in all samples. No atheroma was found. Inflammatory infiltrates were present in aggregated areas and/or diffused into the leaflets with various cell types, especially lymphocytes and monocytes (32/35 [91%]). Neoangiogenesis, usually described in rheumatic valves, was evident in 20 (57%) of the 35 aortic valve leaflets examined. There was a link between the presence of neoangiogenesis and the largest amounts of inflammatory cells (P = .045). This correlation was increased between thin vessels and an abundance of lymphocyte infiltrates (P = .001).

In group 2, a correlation was shown between the amount of calcium and the number of neovessels (P = .048).

The comparison between the morphologic parameters determined for the 2 groups showed that high scores prevailed in both for calcium and sclerosis. However, these amounts were significantly different in the groups, showing a prevalence of inflammation (P = .002) and neoangiogenesis (P = .04) in group 1 and sclerosis (P = .002) and calcification (P = .04) in group 2 lesions.

In patients who were smokers, CP lesions showed an increased amount of calcium deposition (P = .01), and the same also tended to occur in AS (P = .09).

**Immunohistochemical Markers**

In group 1 samples, most endothelial cells of the neovessels were positive for ICAM-1 and VCAM-1 antibodies with variable expression. In group 2 samples, there was lower expression of adhesion molecules. In group 1, there was a positive correlation between inflammatory cells and ICAM-1 expression (P = .047). In group 2 samples, we found a positive correlation between inflammatory infiltrates and ICAM-1 expression (P = .06), between neoangiogenesis and ICAM-1 expression (P = .04), and, in particular, between thin neovessels and ICAM-1 expression (P = .02).

**Discussion**

Our study compares hemodynamic carotid atherosclerosis with symptomatic calcified AS, taking into account the clinical and biologic features of these 2 cardiovascular diseases. The information we gained suggests shared atherosclerotic risk factors and some pathobiologic hallmarks.
Image A. In a carotid lesion, nodular deposits of calcium (violet area), inflammatory infiltrates, and neovessels are observed (H&E, ×100). C. In a valve lesion, widespread areas of calcification surrounded by inflammatory infiltrates are observed (H&E, ×200). In carotid (B, H&E, ×100) and valve (D, H&E, ×100) lesions, nodular inflammatory infiltrates are associated with thin-walled neovessels. In a carotid lesion, immunohistochemical positivity (stained brown) for intercellular adhesion molecule 1 (with 3,3′-diaminobenzidine [DAB] substrate) (E, ×400) and vascular cell adhesion molecule 1 (with DAB substrate) (F, ×400) is observed in the endothelial cells of neovessels.
Major Clinical Findings

In our set of cases, we found that AS increased in prevalence with age in comparison with CP disease, in agreement with the findings of Otto et al\(^\text{23}\) (Table 1). Nevertheless, half of the group 1 patients showed echocardiographic signs of aortic valve sclerosis, which is considered a marker of atherosclerosis and of the early stage of calcified aortic valve disease.\(^\text{23,24}\) Another study confirms that about one third of patients with aortic valve sclerosis developed some degree of aortic AS in a follow-up of 4 years.\(^\text{25}\) The CP cases in our study had coronary artery disease (32%) and peripheral artery disease (26%). In the AS cases, we found a high incidence (89%) of nonhemodynamic CP and a 43% incidence of coronary artery disease associated with peripheral artery disease (14%). These data indicate that more diffuse and advanced atherosclerotic disease is associated with age and are consistent with a similar incidence of coronary artery disease.\(^\text{26}\)

Groups 1 and 2 did not differ in common cardiovascular risk factors such as male sex, family history, hypertension, hypercholesterolemia, smoking, and diabetes, in agreement with the findings of many previous studies.\(^\text{4-9}\) Our data showed that patients who smoke had a large amount of calcium deposition in aortic valves and had a tendency to increased calcification in carotid plaque tissue samples. Ngo et al\(^\text{27}\) demonstrated that smoking and obesity are independent predictors of significant progression of AS and that smoking is associated with atherosclerotic complications, being a major risk factor for acute coronary thrombosis.
Although the results suggest that the different expressions of the disease may be linked to a common physiopathologic model, to the extent that we hypothesize that we are dealing with the same disease in 2 different sites, the state of our knowledge is not sufficient to answer the question of how different factors (genetic predisposition? interaction of risk factors?) can affect the different localization within the cardiovascular tree.

**Specific Tissue Features of Plaques and Valves**

In our study, hemodynamic CPs in previously symptomatic or asymptomatic surgical patients appeared as “stable” lesions with minimal surface thrombosis and showed balanced tissue areas of sclerosis, atheroma, calcification, hemorrhage associated with neovascularization, inflammation, and endothelial activation. Several studies have observed that neoangiogenesis possibly has a role in the development of hemorrhage and thrombosis and rupture of coronary and carotid plaques. Calcification is the prevalent tissue feature in AS, associated with inflammatory infiltrates, neoangiogenesis, and activated endothelium. In 3 samples (Image 2B) of valve tissue, we found endochondral and bone tissue with bone marrow and hematopoietic elements, in agreement with previous studies that correlate calcification and bone tissue with neoangiogenesis. Chronic inflammatory and angiogenic activities in situ are sustained by progressive abnormal flow, resulting in abnormal mechanical stress and tissue damage.

**Common Tissue Features of Plaques and Valves: Role of Angiogenesis and Inflammation**

In our study, hemodynamic CP and severe AS appeared as 2 advanced lesions with an important biologic activity caused by a chronic inflammatory process. In the advanced phases of disease, calcium, inflammation, and neoangiogenesis characterize both tissue lesions, whereas in the earliest phases (fatty streaks and aortic sclerosis), macrophages/macrophages, mast cells, and T lymphocytes accumulate. It has been demonstrated that these small vessels originate from the adventitial vasa vasorum and the main vessel lumen and that endothelial activation may facilitate the entrance of leukocytes into lesions and cause intraplaque hemorrhage. Unlike arterial walls, normal semilunar valve cusps are constituted by avascular tissues, sufficiently thin to allow complete nutrition by diffusion. In valve tissue, chronic inflammation and neoangiogenesis probably do not evolve into thrombosis or hemorrhage also because of the absence of vasa vasorum and the prevalence of fibrotic valve tissue.

Monocyte/macrophages release angiogenic factors under hypoxic conditions; interleukin-8, an endothelial-derived chemokine, is a potent proangiogenetic signal in coronary plaque formation. The neovessel-activated endothelial cells express high levels of adhesion molecules (VCAM-1, ICAM-1, and E-selectin) and are associated with increased inflammatory infiltrates, establishing a self-perpetuating inflammatory process. The relationship between inflammation and angiogenesis in plaque destabilization and intraplaque hemorrhages has been outlined by various authors. In calcified AS, the correlation we found between neovessels and calcium is in agreement with the pathophysiologic hypothesis that neoangiogenesis may condition the active processes of calcification and ossification and the speed of progression of stenosis.

In plaque and valvular tissue, it has been demonstrated that inflammation is associated with osteopontin, osteonecin, osteocalcin, calcium deposits, apolipoprotein B, fibrin, and matrix metalloproteinase 3. This suggests a complex interaction between noncollagenous bone protein, calcium, matrix growth, and degradation that may lead to acute plaque complications such as destructive hemorrhage and thrombosis and may sustain the progression toward calcification or ossification in plaque and valve lesions. In arterial walls and in valve leaflets, mineral metabolism normally is balanced. In the proinflammatory plaque and valve microenvironments, on the other hand, the immunomodulating cytokines released is the prominent vascular response and is involved in sustaining inflammation. The same also may happen in atherosclerosis and in AS, in which the initial endothelium injury by oxidative low-density lipoprotein is followed by inflammatory and repair processes associated with the progression of the lesions.
facilitate the recruitment and development of osteoblast-like cells that are responsible for mineral net deposition.39,40

The correlation between angiogenesis and inflammation may be justified in the presence of a strongly activated endothelium of the neovessels, but quantitatively different orders of chronic inflammation and different phases of the same process coexist inside the plaque and valve, suggesting a “dynamic” process of intratissue remodeling.34 The regulatory stimuli for the inflammatory and angiogenetic responses may be crucial for the equilibrium between plaque “stability” and “instability” and calcium progression.

Conclusions

Through our comparison of hemodynamic CP stenosis and severe calcified AS, we have demonstrated that these 2 different clinical cardiovascular diseases have common atherosclerotic risk factors and have confirmed that more advanced age is associated with more clinically diffuse and advanced atherosclerotic disease. Moreover, CP and AS proved not to be “biologically quiescent” lesions but 2 different sites of similar active processes.

Further knowledge about angiogenetic and inflammatory mechanisms underlying plaque and valve remodeling can provide clues to aid understanding of the molecular targets. We think that a possible limitation to this type of study lies in the objective difficulty of obtaining ex vivo tissues of early to mid-stage human atherosclerotic plaque and valve disease to better understand their common pathogenesis and evolution.

References