Original Article
Protein expression patterns and histologic characteristics of coronary atherosclerosis from sudden cardiac death

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Abstract: To identify the distinct histologic and immunohistochemical characteristics of coronary atherosclerosis in sudden cardiac death patients, we analyzed these characteristics in 2 study groups: a sudden cardiac death group (group A) and a noncardiac death group (group B). The plaques in group A that caused sudden cardiac death showed vulnerable features, including necrosis, thin fibrous cap over lipid core, disruption, hemorrhage, thrombus, cholesterol cleft formation, inflammatory cell infiltration, and vasa vasorum formation, more frequently and severely than in group B, and this difference had statistical significance. Immunoreactivity for CD68, ubiquitin, endothelin-1 was significantly higher in group A than in group B. In addition, immunoreactivity for P2Y$_{12}$, CRP, and ubiquitin was higher in group A than in group B. Therefore, CD68, ubiquitin, and endothelin-1 may play a role in plaque vulnerability as a mediator of inflammation or vasoconstriction leading to sudden cardiac death.

Keywords: Coronary atherosclerosis, sudden cardiac death, immunohistochemistry, antigens, CD68, ubiquitin, endothelin-1

Introduction

Coronary atherosclerosis is a common and significant disease finding in autopsy practice as well as in clinical medicine. More than 42% of all autopsy cases examined by the National Forensic Service (NFS) in Korea in 2011 were deemed natural deaths, and more than half of these natural deaths (51.8%) were caused by heart disease, 76.8% of which was related to coronary atherosclerosis [1]. However, we were unable to find data on specific anatomic changes in the heart of subjects who died of cardiac causes, except for some acute myocardial infarction cases presenting with coronary thrombus, distinct myocardial infarction, or complications of myocardial infarction. And coronary atherosclerosis with moderate or severe stenosis is noted in the heart of noncardiac death cases, especially in advanced age. So, we wanted to determine the histologic and immunohistochemical characteristics of atherosclerosis in sudden cardiac death group and compare these characteristics with those of the noncardiac death group.

A typical atherosclerotic plaque comprises a lipid core surrounded by fibrous tissue. The lipid core can contain foam cells, cholesterol cleft, granulation tissue, smooth muscle cells, myofibroblasts, inflammatory cells, and necrotic material. Secondary degeneration, including plaque disruption, hemorrhage, thrombosis, and medial spasm, can occur within the plaque. These secondary changes play a fundamental role in the pathogenesis of unstable symptoms of coronary heart disease [2].

Virmani et al. [3] examined the pathology of vulnerable plaque that induces unstable angina, acute myocardial infarction, and sudden coronary death. They found that the significant lesion that preceded the plaque's rupture was a thin fibrous cap over a lipid core. The plaques usually ruptured when lesions showed mild (<

50%) stenosis and were observed mostly in the proximal portion of coronary arteries. Plaques susceptible to disruption were generally characterized as having a thin, inflamed fibrous cap over a very large lipid core; > 50% of plaques featured a > 75% cross-sectional area stenosis [3, 4].

The inflammation and vasoconstriction that characterize plaques are important mechanisms of atherogenesis and change, such as plaque disruption [5-7]. As a result, there have been many studies of plaque biology that focused on molecular biomarkers that are possibly related to atherogenesis. Such studies have found that CD68, ubiquitin, CRP, $P2Y_{12}$, and endothelin-1 immunoreactivity are enhanced in atherectomy specimens of patients with unstable angina [8-12].

Herein we reviewed and analyzed the histologic features and molecular biomarkers of immunoreactivity that are possibly related to plaque inflammation and vasoconstriction. We studied 2 groups of subjects: a sudden cardiac death group and a noncardiac death group, using coronary atherosclerotic samples obtained from autopsies.

Materials and methods

Case selection

This study included 2 groups: group A, comprising 68 autopsy cases of acute myocardial infarction or ischemic heart disease, and group B, comprising 39 cases of noncardiac death. All autopsies were conducted at the NFS from January 2011 to December 2013. Acute myocardial infarction was diagnosed when coronary artery thrombus, severe coronary artery atherosclerosis (> 75% of coronary artery cross-section occluded by atherosclerotic plaque), and microscopic findings of acute or recent myocardial infarction were noted. Ischemic heart disease was diagnosed as either moderate (50%-75% of coronary artery cross-section occluded by atherosclerotic plaque) or severe coronary artery atherosclerosis with secondary myocardial changes (e.g., microscopic findings of old infarction, left ventricular hypertrophy, or evidence of chronic heart failure). Subjects that had received a heart stent or undergone angioplasty were excluded from both groups. Subjects included in the noncardiac death

group B had more than moderate coronary artery atherosclerosis and an obvious noncardiac cause of death, such as trauma, asphyxia, intoxication, or cerebral hemorrhage.

Clinical characteristics of selected cases

The sex, height, weight, body mass index (BMI), and heart weight in groups A and B were analyzed. Smoking status and past history of diabetes mellitus, hypertension, and hypercholesterolemia were investigated using questionnaires given to the families of the deceased subjects.

Gross and microscopic examinations

A routine heart examination during autopsy involves weighing the heart and examining the main coronary arteries (left anterior descending artery, left circumflex artery, and right coronary artery), valves, and myocardium. In this study, 3 main coronary arteries were cut serially at 5 mm intervals, and the cut surface was examined. The culprit lesions were selected for making microscopic sections. Based on the microscopic examination findings, coronary atherosclerosis was classified using the American Heart Association (AHA) classification system, and the extent of stenosis due to atherosclerosis in cross-sections of the coronary artery was expressed as a percentage of the obstructed area [2, 13]. Atherosclerotic plaques were classified using the AHA classification system. The examination also verified the extent of stenosis due to plaque, necrosis, thin fibrous cap over the necrotic core (fibrous cap measuring < 65 mm) [3], hemorrhage, disruption (fissures and ulcerations of the lesion surface), thrombus, organizing thrombus, and cholesterol cleft (a space caused by cholesterol crystals dissolving out into sections of tissue embedded in paraffin). Calcification, inflammatory cell infiltration, and formation of vasa vasorum (networks of microvessels that supply the walls of large blood vessels) were classified as mild, moderate, or severe.

Immunohistochemical staining and analysis

The representative specimens were stained with antibodies against CD68 (Dako, Kyoto, Japan), CRP (Novus, Littleton, CO, USA), ubiquitin (Novus), $P2Y_{12}$ (Novus), and endothelin-1 (Novus). Samples were incubated with primary
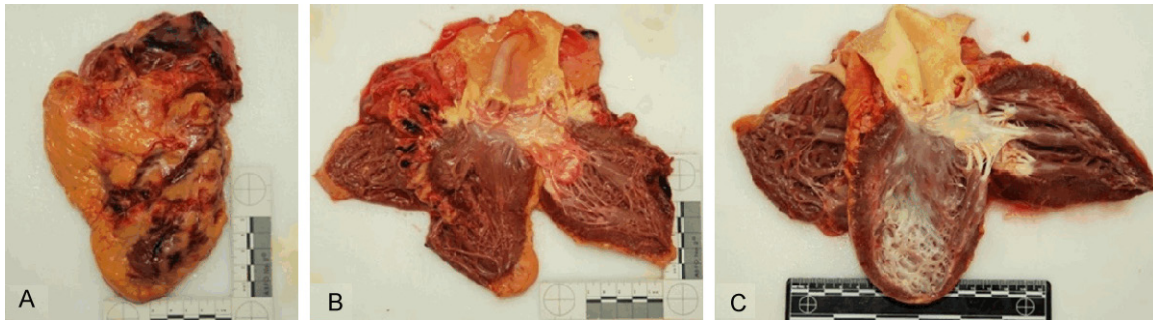
Figure 1. Gross appearance of heart. The heart of case 5 in group A shows cardiomegaly (460 g) and hemorrhage and necrosis of the ventricular wall. These findings were not seen in all cases of acute myocardial infarction (A and B). The heart of case 6 in group A displays cardiomegaly (546 g) and broad fibrosis and thinning of the interventricular septum (C).
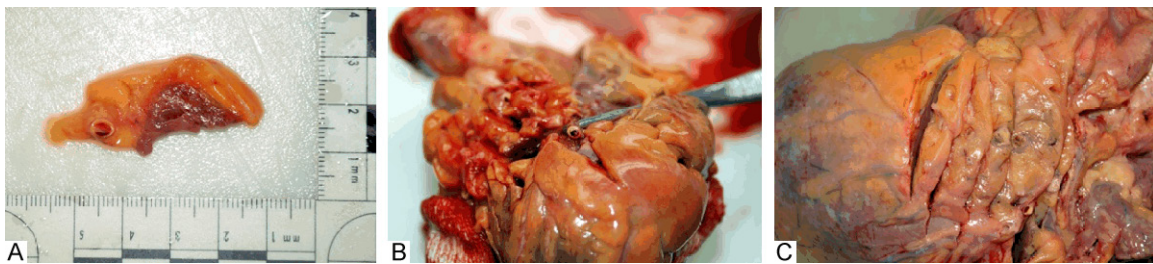

Figure 2. Gross appearance of coronary atherosclerotic plaque. Coronary atherosclerotic plaque with hemorrhage narrows approximately 40% of the cross-section of this coronary artery (A). The coronary artery is almost completely obstructed by atherosclerotic plaque and thrombus (B). Severe coronary atherosclerosis with old hemorrhage and necrosis are noted on gross examination of the coronary arteries (C).

antibodies for 1 hr at room temperature, washed 2 times for 10 min each with phosphate-buffered saline, incubated with EnVision Detection System (Dako) for 30 min at room temperature, and washed again with phosphate-buffered saline.

The results of immunohistochemical staining were graded by scoring them from 0 to 6. The score accounted for the extent of positively stained area and the intensity of staining. The extent of immunohistochemical staining of the lesion was analyzed on a scale of 0 to 3 (0 = positivity in < 5% of the lesion; 1 = positivity in 5%-20% of the lesion; 2 = positivity in 21%-50% of the lesion; 3 = positivity in > 50% of the lesion). The intensity of staining was graded on a scale of 0 to 3 (0 = no stain; 1 = weak; 2 = moderate; 3 = strong).

Statistical analysis

Continuous variables were expressed as mean ± SD, and categorical variables were expressed as frequency. Continuous variables were com-

pared using Student's t-test, and categorical variables were analyzed using the chi-square test. Probability values of $P < 0.05$ were considered significant.

Results

The average ages of subjects in groups A and B were 55.3 and 51.7 years, respectively. Age distribution and sex ratio were similar in both groups. Men composed > 80% of subjects in both groups. BMI and cardiac weight were significantly higher in subjects in group A than in group B.

A survey of families of the deceased subjects asked about the deceased person's history of smoking, diabetes mellitus, hypertension, hyperlipidemia, and family history of sudden cardiac death. This survey was unable to provide the necessary statistical power because many respondents replied that they did not know the answers to questions, and many survey recipients did not respond at all.
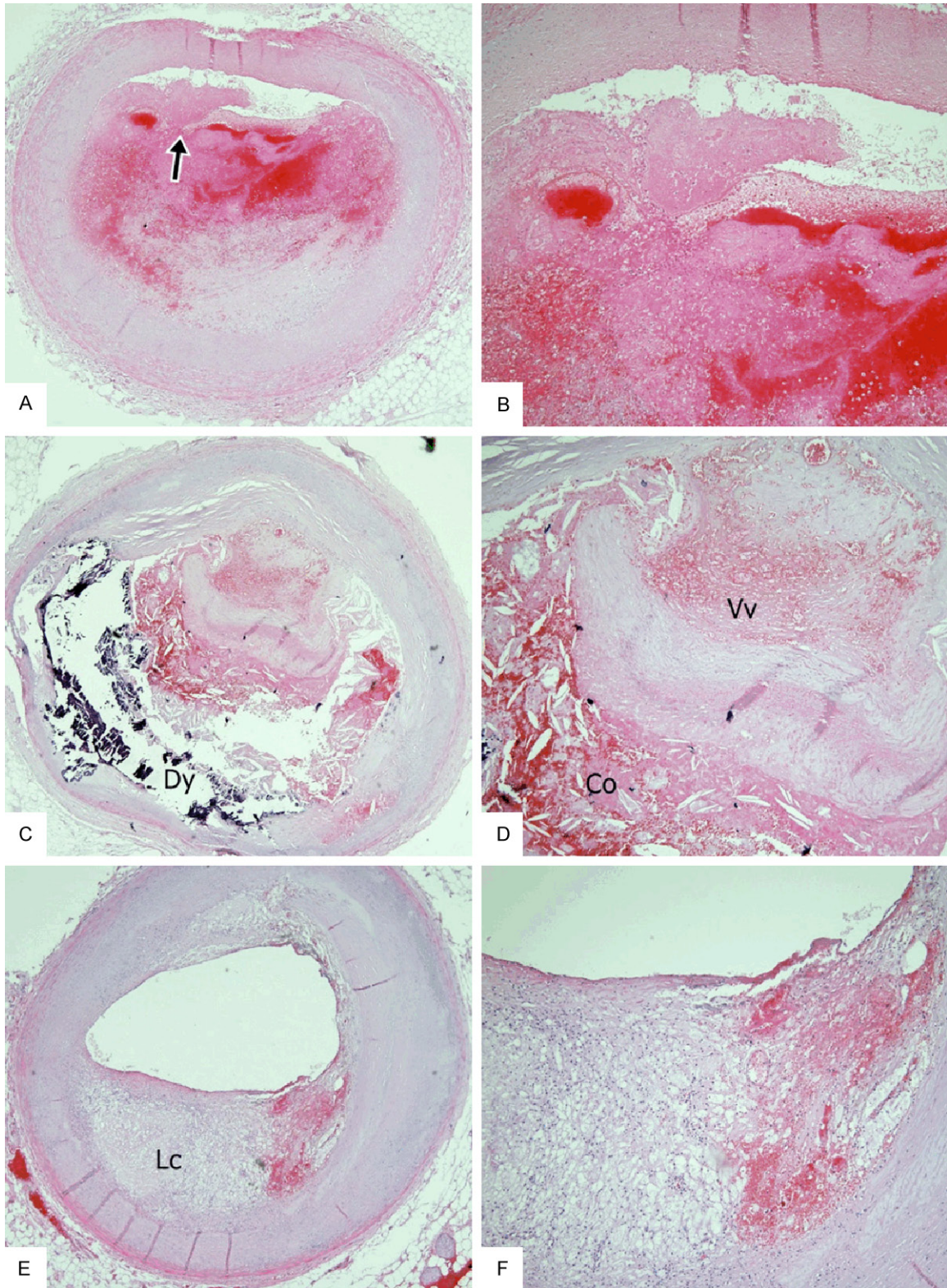
Figure 3. Light microscopic findings of coronary atherosclerotic plaque. Coronary artery of case 55 from group A shows extensive intra-plaque hemorrhage and disruption (arrow) (A and B). Coronary artery of case 8 in group A displays hemorrhage, cholesterol cleft (Co), dystrophic calcification (Dy), and vasa vasorum (Vv) (C and D). An atherosclerotic plaque with a lipid core (Lc) and overlying thin cap (40 μm in the thinnest part) is seen in the plaque of case 59 in group A (E and F) (all hematoxylin-eosin stains; A-E, ×40, B-F, ×100).
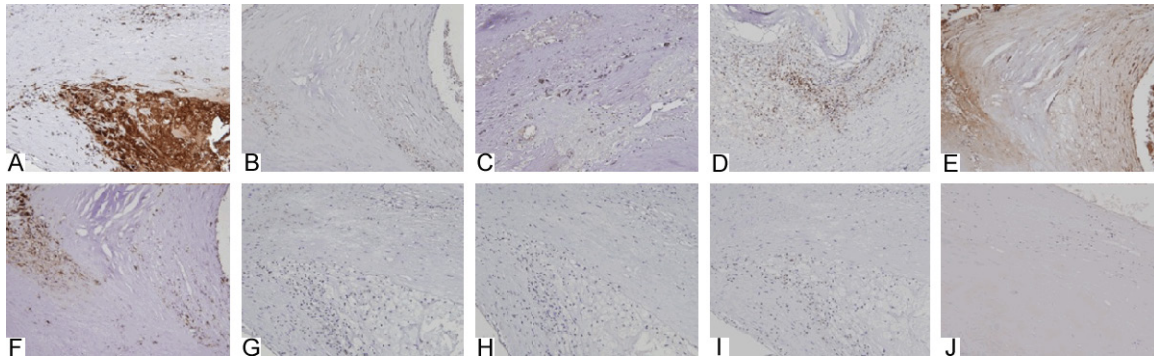
Figure 4. Representative images of CD 68, ubiquitin, tissue endothelin-1, P2Y$_{12}$, and CRP immunohistochemical staining in coronary atherosclerosis from group A (A-E) and group B (F-J) (×200). Immunohistochemistry for CD68 showed positive staining in groups A (A) and B (F); CD68 immunoreactivity was stronger in group A than in group B. In contrast, immunohistochemistry for ubiquitin, tissue endothelin-1, P2Y$_{12}$, and CRP showed positive staining in group A (B-E) but weak positive or negative staining in group B (G-J).

The hearts of subjects in group A showed more prominent cardiomegaly than those in group B. Hemorrhage and necrosis of the ventricular wall were noted rarely, and ventricular fibrosis was seen in some cases (**Figure 1**). On gross examination, coronary atherosclerotic plaques were noted to have fresh or old hemorrhage, thrombus, and necrosis (**Figure 2**). The median cross-sectional areas of stenosis were larger on average in group A (79%). Plaque disruption, superimposing or organizing thrombus, and moderate or severe vasa vasorum formation were observed only in group A. Necrosis, a thin fibrous cap over a lipid core, and hemorrhage were more common in group A than in group B, having statistical significance. Cholesterol cleft formation and inflammatory cell infiltration were more severe in group A than in group B, also reaching statistical significance (**Figure 3**).

The immunohistochemical staining grade (extent and intensity of the stained area of plaque) of CD68, P2Y$_{12}$, CRP, ubiquitin, and endothelin-1 tended to be higher in group A than in group B, but only CD68, ubiquitin, and endothelin-1 showed intergroup differences that reached statistical significance (**Figure 5**).

CD68 stained mainly on plaque macrophages, and cellular areas were stained most strongly. CD68-positive macrophages were noted on the lipid or necrotic core and surrounding areas, the fibrous cap, and unevenly all over the plaque. Ubiquitin, P2Y$_{12}$, and CRP were present in smooth muscle cells, myofibroblasts, inflammatory cells, and the necrotic core of plaques.

In some samples, the plaque shoulder showed increased cellularity and stronger immunoreactivity for ubiquitin, P2Y$_{12}$, and CRP, though not in all cases. Endothelin-1 was present in smooth muscle cells and myofibroblasts in plaque and normal endothelial cells (**Figure 4**). Clinical and immunohistochemical characteristics of study subjects are displayed in **Table 1**.

In group A, immunohistochemical staining grade for CD68 and CRP tended to increase in the advanced atheroma group (identified using the AHA classification system). In group A, CRP immunoreactivity was significantly higher in subjects aged > 50 years than in those aged < 50 years (mean 3.18 ± 1.50 vs. 2.21 ± 1.68, *P* = 0.016, **Figure 6**). Other molecular biomarkers did not show significant differences according to subject age.

Discussion

In both groups, heart weight was greater than in the normal Korean population, based on available data [14]. In addition, cardiac weight and BMI were significantly higher in group A than in group B. Cardiomegaly was an important abnormal feature of heart anatomy in the sudden cardiac death group.

Atherosclerosis of the coronary arteries is a chronic immune system mediated and inflammatory disease that originates with impaired intima. The atheroma's core consists of foamy cells, which are differentiated from migrated monocytes with atherogenic lipoproteins, grad-
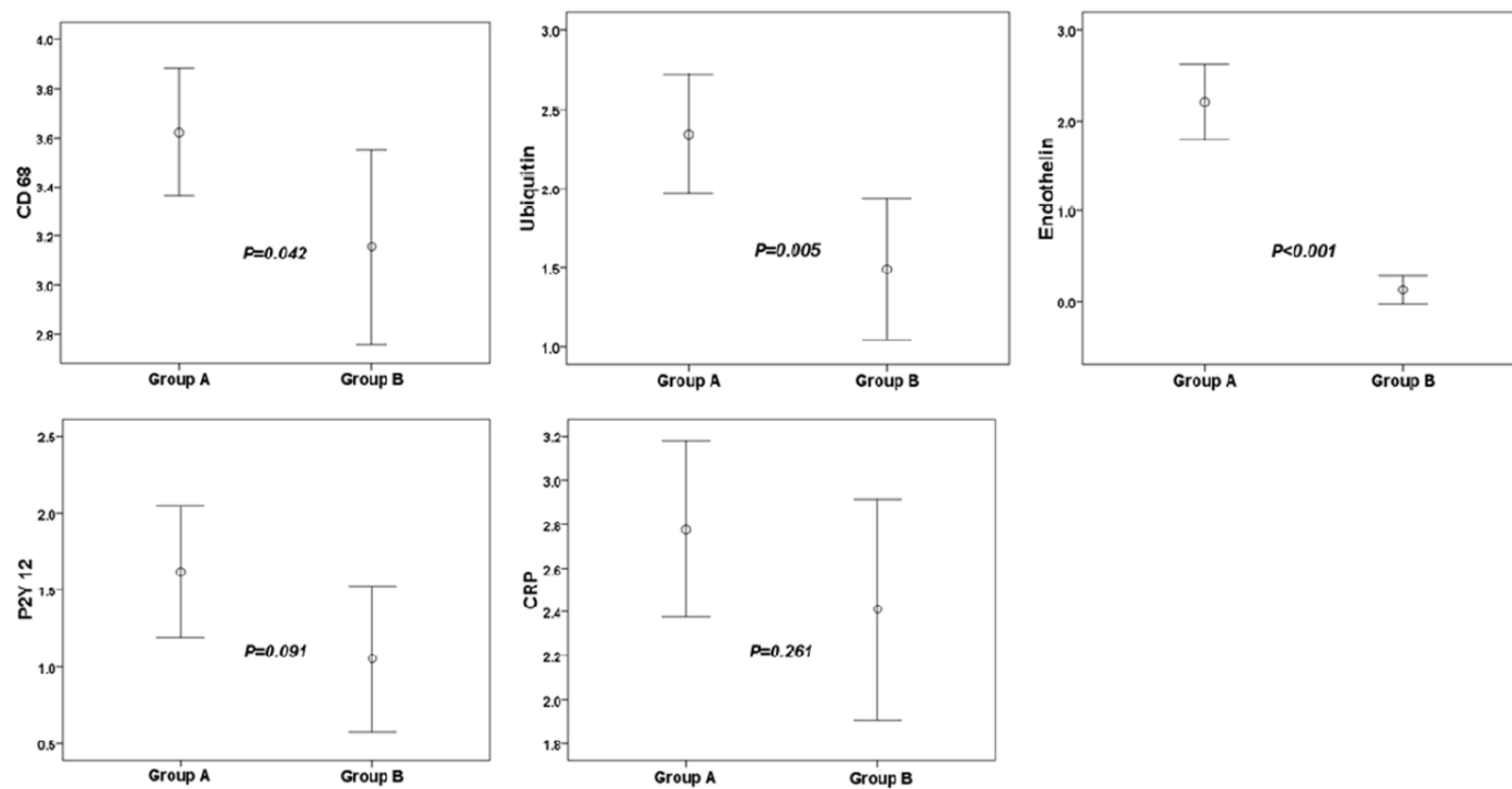
Figure 5. The immunohistochemical staining grade of CD68, P2Y$_{12}$, CRP, ubiquitin, and endothelin-1 tended to be higher in group A than in group B; the CD68, ubiquitin, and endothelin-1 immunohistochemical stain showed statistically significant differences.

Table 1. Clinical and immunohistochemical characteristics of study subjects

Characteristic	Group A (n = 68)	Group B (n = 39)	P value
Age (years)	55.3 (± 14.7)	51.7 (± 12.7)	0.203
Sex			0.398
Male	60	32	
Female	8	7	
Height (cm)	169.1 (± 10.6)	164.8 (± 7.7)	0.009
Male	170.9 (± 7.9)	166.4 (± 7.2)	
Female	158.3 (± 5.5)	157.6 (± 5.7)	
Weight (kg)	84.8 (± 13.5)	57.7 (± 10.7)	0.052
Male	89.1 (± 15.6)	58.2 (± 11.6)	
Female	58.0 (± 9.4)	55.4 (± 4.6)	
BMI* (kg/m^2)	24.9 (± 4.0)	21.4 (± 3.4)	< 0.001
Male	25.1 (± 4.0)	21.1 (± 3.8)	
Female	23.1 (± 3.0)	22.3 (± 0.9)	
Cardiac weight (g)	459 (± 95)	396 (± 88)	0.001
Male	465 (± 93)	389 (± 86)	
Female	415 (± 112)	433 (± 97)	
AHA† classification			0.002
II	2	0	
III	2	2	
IV	4	2	
V	36	34	
VI	24	1	
Histology			
Cross-sectional area of stenosis (%)	79.0 (± 17.3)	58.6 (± 16.9)	< 0.001
Necrosis	25 (37%)	4 (10%)	0.003
Thin fibrous cap over lipid core	19 (28%)	2 (5%)	0.005
Disruption	15 (22%)	0	0.003
Hemorrhage	24 (35%)	1 (3%)	0.003
Thrombus	9 (13%)	0	0.025
Organizing thrombus	10 (15%)	0	0.013
Calcification			0.574
Mild	17 (25%)	12 (31%)	
Moderate	16 (24%)	8 (21%)	
Severe	4 (6%)	1 (3%)	
Cholesterol cleft formation			0.015
Mild	21 (31%)	14 (36%)	
Moderate	17 (25%)	8 (21%)	
Severe	12 (18%)	1 (3%)	
Inflammation			0.007
Mild	30 (44%)	21 (54%)	
Moderate	20 (29%)	4 (10%)	
Severe	5 (7%)	1 (3%)	
Vasa vasorum formation			< 0.001
Mild	30 (44%)	17 (44%)	
Moderate	16 (24%)	0	

ually formed central necrosis, fat, cholesterol crystals, and cell debris [8].

Not every atherosclerotic plaque results in sudden cardiac death. Therefore, we compared the histologic characteristics of atherosclerotic plaques in the sudden cardiac death group (A) with those of the noncardiac death group (B). The culprit plaque in group A showed more severe stenosis than in group B. Necrosis, a thin fibrous cap over a necrotic core, disruption, hemorrhage, thrombus, and organizing thrombus were more common in group A, with statistical significance. A thrombus or organizing thrombus is an important diagnostic feature of acute myocardial infarction. A thin fibrous cap and secondary degeneration (e.g., necrosis, disruption, and hemorrhage) are noticeable histologic findings in sudden cardiac death. Cholesterol cleft formation, infiltration of inflammatory cells, and vasa vasorum formation were more severe in group A than in group B. Cholesterol crystals produced in the necrotic core were able to activate inflammation [8]. The vasa vasorum correlated highly with the extent of inflammatory cells [15] and could be a source of disease progression, leading to endothelial impairment and inflammatory cell migration [16]. Our results suggest that, in coronary atherosclerosis, the vasa vasorum may increase the risk of plaque destabilization by

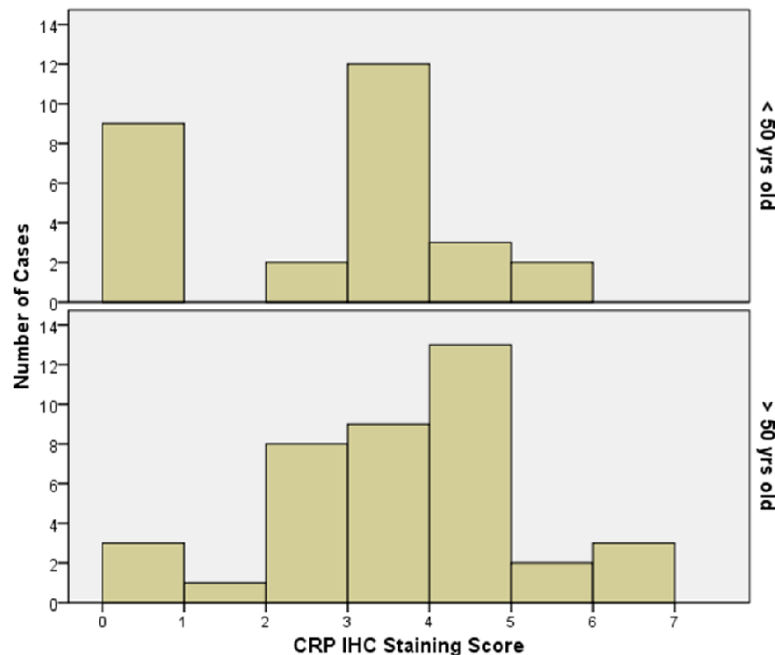|			
Severe	3 (4%)	0	
Immunohistochemical staining scoring			
CD68	3.62 (± 1.23)	3.15 (± 1.23)	0.042
Ubiquitin	2.34 (± 1.52)	1.49 (± 1.37)	0.005
Endothelin-1	2.21 (± 1.69)	0.13 (± 0.47)	< 0.001
CRP	2.78 (± 1.64)	2.41 (± 1.55)	0.261
$P2Y_{12}$	1.62 (± 1.75)	1.05 (± 1.47)	0.091

*BMI: Body mass index †AHA: American Heart Association

Figure 6. In group A, CRP immunoreactivity was significantly higher in subjects aged > 50 years than in subjects aged < 50 years.

stability. Depending on the extent of the inflammatory reaction, CD68 was positive in the middle of the atherosclerotic plaques. In our study, CD68 likewise stained predominantly in cellular, macrophage-rich areas of plaque. Active inflammatory processes involving macrophages are important mechanisms that increase plaque vulnerability, so CD68 may play an important role in increasing plaque vulnerability.

Herrmann et al. [9] showed that in patients with acute coronary syndromes, ubiquitin-mediated proteolytic pathway immunoreactivity was enhanced in unstable infarct-related lesions more than in non-infarct-related lesions. Ubiquitin stained positive predominantly in the plaque's lipid core, shoulder, and fibrous cap regions. These authors suggested that the ubiquitin system has a role in the destabilization and rupture of coronary atherosclerotic plaques in humans. In our study, ubiquitin immunoreactivity also increased significantly in group A more than in group B. Ubiquitin was positive in smooth muscle cells, myofibroblasts, inflammatory cells, the lipid core, and the cellular area of the fibrous cap.

contributing to inflammatory cell infiltration and plaque hemorrhage.

Using immunohistochemical expression of candidate antibodies, we detected functional changes in atherosclerotic plaques that could be related to plaque vulnerability. The CD68 antigen is a membrane glycoprotein strongly expressed by blood monocytes and tissue macrophages. Cojocaru et al. [8] studied the immunohistochemical expression of the anti-CD68 antibody at the level of atherosclerotic plaques. They concluded that the presence of many macrophages in atherosclerotic plaques, together with CD68 immunoreactivity, indicates a chronic inflammatory reaction accompanied by fibroblast proliferation and connective tissue changes that influence plaque

Preprocedural serum CRP levels are powerful predictors of outcomes in patients undergoing percutaneous coronary intervention [17, 18]. In coronary atheromatous plaques, immunoreactivity to CRP is increased in culprit lesions of unstable angina. CRP immunoreactivity is localized to macrophages and stellate smooth muscle cells, as well as to necrotic areas in directional coronary atherectomy samples. Ishikawa et al. concluded that CRP is an important factor in the process of plaque formation, secondary

changes, and vulnerability [10]. In the present study, CRP immunoreactivity tended to be higher in group A than in group B, and immunoreactivity was localized similarly to the findings of the referenced studies. However, we found no statistically significant differences in immunoreactivity between the 2 groups.

$P2Y_{12}$ receptors are present in coronary atherosclerotic plaques and are abundant in the culprit plaques of patients with acute myocardial infarction. In addition, $P2Y_{12}$ receptors may play a role in plaque destabilization [11]. In our study, $P2Y_{12}$ immunoreactivity tended to be higher in group A than in group B, but there was no statistically significant difference in immunoreactivity between both groups.

Coronary artery spasm could be another important mechanism underlying acute coronary syndromes. However, we are not able to visually check arterial spasms at autopsy. Endothelin-1 is a potent vasoconstrictor produced by endothelial cells, human vascular smooth muscle cells, and macrophages that is related to endothelial cell dysfunction. Zeiher et al. found that endothelin-1 immunoreactivity is significantly increased in the atherosclerotic lesions of patients with crescendo angina and angina at rest. The authors concluded that increased endothelin-1 immunoreactivity could be related to coronary artery spasm [12]. These findings may provide insight into the mechanisms of increased vascular reactivity of the culprit lesion in sudden cardiac death [7].

Our results resemble those of previous studies. The immunohistochemical stain grading of endothelin-1 was significantly higher in group A than in group B. We also found that CD68, ubiquitin, and endothelin-1 immunoreactivity significantly increased in the atherosclerotic plaques of the sudden cardiac death group, more than in the noncardiac death group. These findings suggest that CD68, ubiquitin, and endothelin-1 play a role in increasing plaque vulnerability, which can lead to sudden cardiac death.

Using autopsy materials and procedures, we were able to examine the histology and immunoreactivity of coronary artery plaques seen in cases of sudden cardiac death and noncardiac death. However, we did not have access to a detailed medical history of the deceased subjects. In addition, the expression of antibodies could not be confirmed by western blot analysis. Despite these limitations, we believe that the histologic and immunohistochemical characteristics of culprit plaques can be used to determine cause of death. These markers have the potential to improve diagnostic objectivity in daily autopsy practice and improve our understanding of the mechanisms underlying coronary artery atherosclerosis.

Acknowledgements

Disclosure of conflict of interest

None.

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