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Review Article

Expression of Inflammatory Markers CD68 and SMA in Endarterectomy Specimens

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Abstract

Background: Majority of deaths throughout the world are due to cardiovascular causes. Atherosclerosis is the most common pathology involved in the cause of death. It has been widely accepted that inflammation is a key mechanism in atherosclerosis. This study was conducted to evaluate the role of CD68 and SMA inflammatory markers in the pathogenesis of atherosclerosis.

Methods: This cross sectional study was carried out among 21 endarterectomy patients with severe atherosclerosis. Representative 5mm thick bits were sampled from the endarterectomy specimens and processed for light microscopy. Additional 5-micron thin sections were set aside for Immunohistochemistry staining using CD68 and SMA (Dako cytomation) by standard immunohistochemical staining using Biotinylated secondary link antibody.

Results: Among the study participants, majority belonged to 61-65 years of age. Coronary artery was involved in 10 participants, while in 11 participants carotid artery was involved. The lipid pool appeared to be larger in carotid than in coronaries. Cholesterol clefts were seen in both coronary and carotid endarterectomies. A 3+ positivity was only seen in a 36% of carotids.

Conclusion: Our study has helped in the morphological characterization of atheromatous plaques in endarterectomised specimens, which may have significant clinical relevance. Moreover, expression of immunohistochemical markers like CD68 and SMA have proved useful in highlighting the inflammatory nature in atherosclerosis. Additional highlight of the study is neovascularization.

Keywords: Atherosclerosis; CD68; Carotid artery; Immunohistochemistry

Introduction

Among the non-communicable diseases, atherosclerotic heart disease is considered a major health hazard in our country leading to increase morbidity and mortality. Atherosclerosis is regarded as a dynamic and progressive disease arising from the combination of endothelial dysfunction and inflammation. The classic concept of atherosclerosis as a disorder of lipid metabolism and deposition has gained wide acceptance. However, the story of atherogenesis extends beyond dyslipidemia.

In recent years, scientists have shown that inflammation is involved in the initiation (atherogenesis), lesion progression and ultimate complications leading to acute coronary and cerebrovascular catastrophes due to complications like plaque rupture, intra plaque hemorrhage and thromboembolism. The factors leading to the instability of a plaque includes increased accumulation of lipid, the thinness of the fibrous cap and local inflammation.

The role of inflammation in atherosclerosis has been studied to a great extent in recent years and is considered a major factor for plaque vulnerability. Inflammatory mediators like cytokines promote the migration and proliferation of smooth muscle cells leading to their transformation into foam cells. Inflammation also leads to increased neo-vascularization of the plaque. There is need to study the immunohistochemical markers of macrophages and smooth muscle *viz.*, CD68 and SMA respectively in order to analyze the distribution in coronary and carotid endarterectomy specimens. An insight into this will give key exposures and the possible significance of their role in the inflammatory pathogenesis of atherosclerosis.

The available literature is an evidence of studies, which have been done on autopsy specimens and experimental animals with atherosclerosis. There are few studies done on living patients; reason being the difficulty in acquisition of atherosclerotic disease tissue from live patients. This research project is carried out on endarterectomy specimens obtained from live patients with complicated atherosclerotic heart disease.

Objectives

1. To study the morphological characteristics of atheromatous plaques observed in coronary and carotid endarterectomy specimens.

2. To study expression of CD68 and SMA in order to determine the role of these markers in the inflammatory pathogenesis of an atheromatous plaques.

Methodology

Study Setting - This study was carried out as cross sectional study in the Department of Pathology of our tertiary care hospital for a period of eight months.

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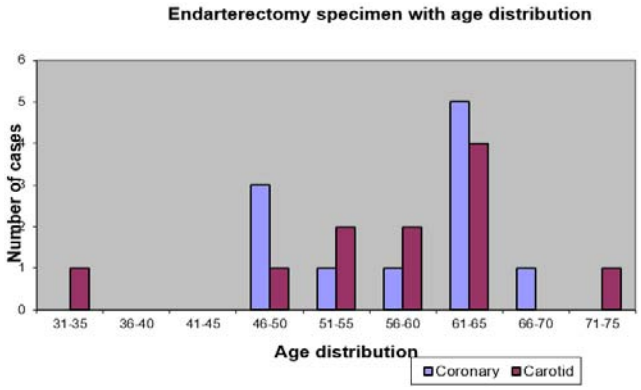

Figure 1: Age distribution of the study participants according to the vessel affected.

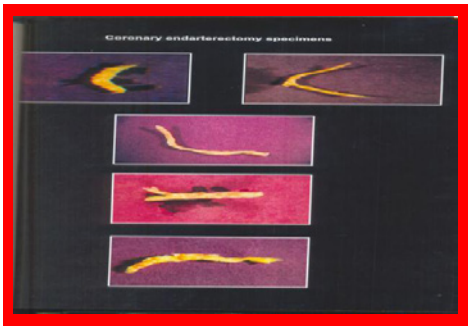

Figure 2: Coronary endarterectomy specimens.

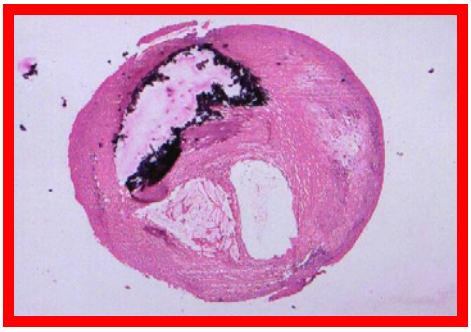

Figure 3: Endarterectomy showing lumen occlusion, lipid pool, foam cells, inflammation and calcification.

Study population - All the patients with atherosclerotic disease who underwent angioplasty for atherosclerotic heart disease during the study period were included for the study. A total of 21 patients were enrolled in the study, of which 10 were coronary endarterectomy patients while 11 were carotid endarterectomy patients.

Selection criteria

Data collection tools: Representative 5mm thick bits are sampled from the endarterectomy specimens and processed for light microscopy. Five-micron thin sections from the paraffin embedded blocks were subjected to routine haematoxylin and eosin and Masson's trichrome stains.

Additional 5-micron thin sections were set aside for Immunohistochemistry staining using CD68 and SMA (Dako cytomation) by standard immunohistochemical staining using Biotinylated secondary link antibody. The control slide for CD68 was cholesterolosis of gall bladder and the control slide for SMA was leiomyoma of the uterus.

Immunohistochemistry

The 2-3 micron sections from paraffin embedded tissue were taken on poly-l-lysine coated slides and rehydrated through two changes of xylene, five minutes each and two changes of 100% alcohol, five minutes each and then rinsed in running tap water. To this, 3% hydrogen peroxide was then applied for 10minutes to inhibit endogenous peroxide activity. It was then rinsed in three to four changes of water, for two minutes each. This was followed by antigen retrieval which was done using citrate solution by the microwave method. Normal horse serum three drops and 10ml of wash buffer was then applied to the section to block nonspecific binding. This was incubated at 37^0C for 20 minutes. Primary antibodies (CD68 & SMA) was then applied and kept for 30 minutes. The slides were washed in Tris buffer for two changes, five minutes each and immersed in this solution for five minutes. Biotinylated secondary link antibody was then applied to the section and kept for incubation for 30 minutes. This was followed by two changes in Tris buffer for over five minutes. Streptavidin conjugated horse radish peroxidase was then applied on to the section and incubated for 30 minutes. It was then washed in Tris for two changes over 5 minutes. The substrate chromogen solution (DAB) was applied for 10 minutes and the color reaction was observed. The slides were then rinsed in water to remove the excess DAB reagent and then placed in running tap water for 30 seconds. This was followed by counterstaining with haematoxylin for 60 seconds. The slides were then re-immersed in running tap water for one minute. The slides were then dehydrated in 95% alcohol for one minute and three changes of 100% alcohol, one minute each. The sections were then cleared in three changes of xylene, 1 minute each and cover slips was applied using DPX mountant.

Operational definition

The American Heart Association grading of atherosclerosis was based on two independent reviewers blind to histopathology. The cross sections were classified as AHA lesion types I/II, III, IV/V, VI, VII or VIII among the study participants.

Light microscopy features studied included lipid pool, foam cells, lumen occlusion, cholesterol clefts, calcification, rupture, intraplaque hemorrhage, thrombosis and fibrous cap thickness. These light microscopic features were graded as + to +++ subjectively depending on their relative proportion to the plaque area as:

+	=	0 - 33% of plaque area
++	=	36 - 67% of plaque area
+++	=	>67% of plaque area

Immunohistochemical assessed expressivity was based on the intensity and distribution of staining as follows + -(0-33%),++ -(33-67%),+++-(67-100%).

Data analysis

The data was entered and analyzed using SPSS ver.15 software. Chi square test was used to evaluate statistical significance. A p value

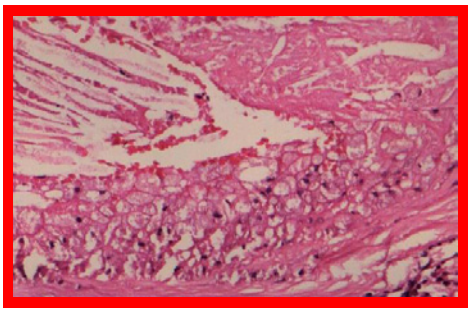

Figure 4: Endarterectomy section showing foam cells, inflammation and lipid pool.

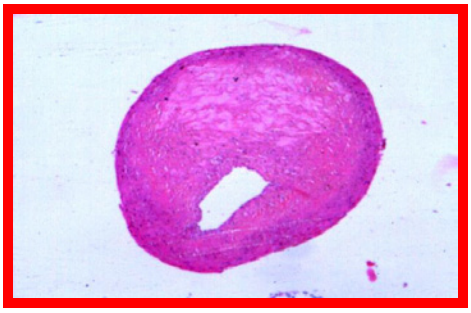

Figure 5: Coronary endarterectomy specimen showing outward remodeling.

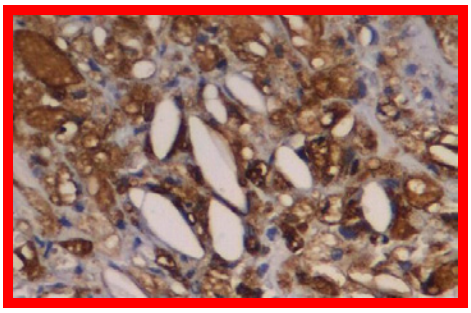

Figure 6: CD68 positivity in foam cells and spillage around cholesterol clefts.

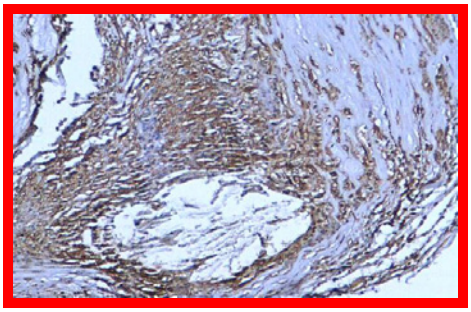

Figure 7: Intense SMA positivity around lipid pool.

<0.05 was taken as statistically significant.

Results

This cross sectional study was carried out among 21 endarterectomy specimens. The background particulars of the study participants are given in (Table 1). The majority of the patients were in the 61-65 age groups with male > female (8:1). The 2nd peak was

Table 1: Background characteristics among the study participants.

S. No	Particulars	Frequency (n=21)	Percentage (%)
1	**Age (in years)**		
	<50	4	19
	51-60	6	28.6
	>60	11	52.4
2	**Vessel type**		
	Coronary artery	10	47.6
	Carotid artery	11	52.4
3	**AHA grading**		
	I	0	0
	II	0	0
	III	0	0
	IV	1	4.7
	V	11	52.4
	VI	9	33.3
	VII	0	0
	VIII	0	0

Table 2: Immunohistochemistry findings among the study participants.

S. No	Parameters	Coronary artery (%)	Carotid artery (%)
1	**Lipid pool**		
	1+	30	9.1
	2+	40	45.5
	3+	30	36.4
2	**Foam cells**		
	1+	37.5	14.2
	2+	44.4	75.6
	3+	25.1	14.2
3	**Cholesterol clefts**		
	1+	50	27.3
	2+	40	18.3
	3+	10	36.4
4	**Calcification**		
	1+	20	45.5
	2+	10	9.1
	3+	40	36.4
5	**CD 68**		
	1+	40	27.3
	2+	50	45.5
	3+	10	27.3
6	**SMA**		
	2+	80	63.6
	3+	20	36.4

Table 3: Particulars regarding rupture among the study participants.

S. No	Sex	Coronary (%)	Carotid (%)
1	Males	37.5	57.4
2	Females	33.33	33.33

seen in the 45-50 age groups. All the endarterectomy specimens had atheromatous plaques and most of them belong to the AHA grades IV-VI.

The age wise distribution of the study participants between coronary and carotid arteries is given in (Figure 1). It was observed that coronary artery was highly affected in the age group of 61-65 years, while in younger age group carotid artery was the most affected.

The immunohistochemistry findings of the study participants are given in (Table 2). The lipid pool appeared to be larger in carotid than in coronaries. Cholesterol clefts were seen in both coronary and carotid endarterectomies. A +++ positivity was only seen in a 36% of carotids whereas in coronary the same was seen only in 10% of cases. In our study calcification was seen in both carotid and coronary endarterectomy specimens. Coronary endarterectomy (40-50%) expressed more positivity than carotid specimens (27-45%) positivity, suggesting the role of inflammation as a contributory factor for vulnerability. Increased density of CD68 expression was noted in the shoulder region of the plaque. About 80% coronary endarterectomies showed a 2+ positivity, whereas only 63% carotids showed ++ positivity. The remaining patients showed 3+ positivity. SMA also shows the presence of intense neo-vascularization in 90% of coronaries and 100% in carotids (Figures 2-8).

In the carotid endarterectomy specimens, the degree of occlusion appeared to be increased in the age group of 60-80years. Among the 4 female patients 2 showed 75-90% occlusion and 17 male patients showed 50-95% occlusion. This appeared to be statistically significant with a p value of 0.038 (Figure 9).

Rupture occurs as a complication of atherosclerotic disease and in our series was more common in males than females. In the coronary endarterectomy 30% of cases (3/10) and 54.5% of carotid endarterectomy showed, rupture (Table 3).

The other significant finding in light microscopic evaluation was outward remodeling in 30% of case and intra plaque hemorrhage was noted in 20% of coronaries and 36% of carotid endarterectomies.

Discussion

Coronary heart disease is a worldwide disease and the mortality rates vary widely in different parts of the world. The highest coronary mortality is seen at present in Northern Europe and English speaking countries like Scotland, Finland etc. On the other hand rates in southern Europe are much lower eg., Italy, France and Japan being the lowest. It accounts for >19 million deaths worldwide annually. On screening the Indian population over the age of 30 years by a 12 leaded ECG, in Chandigarh (urban population), the prevalence was found to be 65.4 and 47.8 per 1000 males and females respectively. In a village in Haryana, the prevalence was 22 and 17.3/1000 males and females respectively [1]. Atherosclerosis is not merely a disease caused by the increasing accumulation of lipids in the arterial walls, but rather a complex process, much of which is inflammatory

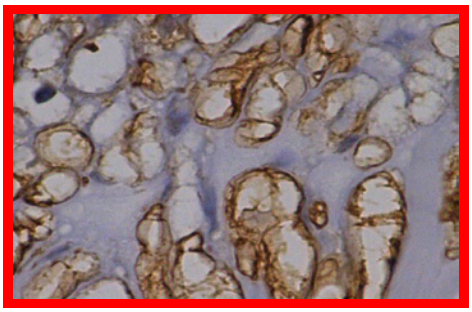

Figure 8: SMA showing neo-vascularization.

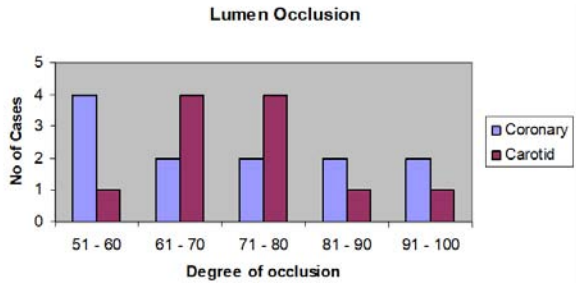

Figure 9: Lumen occlusion among the study participants.

in nature. Inflammation is involved in the process of initiation (atherogenesis), lesion progression and the ultimate complications (plaque disruption and thrombosis) [2,3]. Wolf gang in his study also mentions that vessel wall inflammation [4] is a central feature in the initiation, progression and terminal steps of atherosclerosis that lead to plaque rupture and thrombosis [4].

These morphologic changes are preceded by the dysfunction of activated endothelial cells, which represents the initial step in atherogenesis according to the classic "response to injury" hypothesis. Recent research has shown that inflammation plays a key role in CAD and other manifestations of atherosclerosis. Immune cells dominate in early atherosclerotic lesions and their effector molecules accelerate progression of the lesion [3,5]. Further activation of inflammation can elicit acute coronary syndromes.

Studies have shown that atherosclerotic plaques show marked variability with respect to the distribution of macrophages and smooth muscle cells not only from one lesion to the other, but also within one and the same plaque [6]. This phenomenon is significant because plaque inflammation is widely considered to play a role in plaque destabilization [7] and eventually plaque erosion and rupture. Our study also is in concordance with these workers, since there was a significant distribution of CD68 and SMA in both coronary and carotid endarterectomy specimens. However, the interesting finding in this study was that SMA highlighted not only the tunica media and smooth muscle derived foam cells, but also highlighted with great intensity the neo-vascularization, which was made out in both coronary and carotid endarterectomy plaques.

From this institution, Balakrishnan and Sarah Kuruvilla have vividly depicted the inflammatory nature of atherosclerosis, recently in a light and electron microscopic study of endarterectomy

specimens [3]. In our series, few cases also showed other complications like outward remodeling and intra plaque hemorrhage. In recent years imaging modalities such as Angiography, Angioscopy and newer techniques like Intra Venous Ultra Sound (IVUS), Magnetic Resonance Imaging (MRI), Thermography of plaques, Optical Coherence Tomography (OCT), Near Infrared (NIR) and Elastography have shown considerable promise in the identification of plaques types and their vulnerability *invivo*. These are important in the clinical perspective, since they may bear therapeutic significance.

In summary, our study has helped in the morphological characterization of atheromatous plaques in endarterectomised specimens, which may have significant clinical relevance.Moreover, expression of immunohistochemical markers like CD68 and SMA have proved useful in highlighting the inflammatory nature in atherosclerosis.

Conclusion

The degree of lumen occlusion, density of foam cells was more in carotid endarterectomies, surface distribution of cholesterol clefts was also slightly increased in carotid and this difference was statistically significant.

An important additional highlight of this study was the intense neo-vascularization accentuated by the immunohistochemical marker smooth muscle action. This was seen in 90% of coronary and 100% of carotid endarterectomies.

Limitation

Our objective in using the Immunohistochemistry marker SMA was to look for foam cells derived smooth muscle and their significance in the inflammatory pathogenesis of atherosclerosis. However, the unique finding in this study was that SMA intensely highlighted the neo-vascularization in the majority of plaques in addition to the smooth muscle derived foam cells. The statistical significance cannot be estimated because of the small number of cases.

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