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Original Article

Pathology Section

Quantification of Various Inflammatory Cells in Advanced Atherosclerotic Plaques

VERONICA SOUNDRA VEENA PAUL[1], CHRISTINA MARY PRIYA PAUL[2], SARAH KURUVILLA[3]

ABSTRACT

Introduction: Atherosclerosis, the pathological basis of coronary artery disease is being extensively studied as understanding of the complex processes involved in the formation and progression that can provide an insight into prevention and treatment of the same. This is an autopsy study to identify and quantify various inflammatory cells in advanced atherosclerotic plaques.

Aim: This study aims at identifying and categorizing the various inflammatory cells present in advanced atherosclerotic plaques, noting their distribution in the plaque, quantifying them using histomorphometry and comparing them across plaques of different AHA types.

Materials and Methods: Post-mortem angiogram was performed on 3 heart specimens obtained at autopsy of random Road Traffic Accident (RTA) cases which revealed evidence of coronary artery disease. End-arterectomy was done and the arteries with atherosclerotic plaques were cut into serial sections and made into tissue blocks. Sections from these blocks were stained with H & E stain and the plaques were classified based on AHA classification. 50 advanced atherosclerotic plaques of AHA Type IV and V were chosen for this study and were screened for inflammatory cells, first with H & E stain and then with different immunohistochemical stains for T-lymphocytes, B-lymphocytes and neutrophils. The T-lymphocytes thus identified was further sub-typed into CD4+ and CD8+ cells again using IHC markers and the percentage area of each was measured using histomorphometry. Then, these values were compared between AHA Type IV and AHA Type V lesions.

Results: It was found that the inflammatory cells found in advanced atherosclerotic plaques were predominantly T-lymphocytes as evidenced by their CD3 positivity and they were found to be distributed mainly around the shoulder region and fibrous cap of the plaque. When categorized further, it was found that CD8+ T-cells were always more than CD4+ T-cells in advanced lesions. Meloperoxidase stain for neutrophils was negative in all the plaques examined. The difference in the amount of inflammatory cells between AHA type IV and Type V was not statistically significant.

Conclusion: The study of the amount of inflammatory cells in atherosclerotic plaques and understanding their role in the pathophysiology of advanced plaques may have therapeutic implications.

Keywords: End-arterectomy, Histomorphometric study, Immunohistochemistry of advanced plaques, Inflammation in atherosclerosis

INTRODUCTION

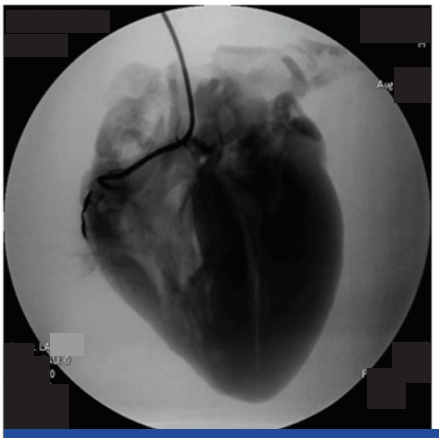
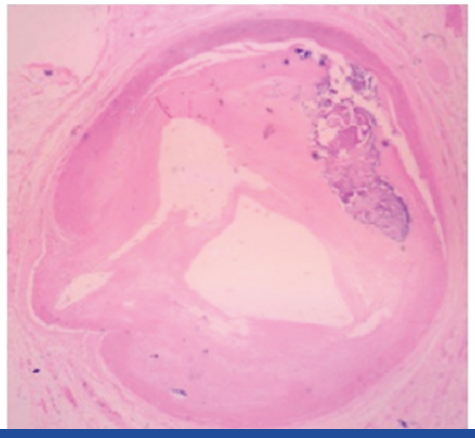
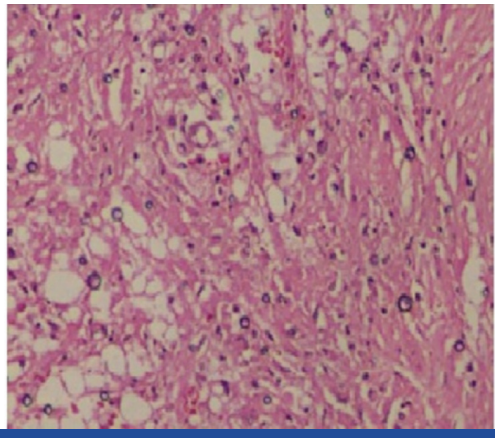
Atherosclerosis is the pathological basis for coronary artery disease which is the leading cause of mortality all over the world. Though formerly it was believed that atherosclerosis was a bland lipid storage disorder, extensive research in the recent years have shown that it is primarily an inflammatory lesion and that inflammation plays a vital role in all stages of the disease from plaque initiation, lesion progression up to ultimate complications such as plaque rupture, intra-plaque hemorrhage and thromboembolism [1].

Most of the studies on atherosclerosis are either autopsy studies or studies on experimental animals as it is difficult to acquire atherosclerotic tissue from live patients [2]. The presence of active inflammation with T-lymphocytes or macrophages is one of the criteria that make a plaque vulnerable to rupture [3]. T-cells are of both the T helper (CD4+) and T killer (CD8+) phenotypes and may be capable of clonal proliferation in response to appropriate antigens. Studies have shown that the majority of T-lymphocytes in atherosclerotic plaques are CD4+ while some other studies show that CD8+ T-cells are the predominant population in advanced atherosclerotic lesions [4]. This is a study on end-arterectomy specimens obtained at autopsy aimed at identifying and categorizing the various inflammatory cells present in advanced atherosclerotic plaques by using appropriate immunohistochemical (IHC) stains and quantifying them by using histomorphometry.

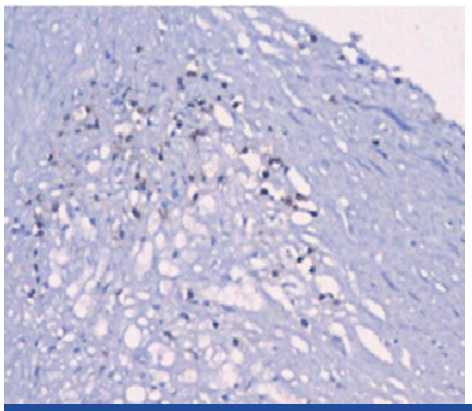
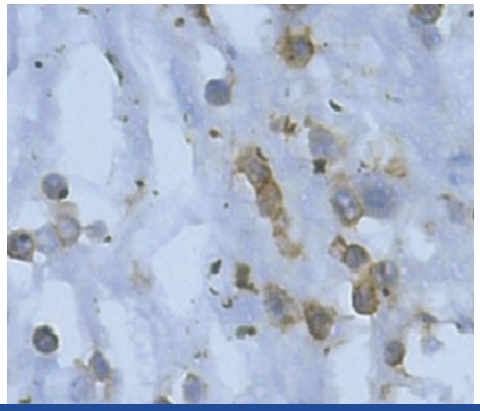
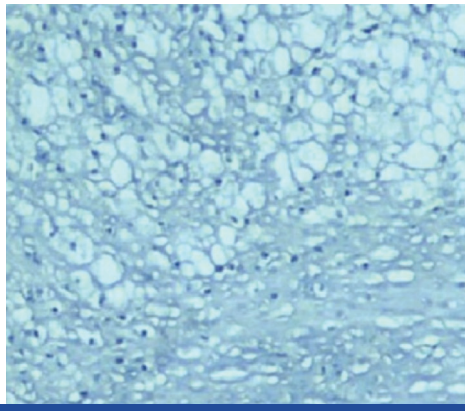
MATERIALS AND METHODS

This is a histomorphometric study to categorize and quantify inflammatory cells present in advanced atherosclerotic plaques using IHC stains, done in Sri Ramachandra Medical College (SRMC), Chennai, between 2007 and 2009. The study was done on autopsy material obtained and processed to tissue blocks from another institute, Madras Medical College (MMC), Chennai. The tissue blocks were cut into sections, stained with H & E, screened for plaques, categorized according to American Heart Association typing in SRMC and 50 advanced atherosclerotic plaques were chosen for the study.

Three heart specimens (selected at random) were collected from autopsies done for cases of accidental deaths. The coronary arteries were perfusion-fixed with 10% neutral buffered formalin at a pressure of 100cm of water against a closed aortic valve for a period of 24 hours. Post mortem angiogram was performed in all three specimens. Post mortem clots were flushed out by infusing saline at 38°C. The arteries were then perfused with an Iso-opaque dye. The findings were recorded on X-ray angiograms in both anterior and lateral views [5] [Table/Fig-1]. The coronary arteries with their branches were dissected intact from the specimen. The coronary arteries and their branches were then divided into 5 mm transverse slices throughout the entire length. Each slice was further fixed in formalin and was embedded in paraffin wax blocks. (Until this step, the process was done in MMC. Since, the details such as age, gender, pre-existing illness etc. do not

[Table/Fig-1]: Post-mortem coronoary angiogram demonstrating 90% occlusion in the mid right coronary artery. **[Table/Fig-2]:** Scanner view of a vessel with a calcified plaque, AHA type Vb, H&E stain x 20. **[Table/Fig-3]:** Low power view of lymphocytes in the plaque, H&E stain x 100.

[Table/Fig-4]: Low power view of CD3 staining of T-lymphocytes in the plaque x 100. **[Table/Fig-5]:** High power view of CD3 staining in the plaque showing cytoplasmic positivity x 400. **[Table/Fig-6]:** Low power view of CD20 stain-Negative in the plaque x 100.

influence the outcome of this study; those parameters were not taken into consideration).

In SRMC, sections were cut from the tissue blocks, were stained with Haematoxylin & Eosin (H&E) stain, and were examined for the presence of plaque and the number of plaques in each vessel was noted. Then the plaques were classified according to the American Heart Association (AHA) classification and early lesions (AHA Type I, II and III) [6] were separated from the advanced lesions (AHA Type IV and above) [7]. Fifty advanced atherosclerotic plaques, AHA Type IV and V were chosen and screened for inflammation on H & E sections as there was no AHA Type VI plaque in any of the specimen chosen for the study. Later, confirmation and categorization of inflammatory cells in the plaque was done using immunohistochemistry (IHC).

The immunohistochemical stains used to identify inflammatory cells were mouse anti-human CD3 clone to identify T- lymphocytes, mouse anti-human CD4 clone and mouse anti-human CD8 clone to further sub-classify them into CD4+ and CD8+ T-lymphocytes respectively, mouse anti- human CD20 clone to identify B-lymphocytes and rabbit anti-human Myeloperoxidase clone to identify neutrophil granulocytes. The IHC stains were done using positive and negative controls simultaneously with the tests. The percentage area of IHC positivity of various stains was then measured using histomorphometry.

RESULTS

The plaques from the advanced lesions consistently showed inflammatory cells and it was observed that the inflammatory cells were predominantly present in the shoulder region of the plaque and in the fibrous cap which was observed on H & E sections [Table/Fig-2,3].

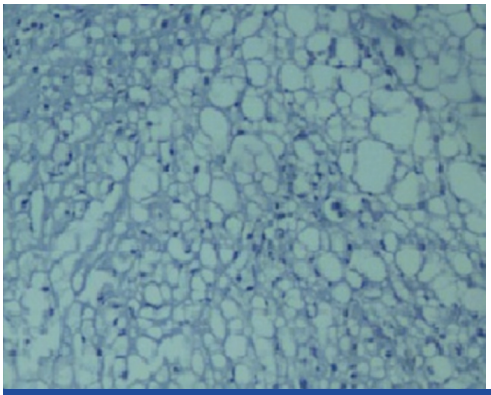
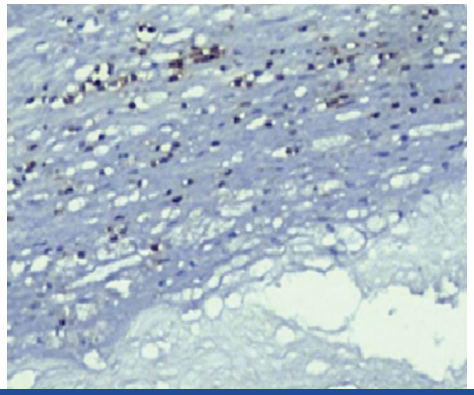
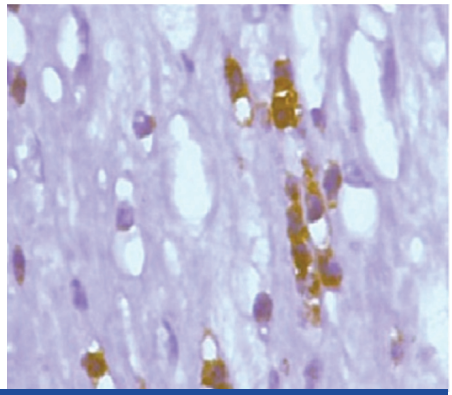
On immunohistochemical examination of advanced lesions using CD3, CD4, CD8, CD20 and Myeloperoxidase, it was found that the inflammatory cells were predominantly T-lymphocytes as they were positive for CD3 [Table/Fig-4,5] and negative for CD20 [Table/Fig-6]. The cells were also negative for myeloperoxidase, a stain for poymorphonuclear neutrophils [Table/Fig-7]. The T-cells were further sub-classified into CD4+ T-lymphocytes [Table/Fig-8,9] and CD 8+ T-lymphocytes [Table/Fig-10,11] and it was seen that the CD8+ T-lymphocyte population was greater than CD4+ T-lymphocyte population in most of the lesions. However, on statistical analysis, it was found that the difference between the amount of CD4+ and CD8+ T-cells in AHA Type IV and Type V lesions was not statistically significant.

In this study, it was observed that plaques with higher concentrations of CD4+ T-cells also had higher amounts of CD8+ T-cells showing a positive correlation on analytical studies (correlation coefficient-0.98). The values of ratio of percentage area of CD4+ to CD8+ T-cells ranged from 0.18:1 to 0.84:1.

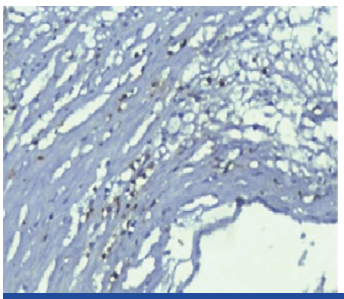
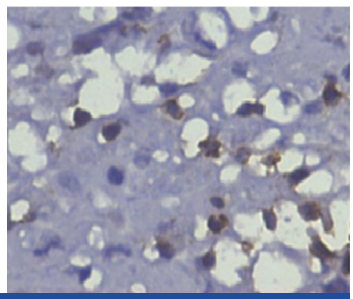
DISCUSSION

It is now proven that atherosclerosis is an inflammatory disease and not just purely accumulation of lipids in the wall of the arteries. Recent studies are focused on understanding how this inflammation is regulated and how it can be influenced to prevent/ treat atherosclerosis. T-lymphocytes, a component of adaptive immunity has a major role in atherogenesis [8]. The presence of T-lymphocytes in human atherosclerotic plaques was first described by Jonasson et al., in 1985 [9]. Xinghua Zhou et al., observed in their study on Apolipoprotein- E- Deficient mice that CD4+ T-cells were prominent in the fibrous cap and sub-endothelially whereas CD8+ T-cells were sparse in advanced atherosclerotic lesions [10].

Alan Daugherty et al., also found that the major class of T-lymphocytes was CD4+ and that they later differentiated into T_h1 and T_h2 lineage in response to the local milieu of cytokines. The principle inducers of T_h1 and T_h2 are IL-12 and IL-10 respectively.

[Table/Fig-7]: Low power view of myeloperoxidase stain-Negative in the plaque x 100. **[Table/Fig-8]:** Low power view of lymphocytes with CD4 stain in the plaque x 100. **[Table/Fig-9]:**High power view of CD 4 stain in the plaque showing cytoplasmic positivity x 400.

[Table/Fig-10]: Low power view of CD stain in the plaque x 100.
[Table/Fig-11]: High power view of CD 8 stain in the plaque showing cytoplasmic positivity x 400.

Comparisons	't' statistic value	p-value
AHA Type Vs CD3 % Area	0.87	0.38
AHA Type Vs CD4 % Area	1.53	0.13
AHA Type Vs CD8 % Area	1.3	0.198
AHA Type Vs CD4/CD8 % Area	1.47	0.15

[Table/Fig-12]: Results of all comparative statistical tests. (using Epinfo6.04d for Dos; tests done for alpha 0.5, power-80%, 2-sided test)

Activated T-lymphocytes functionally defines the cytokines produced with INF-γ secreted by the T_h1 cells and IL-4 from the T_h2 cells [11]. CD4+ T-cells predominantly T_H1 subset has a pro-atherogenic effect as demonstrated by Zhou et al., in a study in which it was shown that transfer of CD4+ T-cells aggravates atherosclerosis in immunodeficient apolipoprotein-E knockout mice [12].

Jan Gewaltig et al., observed that among leukocytes that accumulate in advanced atherosclerotic plaques in humans, CD8+ T-lymphocytes played a quantitatively dominant role. In their study, they demonstrate that intimal CD8+ T-cells increase with increasing severity of the atherosclerotic lesions. A recent functional analysis of T cell activation by lipoproteins showed that high density lipoprotein induces CD8+ T cell proliferation and leaves CD4+ T-cells unaffected [13]. In a study by Ludewig et al., it was shown that CD8+ T-cells in apolipoprotein-E knockout mice can cause the death of arterial cells and accelerate atherosclerosis [2].

In a study by Stemme et al., it was found that majority of the plaque T-cells are memory cells which may be due to preferential recruitment or retention of activated peripheral blood T-cells [14]. It was also observed in another study that CD4+ cells in the plaques showed more activation than in peripheral blood [15]. A vulnerable plaque is one which is susceptible to producing complications in a vulnerable patient. A vulnerable plaque is defined based on some criteria such as active inflammation with macrophages or T-cells, thin fibrous cap (<65μ) with a large lipid core (>40% of the plaque volume), stenosis > 90% etc [16].

Though T-lymphocytes are popularly believed to exacerbate atherosclerosis, it is proven through studies that different subsets of T-helper cells have different effects on the atheromatous plaque [17]. T_{reg} cells are critical for the maintenance of immune homeostasis and their role in atherosclerosis has been established. Depletion of peripheral T_{reg} cells by anti-CD25 monoclonal antibodies increased atherosclerotic lesion size and vulnerability in ApoE-/-mice [18].

In this study, it was observed that $CD3^+$ T-lymphocytes were always found in advanced atherosclerotic plaques though in variable amounts. It was also found that in advanced lesions CD8+ was the more quantitatively dominant subset as against CD4+. Since early lesions were not included in this study, it was not possible to ascertain if there was a difference in the number of CD4+ and CD8+ T- lymphocytes between early and advanced lesions. It was also observed that plaques with higher levels of CD8+ T-cells also had higher concentrations of CD4+ T-cells. It was observed that the differences in number of inflammatory cell subsets among AHA type IV and Type V lesions were not statistically significant [Table/Fig-12]. This study has its limitations because of the small sample size and methods such as morphometry for estimating amount of inflammatory cells (as only a few portions of the plaque are examined and not the entire plaque). However, it does provide some valuable data using inter-plaque and inter-AHA type comparison and the understanding of the role of various T cell subsets in the pathophysiology of atherosclerotic plaques has profound therapeutic implications [19].

CONCLUSION

Advanced atherosclerotic plaques consistently showed the presence of T-cells, mainly CD8+. They were found mostly in the shoulder region and the fibrous cap of the plaque. There was no significant difference in their quantities between AHA Type IV and Type V. The presence of T-cells in large numbers in advanced atherosclerotic lesions suggests a local immune response.

REFERENCES

[1] Libby P, Ridker PM, Maseri A. Inflammation and Atherosclerosis. *Circulation*. 2002;105(9):1135-43.
[2] Ludewig B, Freigang S, Ja¨ggi M, Kurrer MO, Pei YC, et al. Linking immune-mediated arterial inflammation and cholesterol-induced atherosclerosis in a transgenic mouse model. *Proceedings of the National Academy of Sciences, USA*. 2000;97(23):12752-57.
[3] Allard C. Wal V, Becker AE. Atherosclerotic plaque rupture – pathologic basis of plaque stability and instability. *Cardiovascular Research*. 1999;41(2):334-44.
[4] Aukrust P, Yndestad A, Wiggo J. Sandberg, Gullestad L, Damås JK. T-cells in Coronary Artery Disease: Different Effects of Different T-Cell subsets. *Journal of the American College of Cardiology*. 2007;50(15):1459-61.
[5] Coghill SB, Nicoll SM, McKimmie A, Houston I, Matthew BM. Technical methods-revitalising postmortem coronary angiography. *Journal of Clinical Pathology*. 1983;36(12):1406-09.
[6] Stary HC, Chandler AB, Glagov S, Guyton JR, Insull W, Rosenfeld ME, et al. A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. A report from the committee on vascular lesions of the council on arteriosclerosis, american heart association. *Arteriosclerosis. Thrombosis and Vascular Biology*. 1994; 14:840-56.

[7] Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W et al. Definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. *Circulation*. 1995;92:1355-74.
[8] Anna-Karin L. Robertson and Göran KH. T-cells in atherogenesis: For better or for worse?. *Arteriosclerosis, Thrombosis and Vascular Biology*. 2006;26(11): 2421-32.
[9] Jonasson L, Holm J, Skalli O, Gabbiani G, Hansson GK. Expression of class II transplantation antigen on vascular smooth muscle cells in human atherosclerosis. *The Journal of Clinical Investigation*. 1985;76(1):125-31.
[10] Zhou X, Stemme S, Hansson GK. Evidence for a Local Immune Response in Atherosclerosis: CD4+ T-cells Infiltrate Lesions of Apolipoprotein-E-Deficient Mice. *American journal of Pathology*. 1996;149(2):359-66.
[11] Daugherty A, Rateri DL. T-lymphocytes in Atherosclerosis: The Yin-yang of th1 and th2 influence on lesion formation. *Circulation Research*. 2002;90(10): 1039-40.
[12] Zhou X, Nicoletti A, Elhage R, Hansson GK. Transfer of CD4+ T-cells aggravates atherosclerosis in immunodeficient apolipoprotein E knockout mice. *Circulation*. 2000;102(24):2919-22.
[13] Gewaltig J, Kummer M, Koella C, Cathomas G, Biedermann B. Requirements for CD8 T-cell migration into the human arterial wall. *Human Pathology*. 2008;39(12):1756-62.
[14] Stemme S, Holm J, Hansson GK. T-lymphocytes in human atherosclerotic plaques are memory cells expressing CD45RO and the integrin VLA-1. *Arteriosclerosis, Thrombosis and Vascular Biology*. 1992;12(2):206-11.
[15] Grivel JC, Ivanova O, Pinegina N, Blank PS, et al. Activation of T-lymphocytes in atherosclerotic plaques. *Arteriosclerosis, Thrombosis and Vascular Biology*. 2011;31(12):2929-37.
[16] Naghavi M, Libby P, Falk E, et al. From vulnerable plaque to vulnerable patient: a call for new definitions and risk assessment strategies: Part I. *Circulation*. 2003;108:1664-72.
[17] Tse K, Tse H, Sidney J, Sette A, Ley K. T-cells in atherosclerosis. *International Immunology.* 2013;25(11):615-22.
[18] Pastrana JL , Sha X, Virtue A, Mai J, Cueto R, et al. Regulatory T-cells and Atherosclerosis. *Journal of Clinical and Experimental Cardiology*. 2012; suppl. 12:002.
[19] Packard RRS, Libby P. Inflammation in atherosclerosis: from vascular biology to biomarker discovery and risk prediction. *Clinical Chemistry*. 2008;54(1):24-38.

PARTICULARS OF CONTRIBUTORS:
1. Assistant Professor, Department of Pathology, A.C.S Medical College, Chennai, India.
2. Associate Professor, Department of Community Medicine, A.C.S Medical College, Chennai, India.
3. Senior Consultant and Head of the Department, Department of Pathology, The Madras Medical Mission Hospital, Chennai, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Veronica Soundra Veena Paul,
No.18, Lakshmi Street, VGN Mahalakshmi Nagar, Perumalagaram, Velappanchavadi, Chennai- 600077, Tamil Nadu, India.
E-mail: veronica212121@yahoo.com

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