Original Research

Immunohistochemical Expression of VEGF and BAX Proteins in Induced Atherosclerosis of Aorta in Male Wistar Rats

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Abstract

Atherosclerosis is a cardiovascular and fibroproliferative inflammatory disease commonly associated with dyslipidemia. The present study was carried in wistar rats, divided into two groups consisting of 12 rats in each group. Atherosclerosis was induced by addition of 1% cholesterol and 15% saturated oil to 1000 g of standard rat diet and fed to rats for 90 days (Group II). Group I rats fed normal diet for 90 days and kept as control. Endothelial cells, lipid laden macrophages, nucleus and membrane of foam cells, thrombus and structurally modified stromal cells in initiated atherosclerotic lesions has shown distinct immunoreactivity with VEGF and progressive atherosclerotic lesions with endothelial attached thrombus has shown great degree of positivity with BAX protein.

Key words: Atherosclerosis, Aorta, BAX, Immunohistochemsitry, Wistar rats, VEGF

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Introduction

Atherosclerosis is a cardiovascular disease with heterogenous mechanisms of progression and is an oxidative, chronic inflammatory and thrombotic disease refers to fatty deposits on the inner lining of the large to medium sized elastic and muscular arteries and is precipitated by elevated level of low-density lipoprotein cholesterol (LDL-C) in the blood (Srinivasa *et al.*, 2017).

Vascular endothelial growth factor (VEGF) is a potent angiogenesis factor produced by a variety of cells including smooth muscle cells, glioma cells and marcophages. VEGF is reported to induce migration and proliferation of endothelial cells, enhance vascular permeability and modulate thrombogenicity and

considered unique among angiogenic growth factors. VEGF also induces migration of mononuclear phagocytes/monocytes and stimulate their expression of tissue factors (Berse *et al.*, 1992).

VEGF is expressed in atherosclerotic plaques of carotid and coronary arteries. However, role of VEGF in atherosclerotic plaques of carotid and coronary arteries has not been fully elucidated (Couffinhal *et al.*, 1997). VEGF positivity was detected in structurally modified macrophages, smooth muscle cells (SMCs) as well as endothelial cells (ECs) in early hyper cellular atherosclerotic lesions. Whereas in advanced atherosclerotic lesions, all ECs were uniformly positive for VEGF and no VEGF positive SMCs or marcophages underlying the ECs denuded areas and strong VEGF positivity was observed in macrophages and in the ECs of the newly formed micro vessels (Mayumi *et al.*, 1998).

Expression of VEGF in foam cells was detected at thin cytoplasm, nucleus and the extracellular matrix around the cells but not at the foamy component of lipid. VEGF expression in fusiform cells was mainly at the cytoplasm. In addition, foam cells around intra plaque hemorrhage and newly formed vessels showed distinct VEGF immunoreactivity. Nucleus and membranes of foam cells in medium and deep layers showed the distinct immunoreactivity of VEGF. However, elastic and collagenous fibers did not show the VEGF immunoreactivity. Foam cells and some extracellular matrix around newly formed vessels and intra plaque hemorrhage showed particularly distinct immunoreactivity of VEGF (Kanno *et al.*, 2014).

Apoptotic cells in atheromatous plaques ranges from 2-30%[5]**.** Proliferating smooth muscle cells and macrophages undergoes more apoptosis (Bennet *et al.*, 1995, Hank *et al.*, 1995 and Mallet *et al.*, 1997)**.** Recent work by so many laboratories in the world demonstrated the presence of apoptotic cell death in human and experimental atherosclerotic plaques (David *et al.*, 1995). Most of these studies use DNA in situ end-labeling techniques (TUNEL) to detect apoptotic cell death within the plaques. Apoptotic cell death shows a steady decline after lipid lowering, moreover, the pro-apoptotic protein BAX, which is up regulated in both human and experimental atherosclerotic plaques, strongly decreased after lipid lowering, indicate that susceptibility of cells in the plaque undergoing apoptosis (Mark and Arnold, 2000).

Occurrence of apoptosis in all cells of the atherosclerotic plaque, becoming increasingly frequent and important as the plaque develops and contributes to plaque growth, lipid core development, plaque rupture and thrombosis, although the extent to which apoptosis regulates these processes is unknown (Victoria and Martin, 2004). In atherosclerosis, intimal thickening composed primarily of longitudinally oriented SMCs that strongly expressed alpha SMC actin and morphology similar to the medial SMCs. Fatty streaks composed primarily of SMCs that express alpha SMC actin. A significant portion of the same cells showed intracellular fat accumulation, foam cell formation and showed a strong cytoplasmic BAX expression in a study conducted on human atherosclerotic plaques (Mark *et al*., 2013). Hence, the present study was carried to know the expression of VEGF and BAX protein in the atherosclerotic lesion of aorta of wistar male rats.

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Materials and Methods

Procurement of Experimental Animals

Male Wister rats of about 16 weeks of age weighing around 200 g were procured from Sri Venkateswara Agencies, Bangalore. After acclimatization of one week the rats were grouped and housed in standard poly propylene rat cages (three rats per cage) and maintained at 25±1˚C and a 12:12 hour interval light / dark cycle throughout the experimental period of 90 days. The approval of the institutional Animal Ethical Committee was obtained prior to commencement of the experiment.

Chemicals and IHC Kits

Cholesterol extra pure, AR grade (Product code No: 97900), SRL fine chemicals, Indian Scientific, Tirupati, Andhra Pradesh was procured. VEGF and BAX kits procured from Biogenic Ltd, Bangalore. Horse radish peroxidase (HRP), 3, 3' Diaminobenzidine (DAB) solutions, Indian Scientifics, Tirupati.

Experimental Design

A total of 24 healthy wistar male rats were distributed into 2 groups containing 12 rats in each group. Atherosclerosis was experimentally induced by 1% cholesterol and 15% hydrogenated oil in 1000 g standard rat chow diet (High cholesterol diet) for 90 days (Group II). Group I rats fed normal diet for 90 days and kept as control.

Histopathology

Small tissue pieces of thoracic aorta were collected in neutral buffered formalin for routine histopathological processing by paraffin embedding technique and section were stained with Haemotoxylin & Eosin (H&E) (Culling, 1974).

Immunohistochemistry Procedure

Paraffin sections were cut at 3-4 µ thickness and mounted on 3- Aminopropyltriethoxysilane (APES) coated slides and incubated overnight at 37˚C. De-paraffinized through xylene for 15 min for 2 changes and 2 alcohol dips to remove xylene. Washed under running tap water for 10min and rinsing in distilled water for 5min. Heat induced epitope retrieval was carried out by immersing the slides in the citrate buffer and microwaving at 600 W for 20min, before cooling and rinsing in phosphate buffer saline (PBS) for 5min. The slides were kept in the humid chamber and in the 1% hydrogen peroxidase (H_2O_2 diluted in methanol) block solution for 30min to block the endogenous peroxidase. The slides were washed in PBS for 5min for 3 changes. The power block solution (15mM of sodium azide, pH 7.4-7.6) was put on tissue section for 15min. Sections were incubated with monoclonal mouse anti VEGF/BAX antibodies at 1:50 for two hours at room temperature. Washed in PBS for 5 min for 3 changes. Added super enhancer solution and the slides

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were kept in PBS for 5 min for 3 changes. Sections were incubated with secondary antibody with HRP for 30 min. slides were washed in PBS for 5 min for 3 changes. 3,3' Diaminobenzidine (DAB) colouring reagent was prepared by adding one drop of DAB in 1 ml of substrate. The sections were kept in the colouring reagent for 5-8 min. Washed in PBS for 2 min and in tap water for 2 min. The slides were counter stained with Harris haematoxylin for 1 min. The slides were washed in tap water for 5 min, dried and mounted in DPX.

Results and Discussion

Aorta from atherogenic diet fed group II rats showed initiation of atherosclerotic lesions with intimal thickening, endothelial degeneration, scattered sub intimal foam cells in major portion of the aorta (Fig.1), stromal cell (SMCs) proliferation along with structural alteration, thrombus formation in majority of the cases. Immunohistochemical staining with VEGF revealed almost all ECs were uniformly positive both in large and small vessels present in between cardiac muscle fibers and strong positivity of lipid-laden macrophages in the initiated atherosclerotic lesions. Nucleus and membrane of foam cells revealed distinct immunoreactivity with VEGF. The immunoreactivity was not observed in foamy component of the macrophages, elastic and collagen fibers. VEGF positivity was also evident in thrombus and structurally modified stromal cells in initiated atherosclerotic lesions (Fig. 2). Erythrocytes in the lumen of the aorta also stained with VEGF. Control group I rats not showed any initiated atherosclerotic lesions and positivity with VEGF. (Fig.3). VEGF is produced by a variety of cells including marcophages and smooth muscle cells, which induces migration and proliferation of endothelial cells, enhance vascular permeability and modulate thrombogenicity. VEGF is considered unique among angiogenic growth factors (Berse *et al.*, 1992). It also induces migration of mononuclear cells/monocytes and stimulate their expression of tissue factors and playing an important role in the atherosclerosis. VEGF is expressed in atherosclerotic plaques of aorta, carotid and coronary arteries; however, its role in atherosclerotic plaques has not been fully elucidated (Couffinghall *et al.*, 1997). Positive immunohistochemical staining was observed in structurally modified macrophages, SMCs along with ECs in hyper cellular atherosclerotic lesions (Mayumi *et al.*, 1998). Whereas uniform positivity for VEGF was noticed in advanced atherosclerotic lesions and in all ECs of newly formed micro vessels (Kanno *et al.*, 2014).

VEGF demonstration was significantly higher in the hypercellular and in the initiated lesions of atherosclerosis of group II rats than in normal. Results suggest that VEGF is playing a role in the stimulation of EC proliferation, vascular permeability and altered thrombogenicity as well as migration of mononuclear cells there by initiation and progression of atherosclerosis. Distinct pattern of BAX positivity in both groups indicates varying degrees of cellular apoptosis in the atherosclerotic lesions. Progressive atherosclerotic lesions (Fig.4) with endothelial attached thrombus stained positively for BAX protein in the atherogenic

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diet fed group II (Fig.5) rats suggests more number of cells is under apoptosis. Some authors demonstrated around 2-30% apoptotic cells in the atherosclerotic plaques (Hank *et al.*, 1995) and some reported even upto 60% cells are apoptotic (Mark and Arnold, 2000) and the pattern of apoptosis in macrophages and SMCs could be different, more proliferating and intracellularly fat loaded SMCs shows more apoptosis when compared to normal non proliferating SMCs (Mark *et al*., 2013). Expression of BAX positivity in control group also observed but the extent is minimal (Fig.6).

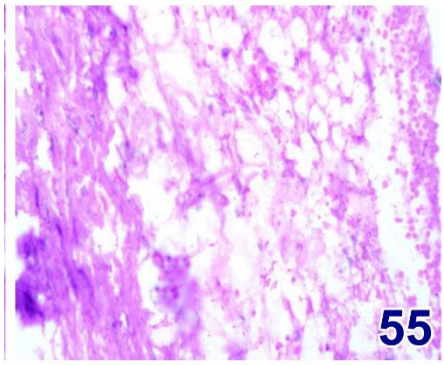

Fig. 1: Aorta:- (Group II): Section showing severe endothelial degeneration, foam cell accumulation with initiated atheromatous plaque. H&E X400

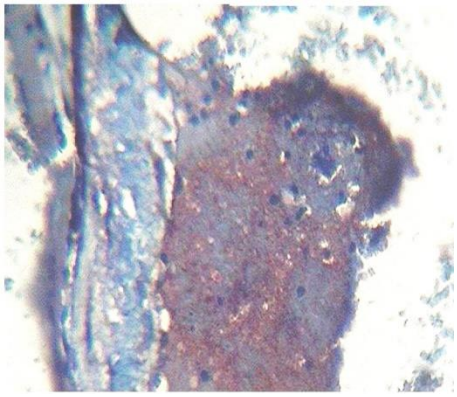

Fig. 2: Aorta:- (Group II): Section of aorta showing endothelial denudation, sub intimal few foam cells with attached thrombus stained positively with VEGF marker. VEGF X400

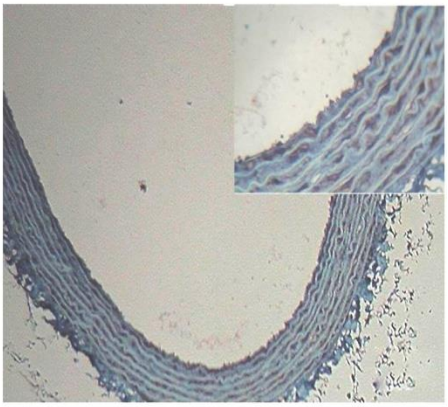

Fig. 3: Aorta:- (Group I): Normal architecture of aorta with endothelial and vascular stromal cell positivity with the VEGF. VEGF X100

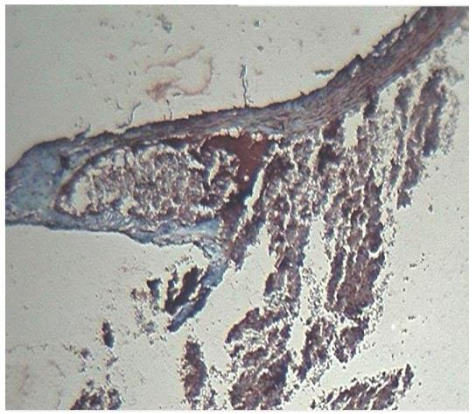

Fig. 4: Aorta:- (Group II): Section of the aorta showing initiated small atheorosclerotic plaque consists of various cells stained positively with Bax protein. Bax X100

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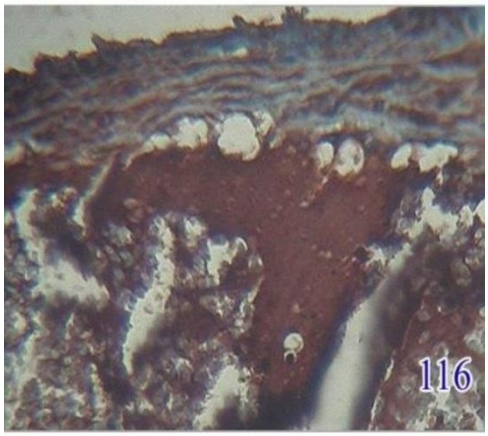

Fig. 5: Aorta: (Group II): Bax positive microthrombus attached to the wall of the lumen of aorta. Bax X400

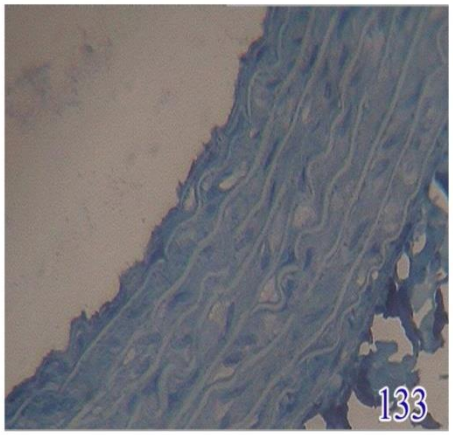

Fig. 6: Aorta:- (Group I): Normal aortic structures without Bax protein positivity. Bax X400

Apoptosis occurs in all cells of the atherosclerotic plaques and it contributes to plaque growth, lipid core development, plaque rupture and thrombosis (Victoria and Martin, 2004). Present findings suggest that, BAX positivity was more in the developed atherosclerotic lesions compared to non-atherosclerotic control group.

Conclusion

VEGF positivity was observed in endothelial cells, thrombus and structurally modified stromal cells of initiated atherosclerotic lesions. Initiated atheromatous plaque showed high levels of positivity with BAX compared with other parts of blood vessels. Need further studies for understanding of various adhesive and cytokine markers which are playing a role in the pathogenic mechanism of atherosclerosis for formulation of various preventive protocols as atherosclerosis is one of the leading causes of mortality in human beings worldwide.

Acknowledgments

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