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Dynamin and perforin are associated with neovascularisation in advanced carotid plaques

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1. ABSTRACT

Intimal plaque neovascularization is associated with the development of symptomatic disease and thrombosis, with new ´leaky´ fragile microvessels prone to haemorrhage. Perforin or pore forming protein is involved in vascular cell death by forming pores in target cells. Enzymes, in particular, granzyme B are secreted by immune infiltrates present in inflammatory plaque regions and have been shown to induce endothelial cell apoptosis. Similarly, dynamin-2 is a GTPase which mediates oxidised low density lipoprotein-induced apoptosis and is also required for granzyme B-mediated exocytosis and apoptosis. Our pilot studies identified increased expression of these proteins in complicated atherosclerotic plaques. Here we demonstrate by immunohistochemistry that both proteins are over-expressed in angiogenic regions of complicated carotid plaques. Dynamin-2 was extensively localised around microvessels and in immune infiltrating cells whilst perforin was localised in immune infiltrating cells, endothelial cells and smooth muscle cells. Over-expression of these proteins may contribute to plaque destabilisation by increasing cellular apoptosis in vulnerable atherosclerotic plaques.

2. INTRODUCTION

Angiogenesis is a recognized feature of the atherogenic process, intimal neovascularization arising most frequently from the dense network of vessels in the adventitia, adjacent to a plaque, rather than from the main artery lumen. New blood vessels may have an active role in promoting plaque metabolic activity and its growth beyond the critical limits of diffusion from the artery lumen (1). Our previous studies and those of others have suggested that haemorrhagic, leaky blood vessels from unstable carotid plaques proliferate abnormally. Immature neovessels may contribute to instability of the plaque by facilitation of inflammatory cell infiltration and haemorrhagic complications. In a study of coronary artery atherogenesis, from patients subjected to heart transplant, lesions with the highest neovessels content were demonstrated to be of type VI and associated with the highest rate of thrombotic episodes (2).

Perforin is a soluble pore-forming cytolytic protein synthesized in cytotoxic T lymphocytes (CTL) and natural killer (NK) cells and is sequestered into secretory cytotoxic granules. It can perforate target cell membranes by forming transmembrane pores in cells that lose membrane integrity and die by colloid osmotic lysis (3).

Table 1. Plaques examined by immunohistochemistry

Patient ID	Age at death	Sex	Plaque type	Plaque description	Dynamin EC	Dynamin SMC	Perforin EC	Perforin EC/SMC
A	69	M	Complicated/ thrombotic	Gross Inflammation, angiogenesis, focal necrosis	XX	XXX	XX	XXX
B	68	M	Stable/non-complicated	Lipid core, mild inflammation	XX X	X	X	X
C	79	M	Complicated/haemorrhagic	Gross inflammation, rupture, angiogenesis, calcification	XX	XX	XX	XXX
D	75	M	Normal	Type 1, thickening of intima	-	-	X	X
E	86	F	Stable/non-complicated	Mild inflammation, lipid core, angiogenesis, focal necrosis	XX	XX	XX	XXX
F	76	F	Normal	Thickening of intima	-	X	-	X
G	56	M	Normal	Thickening of intima	X	X	-	X
H	74	M	Complicated/ thrombotic	Calcification, mild inflammation, Angiogenesis	XX	XX	XX	XX
I	70	M	Complicated/ thrombotic	Gross inflammation, angiogenesis, focal necrosis, calcification, lipidic core	XX	XX	XX	XXX
J	82	M	Stable/non-complicated	Calcification, inflammation, angiogenesis, erosion	XX	XX	XX	XX
K	66	M	Complicated/haemorrhagic	Gross inflammation, calcification, angiogenesis, rupture, lipid core	XX	XX	XX	XXX

-: negative; X weak, XX medium; XXX strong staining

Perforin is a key component of the cytolytic machinery. On secretion it binds to and inserts into the phospholipid bilayer of the target cell plasma membrane, and polymerises to form a pore of ~16nm diameter. The pore induces cell death in one of two ways: (i) by osmotic lysis or (ii) by enabling other lytic granule components, most notably granzyme B, to enter the target cell and initiate apoptosis (4). Perforin is synthesized in the rough endoplasmic reticulum and travels through the golgi apparatus, where post-translational modification occurs and is packaged into cytoplasmic granules. The release of perforin is predominantly controlled by regulated secretion. A second pathway of perforin secretion has been demonstrated in which newly synthesized perforin is released directly from the cell without prior inclusion in granules (5). It has been shown that perforin produced by this constitutive pathway is functional (6). Recent research has shown an involvement of the perforin/granzyme B pathway in mediating apoptosis in atherosclerotic lesions and that granzyme B/perforin may affect the stability of atherosclerotic plaques (7). Dynamin-2 has been shown to facilitate granzyme B endocytosis into target cells via post-translational modifications including tyrosine phosphorylation, thereby promoting apoptosis (8). However, other studies have shown that vascular endothelial cell growth factor-2 (VEGFR-2; KDR) which is also found in vascular atherosclerotic lesions can co-localise with dynamin-2 and that this process is necessary for endothelial cell survival and signalling (9). Similarly, inhibition of dynamin-2 GTPase function reduced bovine aortic endothelial cell growth, whilst nitric oxide, directly promoted dynamin-2 function through cysteine residue nitrosylation, thereby promoting endocytosis and survival signalling (10). The expression and localization of these proteins is therefore of interest as they may have a significant role in determining vascular cell survival and subsequent plaque stability in atherosclerotic lesions.

3. MATERIALS AND METHODS

3.1. Patient samples

Human carotid endarterectomy specimens (n=11) were obtained from patients with a significant degree of carotid stenosis as demonstrated by duplex ultrasonography. Extensive neurological examination and histology was used to classify plaque regions into ulcerated, unstable, fibrous, calcified, haemorrhagic, and inflammatory groups. Full clinical and biochemical data were collected (Table 1). The specimens were opened longitudinally, cut whilst frozen into 2mm segments and fixed in 10% formaldehyde for immunohistochemistry. All patients gave informed consent and the protocol was approved by the appropriate ethical committee.

3.2. Immunohistochemistry

Paraffin embedded sections were deparaffinised and rehydrated in graded ethanol solutions. Slides were then rinsed in distilled water and treated with 0.5% hydrogen peroxide in methanol (30 min at room temperature) to neutralise endogenous peroxidase activity. Sections were placed in antigen unmasking solution in a pressure cooker for 15 min before blocking with diluted serum (20 min at room temperature). Primary anti-perforin and anti dynamin-2 antibodies (Autogen Bioclear, USA) (1:50) were added and sections incubated overnight at 4 oC. After rinsing in PBS, biotinylated secondary antibody (Vector laboratories) was applied for 30 min and the section rinsed in PBS. The standard Vectastain avidin-biotin-peroxidase complex was applied and incubated for 30 min. After rinsing in PBS, colour was developed using DAB peroxidase. Sections were counter stained with haematoxylin before dehydration, cleaning and mounting. Staining specificity was confirmed by using control sections in which the primary antibody was replaced with PBS.

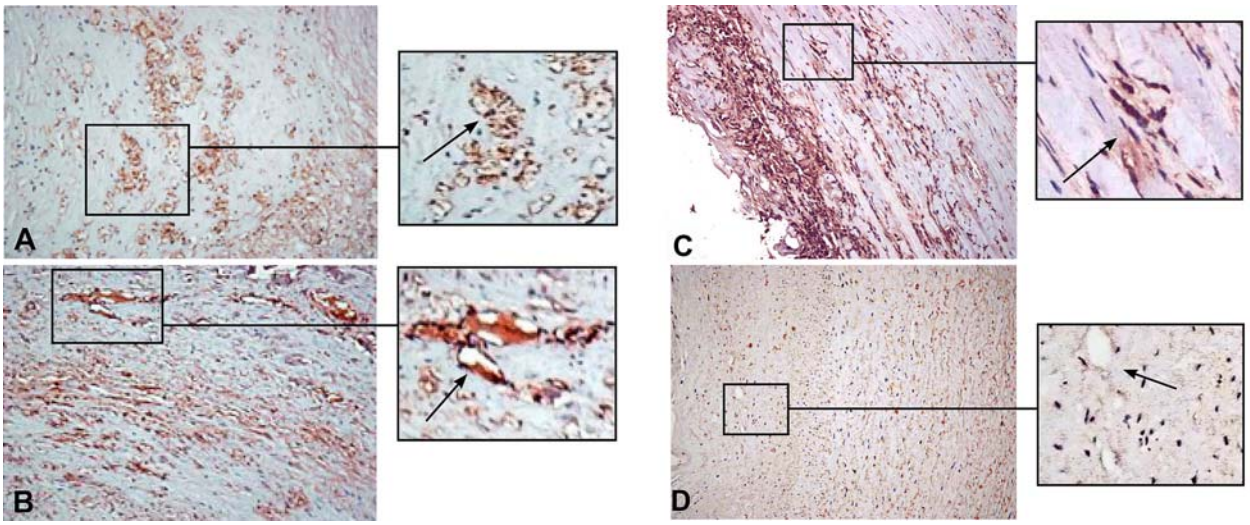

Figure 1. Perforin staining of carotid plaques.A, shows positive staining for inflammatory cells of unstable plaques (patient C shown; arrow shows stained inflammatory cells), B, shows staining of neo-intimal blood vessels (patient A shown; arrow shows stained endothelial cells), C, shows strong staining in smooth muscle cells in stable plaques (patient E shown; arrow shows stained smooth muscle cells. D, shows type I-II plaque with intimal thickening and very weak or no staining of perforin in blood vessels (arrow) and smooth muscle cells (plaque D). Magnification was, x 40 (main pictures) and x 100 insets.

4. RESULTS

4.1. Perforin expression in atherosclerotic plaques

Immunohistochemical analysis demonstrated specific cellular localization of perforin in hemorrhagic and ulcerated regions in individual plaques. There was highly selective staining with this antibody with stained areas being close to unstable regions where ulceration and haemorrhage was visible together with macrophage infiltrates (Figure1A). There was abundant staining around plaque intimal neovessels (Figure1B) and in smooth muscle cells (Figure1C). Non-complicated plaques and plaque regions had much lower expression (Figure1D).

4.2. Dynamin-2 expression and localization

Immunohistochemical analysis demonstrated specific cellular localization of dynamin-2 in hemorrhagic and ulcerated regions of complicated plaques (Figure2). Again, there was highly selective staining with this antibody, with intimal neovessels close to areas of thrombosis being strongly stained (Figure2B), as well as sites of inflammatory/macrophage infiltration (Figure2C). Non-complicated plaques and plaque regions had no noticeable expression of dynamin-2 (Figure2D-E).

5. SUMMARY AND PERSPECTIVES

Angiogenesis is a critical component of plaque pathobiological development and regions of neovascularization are especially prone to rupture and thrombosis. The angiogenic processes are in part initiated by hypoxia generated in the plaque, the specific action of growth factors, particularly vascular EC growth factor (VEGF) and basic fibroblast growth factor (FGF-2), secreted by vascular and inflammatory cells and other factors such as hemodynamic stress. The expression and role of membrane trafficking proteins has not been examined in atherosclerotic lesions. Our pilot data obtained from protein microarray studies demonstrated increased expression of both perforin and dynamin-2 in complicated unstable carotid atherosclerotic plaques, and this prompted us to explore their expression and localization by immunohistochemistry in atherosclerotic lesions (11). Here we showed a dramatic increase in perforin expressing cells in haemorrhagic, inflammatory regions of advanced carotid plaques. There was a particular association with plaque neovessels close to areas of rupture.

Previous work has shown that patients with unstable angina had a population of perforin expressing $CD4^+$ T-cells that were absent in patients with stable angina. B cells, NK and T- lymphocytes and macrophages have all been observed in atherosclerotic lesions (12, 13). Increased expression of perforin may originate partially from its release by NK cells, B cells and T-cells present as infiltrates in plaques. Its expression may contribute to plaque instability due to increased regional cellular apoptosis. Immunohistochemistry localised the expression of perforin to inflammatory cells, cells surrounding neovessels and also smooth muscle cells. The expression of perforin in smooth muscle cells has not previously been identified to our knowledge and may represent a novel finding and mechanism of initiation of smooth muscle cell death. The involvement of perforin in angiogenesis is poorly understood however it is known that the formation of new unstable vessels leads to plaque instability. Perforin expressing $CD4^+$ T-cells isolated from the peripheral blood of patients with atherosclerosis were able to induce endothelial cell apoptosis (12). Granzyme B in combination with perforin has been shown to induce apoptosis of vascular endothelial cells (13). We were also

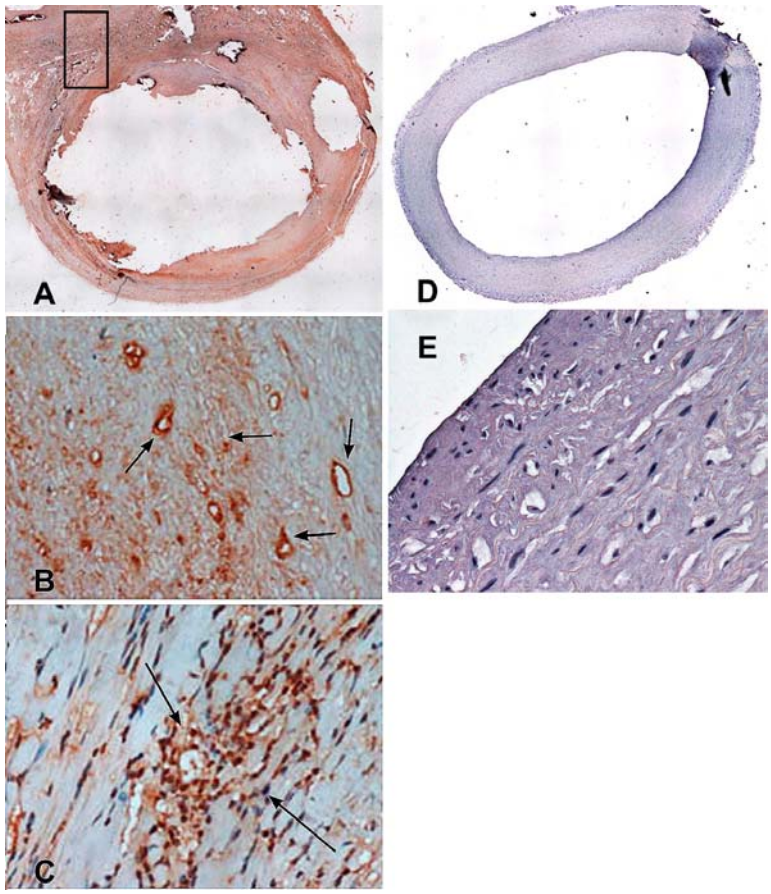

Figure 2. Dynamin-2 staining of carotid plaques.A, shows low power (x10) view of a ruptured plaque (patient K), B, shows staining of neo-intimal blood vessels (x 40, patient K shown; arrow shows stained neovessels), C, shows strong staining in inflammatory infiltrates of the same plaque (x 40, arrows). D and E show stage I-II atherosclerotic artery with media thickening and no observable expression of dynamin-2 (x10 and x40 respectively; plaque D).

able to demonstrate the presence of perforin in vascular smooth muscle cells. Our further studies have confirmed the expression of perforin in cultured vascular smooth muscle cells (unpublished data). Increased apoptosis in intimal and/or medial smooth muscle cells would increase plaque weakness resulting in increased likelihood of rupture.

The co-expression of dynamin-2 in the same regions suggests a potential mechanism for enhancement of granzyme B trafficking which could further promote apoptotic cell death (8), however, since dynamin-2 has also been associated with pro-survival membrane trafficking in vascular endothelial cells, particularly in the presence of cytokines and growth factors the overall outcome is still in question (9, 10). Increased angiogenesis together with apoptosis might be even less beneficial for maintenance of plaque stability.

In summary, although the expression of dynamin-2 and perforin in atherosclerosis may contribute to the pathology of the disease, further investigation of their role in progression of this disease and the mechanisms involved needs to be evaluated by using *in vitro* systems.

6. AKNOWLEDGEMENTS

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Abbreviations : CTL: Cytotoxic T Lymphocytes. NK: Natural Killer cells, VEGF: Vascular Endothelial cell Growth Factor, FGF-2: Fibroblast Growth Factor-2.

Key Words: Angiogenesis, Perforin, Dynamin, Atherosclerosis, Plaque Rupture

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