ULTRASTRUCTURAL ALTERATIONS OF ENDOTHELium COVERING ADVANCED ATHEROSCLEROTIC PLAQUE IN HUMAN CAROTID ARTERY VISUALISED BY SCANNING ELECTRON MICROSCOPE

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Human atherosclerotic plaque morphology at its various stages was extensively documented using light microscopy. However, much less is known of the ultrastructure of the human atherosclerotic plaque, in particular of ultrastructure of endothelial cells in atherosclerosis. Here, we analysed alterations of endothelial cells covering advanced atherosclerotic plaque in carotid artery using scanning electron microscope. Examination was performed on specimens from atherosclerotic lesions of the interior carotid artery, collected from 8 patients who had undergone endarterectomy. We found wide spectrum of pathological alterations of the luminal surface of atherosclerotic plaque. In dominant part of the vessel, endothelial layer was preserved but displayed pronounced irregularities in endothelial architecture including appearance of cuboidal cells. Some endothelial cells were covered by numerous microvilli and/or contained "craters" disrupting continuous surface of the endothelium. Platelets and leukocytes adhering to endothelium were frequently observed. There were also areas of the vessel lumen with endothelial denudation, in which the subendothelial surface containing fibrin proteins and collagen fibrils were visible. Interestingly, signs of proliferation of endothelial cells tending to cover the partially denuded vessel were observed. In summary, in scanning electron microscope, preserved endothelial cells of advanced atherosclerotic plaque displayed pronounced pathology; whether any of these changes represent the ultrastructural correlate of endothelial dysfunction remains to be established.

Key words: atherosclerosis, carotid artery, endothelium, scanning electron microscope.
INTRODUCTION

Atherothrombosis is a major cause of ischaemic heart disease, stroke and peripheral vascular disease and, therefore, the leading cause of death. Recently, the classical view on atherosclerosis as cholesterol-driven pathology has given way to understanding of atherosclerosis as a chronic inflammatory disease of the vascular wall. Still, the mechanisms involved are far from being understood. It is becoming clear, however, that endothelial dysfunction plays a key role in the formation and progression of atherosclerotic plaque (1,2). Indeed, endothelial dysfunction has gained diagnostic, therapeutic, as well as prognostic significance in atherosclerosis (3-5).

Historically, in their original formulation of "response to injury hypothesis" of atherosclerosis, Ross and Glomset proposed that overt endothelial injury was responsible for the formation of atherosclerotic lesion (6). However, subsequent studies in experimental models of atherosclerosis as well as examination of early human atherosclerotic plaques failed to document frank endothelial injury. Today, it is widely accepted that it is not overt endothelial injury but endothelial dysfunction, characterised by the loss or by the dysregulation of homeostatic mechanisms normally operative in healthy endothelial cells, that promotes the development of atherosclerosis (7). Clinically, the loss of endothelial vasculoprotective properties is frequently diagnosed as an impairment of endothelial NO-dependent vasodilation (4), which seems to predict future cardiovascular events (3). Other biochemical changes associated with endothelial dysfunction in atherosclerosis include: impairment of PGI₂ synthesis (8), increased vascular production of superoxide anion (3), decreased t-PA activity (9), increased plasma levels of PAI-1 (10), von Willebrand factor (11), endothelin-1 (12) as well as several markers of inflammation, such as cytokines (e.g. IL-6), soluble adhesion molecules (e.g. P-selectin or sICAM-1), and finally, hs-CRP (13).

In fact, endothelial dysfunction in atherosclerosis is being extensively characterised at a biochemical, and molecular level. In contrast, data on the morphological alterations of endothelial cells in experimental (14-16), or in human atherosclerosis (17,18) are limited. In this study, using a powerful tool such as scanning electron microscope, we attempt to evaluate ultrastructural alterations of endothelial cells covering advanced atherosclerotic plaque in human carotid artery looking for the ultrastructural signs of endothelial dysfunction.

MATERIAL AND METHODS

Specimens from human interior carotid artery (just above bifurcation of common carotid artery), were obtained from patients who underwent endarterectomy. This study was performed on carotid endarterectomy specimens from 8 patients (7 men and 1 woman) aged 58-72 years old. All
8 patients underwent endarterectomy because of the substantial narrowing of the lumen of the interior carotid artery, associated with atherosclerotic plaque. In this study, scanning ME photographs from one representative specimen are shown.

For examination under light microscopy vessel specimens were fixed in 4% buffered formalin (pH 7.4). For examination under scanning electron microscope vessel sections were fixed using a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde, in 0.05M cacodylate buffer at a pH 7.4. Subsequently, vessel tissue was fixed in a mixture of 1.6% K₄Fe(CN)₆ and 2% OsO₄. After fixing, tissue sections were dehydrated in series of alcohols and acetate solutions, critical-point dried, and prepared to examination in scanning electron microscope JEOL 1200EX using standard procedures.

All procedures were carried out according to EU directives and reviewed by local ethical committee.

RESULTS

The major part of the luminal surface of the carotid artery with advanced atherosclerotic plaque was covered by intact endothelium. However, area of endothelial denudation was also detected (Fig. 1, Fig. 2).

Scanning electron microscope examination was performed on the luminal surface of the vessel wall in the region that was situated between the area covered and uncovered by endothelium (Fig. 2).

We found wide spectrum of pathological alterations of the luminal surface of atherosclerotic plaque. Luminal vessel surface was covered by endothelial cells, which were irregularly orientated and morphologically changed, (Fig. 3). In particular many of the endothelial cells had cuboidal appearance protruding into the lumen of the vessel (Fig. 3). Some endothelial cells were covered by numerous microvilli and/or contained "craters" disrupting continuous surface of the endothelium (Fig. 4, Fig. 5). Platelets and leukocytes adhering to endothelium were frequently observed (Fig. 3, Fig. 4). In region of the vessel with endothelial denudation the exposure of underlying connective tissue was visible (Fig. 6). Subendothelial surface was formed by fibrin proteins and collagen fibrils (Fig. 7). In these regions also signs of proliferation and migration of endothelial were sometimes detected. As shown in Fig. 8, newly-formed, endothelial cells tends to cover partially denuded luminal surface of the vessel.

DISCUSSION

In this study, using scanning electron microscope technique, we presented wide spectrum of alterations of endothelial cell covering advanced atherosclerotic plaque in human carotid artery.

Endothelial layer of healthy human artery has a regular pattern with endothelial nuclei uniformly arranged in parallel and spindle-shape endothelial cells (7). This usual polarization of endothelial cells in the direction of flow was lost in endothelium covering advanced atherosclerotic plaque in specimens presented here. It could well be that high-grade stenosis of carotid artery was
**Fig. 1.** Cross-section of human carotid artery under light microscope at low magnification (hematoxylin and eosin staining - H.E., magnification x15). Atherosclerotic plaque occupying a major part of carotid artery circumference and substantially narrowed lumen of the vessel are visible.

**Fig. 2.** Cross-section of carotid artery under light microscope at high magnification (H.E. staining, magnification x100). Endothelial cells covering atherosclerotic plaque are preserved in major part of vessel circumference. However, the region with disrupted endothelium is also visible. Arrows indicate places with preserved endothelium (fig. 3-5) and with disrupted endothelial layer (fig. 6-8). Scanning ME photographs were taken in the area that was situated between the region covered and uncovered by endothelium.
Fig. 3. Scanning ME of the surface of endothelium covering atherosclerotic plaque (magnification x1200). Endothelial cells covering atherosclerotic plaque are irregular in shape. Many of them have cuboidal appearance. Single cuboidal endothelial cell or their clusters protrude into the lumen of the vessel. Platelets sticking to endothelium are visible.

Fig. 4. Scanning ME of the surface of endothelium covering atherosclerotic plaque (magnification x2400). Endothelial cells contains numerous microvilli (indicated by arrows). Aggregated platetels and leukocytes adhering to endothelium are visible.
Fig. 5. Scanning ME of the surface of endothelium covering atherosclerotic plaque (magnification x2400). Surface of endothelial cells is covered by microvilli. Endothelial cell layer is disrupted by numerous craters (indicated by arrows).

Fig. 6. Scanning ME of the surface of vessel wall in the region with endothelial disruption (magnification x2800). Endothelial cells are only partially preserved and contains numerous craters. Subendothelial surface (indicated by arrows) is visible.
Fig. 7. Scanning ME of the surface of vessel wall in the region without endothelial layer (magnification x2800). Subendothelial surface is apparent with fibrous cap formed by fibrin proteins and collagen fibrils.

Fig. 8. Scanning ME of the surface of vessel wall in the region with endothelial disruption (magnification x2400). Area of altered endothelium and subendothelial space covered by fibrous cap are visible. Proliferation of endothelial cells towards areas, which are not covered by endothelium is indicated by arrows.
associated with turbulent (non-laminar) flow, which may have a pronounced effect on endothelial cells morphology leading to changes of endothelial appearance, in particular in down-stream region of the plaque (17). However, striking irregularities of endothelial cells shapes and morphology shown here (fig. 3), could not be entirely explained by effects of turbulent flow. Although in some specimens elongated endothelial cells in parallel alignment were seen (fig. 4), endothelial surface layer of atherosclerotic plaque was dominated by irregularly located endothelial cells which were cuboidal in appearance. Single cuboidal cells or their clusters protruded substantially into the vessel lumen (fig. 3). Cuboidal appearance of the endothelial surface of the atherosclerotic lesion was previously observed in monkeys with experimental atherosclerosis induced by fat-rich diet in monkey (16) as well as in early human lesions (18). This change in shape of endothelial cell was suggested to be due to the large number of accumulated lipid-filled macrophages or foam cells in the intima of the vessel and bulging of the endothelial cells covering them (16,18). This suggestion, however, cannot explain the presence of cuboidal endothelial cells in advanced atherosclerotic plaque in human carotid artery, which possesses a highly characteristic architecture of a fibrous cap overlaying the central core. Rather, we suggest that cuboidal endothelial cells may represent a phenotype of activated endothelial cells. Indeed, cuboid endothelial cells, which were detected in animal models of atherosclerosis (14,16) and in human plaques (17) contained high content of rough endoplasmic reticulum, abundant Weibel - Palade bodies with von Willebrand factor, as well as increased number of mitochondria (14,16,17). These changes are suggestive of an increased level of protein biosynthesis, consistent with cell hypertrophy. Endothelial cells covering advanced atherosclerotic plaque display not only pro-thrombotic phenotype as evidenced by increased content of von Willebrand factor (17) but also pro-inflammatory phenotype as evidenced by increased level of activated NF-kB and increased expression of adhesion molecules (19). It may well be that increased oxidative stress is causally involved in the alterations of endothelial phenotype in atherosclerosis (20, 21).

Interestingly, there is additional evidence, which could support the assumption that cuboidal shape of endothelial cells reflect pro-inflammatory and pro-thrombotic phenotype of endothelium. In a well-established model of atherosclerosis in cholesterol-fed rabbits, endothelial dysfunction is present and include impaired NO-dependent vasodilatation (22), decreased PGI₂ production (8), increased endothelial expression of adhesion molecules, such as ICAM-1 (23), and increased release of von Willebrand factor (14). Interestingly, cuboidal endothelial cells appeared upon cholesterol feeding in this model (15). Approximately, one month after cholesterol withdrawal endothelial cells changed their appearance from cuboidal to flat again (15). Thus, it is tempting to speculate that hypertrophic changes of endothelial cells may represent and adaptive cellular response induced by endothelial cell insult. Whether cuboidal
endothelial cells represent an ultrastructural correlate of endothelial dysfunction with its pro-thrombotic and pro-inflammatory phenotype, still need to be confirmed.

Noteworthy, in several specimens of atherosclerotic carotid artery platelets, platelets microthrombi, or leukocyte were adhering to endothelial surface. These findings are in accordance with previous studies (18) and support the notion of diminished anti-thrombotic and anti-adhesive properties of the dysfunctional endothelium in atherosclerosis.

In addition to changes in endothelial appearance in atherosclerotic plaque, at higher magnifications we observed numerous microvilli covering endothelial surface. We suspect that they might reflect increased permeability of dysfunctional endothelial cells. However, since appearance of microvilli on endothelium has not been hitherto analysed, this remains only a hypothesis to be tested. Moreover, in some parts of lumen surface of the atherosclerotic plaque, integrity of endothelial cells was lost as evidenced by craters or cavities in the endothelial cells. In areas of complete desquamation of endothelium, the exposure of underlying connective tissue and fibrous coat was observed.

It is generally accepted that atherosclerotic plaques are covered by an intact endothelial layer throughout most of the stages of lesion progression (24) and the major cause of the thrombotic events is due to the plaque rapture (25). However, recently it was suggested that plaque erosion without a rupture may also constitute a substrate for acute cardiovascular death (24). Importantly, on erosion sites endothelium is absent (26). Interestingly, apoptosis of endothelial cells was proposed to explain the erosion and desquamation of endothelial layer covering atherosclerotic plaque (27). Surprisingly enough, animal models of atherosclerosis did not confirm existence of areas of endothelial denudation in atherosclerotic vessels (16). They were rather considered to be artefacts of specimen preparation or fixation (16). Although it seems unlikely, we can not exclude that endothelial denudation observed here in the endarterectomy specimens is a manifestation of vasospasm (26) related to surgical procedures or due to the preparation procedures, and it is not an inherent feature of the morphology of advanced atherosclerotic plaque.

We also detected signs of regeneration process in the area of disrupted endothelium covering atherosclerotic plaque. As shown in Fig. 8, newly-formed, endothelial cells tended to cover partially denuded luminal surface of the vessel. Indeed, matrix synthesis and proliferation of endothelium which occur in order to reestablish lost intercellular connections are dominant events in response to vessel injury (28,29).

In summary, preserved endothelial cells of advanced atherosclerotic plaque displayed pronounced pathology at an ultrastructural level. Whether any of these changes represent ultrastructural correlate of endothelial dysfunction remains to be established.
REFERENCES


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