Increased Shedding of Microvesicles from Intimal Smooth Muscle Cells in Athero-Prone Areas of the Human Aorta: Implications for Understanding of the Predisease Stage

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Abstract

Objective: This study evaluated whether a change in the content of matrix microvesicles might occur at the preatherosclerotic stage. Methods: Applying quantitative electron microscopic and immunohistochemical analyses, two areas of grossly normal segments of the thoracic aorta were compared: atherosclerosis-prone (AP) areas, situated at the dorsal aspect of the aorta along the rows of intercostal branch origins, and atherosclerosis-resistant (AR) areas, situated at the corresponding sites of the ventral aspect of the aorta. Results: The electron microscopic analysis showed that there were 1.4 times more microvesicles in AP areas than AR areas (p = 0.019). It was found that matrix microvesicles originated as a result of blebbing and shedding of surface membranes of smooth muscle cells. A quantitative analysis of the expression of ADP-ribosylation factor 6 (ARF6), which is known to be involved in membrane trafficking and microvesicle formation, showed that ARF6 expression was 1.3 times higher in AP areas than that in AR areas (p = 0.006).

There was a positive correlation between the content of matrix microparticles and the expression of ARF6 by intimal smooth muscle cells (r = 0.61; p < 0.0001). Conclusion: The present study supports the concept that alterations of the arterial intima occur at the predisease stage.

Key Words

Human aorta · Intima · Athero-prone areas · Athero-resistant areas · Microvesicles

Introduction

The topographic distribution of atherosclerotic lesions in the human arterial tree has been extensively surveyed [1–5] and athero-prone (AP) as well as athero-resistant (AR) areas of the aorta have been identified and mapped [1, 2, 5]. However, why some areas are prone to lesion formation is not clear, nor have the structural alterations prior to the identification of frank atherosclerotic lesions been well studied [1–7].

In our earlier investigations of the possible changes that might occur at the preatherosclerotic stage of the alteration of the arterial wall we compared several parameters between AR and AP areas of the human aorta [6, 7], selected in accordance with probability-of-occurrence maps [5]. The areas with different predisposition to the
development of atherosclerosis were examined for factors which are thought to relate to putative atherogenic mechanisms such as the accumulation of different class lipids and lipoproteins [6, 7]. An analysis utilizing an antibody to apolipoprotein B and an immunofluorescent technique has revealed that the content of apolipoprotein B was significantly larger in AP areas [6]. AP areas were also found to be characterized by destructive alterations of elastic fibers, ‘vacuolization’ of which was accompanied by the accumulation of neutral lipids and unesterified cholesterol within the damaged fibers [7]. Apart from these differences in the matrix structure and lipid content, the intima of AP areas have been found to contain an increased number of immune-inflammatory cells including monocytes and dendritic cells, which were predominantly localized in the subendothelial space [8–10].

Although the precise mechanisms for initiating the development of atherosclerosis are still insufficiently understood, amongst other molecular structures that have an impact on the pathogenesis, microparticles have recently attracted interest as novel signaling structures [11–13]. These represent microvesicles that are released from cells as a result of exocytic budding of the surface membrane during cell activation or cell death. During this process, the membrane asymmetry is lost and phosphatidylserine, which is normally confined to the inner leaflet, appears on the outer leaflet of the microparticle membrane [11–13]. In addition to the altered surface lipids, microparticles display cell surface markers, specific to the cell type from which they originate [11–13]. As such, microparticle surfaces are enriched by a spectrum of regulatory and triggering molecules [11–13]. It has been reported that microparticles play a role in intercellular communication by means of the exchange of mRNA, miRNA and a variety of proteins between cells and thus, participate in the regulation of important biological processes [11–13]. An important role of microparticles in the initiation of immune responses has also been suggested [11–13].

No previous studies have been undertaken in order to examine a possible role of microvesicles in the initial stages of atherogenesis and this motivated us to investigate whether there might be a difference in the content of microvesicles between macroscopically normal AR and AP areas of the aorta. These aortic areas were further compared regarding the expression of ADP-ribosylation factor 6 (ARF6), a member of the ADP-ribosylation factor family of GTP-binding proteins, which is involved in membrane trafficking and microvesicle formation [14–16].

Methods

Autopsy Material

The material was collected in accordance with the ethical guidelines for consent contained within the Helsinki Declaration and the Medical Research Council’s statement on responsibility in investigation on human subjects. The study was approved by the Ethics Committee of the Institute for Atherosclerosis Research.

The technique of tissue collection and the source of the specimens have previously been described [7, 9]. In the present study archival tissue specimens were used [7, 9]. Segments of descending thoracic aorta, analyzed in the present study, were obtained at autopsy from 13 trauma victims aged between 16 and 40 years. After the adventitia and any adhering adipose tissue had been removed, the aortas were opened longitudinally on their dorsal side between the paired rows of intercostal branch origins. Small pieces (approx. 1.0 cm² of the luminal surface) of tissue without visible fatty streaks or other atherosclerotic lesions were taken from AP areas situated on the dorsal aspect of the aorta along the rows of the intercostal artery origins (fig. 1). Similar small tissue samples were taken from AR areas on the opposing ventral wall. Each tissue specimen was divided into several parts; one part was used for routine processing and embedding in Araldite resin blocks for further electron-microscopic analysis, while other parts from each specimen were processed for further immunohistochemical examination.

The available characteristics of the donors and postmortem intervals are summarized in table 1. There were no clinical records which would indicate that the subjects have had major systematic diseases such as hypertension, diabetes, collagen-vascular diseases or cancer.

Electron Microscopy and Quantitative Analysis of Matrix Microparticles

For further electron-microscopic analysis, up to 10 small tissue fragments (approx. 1 mm³ each) were cut from each tissue specimen in 1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), fixed in the same fixative and routinely processed and embedded in Araldite resin. Ultrathin sections taken from each Araldite block were stained with uranyl acetate and lead citrate and examined with the aid of Hitachi H7000 and Morgagni 268D electron microscopes. From each Araldite block, 10 ultrathin sections were used for the quantitative analysis of matrix microparticles. The quantitative analysis of microvesicles was carried out at ×56,000 magnification. In each ultrathin section, matrix microparticles were calculated in a standard area of 1.92 µm² (10 random areas of the extracellular matrix in each specimen were analyzed), as was used previously [17, 18].

Immunohistochemistry and Quantitative Analysis of ARF6 Expression

Immunostaining Procedures

The tissue blocks containing aortic wall segments were cross-cut into 5-µm consecutive tissue sections. For single immunostaining, after the elimination of endogenous peroxidase activity by 3% H₂O₂, sections were preincubated with normal nonimmune serum and then tested by avidin-biotin complex (ABC) using a standard ABC immunoperoxidase method.

Cell composition of the aortic tissue specimens was analyzed using a set of cell type-specific antibodies. Smooth muscle cells were identified with anti-smooth muscle α-actin (1A4; Dako, No. M0851;
1:400 dilution) and anti-SM22 antibody (Abcam; No. ab89989; 1:250 dilution). T cells were identified using anti-CD3 (Dako; No. A0452; 1:100 dilution); monocytes were identified with anti-CD14 (Dako; No. M0825; 1:20 dilution); macrophages were identified with anti-CD68 (Dako; No. M0876; 1:50 dilution); dendritic cells were identified using anti-S100 (Dako, No. Z0311; 1:500 dilution).

In order to analyze the functional state of cells relevant to microvesicle formation, the expression of ARF6 was examined using anti-ARF6 antibody (Abcam; 1:50 dilution). Briefly, after washing in Tris-phosphate-buffered saline (TPBS), pH 7.6, the sections were incubated with an appropriate biotin-labeled secondary antibody, followed by a treatment with avidin-biotin complex. After washing in TPBS, brown staining was produced by 5-min treatment with 3,3’-diaminobenzidine. All the incubations were completed at room temperature. For negative controls, the first antibodies were omitted or the sections were treated with an immunoglobulin fraction of nonimmune serum as a substitute for the primary antibody. None of the negative control sections showed positive immune staining.

Double immunostaining utilized a combination of anti-ARF6 and anti-smooth muscle α-actin antibodies. Double immunostaining procedures were carried out as described in earlier publications [19]. In brief, after visualization of ARF6, sections were washed with 0.1 M glycine-hydrochloric acid buffer, pH 2.2, and then incubated with anti-smooth muscle α-actin antibody. After rinsing in TPBS, the sections were incubated with biotinylated secondary antibody and then with alkaline phosphatase-conjugated streptavidin (Dako) or with avidin-biotin complex (Dako). A combination of the peroxidase-anti-peroxidase and alkaline phosphatase-anti-alkaline phosphatase techniques were also used. Controls were as for single immunostaining.

Quantitative Analysis of ARF6 Expression
A computerized quantitative analysis of ARF6 expression was carried out at ×400 magnification using the Image-Pro Plus image analysis program (Media Cybernetics, Bethesda, Md., USA). Ten serial sections from each tissue sample were used for the analysis. The expression of ARF6 in each section was measured in pixels per 10 standard microscopic fields (0.04 mm² each) and the results were presented as means from all sections of the sample as used previously [20].

Statistical Analysis
The results for variables were expressed as mean ± SD. Statistical analysis was performed by t test using Prism® 5 (GraphPad Software, San Diego, Calif., USA). Significance was defined at 95% confidence level.

Results

Histological Analysis of AR and AP Areas of the Aorta
No difference in the structural appearance between AR and AP areas was detected in hematoxylin-eosin-
stained sections (online suppl. fig 1; for all online suppl. material, see www.karger.com/doi/10.1159/000339430).

**Analysis of Microvesicles in AR and AP Areas of the Aorta**

Electron microscopic analysis of ultrathin sections showed that nondiseased areas of the aorta contained microvesicles irregularly distributed in the extracellular matrix (fig. 2a, b). In both AR and AP areas there was a marked heterogeneity in the structural appearance of microvesicles, relating to differences in electron density and texture of the core portion of microvesicles (fig. 2a, b). Microvesicles ranged from 90 to 400 nm in diameter and no difference in their structural appearances between AR and AP areas was observed (fig. 2a, b). All microvesicles identified in the sections were surrounded by membranes. A quantitative analysis of microvesicles demonstrated that there were 1.4 times more microvesicles in AP areas than in AR areas (6.246 ± 0.463 vs. 4.454 ± 0.543; p = 0.0192; fig. 2c). Calcifying matrix microvesicles that contained incorporated microparticles of a very high electron density were very rarely observed in both AP and AR areas.

Electron microscopic analysis demonstrated that the vast majority of cells constituting the analyzed intimal areas were presented by smooth muscle cells, readily identifiable by the presence of distinctive features such as myofilaments, basal membrane and basal membrane-associated dense bodies [21, 22]. Examination of the surface of intimal smooth muscle cells revealed the presence of structures along the basal membranes which were identified as shedding microvesicles (fig. 3a, b). The shedding microvesicles displayed different degrees of separation from the cell surface (fig. 3a, b). The structural appear-

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**Fig. 2.** Matrix microvesicles in the intima of grossly normal aorta. **a, b** Matrix microvesicles in the extracellular space of AR and AP areas, respectively; transmission electron microscopy. Scale bar = 400 nm. **c** Quantitative analysis of matrix microvesicles in AR and AP areas.
ances of the shedding microvesicles were analogous to the structural appearances of microvesicles which were freely located in the extracellular matrix (fig. 2a, 3a, b; online suppl. fig. 2a, b).

Expression of ARF6 by Intimal Smooth Muscle Cells
In agreement with our earlier reports [9, 22, 23], immunohistochemical analysis of both AR and AP areas demonstrated that smooth muscle α-actin-positive/SM22-positive cells constituted more than 95% of the total cell population (online suppl. fig. 3), while other cell types expressing CD14, CD68, CD3 or S100 antigens represented only minor cell populations that were mostly confined to the subendothelial sublayer of the intima.

Immunohistochemical staining of tissue sections with anti-ARF6 antibody showed that ARF6 immunopositivity was present in all intimal cells and was restricted to the cytoplasm of cells (fig. 4a). A comparative quantitative analysis showed that ARF6 expression was 1.3 times higher in AP areas than that in AR areas (585.7 ± 33.7 vs. 436.3 ± 36.9; p = 0.0064; fig. 4b). Double immunohistochemistry utilizing a combination of anti-ARF6 and anti-smooth muscle α-actin antibodies confirmed the association of ARF6 expression with smooth muscle cells (fig. 4c).

Correlation between the Content of Microvesicles and the Expression of ARF6 in the Intima
Further analysis demonstrated that there were positive correlations between the content of microvesicles in the extracellular matrix and the expression of ARF6 by intimal smooth muscle cells in both AR and AP areas (r = 0.53, p = 0.005, and r = 0.49, p = 0.008, respectively). The correlation coefficient between the content of matrix microvesicles and the expression of ARF6 in all the analyzed tissue specimens (without separation into AR and AP tissue specimens) was 0.61 (p < 0.0001).

Discussion
Accumulated evidence indicates that the existence of areas with different predispositions to the development of atherosclerosis cannot be simply explained by the effects of different mechanical forces associated with turbulent versus laminar flow or by the altered distribution of pulsatile forces at tethered (branch) sites [1–7, 24–26]. The present study extended the knowledge about the peculiarities of the arterial wall in AP sites and showed, for the first time, that the number of microvesicles (micro-

Fig. 3. Electron microscopic micrographs demonstrating the formation and separation of microvesicles from the surface of intimal smooth muscle cells (a, b). In a small arrows show microvesicles connected to the cell surface, while large arrows show microvesicles located in the extracellular matrix. Scale bar = 300 nm (a) and 200 nm (b).
tle study of lipid composition of human vascular matrix microvesicles, it is known that vascular matrix microvesicles contain approximately equimolar amounts of phospholipids and sterols, of which cholesteryl arachidonate comprises 2.3% [30, 33]. Matrix microvesicles in atherosclerotic lesions also contain bone morphogenic proteins and noncollagenous bone matrix proteins including osteopontin, osteonectin and matrix Gla protein [30]. Annexins are a group of proteins identified in matrix microvesicles [30]. Matrix microvesicles have also been found to be enriched by the presence of calcium-binding S100A9 protein [33]. This chemical and molecular content of microvesicles renders them prone to calcification [30–36]. Calcification of matrix microvesicles in atherosclerosis is recognized as an essential event in the formation of histologically identifiable calcified deposits and the importance of calcifying microvesicles in the progression of atherosclerosis and the formation of unstable plaques has been assessed in a number of studies [35–38]. The present study demonstrates that the number of calcifying microvesicles is very low in both AP and AR areas of grossly normal aorta suggesting that, although microvesicle formation occurs, the microenvironment of healthy arterial walls does not facilitate the processes needed for calcification. Perhaps other triggering factors, which appear only in already formed or forming atherosclerotic lesions, are necessary for the initiation of widespread vascular calcification.

The fact of the presence of microvesicles in healthy arterial walls, as shown in the present study, might be indicative that microparticles in arteries may play roles similar to those documented in other tissues [39, 40]. Recent

**Fig. 4.** ARF6 expression in the intima of grossly normal aorta. a Typical pattern of ARF6 expression; single immunohistochemistry. ABC immunoperoxidase technique utilizing 3,3’-diaminobenzidine as a substrate (brown reaction product). Scale bar = 10 μm. b Quantitative analysis of ARF6 expression in AR and AP areas. c Double immunostaining showing the association of ARF6 expression with smooth muscle α-actin-positive intimal cells. ARF6 antigen was visualized using an AEC substrate kit (red reaction product), while smooth muscle α-actin immunostaining was produced using a Fast Blue substrate kit (blue reaction product). Nomarski technique. Scale bar = 10 μm. Colors refer to the online version only.
reports indicate that in other tissues, shedding microvesicles participate in important biological processes, such as surface-membrane trafficking and the horizontal transfer of proteins and RNAs between neighboring cells which are required for the rapid phenotype adjustments in a variety of conditions [40]. Shedding microvesicles have physiological roles in immune processes via the release of cytokines and in coagulation by mediating the coordinate physiological roles in immune processes via the release of a large number of microvesicles, the release of a large number of microvesicles, the release of a large number of microvesicles into the extracellular space occurs as a result of cell death, including both necrosis and apoptosis [36–38]. Although the stimuli triggering shedding of microvesicles from cell surfaces might be different in the norm and in pathological conditions, molecular processes involved in microvesicle formation are thought to be uniform [11–13, 39, 40]. It is documented that the plasma membrane of cells has an asymmetric distribution of phospholipids [11–13, 39, 40]. Aminophospholipids, phosphatidylserine and phosphatidylethanolamine are specifically sequestered in the inner leaflet of the membrane [11–13]. The transbilayer lipid distribution is under the control of three phospholipidic pumps, including an inward-directed pump, a flippase, an outward-directed pump, or flippase, and a lipid scramblase, responsible for a nonspecific redistribution of lipids across the membrane [11–13]. When cell stimulation occurs, a cytosolic Ca 2+ increase promotes the loss of the phospholipids asymmetry of the plasma membrane and subsequent phosphatidylserine exposure, and this leads to a transient phospholipidic imbalance between the external leaflet at the expense of the inner leaflet resulting in blebbing of the plasma membrane and exocytotic microvesicle release [11–13].

An increased shedding of microvesicles in AP areas, identified in the present study, is likely to be associated with the altered metabolism of intimal smooth muscle cells. This is supported by the results of the examination of ARF6 expression in AR and AP areas of the aorta. ARF6 has a variety of cellular functions that are involved in the regulation of vesicular trafficking, remodeling of membrane lipids and signaling pathways that lead to actin remodeling [14–16]. ARF6 has specifically been implicated in plasma membrane protein recycling and membrane blebbing [14–16]. The present study shows that a difference between AP and AR intimal areas in respect of the number of microvesicles relates to the peculiarities in the functional states of intimal smooth muscle cells in AP and AR areas, in particular, different levels of the expression of ARF6.

Conclusions

Analyzing unique tissue samples of grossly normal segments of aortas, obtained at urgent autopsies, the present study provides proof of the concept that alterations of the arterial intima occur at the predisease stage. The present study showed that there is a difference between AP and AR intimal areas in respect of the number of microvesicles and related this difference to different functional states of intimal smooth muscle cells in AP and AR areas. The findings of this work suggest that an increased shedding of microvesicles from the surface of intimal smooth muscle cells might play a role in the initiation of the development of early atherosclerotic lesions.

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