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Presence of polymorphonuclear granulocytes during the early stage of transplant arteriosclerosis after prolonged ischemia in the rat

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Abstract The presence and function of polymorphonuclear granulocytes has been investigated, in particular, in the microcirculation in many short-term models of ischaemia/reperfusion injury. The aim of this study was to examine the presence of granulocytes in the aorta in a recently established long-term model of transplant arteriosclerosis, based on prolonged cold graft ischaemia time in the rat. Aortic grafts of PVG donors were subjected to two different cold ischaemia times of 1 and 4 h ($n = 5$ in each group) before an orthotopic transplantation to syngeneic recipients. The grafts were explanted shortly after various times post-reperfusion (7.5 min 24 h) and examined with conventional staining, immunohistochemistry and transmission electron microscopy for the presence of granulocytes. The results showed the presence of these cells adherent to the endothelial layer or in the subendothelial layer in grafts with both ischaemia times and with a maximum seen 2 h after transplantation. The internal elastic lamina was interrupted at sites of granulocyte adherence. We concluded that the polymorphonuclear granulocyte may be involved in the ischaemia/reperfusion injury in this model, thus, contributing to the development of accelerated transplant arteriosclerosis.

Key words Polymorphonuclear granulocytes · Aorta transplantation
Ischaemia/reperfusion injury
Transplant arteriosclerosis
Electron microscopy · Rats

Introduction

Little is known about the initiating events and the mechanisms leading to accelerated arteriosclerosis (AA) in transplanted organs. We have recently developed a model of transplant AA following prolonged cold graft ischaemia in the rat [1, 2]. In this model, we have shown that there is a correlation between the length of the cold ischaemia time and the severity of AA, and, further, that development of this type of AA is not accompanied by immunological parameters such as MHC class II expression and presence of T lymphocytes.

As a leading cause for this AA, we have discussed an ischaemia/reperfusion injury. The role of ischaemia/reperfusion damage in the microcirculation [3, 4] and coronary vessels [5] and the prevention of this damage has been extensively studied during storage time and in the period shortly after transplantation. In contrast, its role in the chronic outcome of transplanted organs including arteriosclerotic lesions is poorly investigated. One domi-

nant cell type described in the context of ischaemia/reperfusion damage is the polymorphonuclear granulocyte (PMN). PMNs that infiltrate the post-ischaemic tissue can generate high amounts of reactive oxygen species and thereby cause great tissue damage [6]. In contrast to a well-characterised role of PMNs during reperfusion, their possible presence and role in the development of conventional arteriosclerosis or transplant arteriosclerosis have, so far, not been taken into consideration [7, 8]. The present study was performed to examine whether PMNs are present during the early stage of AA development after a prolonged cold ischaemia time.

Materials and methods

Animals

Male PVG rats (RT1^c) weighing 120–200 g were purchased from Möllegard Farm (Skensved, Denmark). All animals were settled 1 week prior to transplantation and received food and water ad libitum.

Aortic transplantation

The recipient rats were anaesthetised with a chloral hydrate/pentobarbital mixture (195.5/44.6 mg/kg body weight, Eqviticin, Apoteksbolaget, Umeå, Sweden) through intraperitoneal injection. The donor rats were anaesthetised with thiobarbital (Inactin, Byk Gulden, Konstanz, Germany) 120 mg/kg. Aortic transplantation was performed as described before [1–3]. Briefly, a 10–15-mm long abdominal aortic segment from each donor rat was carefully excised. The aortic grafts were stored at 4 °C in a histidine-buffered Frödin perfusion solution (Akademiska Apoteket, Uppsala, Sweden [9] for 1 or 4 h. The recipient's abdominal aortic segment was carefully freed from its bed immediately below the renal arteries to the bifurcation of the abdominal aorta. Clamps were placed on the abdominal aortic segments before extirpation. The graft was then implanted orthotopically with 9-0 nylon suture using single stitches (S&T Neuhausen am Rheinfall, Switzerland). The clamps were removed and the new vascular connection was checked thoroughly for patency.

Animals whose grafts remained from 7.5 min to 2 h were kept under anaesthesia, animals with 24-h grafts were allowed to wake up and were again anaesthetised with thiobarbital on the next day. After the indicated time the grafts were explanted. Additionally, 10-mm long segments, each from the thoracic and abdominal aorta just above the transplanted graft of recipient rats, were taken out to use as negative internal controls. They were divided into two parts each. One part was frozen in a cold isopentane/dry ice mixture, stored at −70 °C and further processed for immunohistochemistry or for conventional May-Grünwald Giemsa staining, and the second part was fixed for electron microscopy as described below.

Immunohistochemical staining

For immunohistochemical detection of granulocytes, we used a staining with ED1 (Serotec Lab., Oxford, England [10]) and with the L26 antibody recognising the CD11b receptor. The ED1 antibody identifies monocytes, macrophages and dendritic cells. The CD11b receptor is part of the mac-1 receptor present on both granulocytes and macrophages but not lymphocytes. ED1-negative and CD11b-positive cells were regarded as granulocytes. The antibody L26 was a kind gift from Dr. Claes Lundberg (Kabi & Pharmacia, Uppsala, Sweden). It is a mouse monoclonal antibody directed against the rat CD11b receptor. It has been protein A purified and is of IgG_2b subtype. Its binding to the CD11b molecule has been characterised on a SDS-polyacrylamide gel followed by Western blotting. In addition, the antibody has been shown to be able to block the adherence of rat granulocytes in vitro (Dr. Claes Lundberg, unpublished data). The E11 antibody directed against human parathyroid cells (1:100 [11]) served as a negative control antibody.

Acetone-fixed 5-mm thick frozen midgraft sections were incubated overnight at 4 °C with the primary antibodies dissolved in phosphate-buffered saline containing 0.1 % bovine serum albumin. The ED1 antibody was diluted 1:3200 and the CD11b antibody, 1:250. A rat-absorbed biotinylated anti-mouse IgG antibody (1:80; Vector, Burlingame, Calif., USA) served as a secondary antibody. For the detection of the secondary antibodies, an avidin-biotin complex (Vector, Burlingame, Calif., USA) method was applied (1:100 [12]). The peroxidase reaction was carried out with 3-amino-9-ethylcarbazole as substrate. Counterstaining was performed with Mayer's haematoxylin and eosin. A peroxidase staining was done solely, as an additional characterisation for granulocytes.

Electron microscopy

Two sections with 2-h reperfusion times were chosen for transmission electron microscopical investigation. The tissue was fixed immediately after removal in 3% buffered glutaraldehyde at 4 °C overnight, followed by 1 % osmium tetroxide for 1.5 h at room temperature. After fixation, it was contrasted in 1 % uranyl acetate overnight, dehydrated in ethanol, immersed in propylene oxide and embedded in epoxy resin agar 100 (Agar Aids Ltd. Standsted, England). The polymerisation took place at 60 °C [13]. The 50-nm ultra-thin sections were contrasted with Reynold's lead and examined in a Philips 201 electron microscope.

Quantification of endothelial adherent blood leucocytes

In all, ten transplantations were performed, five with an ischaemia time of 1 h, and five with 4 h of ischaemia time, and each with a different reperfusion time ranging from 7.5 min to 24 h. All CD11b- or ED1-positive cells that were adherent to the endothelium or present in the subendothelial space were counted. This was performed by two investigators, independently, and unaware of the ischaemia time or postoperative observation time. The results are graphically displayed in Fig. 1.

Results

Grafts with short reperfusion times did not show any difference to negative control vessels. They displayed normal histology. No endothelial adherent cells were found (see Fig. 1). After 15 and 30 min, only a few individual CD11b or ED1 were observed. The 30 min grafts also contained a few CD11b-positive cells in the

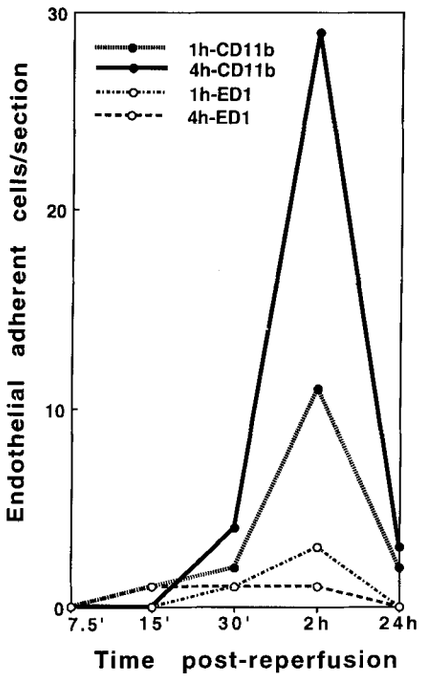

Fig. 1 Numbers of CD11b- and ED1-positive cells in a single section of aorta grafts with various cold ischaemia and reperfusion times. The great majority of cells were CD11b-positive and ED1-negative. After 30 min, higher numbers of CD11b-positive cells were found in the grafts with 4-h ischaemia time than with 1-h ischaemia time

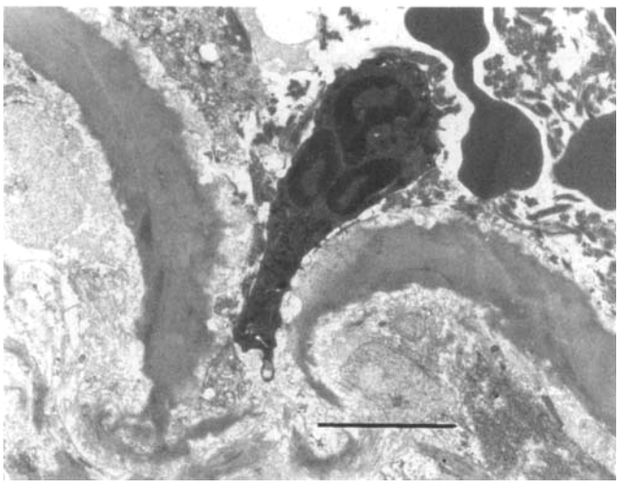

Fig. 3 Transmission electronic micrograph showing a polymorphonuclear granulocyte (PMN) situated within the internal elastic lamina. The internal elastic lamina was thickened and interrupted at the site of the granulocyte. Beside the PMN, a macrophage-containing secondary lysosome was attached to an endothelial cell. Around the granulocyte, clots of fibrils can be identified (original magnification × 12000; *bar* = 3 mm)

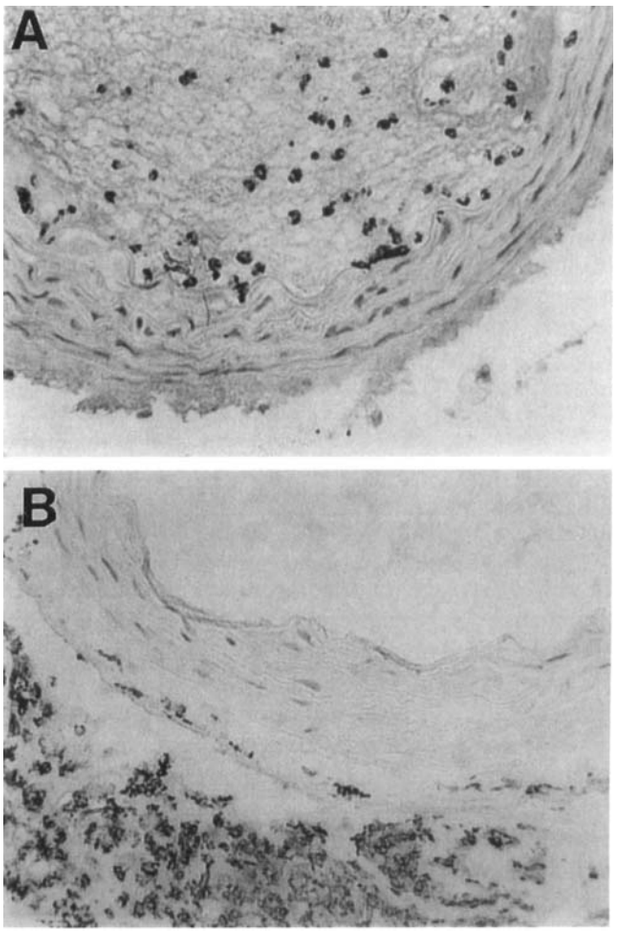

Fig. 2A, B Light microscopic picture of immunostainings for CD11b of grafts with 4 h of ischaemia time and **A** 2 or **B** 24 h of reperfusion time (original magnification × 250). After 24 h, the intimal layer almost completely lacked the presence of these cells. In sharp contrast, the adventitial layer consisted of many CD11b-positive cells

adventitia. The highest number of cells adherent to the endothelial layer was seen after 2 h of reperfusion time in both grafts with 1 and 4 h of cold ischaemia time. The 1-h ischaemia graft displayed 11 CD11b- and 3 ED1-positive cells, whereas the 4-hour ischaemia graft contained 29 CD11b-positive cells and 1 ED1-positive cell (Fig. 2A). The media did not possess any cells with the exception of the subendothelial layer; the adventitia contained a moderate amount of CD11b-positive cells but no ED1-positive cells. After 24 h of reperfusion time, the number of CD11b-positive cells was reduced to two and three in the 1- and 4-h ischaemia grafts (Fig. 2B). No ED1-positive cell were seen. In the adventitia of both grafts, however, high numbers of ED1- and CD11b-positive cells were seen (Fig. 2B). The peroxidase staining and the

conventional May-Grünwald Giemsa staining revealed a similar picture.

In the transmission electronic micrographs, a thick amorphous basal lamina was observed. The lamina was interrupted at the site where granulocytes closely adhered to the endothelium (Fig. 3). Occasionally, macrophages attached to endothelial cells (Fig. 3). A PMN was seen in between the endothelial layer and had a protruding shape heading towards the media. Most of the cytoplasmic granules were in the apical part of the granulocyte. Around the granulocyte, small clots of fibrils were detected. Blood erythrocytes within these clots were deformed (Fig. 3). The media contained smooth muscle cells with swollen round mitochondria lacking cristae.

Discussion

Three findings were noted in this study. First, PMNs were present in the early stage of the development of transplant AA with a maximal number of endothelial-adherent PMNs after 4 h of ischaemia and a 2-h reperfusion time. Second, grafts with 1 h of ischemia contained endothelial-adherent PMNs showing a similar time curve as the 4-h ischaemic grafts. Third, the elastic internal lamina at the site of PMN adherence was thickened and interrupted.

Although the pathophysiological role of PMNs during an ischaemia/reperfusion injury is well established [3, 4, 14], its implication for the long-term development of arteriosclerotic changes has not been discussed [7, 8]. During reperfusion, PMNs accumulate, migrate into the surrounding tissue and may cause deleterious effects by the production of oxygen-free radicals as well as by the release of proteolytic enzymes. Thereby, they undergo necrosis [6, 14]. This might be the reason why these cells are not observed and reported during the development of transplant arteriosclerosis [1, 2, 8, 15]. The importance of the presence of PMNs in our model still has to be elucidated. Recent data by Land and colleagues, however, strongly support the notion that early appearing oxygen-radical producing leucocytes have an impact on graft arteriosclerosis. They treated perioperatively transplanted patients with superoxide dismutase, and, several years following transplantation they observed beneficial effects of this regimen [16].

In our model, we observed the maximum number of endothelial-adherent cells after 2 h of reperfusion. As our next observation time was 24 h, the maximum could have occurred later. However, this was later than reported in an analogous situation in the microvascular endothelium where the maximum leucocyte adherence is reached within the 1st hour [17]. Most of the pathophysiological studies of leucocyte adherence are done mainly in the microcirculation [3, 4] and the situation in the aortic vessel may differ considerably where there are higher blood flow rates and possibly different patterns of adhesion molecule expression.

One striking difference in the transplant arteriosclerosis in the our rabbit model was the destruction of the internal elastic lamina. In an elegant study, Foegh and colleagues have shown that the endothelium and the internal elastic lamina are intact during the development of transplant arteriosclerosis [18]. Our findings might be explained by the severe damage already experienced by the tissue during the prolonged ischaemia time. The lack of cristae in the mitochondria of smooth muscle cells are a clear indicator of injury. Therefore, the cells are more vulnerable to addition reperfusion damage. Our electron microsopic finding showed cytoplasmic granules situated at the apical site of the PMNs suggesting that enzymes released from those granules might have contributed to the injury observed in the internal elastic lamina.

The high content of granulocytes in the adventitia was remarkable. It has to be mentioned that the surgery in the rat was not performed under sterile conditions and this may partially explain the strong inflammation in this vessel layer. Further, the ischaemic damage affects not only the intime but the whole vessel including the adventitia. Damage exclusive to the adventitial layer in the aorta has been proven to be sufficient to cause intimal thickness [19, 20]. Even though ischaemia/reperfusion damage in the microcirculation is shown to occur first at the endothelial level, the strong inflammation in outer layer of the aortic grafts after transplantation warrants further investigation.

In summary, this study demonstrated the presence of PMNs in the early stage of transplant arteriosclerosis and suggested a possible role for granulocytes in the development of AA.

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