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Evidence that dendritic cells infiltrate atherosclerotic lesions in apolipoprotein E-deficient mice

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Summary. Earlier we reported that atherosclerotic lesions of apoE-deficient mice contained cells which stained positively with anti-S-100 antibody and that cells exhibiting the ultrastructural features of dendritic cells were present in the aortic lesions. These observations suggested that dendritic cells might be involved in mouse atherosclerosis. By employing DEC-205 and MIDC-8 antibodies specific for dendritic cells, the present study has established that dendritic cells indeed accumulate in atherosclerotic lesions of apoE-deficient mice. Finding dendritic cells infiltrating atherosclerotic lesions in apoE-deficient mice offers the possibility of investigating the migratory routes of dendritic cells and their involvement in T-cell activation.

Key words: Atherosclerosis, Apolipoprotein E-deficient mice, Dendritic cells

Introduction

Dendritic cells constitute a family of cells able to induce primary immune responses (Banchereau and Steinman, 1998; Lotze and Thomson, 1999). They are thought to arise from a common $CD34^+$ progenitor in the bone marrow from where they migrate via the blood stream to settle in different peripheral tissues (Banchereau and Steinman, 1998; Lotze and Thomson, 1999). After engulfing antigen, dendritic cells migrate via afferent lymph to activate T-cells (Banchereau and Steinman, 1998; Lotze and Thomson, 1999). Dendritic cells express high levels of both class I and class II MHC molecules and co-stimulatory molecules which explains their unique ability to activate naive T-cells (Banchereau and Steinman, 1998; Lotze and Thomson, 1999).

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Using cell-type specific antibodies, we found that in man, dendritic cells reside in the normal non-diseased arteries and become activated during atherogenesis (Bobryshev and Lord, 1995, 1999; Bobryshev et al., 1997; Lord and Bobryshev, 1999; Bobryshev, 2000). Some dendritic cells cluster with T-cells directly within human atherosclerotic lesions while others are thought to migrate to regional lymph nodes to activate T-cells (Bobryshev and Lord, 1998; Bobryshev, 2000). Investigating dendritic cells in atherosclerosis has been limited, however, because the presence of dendritic cells in the artery wall has been proved only in human specimens obtained at operations and post-mortem (Bobryshev and Lord, 1995, 1998, 1999; Bobryshev et al., 1997; Lord and Bobryshev, 1999).

To further our understanding of the significance of dendritic cells in atherosclerosis, an animal experimental model is necessary. ApoE-deficient mice represent one of such popular models as apoE-deficient mice develop advanced atherosclerotic plaques with features characteristic of those seen in humans, including the formation of a necrotic core and calcification (Nakashima et al., 1994; Reddick et al., 1994). Earlier we reported that atherosclerotic lesions in apoE-deficient mice contain cells which stain positively with anti-S-100 antibody (Bobryshev et al., 1999a). Unfortunately, anti-S-100 antibody is not specific for dendritic cells since this antibody stains a variety of cell types (Zimmer et al., 1995; Lotze and Thomson, 1999). The detection of $S\text{-}100^+$ cells in atherosclerotic lesions of apoE-deficient mice therefore did not prove that the positively stained cells were dendritic cells even though cells exhibiting the ultrastructural features of dendritic cells were detected in apoE-deficient mouse aortic lesions by electron-microscopic examination (Bobryshev et al., 1999b).

Very recently, specific antibodies to mouse dendritic cells became commercially available. By employing these antibodies, the present study aimed to determine whether dendritic cells indeed accumulate in atherosclerotic lesions of apoE-deficient mice.

Materials and methods

Aortic specimens

For this study arterial samples were obtained from 10 apoE-deficient C57BL/6X129 mice maintained in microisolator cages on the PMI autoclavable rodent diet No. 5010 for eight months. All the procedures were carried out in accordance with the institutional guidelines for laboratory animal care. The mice were anaesthetised with methoxyfluorane. Macroscopical examination showed that the aortas contained well developed atherosclerotic plaques as well as early intimal lesions surrounded by non-diseased segments of the arterial wall. For histological and immunohistochemical analyses, aortic segments were taken from the region of the proximal aorta (ascending aorta and arch). Non-fixed tissue samples and the samples fixed in periodate-lysine-paraformaldehyde solution were embedded in OCT compound Tissue-Tek II (Miles Laboratories) as previously described (Bobryshev et al., 1997). Small tissue samples were fixed in 2.5% glutaraldehyde in 0.1M cacodilate buffer (pH 7.4) for electron microscopic examination.

Antibodies

DEC-205 (NLDC-145) and MIDC-8 are specific antibodies for identifying mouse dendritic cells (Kraal et al., 1986; Breel et al., 1987; Jiang et al., 1995; Swiggard et al., 1995; Inaba et al., 1995). In the present study DEC-205 (NLDC-145) and MIDC-8 antibodies we used, both purchased from Serotec. NLDC-145 recognises DEC-205, a 205-kDa integral membrane glycoprotein expressed at high levels by mouse dendritic cells (Kraal et al., 1986; Breel et al., 1987; Jiang et al., 1995; Swiggard et al., 1995; Inaba et al., 1995). The antigen DEC-205 has been found on Ia^+ *interdigitating cells* in T-cell areas of all secondary lymphoid organs and is present on *veiled cells*. In non-lymphoid organs, the antigen has been identified on *Langerhans cell*s in the mouse skin. DEC-205 is absent in bone marrow cells as well as in non-dendritic blood cells (Kraal et al., 1986; Breel et al., 1987; Jiang et al., 1995; Inaba et al., 1995: Swiggard et al., 1995). Another antibody, MIDC-8, recognises the antigen located in the cytoplasm of mouse dendritic cells (Breel et al., 1987). This antigen is present in *interdigitating cells* in T-cell dependent areas of secondary lymphoid organs, the medulla of the thymus, *veiled cells*, and a small content was identified in *Langerhans cells*. MIDC-8 antigen is absent from non-dendritic blood cells and macrophages (Breel et al., 1987). In the present study, both the rat anti-mouse DEC-205 antibody and rat anti-mouse dendritic/interdigitating cell antibody (MIDC-8) were used in a dilution 1:10 according to the manufacturer's recommendations.

Anti-CD3 (PharMingen; 500A2; Dilution 1:10) was used for identifying T-cells. Antibody MOMA-2 (Serotec; Dilution 1:20) was used to identify macrophages. Smooth muscle cells were stained with anti-α-smooth muscle actin (Sigma Immunochemicals; Dilution 1:60).

Immunohistochemical procedures

The presence of dendritic cells was examined using biotin-streptavidin and 3-amino-9 ethylcarbazole (AEC) substrate-chromogen system (Dako) which produces a red staining of antigens. In brief, after blocking endogenous peroxidase with 0.3% hydrogen peroxide and treatment with an appropriate normal serum, frozen sections were incubated with an appropriate biotinalated secondary antibody and peroxidase-conjugated streptavidin (Dako) as described earlier (Bobryshev et al., 1997). The peroxidase-oxidase reaction was developed with AEC substrate kit. All immunohistochemical procedures were carried out at room temperature. For positive control, mouse spleen tissue was used. For negative controls, the first antibody was omitted or the sections were treated with an immunoglobulin fraction of non-immune goat serum as a substitute for the primary antibody. None of the negative control sections showed positive immune staining. Counterstaining was performed with Mayer's haematoxylin.

Electron microscopy

For electron microscopic analysis, the aortic samples were fixed in 2.5% glutaraldehyde in 0.1M cacodilate buffer (pH 7.4), postfixed in 1% osmium tetroxide in cacodilate buffer, dehydrated in graded ethanol and propylene oxide and then embedded in Araldite resin. Serial ultrathin sections were cut on a LKB-III ultratome. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with the aid of a Hitachi H7000 electron microscope at an accelerating voltage of 75 kV.

Results

Histological data

Histological examination showed the presence of multiple atherosclerotic lesions surrounded by non-altered segments of the aortic wall. The atherosclerotic lesions were represented by a variety of both early and well-developed plaques. The atherosclerotic lesions contained large amounts of lipids visualised by staining with oil red O, the lesion structures corresponding to those described by us earlier (Bobryshev et al., 1999a).

Histochemical observations

Aortic sections stained with DEC-205 antibody contained no immunopositive cells in non-diseased parts of the vessels (Fig. 1A) but $DEC\text{-}205^+$ cells were present

in the subendothelial space in intimal thickenings (Fig. 1B,C). These positively stained cells were usually spindle-shaped in section and were spread along the internal basal lamina (Fig. 1B,C). DEC-205$^+$ cells were detected in all the aortas studied.

To see whether DEC-205$^+$ cells might originate from the blood stream, we specifically looked for DEC-205$^+$ cells attached to the luminal endothelial surface and these cells were indeed found. They were observed in areas of swollen intima (Fig. 1D) but there were no DEC-205$^+$ cells attached to the endothelium in non-diseased segments of aortas. DEC-205$^+$ cells attached to the luminal surface of atherosclerotic lesions exhibited a round or oval shape (Fisg. 1D, 2A) and their penetration of swollen intima was observed (Fig. 2A). Within the atherosclerotically altered aortic segments, oval- and irregularly-shaped DEC-205$^+$ cells were detected not only in the intima but in both the media (Fig. 2B) and the adventitia (Fig. 2C) as well.

By reconstructive analysis of serial sections of advanced atherosclerotic lesions, we estimated that more than 80% of plaques contained DEC-205$^+$ cells. In

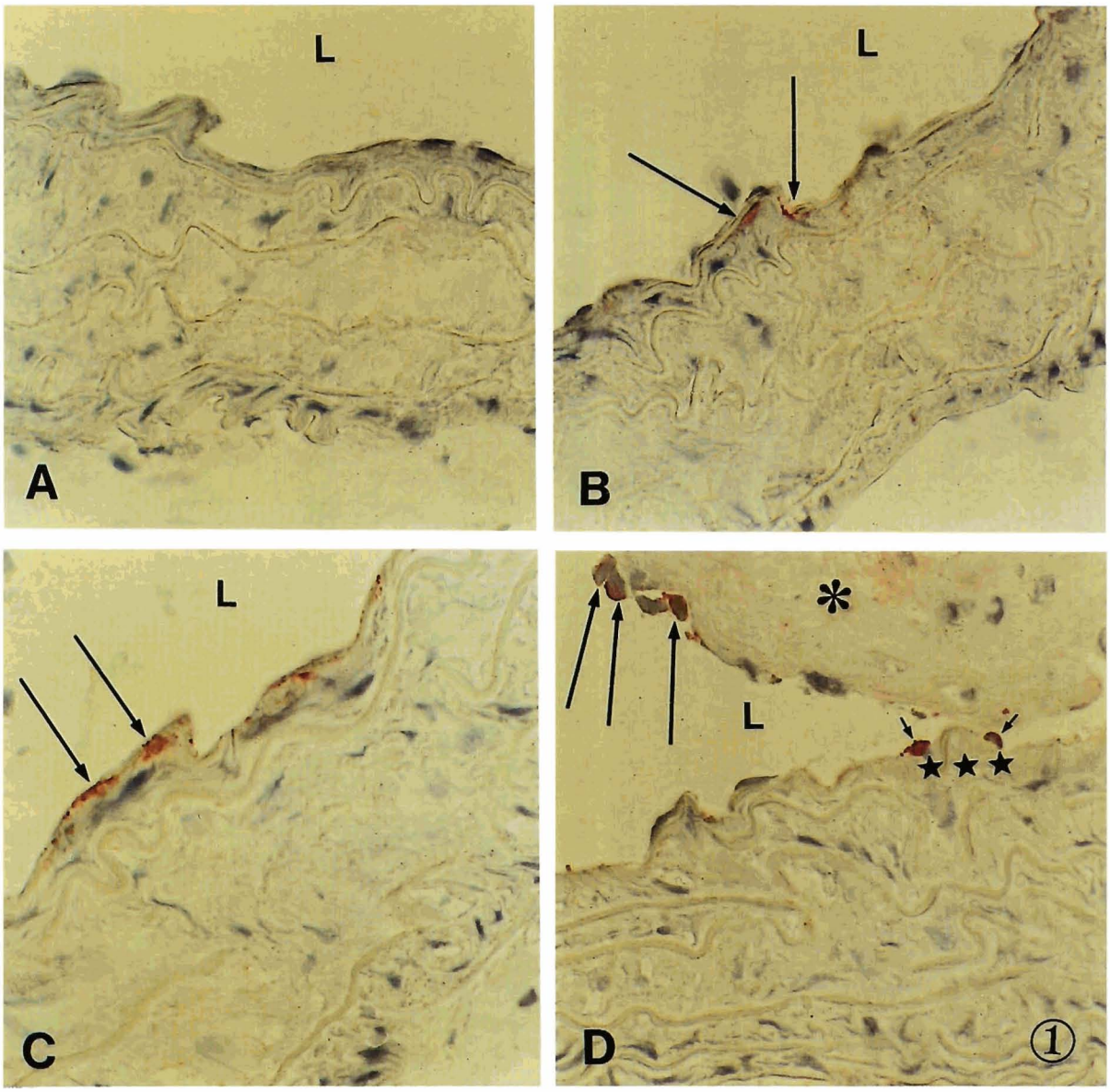

Fig. 1. Identification of dendritic cells with DEC-205 antibody in the aortic wall of apoE-deficient mice. **A.** Non-diseased segment showing no immunopositivity for DEC-205 antibody. **B and C.** DEC-205$^+$ cells (arrows) located along the endothelium in intimal thickenings. **D.** Small arrows show DEC-205$^+$ cells attached to the luminal surface of an intimal thickening (stars) while large arrows show DEC-205$^+$ cells attached to the surface of an atherosclerotic plaque (asterisk). L: lumen of the aorta. Immunoperoxidase technique; visualisation was produced with AEC substrate kit; counterstaining with Mayer's haematoxylin. x 400

general, more DEC-205$^+$ cells were seen attached to the luminal surface in the aortic segments containing atherosclerotic plaques than in those with intimal thickenings (Figs. 1D, 2D). Along the surface of atherosclerotic plaques, DEC-205$^+$ cells formed clusters (Fig. 2D,E). Some clustering, round-shaped DEC-205$^+$ cells seemed to be only slightly attached to the plaque surface through fibrin-like material while others were spread out on the plaque luminal surface (Figs. 2D, 3A).

Within atherosclerotic plaques, there were more DEC-205$^+$ cells than in intimal thickenings (Fig. 1C vs Fig. 3B) and DEC-205$^+$ cells often surrounded foam cell aggregates or, alternatively, were intermingled with foam cells (Fig. 3B). Staining with antibody MOMA-2 demonstrated that most foam cells were of macrophage origin. In the deep portion of atherosclerotic plaques, DEC-205$^+$ cells were often located on the border with the media, along the internal elastic lamina (Fig. 3C).

The use of another antibody specific to mouse dendritic cells, MIDC-8, showed that MIDC-8$^+$ cells were present in all the aortas studied. A comparison of parallel consecutive sections stained with both DEC-205 and MIDC-8 antibodies showed that the patterns of distribution of DEC-205$^+$ cells and those of MIDC-8$^+$ cells were similar or nearly identical, which indicated that both antibodies stained the same cells (Fig. 4A-D).

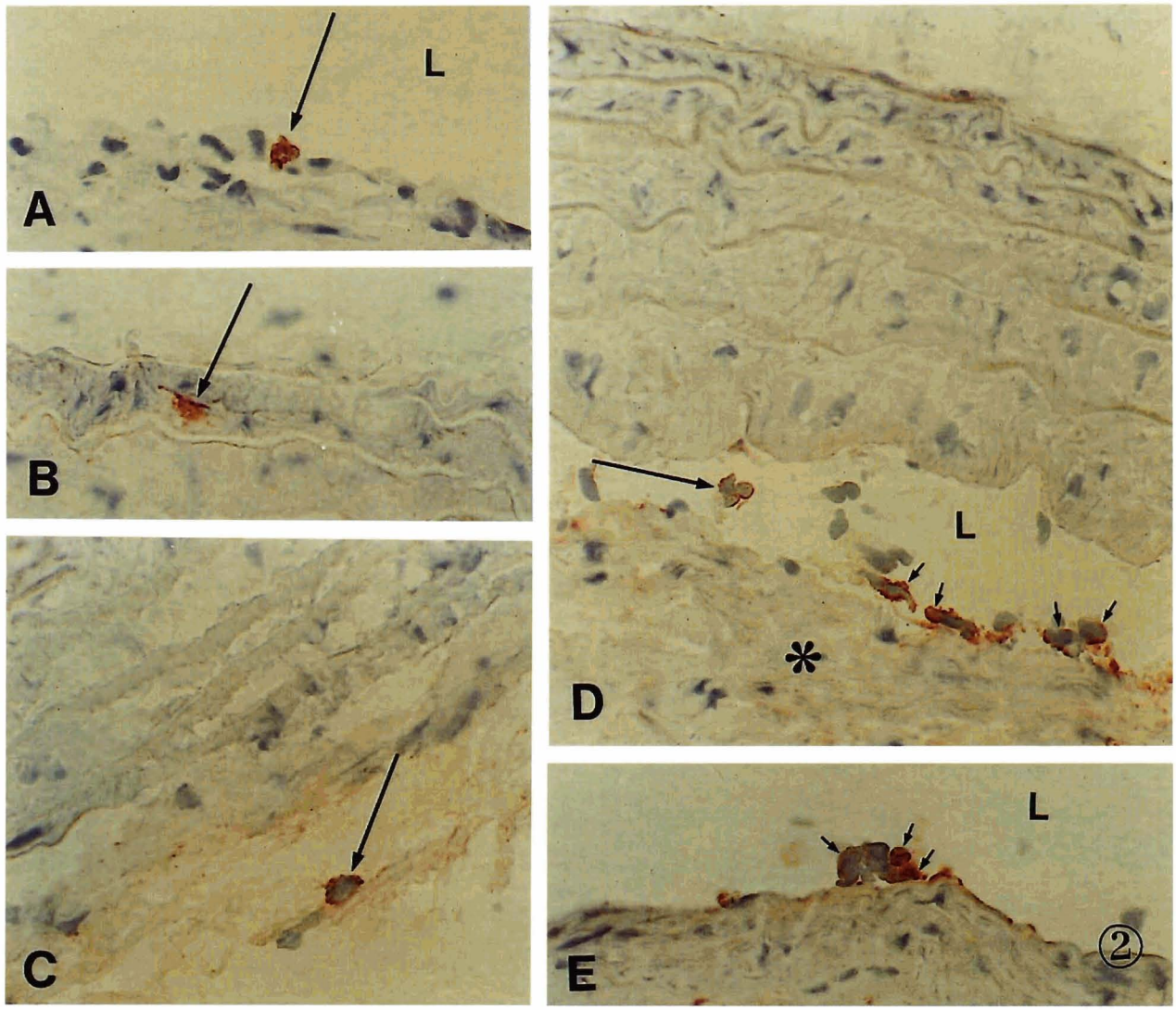

Fig. 2. Distribution of DEC-205$^+$ cells in atherosclerotic aortas of apoE-deficient mice. **A.** DEC-205$^+$ cell (arrow) incorporated within the superficial portion of atherosclerotically altered intima. **B.** A stellate shaped DEC-205$^+$ cell (arrow) located between elastic fibres in the deep part of the media. **C.** DEC-205$^+$ cell in the adventitia shown by arrow. **D.** Small arrows show DEC-205$^+$ cells attached to the suface of an atherosclerotic plaque while the large arrow shows a cluster of DEC-205$^+$ cells attached through fibrin-like material to the surface of the plaque. Note the absence of DEC-205$^+$ cells along non-diseased segment of the aorta. **E.** A group of DEC-205$^+$ cells on the surface of an atherosclerotic plaque. L: lumen of the aorta. Immunoperoxidase technique; visualisation was produced with AEC substrate kit; counterstaining with Mayer's haematoxylin. x 400

To see whether DEC-205$^+$/MIDC-8$^+$ cells co-localised with lymphocytes in atherosclerotic lesions, sets of parallel consecutive sections stained with DEC-205, MIDC-8 and anti-CD3 antibodies were compared. We found that non-atherosclerotic aortic segments were completely free of CD3$^+$ cells and that atherosclerotic lesions contained only a few CD3$^+$ cells or did not contain this cell type at all. We were not able to establish co-localisation between DEC-205$^+$/MIDC-8$^+$ cells and rare CD3$^+$ cells.

Electron-microscopic observations

We previously observed cells with the ultrastructural appearance of dendritic cells are present in atherosclerotic lesions of apoE-deficient mice (Bobryshev et al., 1999b). To ensure that the aortic specimens used in the present work contained cells exhibiting ultrastructural features typical of dendritic cells, electron microscopic analysis was carried out. Cells with typical ultrastructural features of dendritic cells were detected in atherosclerotic lesions taken from all the aortas. These ultrastructural features included, in particular, the presence of a unique tubulovesicular system characteristic of dendritic cells (Lotze and Thomson, 1999). Cells containing characteristic cisterns of the tubulovesicular system lacked lysosomes and phagolysosomes. As the structural characteristics of cells exhibiting these features were illustrated and described by us in detail earlier (Bobryshev et al., 1999b), the electron-microscopic findings are not documented in the present report.

Discussion

Using an immunohistochemical technique, the present study has established the presence of dendritic cells in atherosclerotic lesions in apoE-deficient mice. This finding is important because apoE-deficient mice might provide a useful model for investigating the contribution of dendritic cells in atherogenesis. The significance of dendritic cells in atherosclerosis could be tested by crossing of apoE-deficient mice with dendritic cell-deficient mice but the production of dendritic cell-

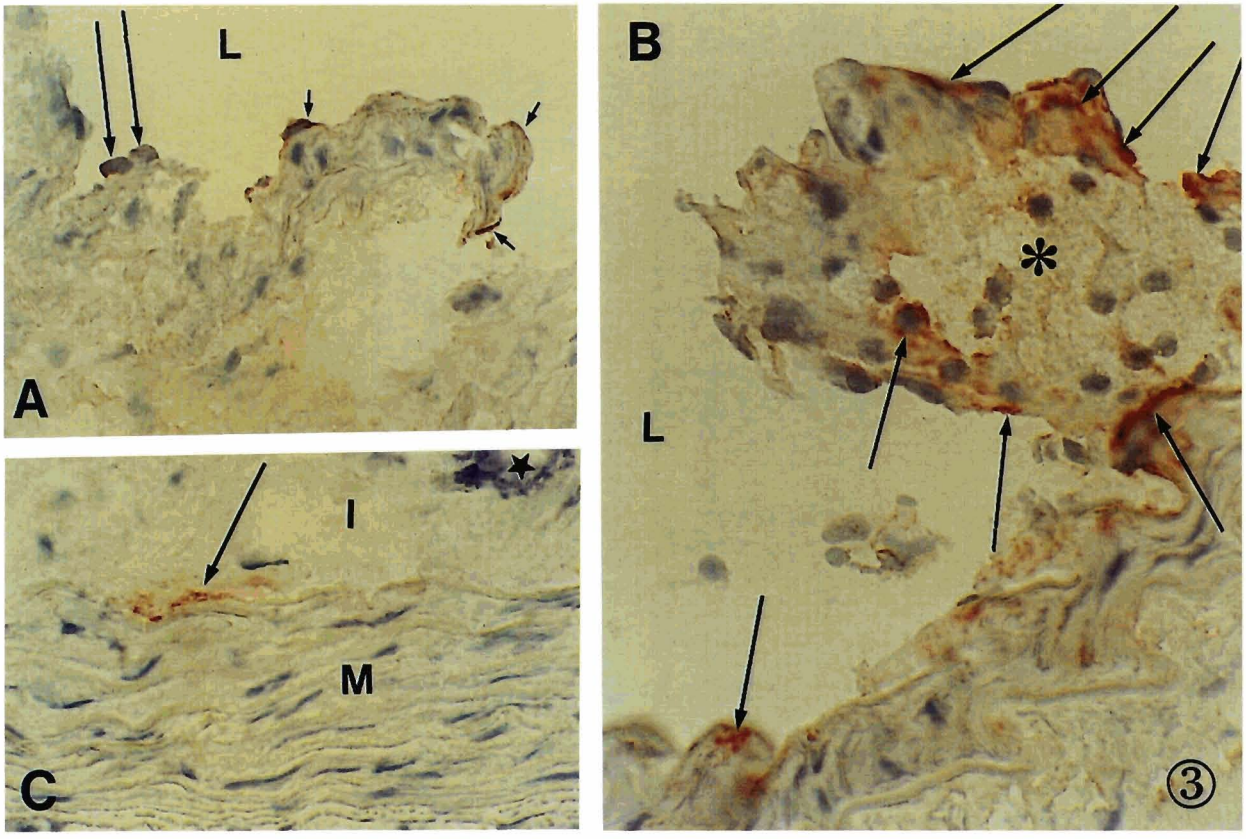

Fig. 3. DEC-205$^+$ cells in atherosclerotic plaques of apoE-deficient mice (A-C). In **A** large arrows show round-shaped DEC-205$^+$ cells attached to the luminal surface of a plaque while small arrows indicate DEC-205$^+$ cells spread out along the plaque surface. **B** shows the distribution of dendritic cells (arrows) in a foam cell-rich plaque. The asterisk indicates an area consisting only of foam cells. Note that DEC-205$^+$ cells are not transformed into foam cells. **C.** DEC-205$^+$ cell (arrow) located in the deep portion of the plaque closely attached to the internal elastic lamina. I: intima; M: Media. Star indicates a calcification area. L: lumen of the aorta. Immunoperoxidase technique; visualisation was produced with AEC substrate kit; counterstaining with Mayer's haematoxylin. x 400

deficient mice has been hampered because known markers for dendritic cells overlapped with the markers expressed by other cell types (Lotze and Thomson, 1999). The recent identification of the genes specifically expressed by dendritic cells (Ariizumi et al., 2000) will likely facilitate the production of dendritic cell-deficient mice and ultimately lead to evaluating the role of dendritic cells in atherosclerosis. At present, there are several mouse models in which dendritic cell function is affected (Lotze and Thomson, 1999). RelB, a member of

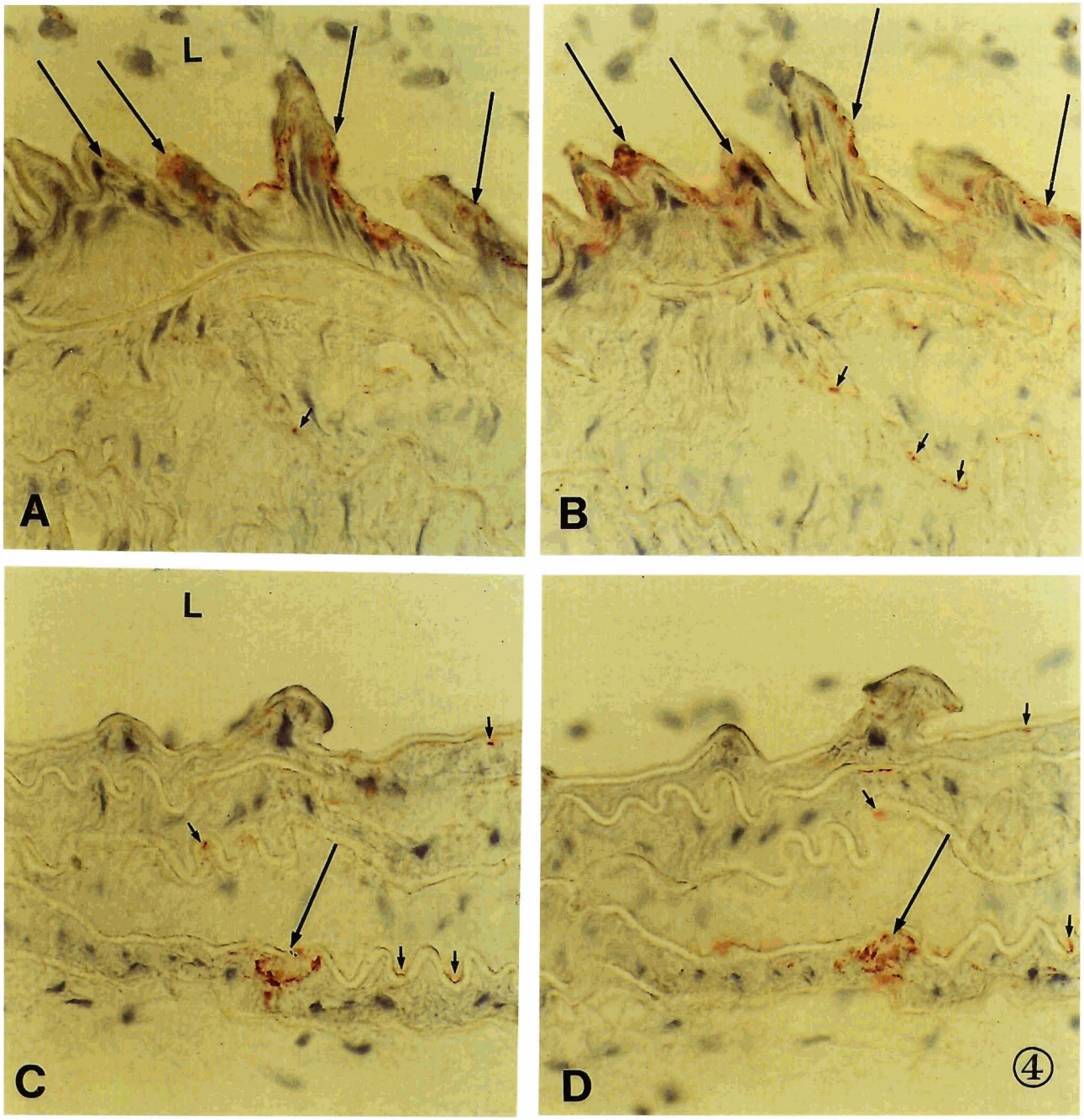

Fig. 4. Parallel consecutive sections (A vs B; and C vs D) of apoE-deficient mouse aortas stained with either DEC-205 antibody (A and C) or MIDC-8 antibody (B and D). Note that the patterns of the distribution of DEC-205$^+$ cells are almost identical to those of MIDC-8$^+$ cells indicating that both antibodies stain the same cells. In (A-D), large arrows show DEC-205$^+$/MIDC-8$^+$ cells located in the intimal layer (A, B) and in the deep portion of the media (C, D). Small arrows identify dot-like DEC-205 or MIDC-8 immunopositive material, presumably corresponding to cross sections of dendritic cell processes. L: lumen of the aorta. Immunoperoxidase technique; visualisation was produced with AEC substrate kit; counterstaining with Mayer's haematoxylin. x 400

the NF-kappaB/Rel family, is highly expressed in dendritic cells and RelB-knockout mice (Burkly et al., 1995; Lotze and Thomson, 1999) can be used for crossing with apoE-deficient mice but the interpretation of such a crossing might be difficult.

Atherosclerosis is a chronic inflammatory disease with antigen specific T-cell activation (Hansson, 1993; Wick et al., 1997; Ross, 1999). T-cell activation depends on the interaction of T-cell receptors with antigen presented by MHC molecules but how T-cells acquire antigens in atherogenesis is still unknown (Hansson, 1993; Wick et al., 1997; Ross, 1999). The principal functions of dendritic cells in other circumstances are antigen processing and antigen presentation (Banchereau and Steinman, 1998; Lotze and Thomson, 1999) and it is most likely that dendritic cells in atherosclerosis function similarly. In atherosclerotic lesions, dendritic cells probably collect immune-significant information and then migrate to regional lymph nodes to present antigen to T-cells (Bobryshev and Lord, 1998). Finding dendritic cells infiltrating atherosclerotic lesions in apoE-deficient mice offers the possibility of investigating the migratory routes of dendritic cells and their involvement in T-cell activation.

Atherosclerosis displays features of immune activation both locally and systemically (Nicoletti et al., 2000). The immune reactions which take place locally in atherosclerotic lesions likely represent only a small portion of the immune reactions occurring systemically in atherosclerosis (Nicoletti et al., 2000). Although mouse atherosclerosis models are useful for investigating some aspects of human atherosclerosis, mouse atherosclerosis seems not to completely mimic the immune-inflammatory aspects of human atherosclerosis (Dansky et al., 1997; Nicoletti et al., 2000). In particular, in contrast to atherosclerosis in man where the accumulation of large numbers of both macrophages and T-cells in the arterial intima is a characteristic of the disease, mouse atherosclerotic lesions contain few T-lymphocytes (Dansky et al., 1997). The present study demonstrated a low infiltration of T-lymphocytes into the aortic wall in apoE-deficient mice. If dendritic cells are involved in T-cell activation in apoE-deficient mice, this seems not to occur in the arterial wall but, mostly likely, occurs in the secondary lymphoid organs.

Several widely different mechanisms appear to lead to the development of atherosclerotic lesions. At one end of the spectrum are cases depending more or less exclusively on high blood cholesterol levels, such as hereditary familiar hypercholesterolaemia due to a deficiency of LDL-receptors in humans, and similar conditions in LDL-receptor-/- or apoE-/- mice. Transplant atherosclerosis is mainly T-cell dependant and represents the other end of the spectrum. Between these extremes there is "common" atherosclerosis, where it seems that inflammatory processes start the disease and foam cell-rich lesions plaques develop later.

Mouse atherosclerosis cannot completely mimic the immune-inflammatory aspects of human atherosclerosis because the normal arterial wall in mice lacks a cell-rich intima, unlike this important structural feature of human arteries. The present study demonstrated that the non-diseased aortic intima in apoE-deficient mice is free of dendritic cells. In man, in contrast to mice, *vascular dendritic cells* seem to reside in the intima of normal non-diseased arteries and become activated during atherogenesis (Bobryshev and Lord, 1995; Bobryshev et al., 1997; Bobryshev and Watanabe, 1997; Lord and Bobryshev, 1999; Bobryshev 2000).

Dendritic cells observed in the atherosclerotically altered intima in apoE-deficient mice probably originate from blood dendritic cells. In man, during plaque formation, in addition to *vascular dendritic cells* resident in the normal arterial wall, *blood dendritic cells* may also infiltrate from the blood (Bobryshev and Lord, 1998; Bobryshev et al., 1999c). The frequent location of dendritic cells within areas of neovascularisation and within the lumen of microvessels in human atherosclerotic plaques favours the possibility that in man, *blood dendritic cells* might penetrate plaques through neovascularisation (Bobryshev and Lord, 1998; Bobryshev et al., 1999c). Using apoE-deficient mouse might help resolve this issue as *in vivo* transfer of labelled dendritic cells could help trace the migratory routes of dendritic cells. Murine knockout models have provided and still afford important clues in understanding atherogenesis, but it is certain that the results cannot be directly transferable to those events leading to "common" atherosclerosis in humans. Investigating *vascular dendritic cells* residing in normal human arteries will require a search for other models and other approaches.

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