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Blood-borne cells involved in arterial repair upon experimental incision injury

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Summary. We had previously shown that microscopically detectable infiltration of dendritic cells and expression of Hsp47 in tissue lysates occur during repair upon experimental arterial injury. We have further analysed here the cell types involved in the repair process by histology, electron microscopy and immunofluorescence. Rat carotid arteries were subjected to brief crushing and full thickness incision and were analysed up to 21 d thereafter. Adhesion and activation of platelets occurred 3 h after surgery. A neointima had formed 7 d after surgery, where immature cells entered from the lumen and gave rise to cells rich in organelles of the secretory pathway and endowed with bundles of phalloidin-binding microfilaments. Alpha smooth muscle-positive, secretory and contractile smooth muscle cells were found in the neointima 14 and 21 d after injury. Seven to 21 d after surgery, endothelial cells appeared immature and the newly formed tissue contained MHC-II positive, CD43 positive dendritic cells which clustered with lymphocytes, a few macrophages containing apoptotic remnants and cells labelled for Hsp47. Thin elastic fibrils appeared in the neointima 21 d after injury. The results suggest that the response to acute arterial incision injury is mediated by blood borne cells which differentiate along multiple pathways; the process evolves without reaching stabilization within the observed time lapse; the secretion of extracellular matrix is marked by the expression of Hsp47; and the constant presence of dendritic cells clustered with lymphocytes makes these cells candidate to a pivotal role in the tissue response to injury.

Key words: Dendritic cells, Endothelial cells, Hemangioblasts, Hsp47, Smooth muscle cells

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Introduction

Restenosis is a major problem upon open-field and percutaneous vascular surgery and may even result in vascular occlusion; injury and repair processes of the vessel wall play a major role in these acute conditions as well as upon trauma. The same final events, stenosis or occlusion, can occur in chronic diseases such as graft versus host disease and atherosclerosis, where the processes and mechanisms may be in part similar to those upon acute injury, including major inflammatory alterations (Ross, 1999; Millonig et al., 2001). Detailed knowledge on the cell types involved in injury repair of the arterial wall and on the evolution of this population with time are needed to design and refine strategies aimed at controlling the process and achieving proper healing of surgical and spontaneous vascular lesions while preventing complications caused by excess repair tissue formation (Forte et al., 2007).

Vessel injury induces an inflammatory response characterized by endothelial loss, platelet activation, release of thrombogenic, vasoactive and mitogenic factors and exposure of adhesion molecules (Welt and Rogers, 2002). These events result in leukocyte infiltration, smooth muscle cell activation and proliferation, extracellular matrix production within the vessel wall and endothelial regeneration. A role in the response to vessel injury has been proposed for a long time for macrophages and, more recently, for other immune system cells, namely lymphocytes, dendritic cells and - at least in humans - mast cells (Kaartinen et al., 1994). Many studies have also led to the view that circulating bone marrow-derived progenitor cells can be a major source of neointimal endothelial cells, extracellular matrix secreting cells and smooth muscle cells (Feigl et al., 1985; Sata et al., 2002; Hibbert et al., 2003).

The cells implicated in arterial injury-repair processes may be expected to be equipped with appropriate molecular devices to thrive in the local

microenvironment and to perform specific functions. Among the molecules possibly relevant to these purposes, heat shock proteins (Hsps) can prevent the accumulation of misfolded proteins in a stressful microenvironment (Welch and Suhan, 1986). Among the latter group of proteins, Hsp47 is a specialized chaperone for collagen (Satoh et al., 1996) and is involved in the attachment to the extracellular matrix and in the fibrosis process (Razzaque and Taguchi, 1999).

In an experimental model of surgical injury in the rat that mimics events occurring upon acute arterial disease we were able to show that the formation of a neointima upon injury is accompanied by the activation of the transcription factor NF-kappaB, the de novo expression, or increase above basal levels of some heat shock proteins, and the infiltration of dendritic cells (Rinaldi et al., 2006). The presence of MHC-II expressing, immune system dendritic cells and of their putative, not yet dendritic precursors within the arterial wall had been reported since the late 1990s, first on the basis of electron microscopic analyses (Bobryshev and Lord, 1995). While these cells have been described in low numbers in the apparently healthy arterial wall of humans, where they increase during atherogenesis and in response to acute injury (Skowasch et al., 2003), they have been found in mouse and rat arteries only during experimental atherogenesis (Bobryshev and Lord, 2002; Ozmen et al., 2002) and in response to acute injury, in particular balloon (Bauriedel et al., 2003) and incision injury (Rinaldi et al., 2006).

In the study cited above (Rinaldi et al., 2006) we had addressed molecular events and the behaviour of dendritic cells; we have now extended the study to analyse which other cell types are involved in the injury repair process, which relationship they have to dendritic cells and how they express Hsp47.

Materials and methods

Animals and experimental treatment

Adult male Wistar Kyoto rats (200-250 g; MHC-II haplotype RT1l) were obtained from Harlan Italy (San Piero al Natisone, Italy). The investigation conforms with the Guide for the *Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996) and the research protocols were approved by the Animal Care and Use Committee of the Second University of Naples where living animals were housed and treated. Animals were maintained on a 12 hour light/dark cycle at 24°C and a relative humidity of 60%, with food and water ad libitum; they were adapted to the laboratory housing for one week before experimentation. The rats were anaesthetized with intraperitoneal ketamine hydrochloride (8 mg/100 g) for both surgery and sacrifice. At surgery, sterile techniques were used and a single dose of ticarcillin (5 mg/100 g) was injected immediately after anaesthesia.

Both carotid arteries were reached through an anterior median incision of the neck. One carotid artery was subjected to a crushing lesion with a plastic clamp applied for 10 sec, followed by a full thickness, longitudinal incision 0.5 mm in length. Haemostasis was obtained with a single 8.0 polypropylene stick on the adventitia (Forte et al., 2001). The other carotid artery was left untreated, as control.

Twenty-seven animals were sacrificed and the carotid arteries excised at different time points after surgery. Arteries were excised also from five untreated animals, as a further control.

The number of animals used for microscopy differed from that used for immunofluorescence, because in each case we wanted to confirm the reproducibility of the results in at least three animals and we needed more samples to achieve this goal for immunofluorescence, given the need to label for several antigens with the respective controls and the amount of tissue needed for each slide – with more than one cryosection per slide – as compared with what was enough for light and microscopic observations on plastic sections.

Light and electron microscopy and morphometry

Microscopic analyses were performed 3 h and 7, 14 and 21 days after surgery (3 animals each time point). Samples were fixed in 2% formaldehyde and 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.4, osmicated and embedded in epoxy resin. Sections 1-2 µm thick were stained with alkaline toluidine blue and examined by light microscopy. Sections about 70 nm thick were stained with lead acetate and uranyl acetate and observed in a Jeol JEM 1010 electron microscope (Tokyo, Japan), at 80 kV.

All the cells in electron micrographs between 7 and 21 d after surgery were counted and the tissue surface measured by point counting in order to compute the cell density, which was expressed as the number of cell profiles per 1000 μm^2. Each photomicrograph was used as a sample unit for statistics. The mean and the standard error (SE) are given in the results; the data were subjected to analysis of variance. One hundred cells within the repair tissue were scanned at electron microscopy at each time point between 7 and 21 d after injury and classified according to morphology. The results were evaluated by non parametrical chi square test (Lentner et al., 1982). In all comparisons $p<0.001$ was assumed as significant.

Immunofluorescence

Rats sacrificed 7, 14 and 21 d after surgery (5 animals each time point) were used for immunofluorescence analyses. Samples were fixed with periodate-lysine-paraformaldehyde mixture in 0.1 mol/L phosphate buffer, pH 7.4 (McLean and Nakane, 1974; Pieri et al., 2002), for 1 h, washed in the same buffer for 24 h at room temperature, cryoprotected with 30% sucrose and snap frozen. Cryosections were laid on poly-

L-lysine-coated slides. After blocking non-specific binding sites with 10 mg/mL bovine serum albumin (Sigma) in 0.1 mol/L phosphate buffer, pH 7.4, primary reagents were applied overnight at 4°C at the indicated dilutions. Primary reagents were tetra methyl-rhodamine isothiocyanate-conjugated phalloidin, 1:4 (Sigma, Milan, Italy) and mouse monoclonal antibodies against the following antigens: rat MHC-II-polymorphic, 1:100 (Serotec, Kidlington, Oxford, UK; clone F17-23-2, reacting with RT1l and RT1n); rat monocyte/macrophage, 1:100 (Biosource, Camarillo, CA; clone ED1, reacting with a 90-100 kDa single chain glycoprotein, possibly homologue of human CD68 and mouse macrosialin); rat (and human) Hsp47, 1:200 (Stressgen, Victoria, Canada); rat CD43, 1:50 (Oxford Biotechnology, Kidlington, Oxford, UK); rat (and human) alpha-smooth muscle actin, 1:200 (Sigma). Fluorescein isothiocyanate-labeled, goat anti-mouse, polyclonal antibodies (Sigma; 1:50, 60 min at 37°C) were used as secondary ones. Omission of primary antibodies or substitution with irrelevant ones were used as negative controls for immunofluorescence. The slides were mounted with Gel/Mount (Biomeda, Foster City, CA), observed in an Axioskop microscope equipped for epifluorescence (Zeiss, Oberkochen, Germany) and captured with an Axio Vision 4 system, consisting of a digital multichannel fluorescence module and dedicated software (Zeiss). In cases of double staining, the pictures were reproduced in pseudocolor and superimposed with either Axio Vision 4 or Photoshop 5.0 (Adobe Systems, San Jose, CA) software.

Omission of primary antibodies and substitution with isotype matched, irrelevant ones were used as negative controls for immunofluorescence.

Results

The results were consistent among animals in the same experimental conditions. The negative control slides for immunofluorescence resulted all unstained.

Control arteries

The intima was restricted to the endothelium, which was rich in transcytosis vesicles and adhered to the lamina elastica interna by means of a thin basement

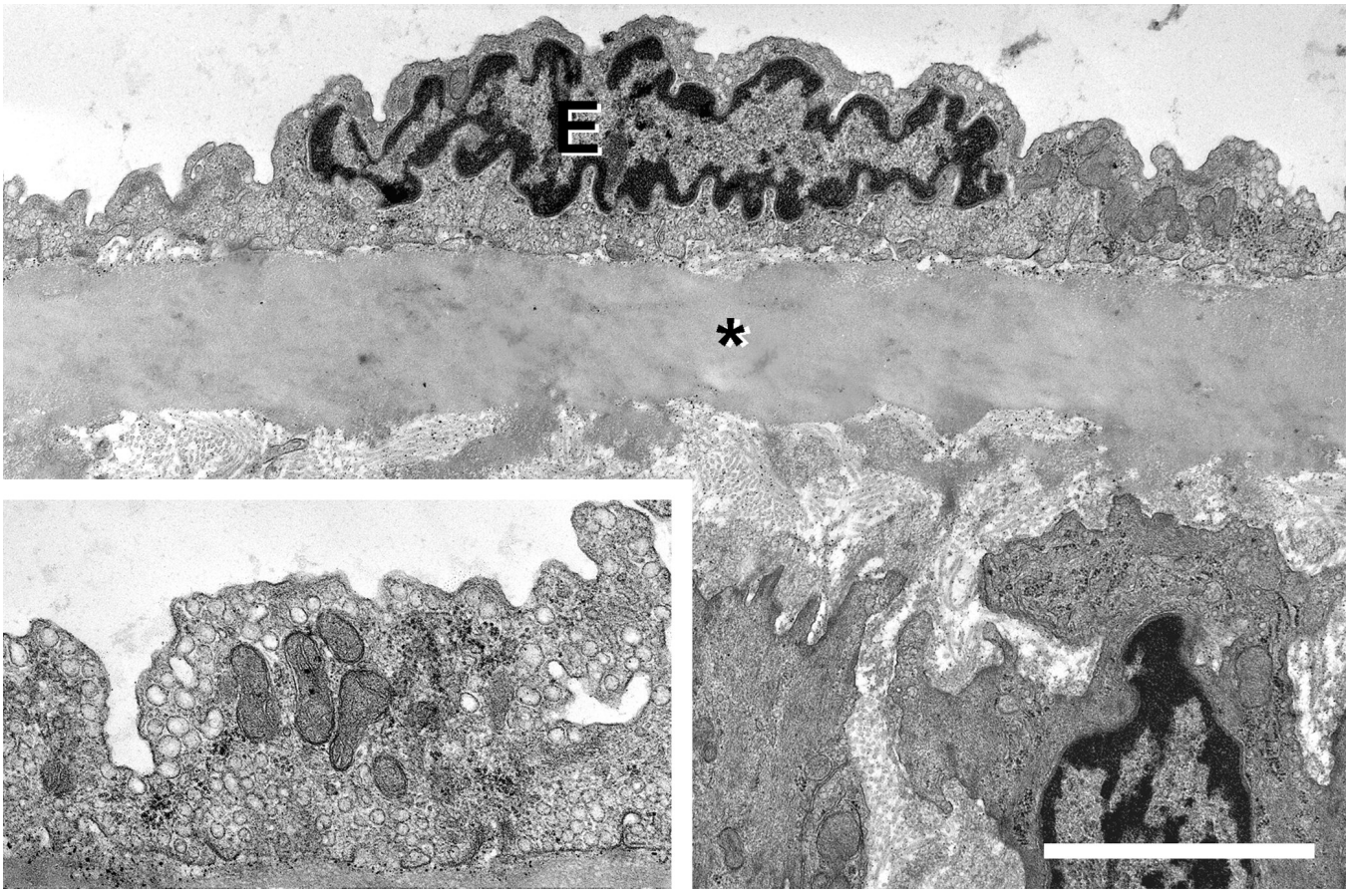

Fig. 1. Electron microscopy of a sham operated artery 14 d after injury. The endothelium (E, and inset) is rich in transcytosis vesicles, devoid of microfilament bundles, and very close to the innermost elastic lamina (asterisk). Scale bar: 2 μm; inset, 0.8 μm.

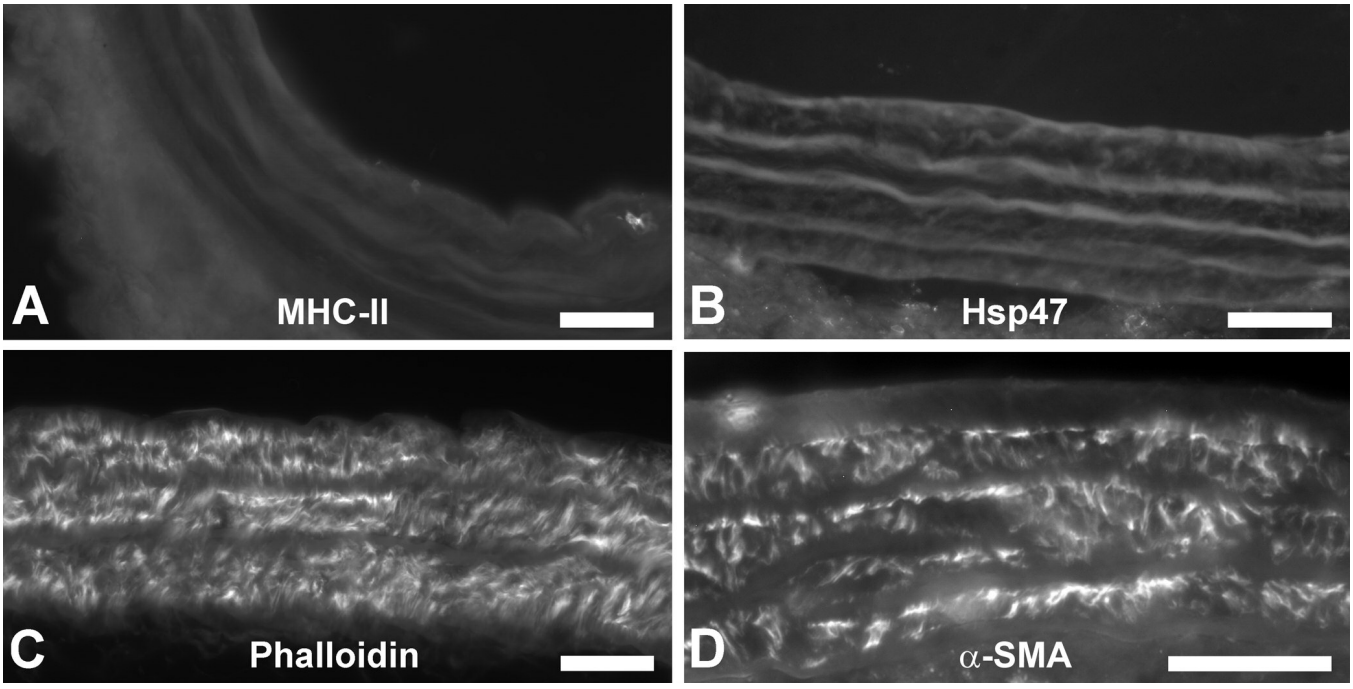

Fig. 2. Immunofluorescence of a sham operated artery 14 d after injury. **A.** The arterial wall is virtually devoid of MHC-II positive cells; one rare exception is shown near the right margin of the panel. **B.** No cells appear stained for Hsp47. **C, D.** Smooth muscle cells in the media are labelled by phalloidin **(C)** and by antibodies against alpha-smooth muscle actin **(D)**. Scale bar: 30 μm.

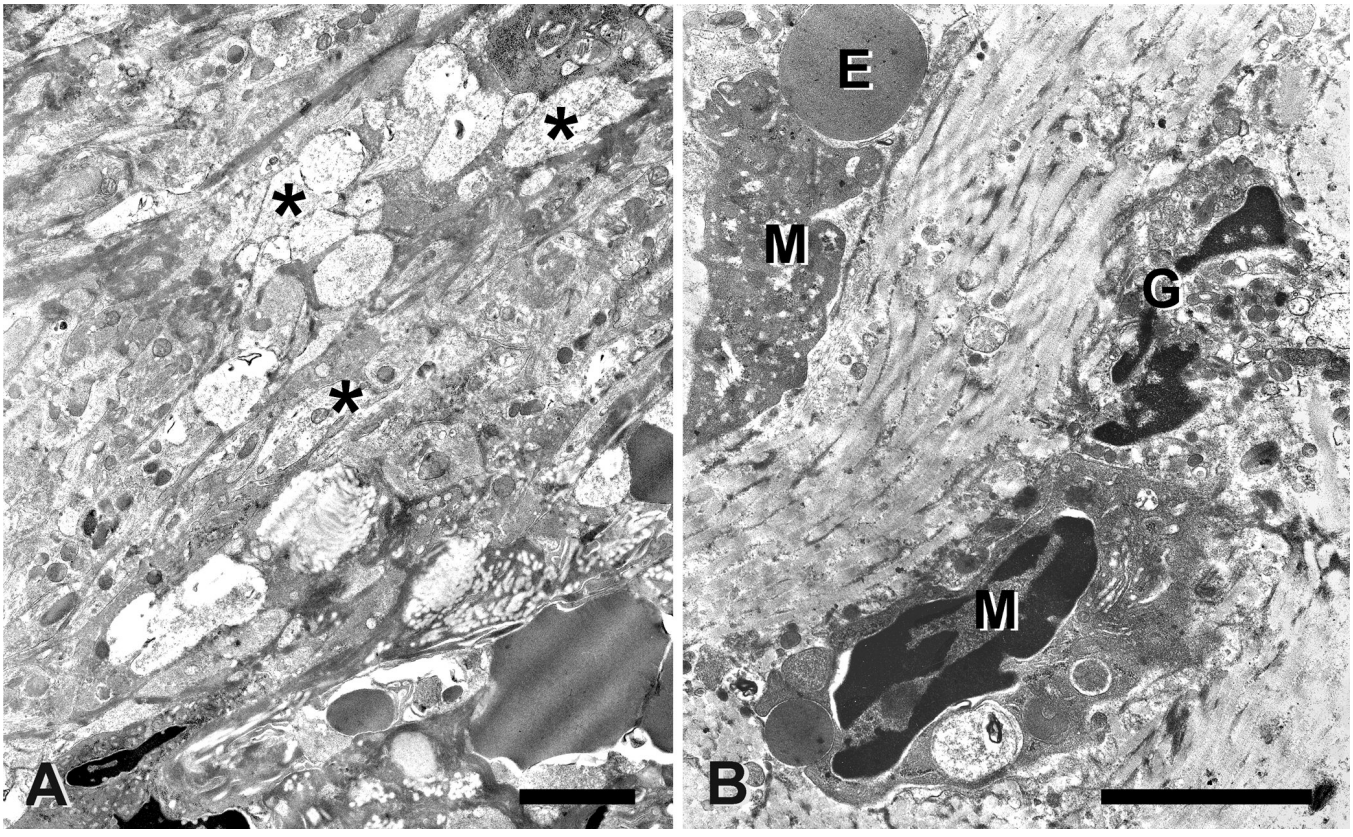

Fig. 3. Electron microscopy of an operated artery 3 h after injury. **A.** Multiple layers of platelets adhere to the arterial wall; many of them are degranulated (asterisks). **B.** The arterial wall is infiltrated by erythrocytes (E), granulocytes (G) and macrophages (M). Scale bar: 2 μm.

membrane (Fig. 1). Three layers of smooth muscle cells in the media alternated to four elastic laminae; the smooth muscle cells were very rich in microfilament bundles, except a strict minority with features of secretory smooth muscle cells, i. e. abundant rough endoplasmic reticulum, large Golgi apparatus, and microfilament bundles restricted to cell projections. Bundles of tubular microfibrils and of collagen fibrils were found in the extracellular matrix among smooth muscle cells (Fig. 1).

By immunofluorescence, exceptional cells were labelled for MHC-II in the intima and no cell was labelled for Hsp47. Smooth muscle cells in the media were labelled by phalloidin and anti-alpha-smooth actin antibodies, as expected (Fig. 2).

Injured arteries

Three hours after surgery multiple layers of platelets adhered to the injury site; many of them appeared to have released their granules (Fig. 3A). Some granulocytes and macrophages had entered the tissue and the latter phagocytosed extravasated erythrocytes (Fig. 3B).

Seven days after surgery a neointima had formed. The endothelium was incomplete and the newly formed tissue appeared rich in cells, mostly elongated, immersed in an extremely loose extracellular matrix devoid of

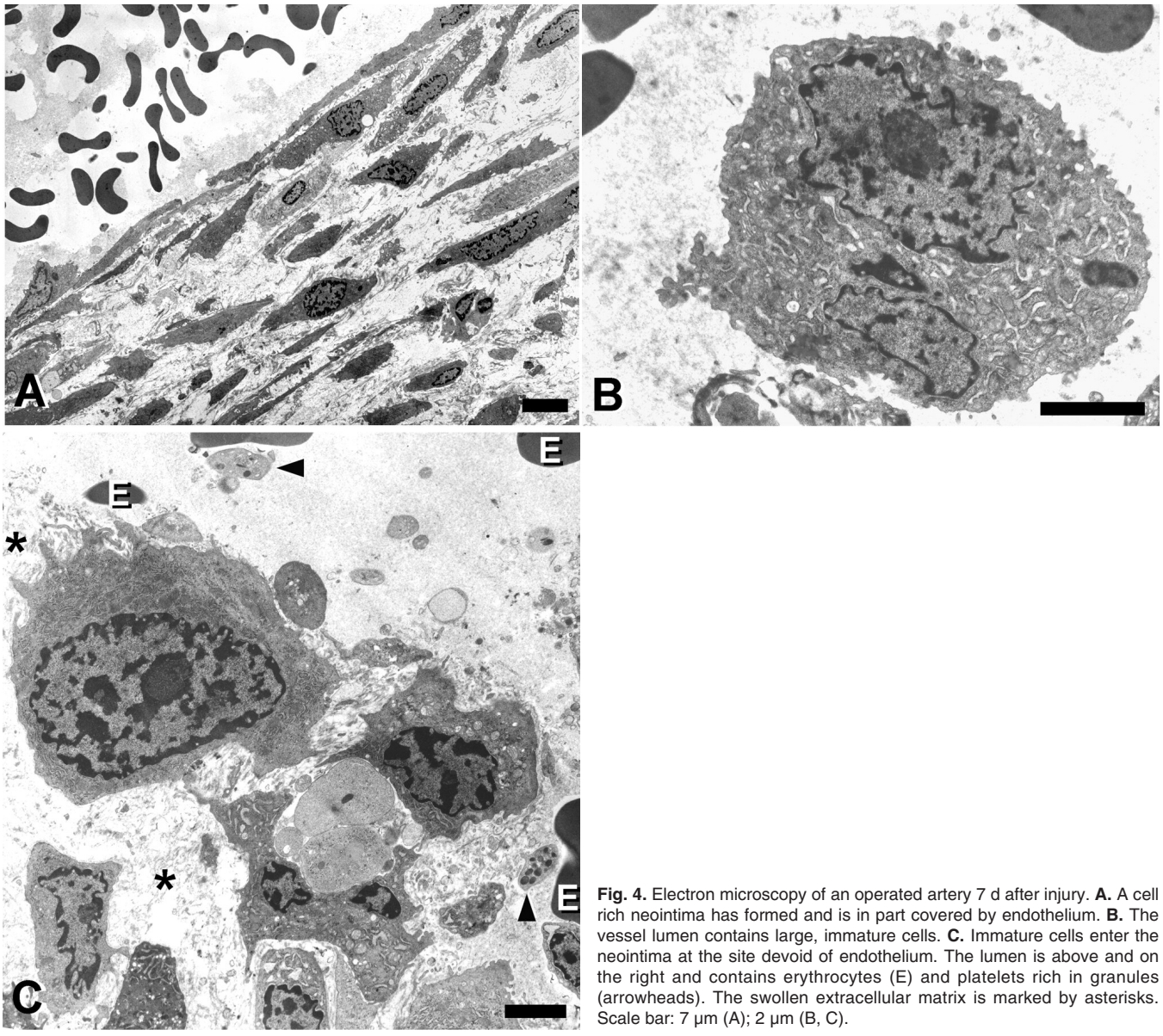

Fig. 4. Electron microscopy of an operated artery 7 d after injury. **A.** A cell rich neointima has formed and is in part covered by endothelium. **B.** The vessel lumen contains large, immature cells. **C.** Immature cells enter the neointima at the site devoid of endothelium. The lumen is above and on the right and contains erythrocytes (E) and platelets rich in granules (arrowheads). The swollen extracellular matrix is marked by asterisks. Scale bar: 7 μm (A); 2 μm (B, C).

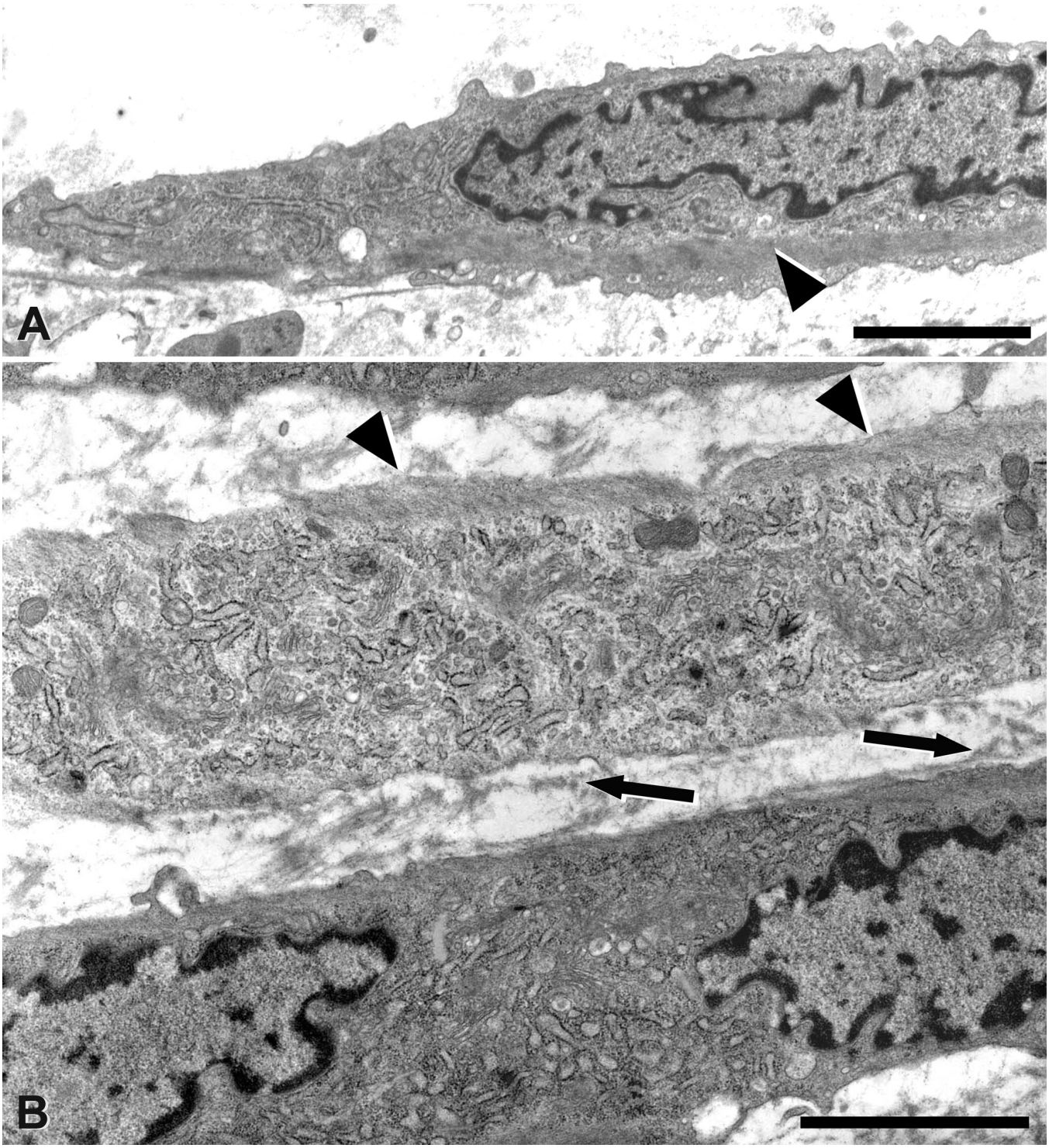

Fig. 5. Electron microscopy of newly formed tissue 7 d after injury. **A.** Detail of an immature endothelial cell, rich in rough endoplasmic reticulum and containing a microfilament bundle near the basal surface (arrowhead). **B.** Detail of two cells in the neointima rich in endoplasmic reticulum and, the one above, with large Golgi apparatus and bundles of microfilaments (arrowheads). Patches of basal lamina are indicated by arrows. Scale bar: 2 μm.

elastic components (Fig. 4A); the infiltration of cells extended to the media and the adventitia. Large, roundish cells were found at the periphery of the lumen; they were rich in organelles, especially rough endoplasmic reticulum (Fig. 4B). Similar cells were found also at the surface of the neointima in sites devoid of endothelium (Fig. 4C). Most endothelial cells were rich in rough endoplasmic reticulum, contained basal bundles of filaments and were poor in transcytosis vesicles (Fig. 5A). Most of the elongated, infiltrating cells contained rough and smooth endoplasmic reticulum, well developed Golgi apparatus but only very few, small lysosomes, and peripheral bundles of microfilaments; they were not surrounded by a basal lamina except for very restricted areas along their membrane (Fig. 5B). Deep in the neointima, and in the media and adventitia, some infiltrating cells were dendritic in shape and organized in a mesh containing lymphocytes (Fig. 6A). Lymphocytes were not found separate from dendritic cells. A few macrophages, containing remnants of apoptotic bodies, were found in the endothelial position (Fig. 6B) and within tissue.

The cell density in the newly formed tissue (number/1000 μm^2; mean ± SE) was 17.74±0.95.

By immunofluorescence, some cells in the neointima and the media, often with a dendritic shape, were labelled for MHC-II (Fig. 7A); MHC-II expressing, mostly dendritic cells were located also in the adventitia in the lesion area. The cells with a dendritic shape in the neointima and elsewhere were labelled also by an antibody against CD43 (Fig. 7B). Very few, roundish cells in the neointima reacted with the monocyte/macrophage specific antibody used (Fig. 7C). Several cells in the injured area were intensely labelled for Hsp47 (not shown). Phalloidin reactive cells were found in the endothelial position and in the superficial layer of the neointima, while only very few cells in the neointima were faintly reactive for alpha-smooth muscle actin (not shown).

Fourteen and twenty-one days after surgery the neointima had enlarged, especially because of increase in the amount of extracellular matrix, which appeared progressively richer in collagen fibres; 21 days after surgery some thin elastic fibres were also found (Fig. 8).

The endothelial lining remained incomplete until the end of the experiment and was made of endothelial cells rich in microfilaments and in organelles of the secretory pathway and poor in transcytosis vesicles (Fig. 9A).

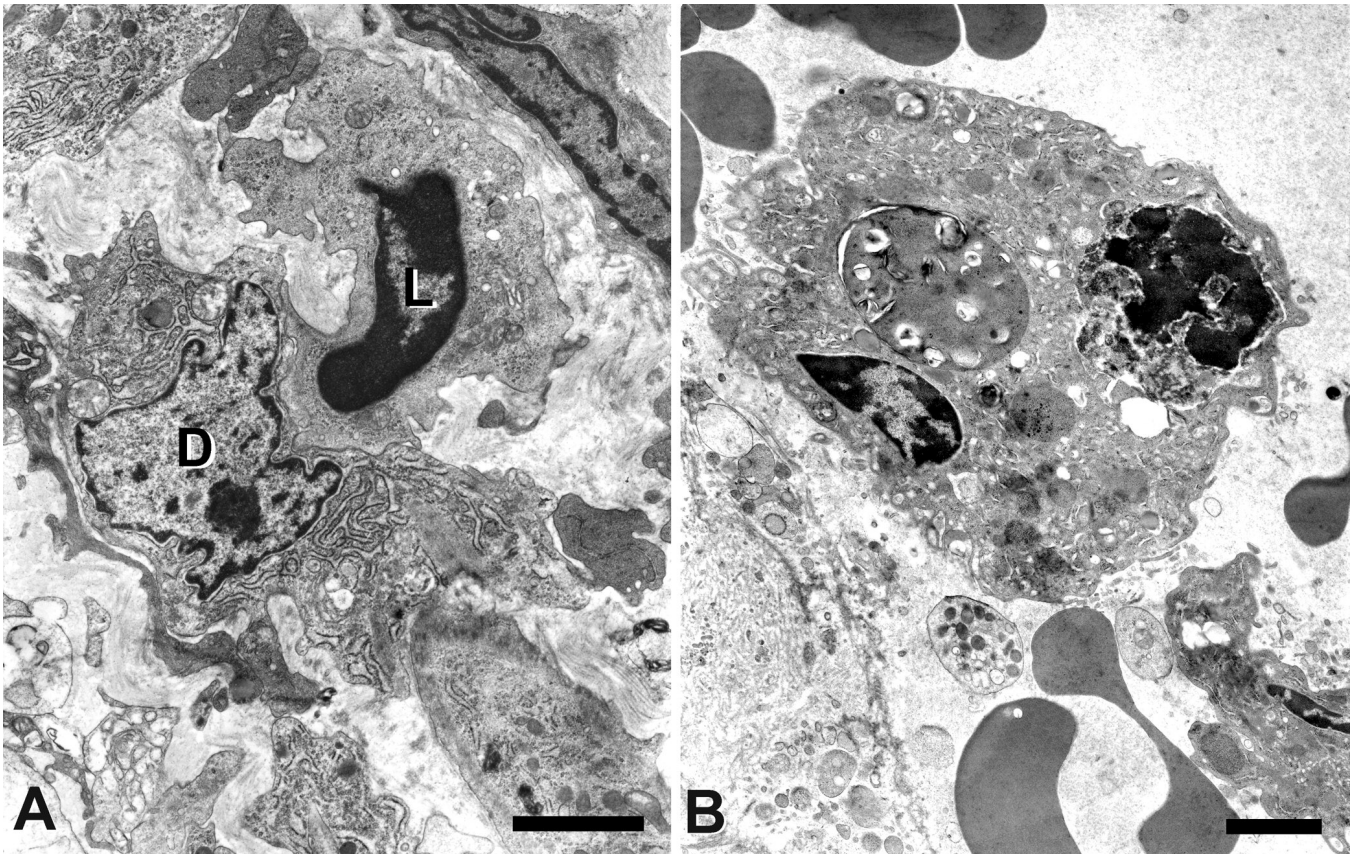

Fig. 6. Electron microscopy of newly formed tissue 7 d after injury. **A.** Dendritic cells (D) associate into a mesh containing lymphocytes (L). **B.** Detail of a macrophage on the neointimal surface containing remnants of apoptotic bodies. Scale bar: 2 μm.

Large, roundish, organelle-rich cells were found beneath the endothelium until 21 d after surgery (Fig. 9A). The extracellular matrix remained loose only immediately below the luminal surface (Fig. 9A), while deeper it became enriched in collagen fibres (Fig. 9B,C); the thin elastic fibres, which appeared 21 d after surgery, had only a relatively little amount of amorphous elastin matrix (Fig. 9B). Cells rich in organelles of the secretory pathway and containing variable amounts of microfilaments were found until the end of the experiment (Fig. 9A-C); some of these cells were especially rich in microfilaments and had the features of contractile smooth muscle cells (Fig. 9C). Multiple, waved, discontinuous sheets of basal lamina like material were found both around these cells, most numerous 14 d after surgery (Fig. 9C), and under endothelial cells (Fig. 9A). Dendritic cells in contact with lymphocytes (Fig. 9D) were also found at all experimental time points, as well as a few macrophages and signs of apoptosis. The cells with signs of apoptosis were most numerous 7 d after surgery (3.44% cells) and decreased thereafter (1.35%, 14 d after surgery; 0.69% 21 d after surgery). Because of the low numbers of these cells in electron photomicrographs a statistical analysis was not performed. Some cells in apoptosis 7 d after surgery were still recognizable as cells with no lineage specificity, secretory muscle cells and macrophages; all the other cells were in such advanced stages of apoptosis that they could not be tributed to any cell type.

Morphometrical analysis at electron microscopy confirmed the long lasting presence of cells without specific lineage features, of dendritic cells and of lymphocytes, the scarcity of macrophages and the late appearance of contractile smooth muscle cells in the repair tissue (Fig. 10). The cell classification used for this analysis is detailed in the caption to figure 10. The cell density in the newly formed tissue (number/1000 μm^2; mean ± SE) was 14.42±0.93, 14 d after surgery, and 30.45±1.85, 21 d after surgery. The value 21 d after surgery was significantly different from both 7 and 14 d after surgery ($p<0.001$).

By immunofluorescence, dendritic shaped cells labelled for MHC-II and CD43 were still numerous in the neointima until 21 d after surgery, and cells reacting with the monocyte-macrophage specific antibody were rare (not shown). Many cells in the neointima were

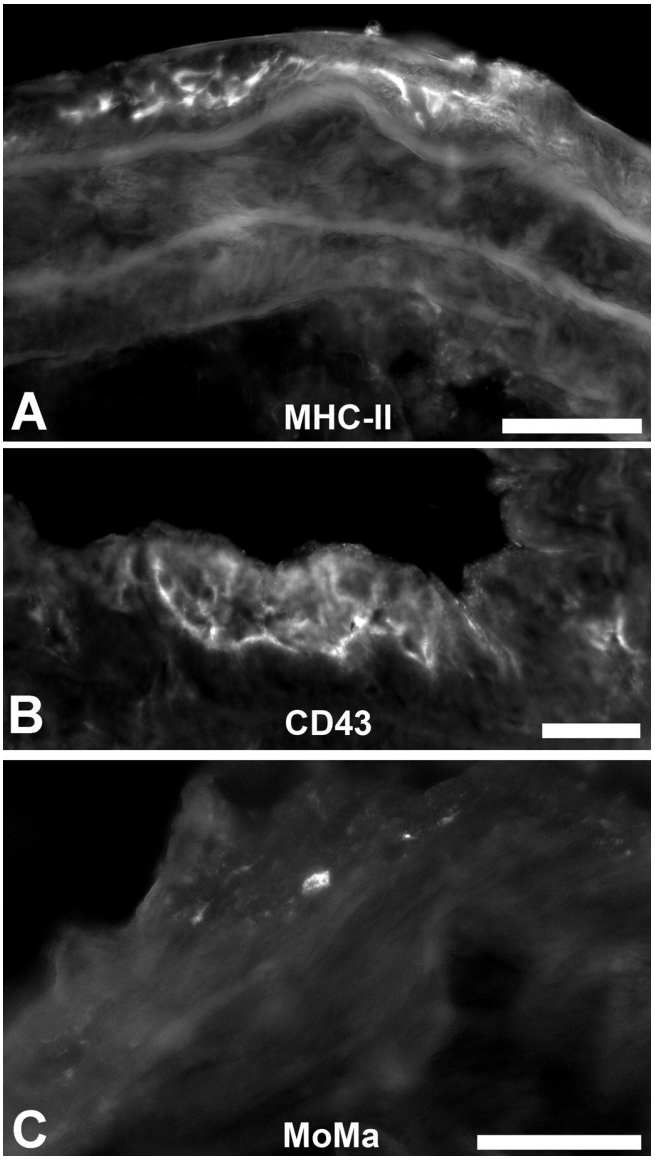

Fig. 7. Immunofluorescence of neointimal cells 7 days after injury. **A, B.** Dendritic cells in the neointima are shown by MHC-II **(A)** and CD43 **(B)** immunolabelling. **C.** An oval cell in the neointima is labelled for a monocyte-macrophage (MoMa) specific antigen. Scale bar: 30 µm.

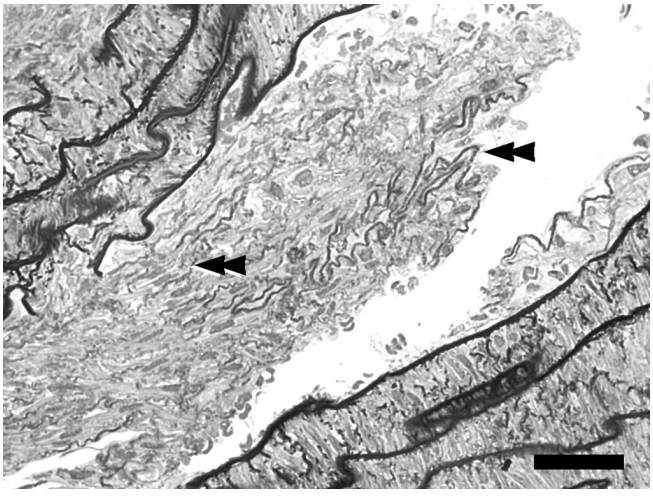

Fig. 8. Light microscopy of neointimal tissue 21 d after injury. The neointima has enlarged by increase in the amount of extracellular matrix, which contained also thin, pale stained elastic fibres (double arrowheads). Scale bar: 30 µm.

reactive for Hsp47 (Fig. 11A). Many phalloidin reactive cells were found in the endothelial position and in the neointima (Fig. 11B). Several cells in the neointima were reactive for alpha smooth muscle actin; the antibodies against alpha smooth muscle actin also labelled smooth muscle cells in the media, as expected (Fig. 11C).

Discussion

This study has moved from the previously reported findings of the electron microscopically detectable infiltration of dendritic cells and the biochemically detectable expression of Hsp47 during repair upon arterial incision injury in the rat (Rinaldi et al., 2006). The present results indicate that: (1) the response to acute arterial injury is mediated, among others, by newly differentiating endothelial and smooth muscle cells; (2) the appearance of the latter cells is preceded and accompanied by that of cells with features not typical of any specific cell lineage and apparently influxing from blood; (3) the differentiation of smooth muscle cells proceeds through several steps identifiable by combined electron microscopical and immunofluorescence analysis; (4) many neointimal cells express Hsp47 concomitant with the start and continuation of extracellular matrix secretion; (5) dendritic cells and lymphocytes represent a considerable fraction of infiltrating cells (no less than one fourth) from the early stages; and (6) the process of cell recruitment and differentiation goes on for several weeks.

We interpret the results as indicating that the organelle-rich, mononuclear cells in the lumen enter the arterial wall and give rise to different cell populations,

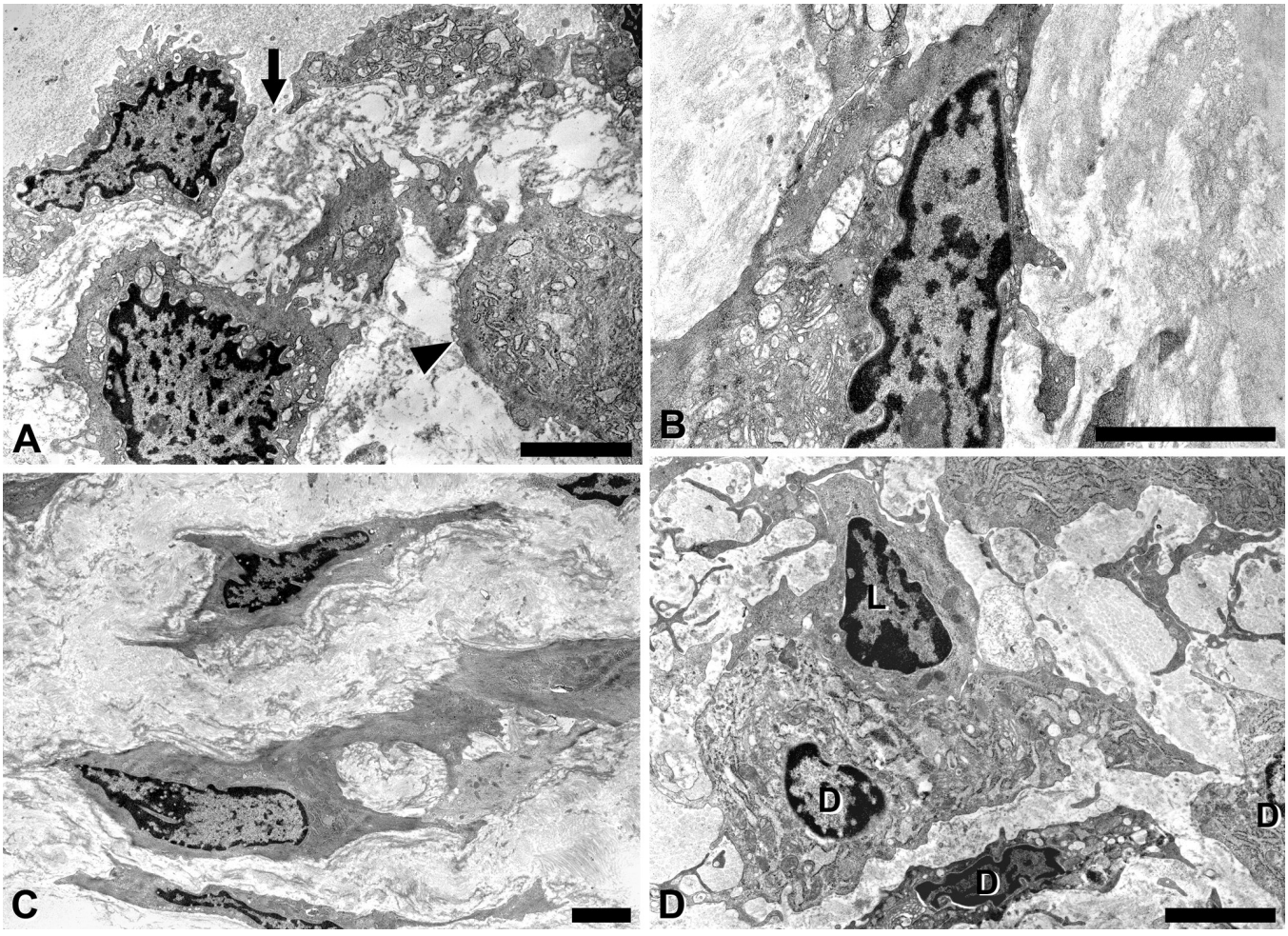

Fig. 9. Electron microscopy of newly formed tissue 14 and 21 d after injury. **A.** 21 d after surgery, the endothelium is still incomplete (arrow) and made of immature cells; the extracellular matrix near the endothelium is loose and hosts immature cells with microfilament bundles (arrowhead) and multiple layers of basal lamina like material among cells. **B.** Tiny elastic fibres appear 21 d after injury, here near a cell with prominent Golgi apparatus. **C.** Smooth muscle cells with variably developed endoplasmic reticulum and Golgi apparatus were found already 14 d after surgery. Note the multilayered, basal lamina-like, pericellular deposits. **D.** Mesh of dendritic cells (D) and lymphocytes (L) are found even 21 d after surgery. Scale bar: 2 μm.

possibly because they are heterogeneous themselves in their differentiation potential. These precursors, possibly together with autochthonous ones (Spyridopoulos and Andres, 1998), differentiate into endothelial cells, dendritic cells, fibroblasts and, later on, secretory, and eventually contractile smooth muscle cells. The finding of precursor cells in the lumen suggests an enhanced mobilization of these cells, as has been shown for hemangioblasts upon ischemia (Takahashi et al., 1999; Sata et al., 2002; Bailey and Fleming, 2003; Hibbert et al., 2003; Hristov and Weber, 2004; Roufosse et al. 2004), for dendritic cell precursors upon major trauma and burns (Gothelf et al., 1988, 1989), and for so-called peripheral blood fibrocytes upon burns (Yang et al., 2002). As far as we could recognize the features of the cells undergoing apoptosis, we found that immature cells with no lineage specificity, secretory muscle cells and macrophages underwent apoptosis as well; we cannot exclude that this process affected also dendritic cells and lymphocytes, because in several instances the cell type specific features were no more recognizable at electron microscopy.

The findings after surgery indicate that platelets adhere to the arterial wall and become activated, which has the release of granules as a morphological counterpart. The secretion of molecules stored in granules and possibly of other mediators can originate signals which trigger monocyte arrest on activated endothelium (von Hundelshausen et al., 2001) and the recruitment of bone marrow derived progenitor cells to sites of vascular injury (Massberg et al., 2006). This effect is synergic with that of stromal cell-derived factor (SDF)-1alpha, which is released upon apoptosis of arterial wall cells in response to injury (Zernecke et al., 2005).

The differentiation of endothelial cells from hematogeneous precursors is supported by several lines of evidence (Bailey and Fleming, 2003; Hibbert et al., 2003), however, neighbouring endothelial cells may also give rise to new cells (Spyridopoulos and Andres, 1998; Shizukuda et al., 1999; Davis et al., 2003). The cytological features of the endothelial cells seen here until the end of the experiment correspond to those of

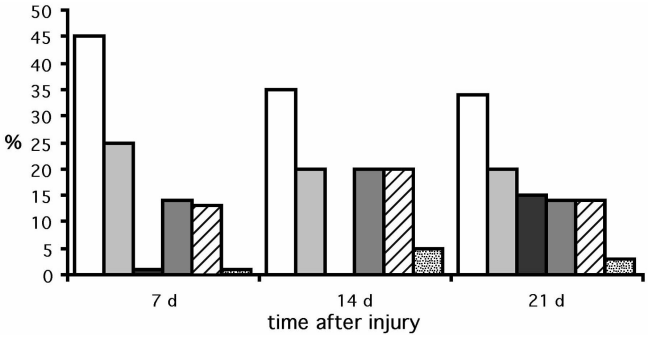

Fig. 10. Cells within repair tissue. Data are expressed in per cent. For this analysis, roundish, monocyte like cells, as well as elongated, variably organelle rich cells and cells with intermediate features were counted as cells with no lineage specificity (white columns); microfilament bundle rich cells were considered to be of muscle cell lineage although we did not test the expression of alpha-smooth muscle actin at electron microscopy and were classified as secretory (pale grey columns) or contractile (black columns) depending on whether microfilaments occupied less or more than half the cytoplasm. Dark grey columns: dendritic cells; hatched columns: lymphocytes; dotted columns: macrophages. The differences in the distribution of cell types among time points were significant ($p<0.001$).

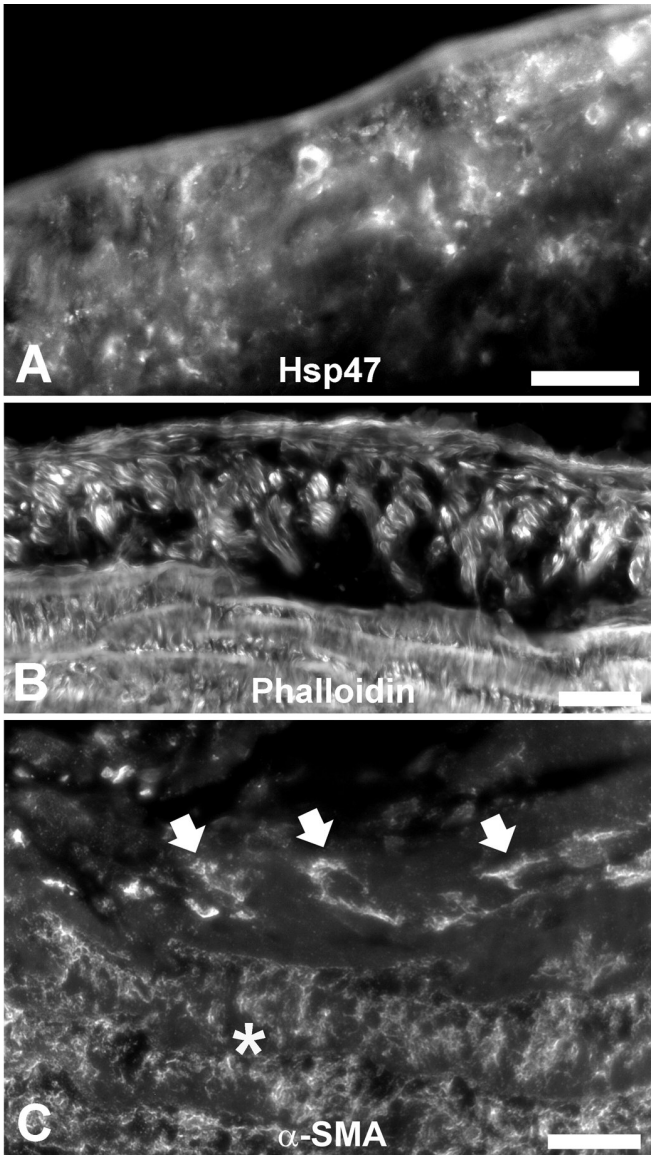

Fig. 11. Immunofluorescence of neointimal cells 14 days after injury. **A.** Cells in the neointima are labelled for Hsp47. **B, C.** Many cells in the neointima contain filamentous actin labelled by phalloidin (**B**), but only a few are labelled for alpha-smooth muscle actin (arrows in **C**), which on the contrary is expressed by all muscle cells in the media (asterisk). Scale bar: 30 μm.

immature endothelial cells as can be seen during angiogenesis (Amselgruber et al., 1999; Ratajska and Fiejka, 1999); given that endothelial cells are usually quick to differentiate (Bertipaglia et al., 2003; Hristov and Weber, 2004), this finding may indicate that endothelial cells undergo protracted turn-over in these conditions because of continuous damage under locally stressful conditions.

The presence of filamentous actin in neointimal cells was shown by electron microscopy and by the reactivity with fluorescent phalloidin, which tags filamentous actin independent of the molecule isoform (Allen et al., 1996) and the cell type (Ross et al., 1999; Capani et al., 2001a,b; Koizumi et al., 2004; Manni et al., 2004). Seven days after injury, part of the cells was very rich in microfilaments at electron microscopy, so that we classified them among secretory smooth muscle cells for morphometry, but the reactivity for alpha-smooth muscle actin started only 14 d after injury. Therefore we could detect a progression from microfilament rich, myofibroblast-like cells (Chaponnier and Gabbiani, 2004) to alpha-smooth actin positive, secretory smooth muscle cells and then to contractile smooth muscle cells in the repair tissue. Myofibroblasts, secretory smooth muscle cells and contractile smooth muscle cells in the arterial wall have long been considered to derive from pre-existing smooth muscle cells of the media (e.g., Ryan et al., 2003), but they can also differentiate de novo from circulating precursors (reviewed by Chaponnier and Gabbiani, 2004), some of which may be in common with endothelial cells (Bailey and Fleming, 2003; Hibbert et al., 2003). Neointimal smooth muscle cells, in turn, have been shown to display a pro-inflammatory phenotype which depends on NF-kappaB activation and leads to the secretion of chemokines stimulating the recruitment of monocytes and memory T cells (Raines et al., 2004; Zeiffer et al., 2004).

The steps and timing of neointima formation found in this study are similar to those seen upon balloon catheter injury in the rat (Ryan et al., 2003), while the distribution of neointimal tissue varies with the localization of injury itself (all around the lumen in balloon injury, on one side only in incision injury). In our opinion, this adds value to the model used here as a way to give information on molecular and cell mechanisms of the intimal response not only to incision, which is the experimental equivalent of vascular surgery, but also to injury restricted to the intima, such as occurs in endoarterial surgery and atherosclerosis. Results in the pig coronary artery after stent implantation and percutaneous transluminal angioplasty (only the latter procedure leading to media disruption) support the similarity in the repair mechanisms among different types of injury (Christen et al., 2001).

The appearance of Hsp47 expressing cells in this study matched in time with the finding of the same protein in tissue lysates (Rinaldi et al., 2006) and with the secretion of extracellular matrix (present data); Hsp47 is a glycoprotein that has been found to associate with procollagen in the endoplasmic reticulum and is considered to act as a collagen-specific chaperone (Rocnik et al., 2001). Our data are in agreement with a previous study that demonstrated, in rat carotid artery, an over expression of Hsp47 in smooth muscle cells after balloon injury associated with overproduction of collagen and intimal thickening (Murakami et al., 2001). A relationship between Hsp47 expression and fibrosis has been identified in human coronary arteries, where Hsp47 was localized to the fibrous cap of the atherosclerotic plaque (Rocnik et al., 2000). The present results lead us to propose Hsp47 as a sensitive specific marker of the early phases of the fibrosing process.

We found cells, at least some of which dendritic in shape, expressing CD43. This membrane molecule can act as a passive repulsive barrier between cells, or as an adhesion molecule to CD54 (Horejsi, 1999) and to the lectins galectin-1 (Rabinovich et al., 2002) and E-selectin (Matsumoto et al., 2005, 2007; Fuhlbrigge et al., 2006). CD43 on T lymphocytes may mediate interactions with antigen presenting cells adding efficiency to TCR mediated signaling (Fierro et al., 2006), inhibiting TCR mediated apoptosis (He and Bevan, 1999), and mediating the intracellular activation of the transcription factors AP-1, NK-AT and NF-kappaB (Santana et al., 2000). CD43 is expressed by dendritic cell precursors and "immature" dendritic cells (Xia et al., 1991; Fanales-Belasio et al., 1997; Naik et al., 2006; Yrlid et al., 2006; Velten et al., 2007) and its cross-linking leads to dendritic cell activation and increased ability to stimulate T lymphocytes (Fanales-Belasio et al., 1997; Corinti et al., 1999; Delemarre et al., 2001; Velten et al., 2007).

The presence of dendritic cells in the newly formed tissue and the fact that they are in part fully mature, as indicated by the contact with lymphocytes shown at electron microscopy, had been the object of a previous paper (Rinaldi et al., 2006). Here we show that dendritic cells are in part immature, as shown by CD43 expression (Fanales-Belasio et al., 1997; Corinti et al., 1999; Delemarre et al., 2001). This finding indicates that an influx of precursors and differentiation of cells continues to occur in the injured arterial wall for a long time and is part of a vicious cycle leading to progressive thickening of the neointima. These cells can regulate the recruitment of blood borne cells and their differentiation, migration and function within the vessel wall by several mechanisms: the presentation of antigens to lymphocytes, including antigens possibly derived from damaged tissue and from cells undergoing apoptosis (Schultz and Harrington, 2003); the secretion of several cytokines (Foti et al., 1999; Sallusto et al., 1999; Zhu et al., 2000); and the stimulation of lymphocytes to secrete more of such molecules (Kim et al., 2003) and to activate other cell types to secrete further amounts of cytokines, coagulation tissue factor and matrix metalloproteinases (Monaco et al., 2004).

More specifically, dendritic cells can synthesize and secrete nitric oxide (Morhenn, 1997) and many

cytokines and growth factors which have non immune cells among their targets: IL-1beta, IL-6, IL-8, IL-10, IL-12, TNF-alpha, type I interferon, IFN-gamma, MCP-1, MIP-1, MIP-2, RANTES (Foti et al., 1999; Sallusto et al., 1999; Zhu et al., 2000). They can also specifically influence the regeneration of vascular tissue since they express the VEGFR-3 ligands VEGF-C and VEGF-D (Baluk et al., 2005). Therefore, in the arterial wall dendritic cells may provide coordinated signals that are essential for the recruitment and differentiation of other cell precursors and for the regulation of both cell survival and apoptosis during tissue repair (Bauriedel et al., 2003).

The results of this study upon incision injury differ from those upon balloon injury (Bauriedel et al., 2003) because in the latter model the cell density and the number of dendritic cells in the neointima peaked 4 d after injury and then decreased. Here dendritic cells did not decrease appreciably between 7 and 21 d after injury and the cell density increased with time. On the contrary, in both experimental models apoptosis peaked relatively early after injury and then decreased and the same was true for Hsp47 (which for incision injury had been reported previously: Rinaldi et al., 2006), although the latter decrease was more marked upon incision than upon balloon injury. These differences suggest that full thickness incision of the arterial wall stimulates more protracted influx and differentiation of cells than injury limited to the intima. The more frequent finding of apoptosis at relatively early time points upon injury in both experimental conditions suggests that the microenvironment becomes progressively less stressful for cells while repair proceeds.

The results of animal studies, like this one, cannot fully account for what happens in humans upon surgery or during atherosclerotic disease, for several reasons. Laboratory rodents are characterized by small size, relatively low blood arterial pressure, limited lipid load and relatively short life span. Also, the cell populations of the arterial wall, at rest and upon injury, do not correspond exactly between humans and rats, since a few dendritic cells are found in the intima of human arteries even in the absence of pathology (Bobryshev and Lord, 1995), together with a few mast cells (Jeziorska et al., 1997), and both types increase in number in pathological conditions such as atheroma, while mast cells have not been found to participate at all to the newly formed tissue in the present study.

Nonetheless, the results of this study may be of relevance to humans inasmuch they draw attention on therapies directed to reduce the immune cell recruitment and activation within lesions of the arterial wall as potentially useful tools for the prevention of restenosis upon vascular surgery and of progression and complication of atherosclerosis. Also, these results raise the question whether the prophylactic effects of non-steroid antinflammatory drugs in these conditions may be exerted also through an inhibition of immune cell mediated inflammation, besides their well known inhibition of platelet aggregation.

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