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Ultrastructural localisation of nitric oxide synthase, endothelin and binding sites of lectin (from *Bandeirea simplicifolia*) in the rat carotid artery after balloon catheter injury

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ABSTRACT

An immunocytochemical and cytochemical study has been made on the ultrastructural localisation of type III (endothelial) nitric oxide synthase, endothelin-1 and the binding sites of lectin from *Bandeirea simplicifolia* to the endothelium surface-associated glycoproteins in the rat left common carotid artery at 1 and 28 d after Fogarty embolectomy balloon catheter-induced injury. Controls were carotid arteries from sham operated rats. In the controls, the immunoreactivity to nitric oxide synthase-III and endothelin-1 was localised in different proportions in vascular endothelial cells (36.9 % ± 4.3 and 7.6 % ± 2.7, respectively); immunoreactivity was confined to the cytoplasm and the membranes of intracellular organelles and structures. In contrast, staining with lectin was localised on the luminal surface of all endothelial cells. 1 d after injury, platelets were adherent to the endothelium-denuded intima. Some of the platelets displayed immunoreactivity to nitric oxide synthase-III and endothelin-1 and were stained with lectin. 28 d after injury, a neointimal thickening of substantial size was present. Subpopulations of the regrown endothelial cells covering the luminal surface of the neointima showed positive immunoreactivity to nitric oxide synthase-III and endothelin-1 but there was a significant decrease in the proportion of nitric oxide synthase-III-containing endothelial cells (17.2 % ± 1.9; $P < 0.001$) and a significant increase in the proportion of endothelin-1-containing endothelial cells (36.9 % ± 4.7; $P < 0.001$) compared with the controls. Staining with lectin was associated with the cell membrane of all endothelial cells and in addition with cells located 'deeper' in the neointima which showed lectin-positive plasmalemma, Golgi complex and multivesicular bodies/lysosomes. In conclusion, regenerated endothelial cells of the neointima showed reduced population (2–fold) of nitric oxide synthase-III- and increased population (5–fold) endothelin-1-positive cells. The subendothelial location of some lectin-stained cells after balloon catheter injury indicates the heterogeneity of the neointima and suggests that some of these cells are involved in early angiogenesis. 24 h and 28 d after injury some platelets showed positive immunoreactivity for nitric oxide synthase-III and endothelin-1.

Key words: Vasculature; endothelium; vasoactive agents; glycoproteins; angioplasty.

INTRODUCTION

Neointimal thickening (restenosis) of the arterial wall appears following percutaneous transluminal angioplasty, the procedure used to relieve arterial stenosis to increase the luminal diameter and to improve blood flow (McBride et al. 1988). This procedure also denudes endothelium from the intima of the arterial wall. The mechanisms and/or factors responsible for postangioplasty restenosis are not yet fully understood

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but a cascade of cytokines and growth factors are released in response to this injury and these stimulate the various cellular responses (Ross, 1993). It has been suggested that platelet derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) stimulate the smooth muscle cells of the media to proliferate abnormally and migrate towards the injured intima (Lindner et al. 1991). bFGF has also been shown to enhance vascular endothelial cell proliferation/regrowth and increases intimal thickening in response to balloon catheterisation (Lindner et al. 1990). The pathogenesis of restenotic lesions initiated by endothelial loss is thus a complex process involving a variety of factors derived from damaged endothelium and subendothelial extracellular matrix and/or basement membrane, vascular smooth muscle, platelets, monocytes/macrophages and plasma (see Shirotani et al. 1993). Most recent data by Wheeler et al. (1995) indicate that vimentin (a vital protein of the cytoskeleton) from damaged endothelium is a primary factor contributing to the rapid and diffuse spread of arterial disease. Additionally adventitial damage (Milner et al. 1995) may also participate in these changes (Booth et al. 1989).

Healthy intact endothelium plays a crucial role in maintaining the normal physiological responses of the blood vessel wall to changes in blood pressure and flow (Furchgott & Zawadzki, 1980; see Ralevic & Burnstock, 1995). Vasoactive agents have been shown to act via endothelial cells, causing relaxation of vascular smooth muscle via endothelium-derived relaxing factor(s) (EDRFs) or contraction via endothelium-derived contracting factor(s) (EDCFs) (see Lincoln et al. 1991; Shepherd & Vanhoutte, 1991). The EDRF, identified as nitric oxide (NO), is synthesised from L-arginine by NO-synthase (NOS) (Palmer et al. 1987). The L-arginine: NO system plays an important role in the regulation of blood pressure and flow (Moncada et al. 1991; Shepherd & Vanhoutte, 1991). The role of endothelin-1 (ET-1) which is also synthesised in endothelial cells is mostly related to its potent vasoconstrictor and mitogen activity for vascular smooth muscle (Yanagisawa et al. 1988; Hirata et al. 1989)

Immunoreactivity to NOS [type I (NOS-I, neuronal/brain) and type III (NOS-III, endothelial), see Förstermann et al. 1991] and ET-1 have been observed in the animal and human endothelium of different vascular beds (Bredt et al. 1990; Miyauchi et al. 1990; Loesch et al. 1991, 1993, 1994; Tomlinson et al. 1991; Klimaschewski et al. 1992; Springall et al. 1992; Loesch & Burnstock, 1993, 1995; Nozaki et al. 1993; Pollock et al. 1993; Sexton et al. 1995, 1996). Using polyclonal antibody to NOS-I, immunoreactivity for NOS has also been observed in vascular smooth muscle of the coronary and pulmonary arteries of newborn rats (Loesch & Burnstock, 1995). In blood vessels, the ET-1-labelling is a characteristic feature of the endothelium but not the vascular smooth muscle (see Loesch & Burnstock, 1995).

Lectins/isolectins have been shown to bind selectively to vascular endothelium (and some epithelial cells) (see Jackson et al. 1990; Coffin et al. 1991). Human endothelium, for example, has the ability to bind to the lectin UEA I (from *Ulex europaeus I*); this lectin binds to L-fucose residues on endothelial cells (Holthofer et al. 1982; Hormia et al. 1983; Jackson et al. 1990). Studies of various mouse tissues indicate that endothelial cells display a highly specific surface glycosylation pattern (Laitinen, 1987). Endothelium surface-associated alpha-D-galactosyl residues (part of glycocalyx glycoproteins) preferentially bind to lectins belonging to the *Griffonia simplicifolia* group (Laitinen, 1987).

In the present study we have examined the ultrastructural distribution of immunoreactivity to NOS-III and ET-1 and stained the intima of rat left carotid artery (LCA) with lectin, 1 and 28 d after Fogarty balloon embolectomy catheter-induced vascular injury in vivo (rat carotid model of neointimal thickening (Clowes et al. 1983)).

MATERIALS AND METHODS

Adult male Wistar rats ($\sim$ 300 g, n = 11) were used in this study. LCA angioplasty was performed in 8 rats as previously described (Clowes et al. 1983). General anaesthesia with midazolam (1 mg/kg, Roche, Hertfordshire, UK) and fentanyl (1 mg/kg, Janssen Pharmaceutica, Gell, Belgium) was followed by neck dissection and exposure of the LCA. The external carotid artery was ligated distally and excised proximally for access of the FG2-Fogarty balloon embolectomy catheter (Baxter Healthcare, Santa Anna, USA) which was passed 3 times along the LCA while distended with 20 µl saline. Sham operated control rats (n = 3) underwent anaesthesia then neck dissection and ligation of the external carotid artery. Conditions of these procedures were applied under the current Animals (Scientific Procedures) Act, 1986, UK. At 1 (n = 4) and 28 (n = 4) d after surgery, the rats were reanaesthetised and then killed by exsanguination through an aortic canula which was subsequently used for perfusion in situ at 100 mmHg with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 for 15 min. The

LCA was removed and placed in fixative overnight at 4 °C. The proximal segment (that nearest to the aortic arch) of LCA (~ 1 cm long) was rinsed in the phosphate buffer and processed for the preembedding electron-immunocytochemistry of NOS-III and ET-1 and electron-cytochemistry of lectin.

Immunocytochemistry

Longitudinal strips (~ 5 mm long) of LCA were processed for peroxidase-antiperoxidase (PAP) immunocytochemistry as previously reported (Loesch & Burnstock, 1988, 1995). The mouse monoclonal anti-NOS-III antibody (Transduction Laboratories, Lexington, USA), goat-antimouse immunoglobulin G serum (Sigma, Dorset, UK) and a mouse PAP complex (Sigma) were used at dilutions of 1:250 (1 μg/ml), 1:40 and 1:200, respectively. The rabbit polyclonal anti-ET-1 antibody (Cambridge Research Biochemicals, CRB, Cambridge, UK), goat-antirabbit immunoglobulin G serum (Biogenesis, Bournemouth, UK) and a rabbit PAP complex (DAKO, Glostrup, Denmark) were used at dilutions of 1:1000, 1:50 and 1:75, respectively. After the immunoprocedure, specimens were postfixed in 1% osmium tetroxide, dehydrated in a graded series of ethanol and propylene oxide and embedded in Araldite. Ultrathin circumferential sections were stained with uranyl acetate and lead citrate and subsequently examined with a JEM-1010 electron microscope.

Controls for immunocytochemistry

The mouse anti-NOS-III monoclonal antibody [(N30020), isotype: IgG1, specificity: human and rat NOS-III)] was manufactured and characterised by Transduction Laboratories, Lexington, USA (distributed by Affiniti, Exeter, UK). To avoid the possibility of cross-reactivity, the antibody was used at an optimal dilution of 1 μg/ml according to the manufacturer's directions.

The rabbit polyclonal antibody to synthetic human/porcine ET-1 (CRB) has been used in immunocytochemical labelling of ET-1 in endothelial cells (Loesch et al. 1991; Loesch & Burnstock, 1995) as well as in immunoassay to detect ET-1 in the perfusate of freshly harvested endothelial cells (Milner et al. 1990). Preabsorption of ET-1 antibody with its respective antigen (synthetic human ET-1, from CRB) at a concentration of 10^{-4} M eliminated positive labelling in human and animal vascular endothelial cells (Loesch et al. 1991; Loesch & Burnstock, 1995; Sexton et al. 1996). An inhibition enzyme-linked immunosorbent assay (ELISA) showed that this antibody cross-reacted with proendothelin 39 (7%), ET-2 (15%) and ET-3 (100%) (Bodin et al. 1992). Preincubation of ET-1 antibody with 10 nmol of human ET-1, ET-2 or ET-3 per ml of optimally diluted antibody was sufficient to abolish immunostaining (CRB).

In the present PAP study, no immunolabelling was observed when the NOS-III and ET-1 antibodies were omitted from the incubation medium and/or replaced with nonimmune normal goat serum and nonimmune normal rabbit serum (both from Nordic Immunology, Tilberg, The Netherlands), and nonimmune normal mouse serum (BioCell, Cardiff, UK) as well as when goat-antirabbit immunoglobulin G serum (Biogenesis) and goat-antimouse immunoglobulin G serum (Sigma) were omitted. Preabsorption experiments with human endothelial cell lysate [recommended as a positive control in Western blotting studies (Transduction Laboratories)] indicate a substantial reduction in eNOS immunoreactivity in rat carotids.

Cytochemistry

Longitudinal strips (~ 5 mm long) from the proximal segments of LCA were incubated overnight at 4 °C with the peroxidase-labelled lectin from *Bandeirea simplicifolia* (*Griffonia simplicifolia*; see Laitinen, 1987) BS-I isolectin B_4 (Sigma), at a concentration of 100 μg/ml in 0.05 M Tris-buffered saline (TBS) at pH 7.6 (DAKO, Carpinteria, USA). Peroxidase was detected with 3,3′-diaminobenzidine (Sigma). Specimens were postfixed in 1% osmium tetroxide, dehydrated in a graded series of ethanol and propylene oxide and embedded in Araldite. Ultrathin circumferential sections were stained with uranyl acetate and lead citrate and subsequently examined with a JEM-1010 electron microscope. Controls were specimens incubated in TBS.

Measurements

Limited quantitation has been made on endothelial cells. In order to establish the percentage of endothelial cells positive and negative for NOS-III and ET-1, these cells were counted in ultrathin sections taken from different levels of specimens separated from each other by a distance of ~ 20 μm (average size of endothelial cells) to avoid double counting of the same cells. Endothelial cells were counted by

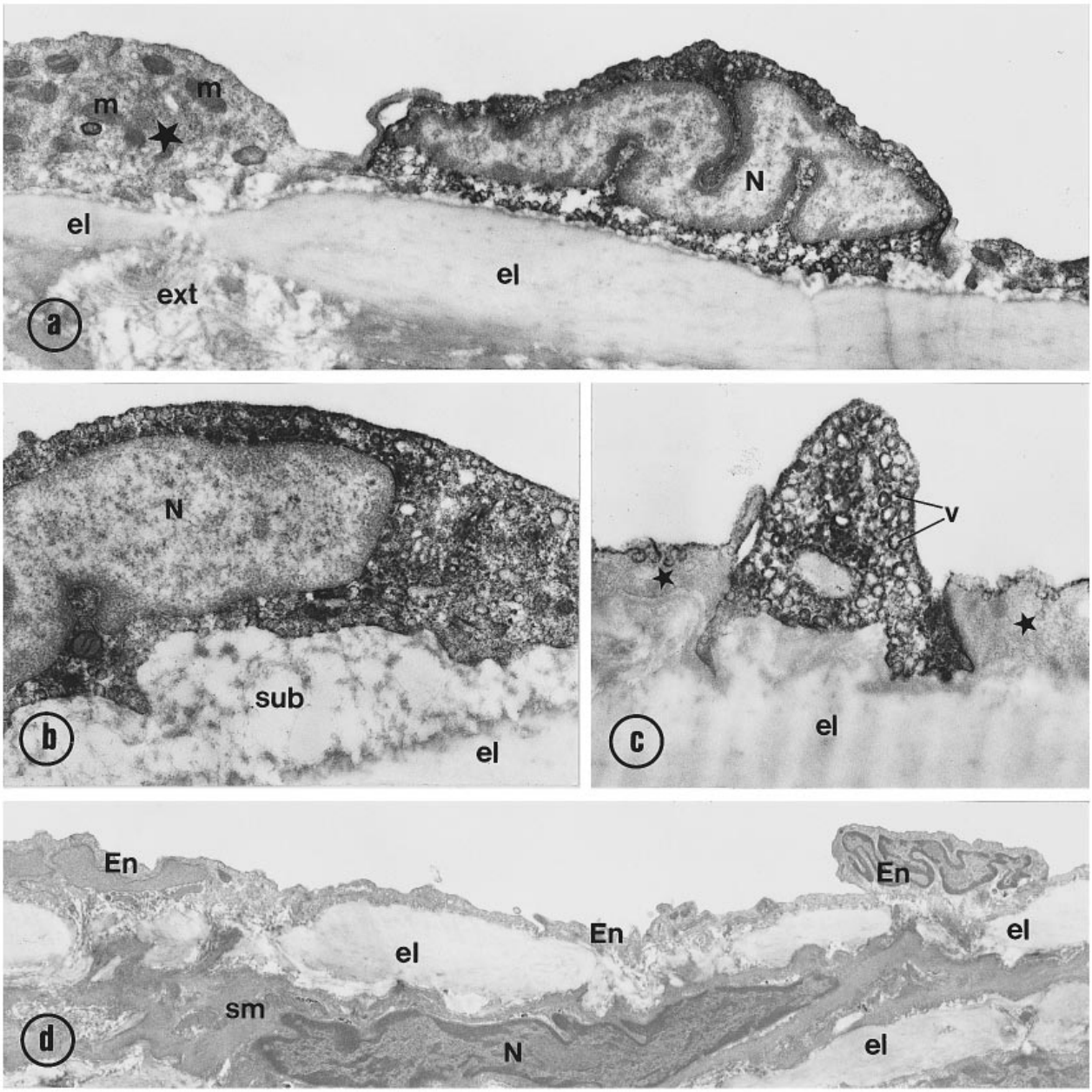

Fig. 1. Control/sham LCA of rat immunolabelled for NOS-III (*a*) and ET-1 (*b*, *c*), and an immunocytochemical control specimen (*d*). (*a*) Note 1 endothelial cell showing cytoplasmic labelling for NOS-III ('black label'). Neighbouring endothelial cell (black star) is NOS-III-negative. N, nucleus; m, mitochondria; el, elastic lamina; ext, extracellular matrix ($\times 13600$). (*b*) Fragment of endothelial cells showing cytoplasmic labelling for ET-1. sub, subendothelial extracellular matrix ($\times 23000$). (*c*) Note the ET-1-positive endothelial cell process filled with cytoplasmic vesicles (v); 2 neigbouring endothelial cells (stars) are ET-1-negative ($\times 27000$). (*d*) Note unlabelled endothelial cells (En) in a control specimen (omission of primary antibody). sm, smooth muscle ($\times 7500$).

examination with the electron microscope (JEM 1010). Results are expressed as mean $\pm$ standard error of the mean (S.E.M.) of LCA preparations from 3 control/sham rats and from 4 rats 28 d after injury. Data were compared using Student's t test and a P value of < 0.05 was taken as significant. Due to the uneven distribution of labelled cells in the intima (see e.g. Tomlinson et al. 1991) as well as the fact that the size of endothelial cells could be changed after injury and that relatively small fragments of the vessels were examined ultrastructurally, these data are therefore only a guide to the proportions of immunoreactive endothelial cells.

RESULTS

Control/sham rats

The ultrathin sections of the control LCA displayed a continuous layer of intimal endothelial cells. The

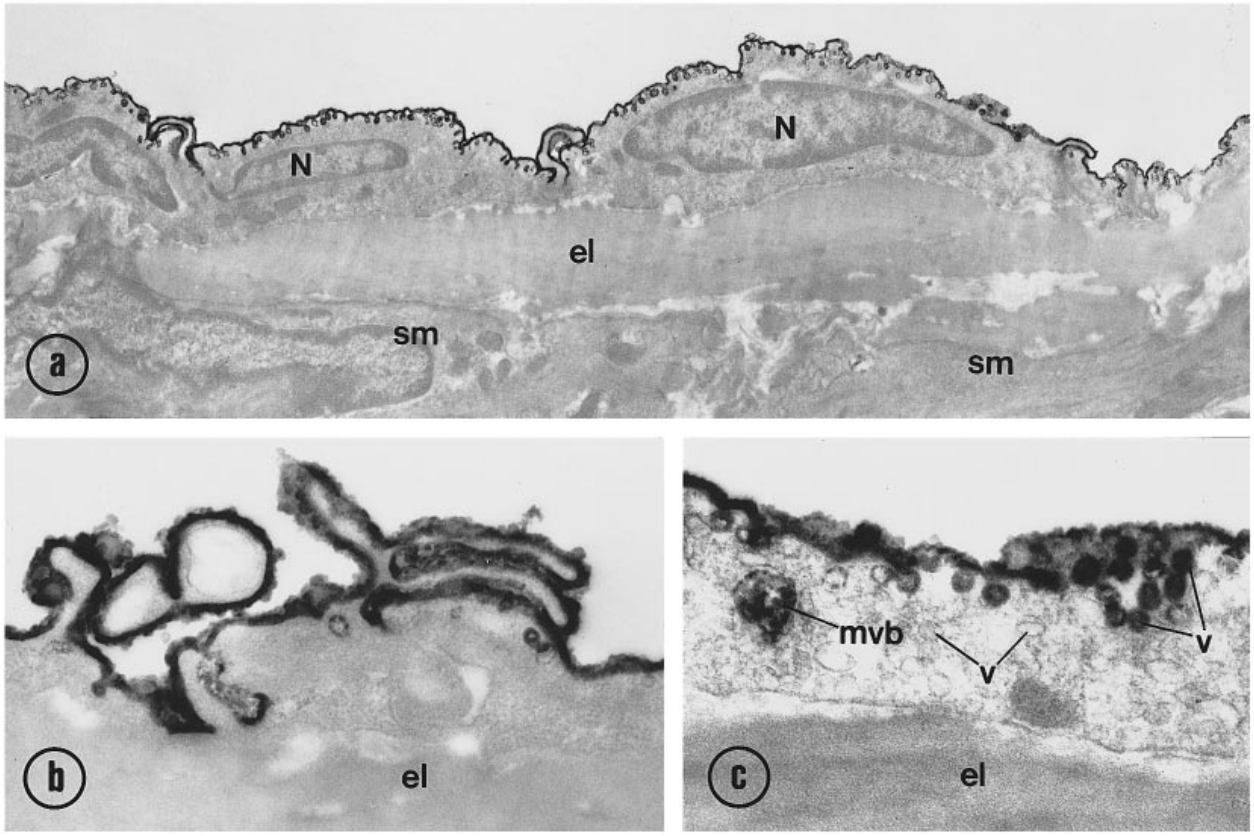

Fig. 2. Control/sham LCA of rat stained with lectin. (*a*) Note the stain associated with the luminal surface of endothelial cells. N, nucleus; el, elastic lamina; sm, smooth muscle ($\times 10000$). (*b*) Higher magnification example of luminal plasmalemma of endothelial cells showing intense stain with lectin ($\times 36800$). (*c*) Note numerous subplasmalemmal/cytoplasmic vesicles (v) and 1 multivesicular body (mvb) stained with lectin; unstained cytoplasmic vesicles can also be seen ($\times 45000$).

LCA labelled with anti-NOS-III and ET-1 antibodies revealed the presence of NOS-III and ET-1-positive endothelial cells (Fig. 1*a-c*). Not all endothelial cells were immunoreactive. More than 30% of the endothelial cells were positive for NOS-III (36.9% $\pm$ 4.3; 118 out of a total of 411 endothelial cells examined in 3 rats; number of fields observed = 22), but less than 10% of endothelial cells were positive for ET-1 (7.6% $\pm$ 2.7; 19 out of a total of 388 endothelial cells examined in 3 rats; number of fields observed = 15). Immunoreactivity for NOS-III and ET-1 was distributed in the cytoplasm and in association with the membranes of intracellular organelles and structures, such as mitochondria, endoplasmic reticulum and cytoplasmic vesicles. No labelling for NOS-III and ET-1 was observed in the vascular smooth muscle of the media. Endothelial cells of LCA processed as an immunocytochemical control showed absence of immunoreactivity (Fig. 1*d*).

All endothelial cells observed in LCA of control rats showed staining with lectin (Fig. 2*a-c*) but smooth muscle cells were negative. The stain was associated with the plasmalemma of endothelial cells dominating on the luminal aspect of the cells. The lectin staining also appeared in subplasmalemmal vesicles (Fig. 2*c*). No lectin-staining was observed in cytochemical control preparations.

24 hours after injury

In contrast to the control rats, 1 d after balloon catheter-induced injury, no vascular endothelial cells were present in the LCA, but the intima was covered with platelets (Fig. 3*a-d*); no nucleated cells were observed. The platelets were tightly adherent to the subendothelial matrix, covering the whole length of the intima. The cytoplasm of some platelets was immunopositive for NOS-III (Fig. 3*a*; $\sim$ 13.9%; 28 out of a total of 208 platelets examined in 4 rats) and ET-1 (Fig. 3*b*; $\sim$ 14.3%; 24 out of a total of 168 platelets examined in 4 rats). Immunoprecipitate was commonly seen on the surface of immunopositive and immunonegative platelets (Fig. 3*b*).

Cytochemical staining with lectin was mostly associated with the platelet plasmalemma, but

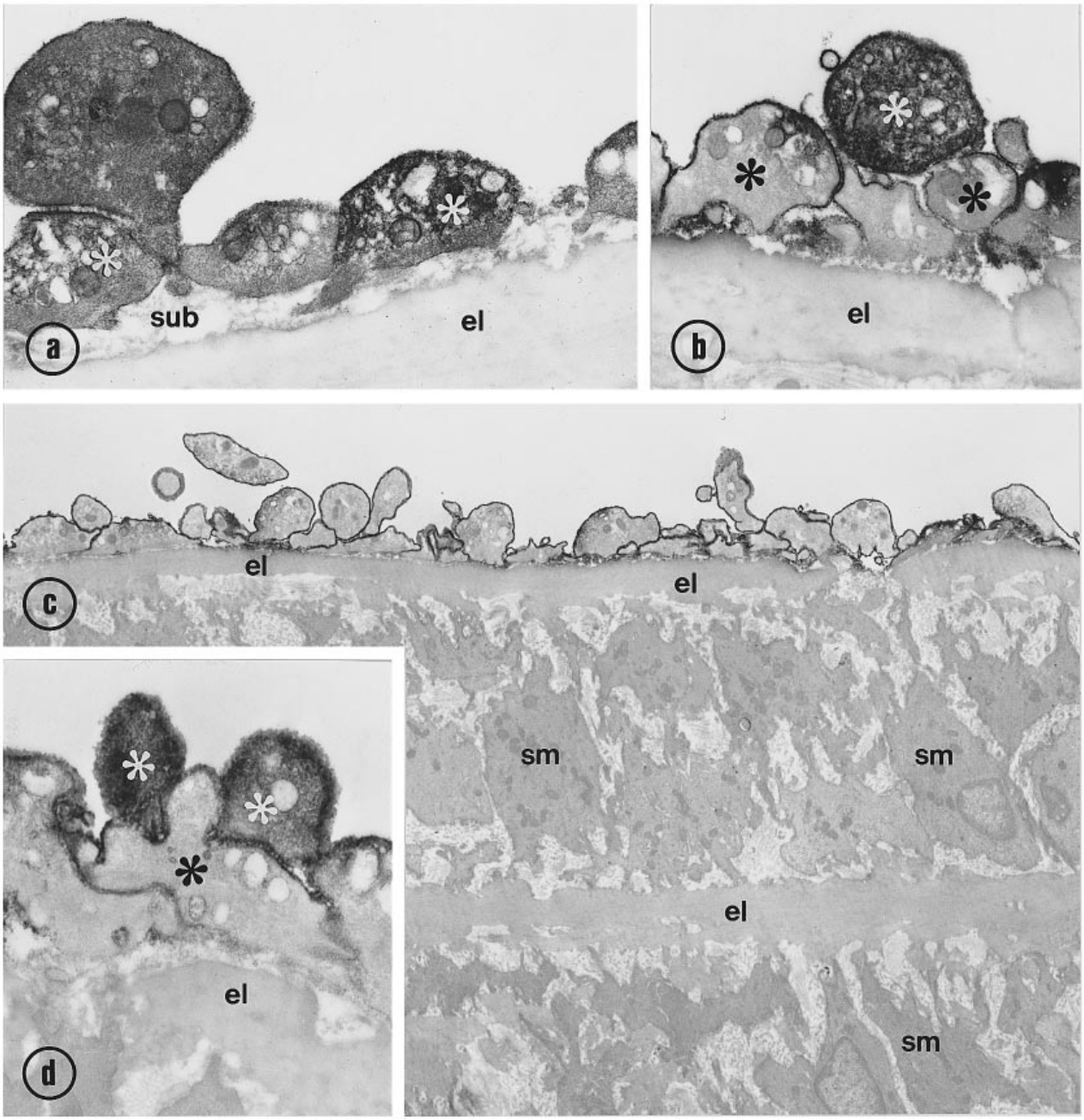

Fig. 3. Rat LCA 24 h after balloon catheter-induced injury immunolabelled for NOS-III (*a*) and ET-1 (*b*), and stained with lectin (*c*, *d*). Note the presence of platelets covering the luminal surface of intima. (*a*) Note at least 2 NOS-III-positive (white asterisks) platelets. sub, subendothelial extracellular matrix; el, elastic lamina (×20000). (*b*) Note ET-1-positive (white asterisk) and ET-1-negative platelets (black asterisks) (×18000). (*c*) Note the lectin-negative cytoplasm of the platelets; lectin-positive stain is associated with the platelet plasmalemma. sm, smooth muscle (×6000). (*d*) Examples of platelets showing intense cytoplasmic stain with lectin (white asterisks) (×20000).

occasionally localisation through the cytoplasm was also observed (~ 3.3%; 7 out of a total 209 platelets examined in 4 rats; Fig. 3*c*, *d*).

28 days after injury

In contrast to the control LCA and LCA 1 d after balloon catheter-induced injury, by 28 d after injury, neointimal thickening was present in the LCA. The neointima was made up of multiple layers of smooth muscle-like cells, extracellular matrix and elastic tissue/lamina. The elastic tissue, however, was mostly distributed in patches. The luminal surface of neointima was made up of a single or at times even double layers of endothelial-like cells. Micrographs of NOS-III- and ET-1-immunoreactivity in the injured arteries are presented in Figures 4 and 5, respectively. A subpopulation of regrown endothelial cells showed cytoplasmic immunoreactivity for NOS-III (17.2% ± 1.9; 58 out of a total 375 endothelial cells

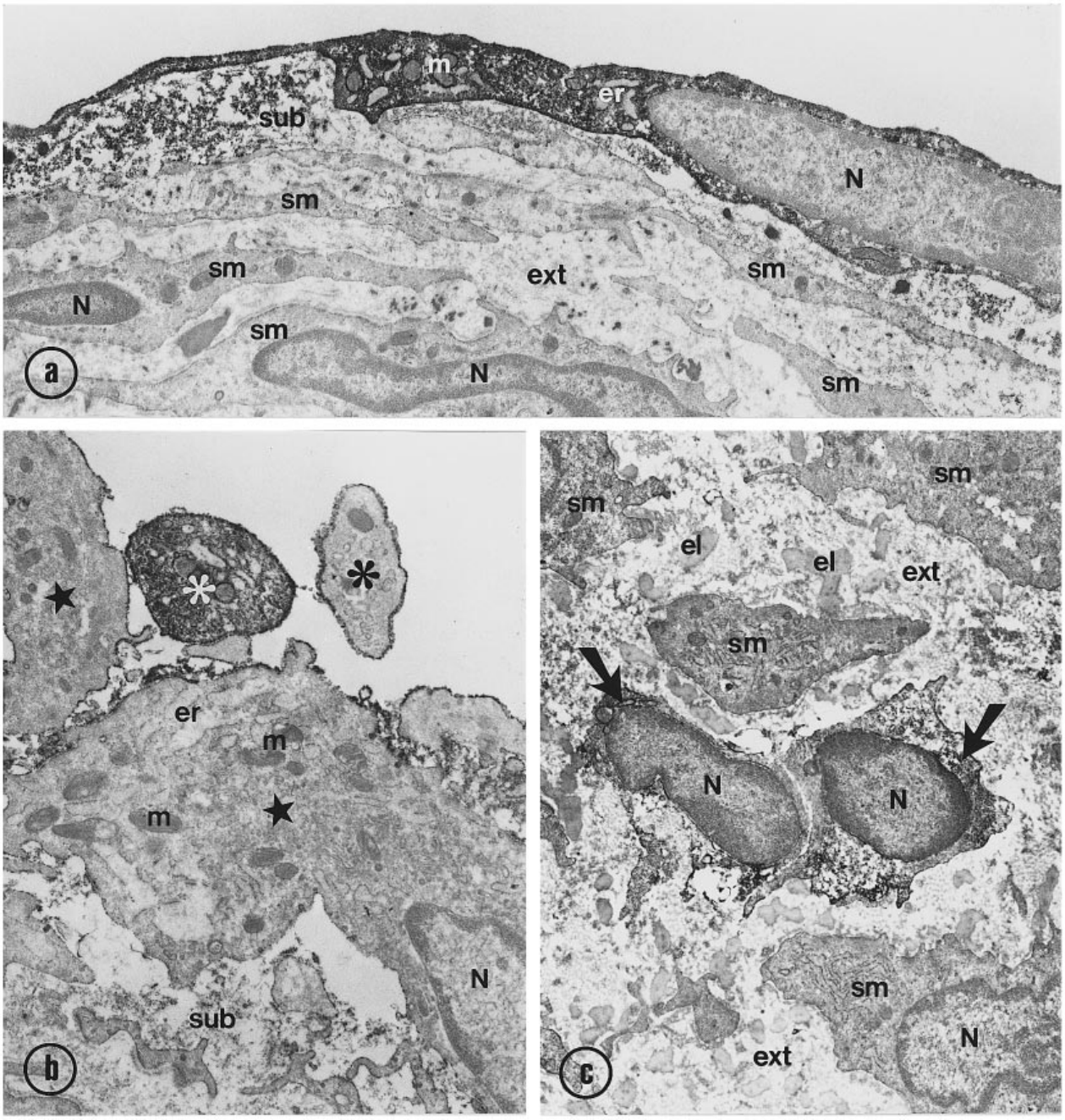

Fig. 4. Rat LCA 28 d after balloon catheter-induced injury immunolabelled for NOS-III. (*a*) Note the NOS-III-positive endothelial cell body lining the surface of neointima. N, nucleus; m, mitochondria; er, endoplasmic reticulum; sub, subendothelial extracellular matrix; sm, smooth muscle; ext, extracellular matrix (×11500). (*b*) Note NOS-III-negative endothelial cells (black stars); 1 NOS-III-positive (white asterisk) and 1 NOS-III-negative (black asterisk) platelet can also be seen (×12500). (*c*) Fragment of neointima showing 2 NOS-III-positive cells (arrows) among NOS-III-negative smooth muscle cells. el, elastic lamina (×9700).

examined in 4 rats; number of fields observed = 16; Fig. 4*a*, *b*) and immunoreactivity for ET-1 (36.9% ± 4.7; 120 out of a total 306 endothelial cells examined in 4 rats; number of fields observed = 16; Fig. 5*a-c*). The percentage of NOS-III-positive endothelial cells was significantly decreased ($P < 0.001$), whilst the percentage of ET-1-positive endothelial cells was significantly increased ($P < 0.001$) in the LCA at 28 d when compared with control rats. The NOS-III- and ET-1-positive endothelial cells were rich in intracellular organelles and intracellular structures including granular endoplasmic reticulum (Figs 4*a*, *b*, 5*a*, *b*). In some areas of neointima, ET-1-positive platelets were also present at the lumen alongside the ET-1-positive and/or ET-1-negative endothelial cells (Fig. 5*c*). Some platelets also were immunoreactive for NOS-III (Fig. 4*b*).

Occasionally, NOS-III-immunoreactivity was

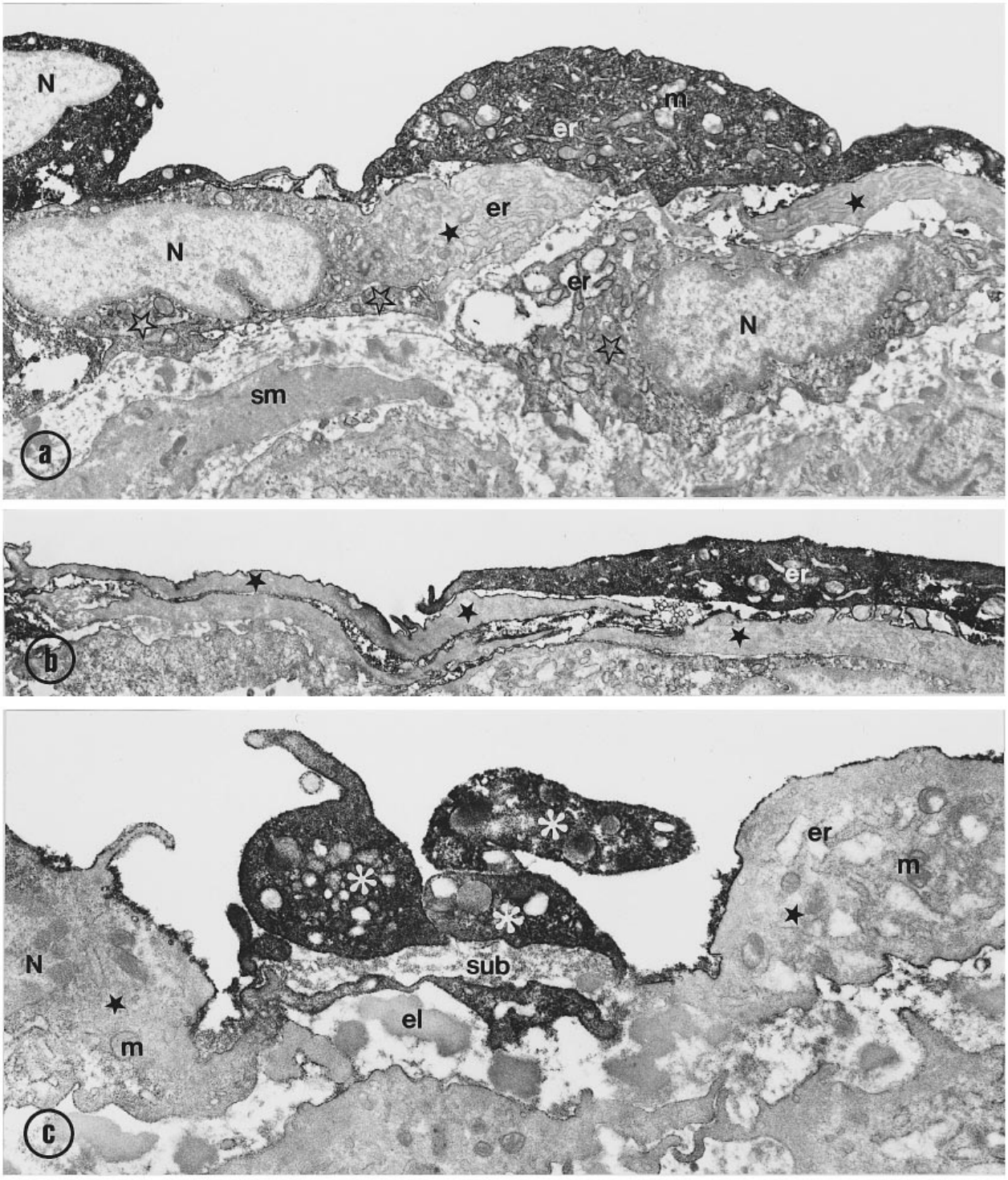

Fig. 5. Rat LCA 28 d after balloon catheter-induced injury immunolabelled for ET-1. (*a*) Note ET-1-positive endothelial cells covering the luminal side of neointima; 2 located subendothelially (transparent stars) are also slightly ET-1-positive. Black stars indicate the ET-1-negative endothelial cells. N, nucleus; er, endoplasmic reticulum; m, mitochondria; sm, smooth muscle (×8800). (*b*) Note 'flattened' ET-1-positive and ET-1-negative endothelial cells (×9000). (*c*) Note ET-1-positive platelets (white asterisks) adhere to the subendothelial extracellular matrix (sub) at the surface of the neointima; 2 ET-1-negative endothelial cells can also be seen. el, elastic lamina (×18000).

found in cells localised distant from the lumen in the subendothelial layer of the neointima (Fig. 4*c*) but cells immunopositive to ET-1 were absent.

Cytochemical examination of the LCA revealed that the regenerated cells at the luminal aspect of the neointima showed a positive stain with lectin. As in

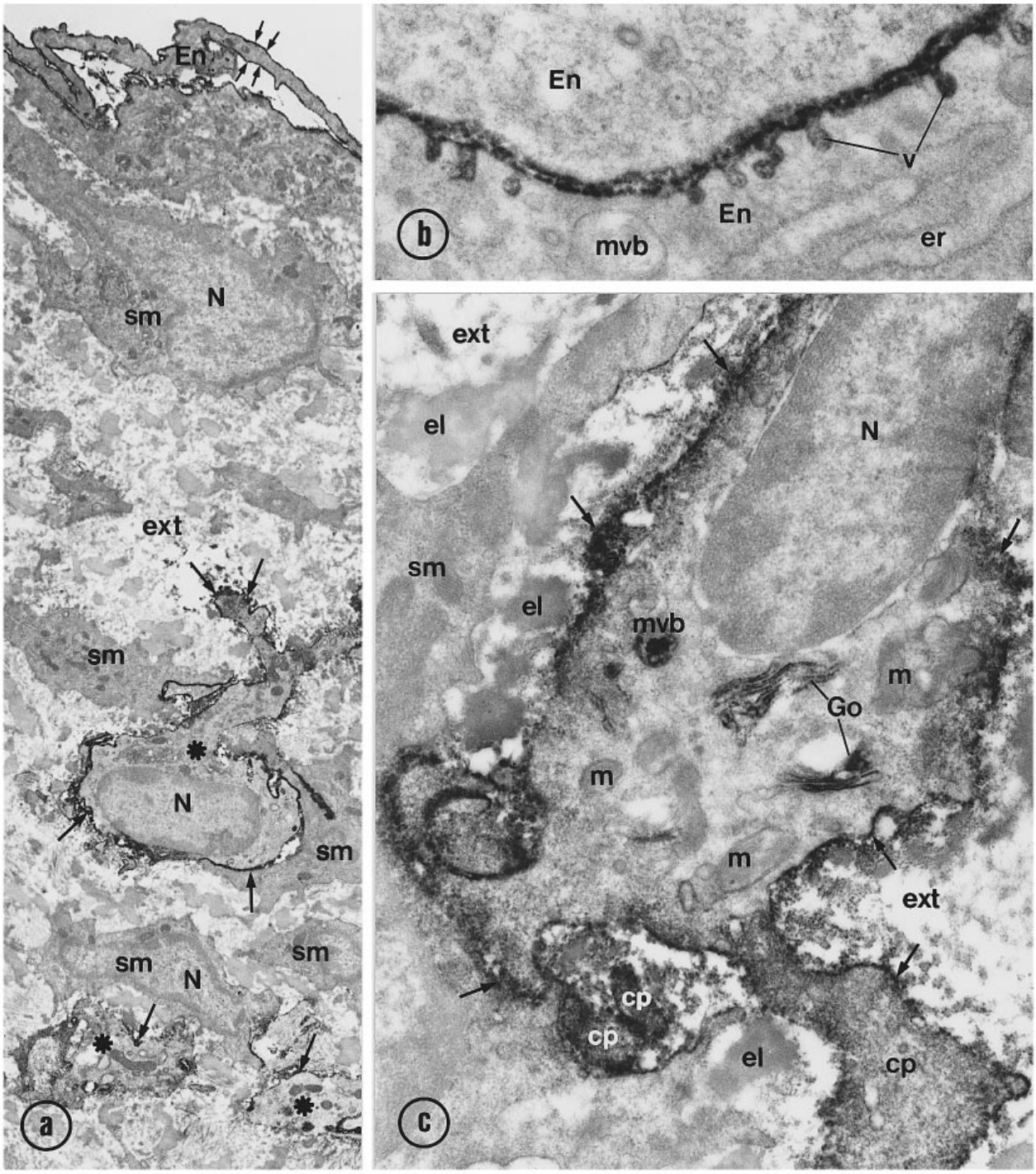

Fig. 6. Rat LCA 28 d after balloon catheter-induced injury stained with lectin. (*a*) Note the positive staining (arrows) around the surface plasmalemma of regrown endothelial cells (En) at the lumen, and around the plasmalemma of cells (black asterisks) localised subendothelially among unstained smooth muscle (sm). N, nucleus; ext, extracellular matrix (× 7000). (*b*) Higher magnification example of 2 endothelial cells (En) showing intense lectin-stain of intercellular space/junctions and subplasmalemmal vesicles (v). er, endoplasmic reticulum; mvb, multivesicular body (× 55300). (*c*) Higher magnification of a lectin-stained cell located within the smooth muscle cells of the neointima. Note the staining associated with the cell membrane (arrows), cisterns of the Golgi complex (Go) and multivesicular bodies (mvb); staining can also be seen in the cytoplasm of associated cell processes (cp). m, mitochondria; el, elastic lamina (× 30000)

the controls, the lectin stain was mostly associated with the surface membranes of endothelial cells, including membranes involved in intercellular junctions (Fig. 6*a*, *b*). In contrast to the controls, however, by 28 d after injury, the lectin-positive cells were also located amongst the lectin-negative cells (probably smooth muscle cells) of the subendothelial part of the neointima (Fig. 6*a*). These cells contained

Golgi complex and multivesicular bodies/lysosomes which displayed strong staining with lectin (Fig. 6*c*).

DISCUSSION

In agreement with the study of rat carotid angioplasty by Fingerle et al. (1989), our immunocytochemical and cytochemical study showed that the vascular endothelium had been removed by the balloon catheter-induced injury to the rat LCA, resulting in platelet adhesion to the endothelium-free intima by 24 h after the injury. This was followed by the process of neointimal thickening as examined at 28 d after the injury. At this time the neointima was covered with regrown endothelial cells showing positive cytoplasmic immunoreactivity for NOS-III and ET-1 as well as staining with lectin from *Bandeirea simplicifolia*. Lectin-positive cells were also located within the smooth muscle cells of the neointima, displaying stained Golgi complex and multivesicular bodies/lysosomes.

The intracellular distribution of endothelial immunoreactivity to NOS-III and ET-1 examined both in the control and injured LCA (at 28 d) was similar to that previously observed in other electron-immunocytochemical studies of NOS-III, NOS-I and ET-1 in various vascular beds (see Loesch et al. 1991, 1993, 1994; Loesch & Burnstock, 1993, 1995, 1996*a*, *b*; Sexton et al. 1995, 1996; Gorelova et al. 1996). The present finding of immunoreactivity for NOS-III and ET-1 in the LCA 28 d after injury strongly supports the endothelial nature of the cells covering the luminal surface of the neointima. However, the proportion of immunoreactive cells was altered in these regenerated endothelial cells; the endothelial immunoreactivity for NOS-III was decreased, but for ET-1 increased in LCA 28 d after injury compared with the sham controls. These results suggest that there is a considerable change in the endothelial contribution of NO and ET-1 28 d after balloon induced-injury with a predominance of endothelial cells synthesising the vasoconstrictor ET-1 rather than the vasodilator NO. These findings support pharmacological data demonstrating impaired endothelial-mediated dilatation in vessels (porcine coronary arteries) with regenerated endothelium (Shimokawa et al. 1989). Bioassay studies of atherosclerotic arteries demonstrated that decreased endothelium-dependent relaxations were due mainly to a reduced release of EDRF/NO (Vanhoutte, 1991). In contrast, Joly et al. (1992) reported induced NOS activity in the medial smooth muscle of rat carotid arteries 6–24 h after balloon catheter-removal of the endothelium. Increased production of NO in the smooth muscle of injured LCA, may serve to inhibit platelet adhesion and aggregation and adhesion of neutrophils at the sites of injured intima (Joly et al. 1992).

Interleukin-1 β, which induces NOS activity in the catheterised rat LCA (Joly et al. 1992) is released from blood cells at the sites of vascular injury (Dinarello, 1985; Davies, 1986; Nathan, 1987). In injured vessels, neutrophils, macrophages, platelets and fibroblasts may be involved in generation of NO (Joly et al. 1992). It is now clear that NO generated by endothelial cells and platelets provides a mechanism to regulate vascular tone as well as the platelet aggregation and adhesion (Radomski & Moncada, 1993). Employing molecular and biochemical techniques, Sase & Michel (1995) identified constitutive NOS-III in human platelets. In the present study, immunoreactivity for NOS-III and ET-1 was observed in a number of platelets adherent to the neointima 24 h after the injury. Whilst it is well known that platelets stimulate production of NO and ET-1 in endothelial cells (Lüscher, 1993), to our knowledge localisation of ET-1 in platelets themselves has not been described. This may be peculiar to platelets in situ.

Staining with lectin indicated the endothelial character of regrown cells covering the luminal side of neointimal thickening. The subendothelial location of some lectin-positive cells amongst the smooth muscle-like cells indicates a heterogeneity in LCA neointima 28 d after the injury. The neointimal location of lectin-positive cells may also suggest that some endothelial cells are involved in angiogenesis. The phenomenon of angiogenesis within a neointima is not unknown. For example, Nabel et al. (1993) demonstrated angiogenesis in the neointima of porcine iliofemoral arteries after FGF-1 gene transfer; this angiogenesis was stimulated by FGF-1 locally transfected into the vessel wall (Nabel et al. 1993). Angiogenesis in injured blood vessels, for instance in those with atherosclerotic plaques, may play a substantial role in facilitating blood supply to the hyperplastic intima/neointima (Barger et al. 1984; Nabel et al. 1993). In our present study the striking finding was a 5-fold increase in the number of ET-1-positive endothelial cells lining the neointima by 28 d after the injury, but lack of ET-1 labelling within the subendothelial neointimal cells (e.g. within those displaying the lectin stain). This may suggest that endothelial cells involved in neointimal angiogenesis do not express ET-1. Lack of detection of ET-1-positive cells in the subendothelial layer is unlikely to be due to inaccessability of the anti-ET-1 antibody since in these same

specimens NOS-III-positive cells were detected using the same immunoprocedure. By 28 d after injury the LCA showed a significant decrease in NOS-III immunoreactivity in endothelial cells lining the luminal side of neointima, and NOS-III-positive cells were also present amongst subendothelial cells of neointima. Thus immunoreactivity for NOS-III and also strong staining with lectin in subendothelial cells of neointima may be a feature of endothelial cells and the initial process of angiogenesis. Further electron-immunocytochemical studies are needed to follow up the present observations.

In summary, regenerated endothelial cells of the neointima following balloon catheter-induced injury show a different profile of immunoreactivity to NOS-III and ET-1 from controls; fewer cells contain NOS whilst more contain ET-1. Some cells in the sub-endothelial layer of the hyperplastic neointima display characteristics of endothelial cells rather than smooth muscle cells (or fibroblasts), i.e. selective lectin staining and may be involved in early angiogenesis.

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