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ARTICLES

Monocyte Chemoattractant Protein-1 Gene Expression in Injured Pig Artery Coincides With Early Appearance of Infiltrating Monocyte/Macrophages

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Abstract Monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) are potent chemokines which attract circulating monocytes and neutrophils respectively to inflamed tissues. JE/MCP-1 gene expression has been previously studied in rabbit aortae after endothelial denudation and the rapid appearance of this transcript was thought to precede emigration of phagocytes. We now report MCP-1 gene expression following de-endothelialization of iliac arteries in the pig, a species which can develop spontaneous atherosclerosis. Using Northern blot analysis, we demonstrated that MCP-1 mRNA was rapidly induced in pig arteries at 2 h and continued to increase to reach a maximum at 8 h before returning to low levels at 16–24 h after injury. The increase seen for MCP-1 mRNA at 8 h was also observed for IL-8 mRNA but was not apparent for growth-related gene expressions, urokinase-type plasminogen activator (u-PA), and plasminogen activator inhibitor-1 (PAI-1). Since smooth muscle cells, endothelial cells, and phagocytes are all capable of expressing MCP-1, we examined pig arteries for immunostaining using a monoclonal antibody to human MCP-1 (5D3-F7). At 8 h after injury, the predominant cell type staining positive for MCP-1 was the monocyte/macrophage. Staining was also observed in occasional scattered neutrophils, but MCP-1 protein could not be detected in smooth muscle cells or on extracellular matrix within the sensitivity constraints posed by our methodology. Our results are consistent with invading monocyte/macrophages having a major input into the production of this chemokine in the arterial-wall following injury. The fact that MCP-1 expression accompanied monocyte/macrophage presence in damaged artery, rather than preceding it, is suggestive that continued MCP-1 expression is required for functions other than chemoattraction.

Key words: monocyte chemoattractant protein-1, gene expression, pig artery, balloon injury, monocyte/macrophages

INTRODUCTION

Acute and chronic inflammatory responses are processes characterized by localization of leukocytes within inflamed tissues. The events which enable the emigration of cells responsible for immunity into an area of inflammation are being elucidated and this information is forming the basis of a model of the molecular events associated with leukocyte extravasation [Butcher, 1991; Springer, 1994; Furie and Randolph, 1995]. The first step is a slight tethering of free flowing leukocytes which causes a slower, rolling motion by these cells along a section of vasculature, a change which is mediated by selectins. As a consequence, leukocytes are increasingly exposed to chemoattractant proteins called chemokines which are expressed on the inflamed lining of the vessel wall. The binding of these chemokines to specific receptors on the surface of leukocytes initiates an upregulation of integrin adhesiveness and provides a mechanism for leukocyte traction during migration. Localized gradients of chemokines then stimulate the directional movement of leukocytes into the inflamed tissue. Thus in the current model of leukocyte extravasation, chemokines play important roles in at least two steps of the multistep process [Butcher, 1991; Springer, 1994; Furie and Randolph, 1995].

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The chemokines are members of a large superfamily of structurally related proteins which is subdivided into two branches according to the spacing of the first two cysteine moieties [Schall, 1991; Miller and Krangel, 1992]. In the CC branch of the cytokine family the first two cysteines are adjacent, whereas in the CXC branch these cysteines are separated by an intervening amino acid. Monocyte chemoattractant protein-1 (MCP-1), a member of the CC branch, is a potent chemoattractant for monocytes but does not attract neutrophils [Yoshimura et al., 1989a]. Normal cells only express very low levels of MCP-1 (or its murine homologue, JE) in an unstimulated state. Endothelial cells, smooth muscle cells (SMC) and monocytes all rapidly induce JE/MCP-1 when exposed to inflammatory cytokines and growth factors [Yoshimura et al., 1989b; Rollins et al., 1990; Taubman et al., 1992]. In addition to chemoattractant properties, MCP-1 has the capability to regulate monocyte functions including induction of the respiratory burst, adhesion molecule expression, and cytokine production [Rollins et al., 1991; Jiang et al., 1992]. Interleukin-8 (IL-8) is a member of the CXC branch of the cytokine family and is a powerful and specific chemoattractant for neutrophils with no attractant activity towards monocytes [Yoshimura et al., 1987]. Like MCP-1, IL-8 is able to regulate a number of functions on its target cell and IL-8 is also produced by a variety of cells reacting to a phlogistic stimulus [Miller and Krangel, 1992; Hebert and Baker, 1993]. The release of a profile of chemotactic cytokines at a site of inflammation appears likely to contribute to extravasation of a defined mix of monocytes, neutrophils, and other immune cells.

The recruitment of circulating leukocytes into the vessel wall is believed to be an early event in atherogenesis and may be regulated by local generation of chemokines [Ross, 1993; Schwartz et al., 1993]. JE/MCP-1 gene expression is also rapidly induced in rabbit aorta following balloon injury and the appearance of this transcript appeared likely to precede emigration of immune cells [Taubman et al., 1992]. Macrophages are located in small numbers in the intima of rat arteries following this type of mechanical injury [Ferns et al., 1991]. Our laboratory had previously observed substantial monocyte/macrophage infiltration in pig arteries following balloon catheter de-endothelialization and the present investigation was undertaken to examine chemokine gene expression in this animal model. We found that MCP-1 mRNA (and IL-8 mRNA) is greatly increased at a timepoint after injury which coincides with the early appearance of monocyte/macrophages in the arterial wall. This temporal relationship, when considered together with our demonstration that monocyte/macrophages in injured pig arteries stain positive for MCP-1 protein, provides evidence these phagocytes have a major input into chemokine production during the inflammatory response in pig arterial tissue.

MATERIALS AND METHODS

Balloon Catheter Denudation of Pig Arteries

Male cross-bred pigs (Landrace and Large White breeds) aged 9–11 weeks (about 20 kg) received a general anaesthetic by using a combination of oxygen plus nitrous oxide and halothane. Following exposure of the right profunda femoris artery, a 5F Fogarty embolectomy catheter (Baxter) was passed up to the aortic bifurcation and inflated to an intraballoon pressure of 700 mm Hg. The inflated balloon catheter was pulled down the right iliac artery over a period of 20 s while rotating to compensate for any asymmetrical inflation. The procedure of advancing and withdrawing the balloon catheter was repeated two more times and the right profunda femoris artery was then ligated. The left iliac artery remained undamaged. The pigs were examined regularly until fully recovered from anaesthesia and returned to their housing where they were fed a normal diet. Animals used in this study were treated in accordance with the National Health and Medical Research Council guidelines for animal research and the experimental procedures were as approved for this project by the Animal Welfare Committee of the University of Western Australia.

Harvesting of Arteries

Pigs which were used in studies of chemokine expression and phagocyte localization were sacrificed within 7 days of balloon injury to arteries. Shortly before the time points specified in this study (2 h, 8 h, 24 h, 2 days, 4 days, and 7 days), pigs received a general anaesthetic as before and a laparotomy was performed. The infra-renal aorta and distal arteries were perfused with 500 ml of isotonic saline, the effluent being drained by the inferior vena cava. The right and left common iliac arteries were har-

vested and the adventitia was dissected away. The arteries were cut transversely and longitudinally into smaller specimens, with several being frozen in liquid nitrogen for mRNA analysis and the remainder being kept for detection of phagocytes. For the latter investigation, transverse and longitudinal cuts of artery were placed in OCT freezing preparation and gradually frozen to the temperature of liquid nitrogen. These specimens were stored at −80°C and subsequently analyzed by immunohistochemistry and enzyme histochemistry. Other pieces of artery were immersed in 2.5% glutaraldehyde in 0.05M cacodylate buffer (pH 7.4), sectioned, and processed for electron microscopy.

When arterial intimal thickening was being studied, pigs were treated slightly differently following sacrifice at 3 week after balloon injury. A laparotomy was performed and the infra-renal aorta and distal arteries were perfused in situ with 4% paraformaldehyde in phosphate-buffered saline. The harvested arteries were tied to a flat cardboard base and immersed for 24 h in 4% paraformaldehyde solution before being embedded in paraffin. Sections were stained with hematoxylin and eosin using standard methodology.

A total of five independent series of pigs were studied in this investigation.

RNA Isolation and Northern Blot Analysis

Total RNA was extracted from arteries using a commercial kit based on the method of Chomczynski and Sacchi [1987]. Frozen arteries were ground to a fine powder under liquid nitrogen and added to Ultraspec solution (Biotecx Laboratories, Houston, TX). The suspension was homogenized by four high speed bursts (15 s) using a Janke and Kunkel tissue disintegrator (Ultra-Turrax T25) and total cellular RNA was then isolated using the Biotecx RNA isolation protocol. The RNA pellet was dissolved in 25 μl 1 mM EDTA and aliquots containing 20 μg of total RNA were heat-denatured and run on formaldehyde gels (1% agarose) containing ethidium bromide. The electrophoresis buffer was 20 mM MOPS (3-[N-morpholino]propanesulfonic acid), 5 mM Na Acetate, and 1 mM EDTA, pH7.0. RNA from normal artery and from injured arteries covering the time span from 2 h to 1 week after balloon injury were loaded on the same gel. RNA was then capillary-transferred overnight to Hybond N^+ (Amersham, UK) and gels were examined under UV light to ensure complete RNA transfer had occurred. After brief alkali fixation (5 min, 50 mM NaOH), membranes were washed and prehybridized for 2 h at 42°C in hybridization solution containing 50% formamide (Amersham Hybond-N^+ protocol). DNA probes were labelled with ^{32}P-dCTP by nick translation of plasmids or random primer extension of inserts using kit protocols (Amersham, UK). Heat-denatured (100°C, 10 min) probe was added to hybridization solution prior to overnight incubation at 42°C. Recommended washing protocols (Amersham, UK) were followed and membranes were exposed to Cronex X-ray film (DuPont, Australia) at −70°C in cassettes using intensifying screens.

DNA Probes

DNA probes used were as follows: MCP-1, a 0.7kb EcoR1 fragment of MCP-1 [Yoshimura et al., 1989b]; IL-8, a 0.75kb Pst1 fragment of 3-10C [Schmid and Weissmann, 1987; Rathanaswami et al., 1993]; u-PA, pHUK-8 (American Type Culture Collection, Bethesda, MD; ATCC 57329); PAI-1, a 6.4kb EcoR1 fragment of pPAI-E6.9 (ATCC 59657); and the plasmid 36B4 originally called pN1 [Masiokowski et al., 1982].

Densitometry

Autoradiograms were scanned using a Molecular Dynamics computing densitometer interfaced with an Image Quant software system. Transcript signals were normalized by referring to the ubiquitously expressed 36B4 mRNA band. 36B4 is human acidic ribosomal phosphoprotein PO [Laborda, 1991] and has been used as an internal reference in recent study of cardiovascular gene expression [Russel et al., 1993], where GAPDH estimation as a reference gene may be unsatisfactory [Knoll et al., 1994]. The value for each transcript at 8 h was then divided by the value for that particular transcript at 2 h after injury to provide an indication of relative changes in gene expression during this period of arterial repair.

Electron Microscopy

Arteries were sectioned and processed for electron microscopy as described elsewhere [Robertson et al., 1990].

Immunohistochemistry

Frozen section of arteries (8–10 μ) were briefly airdried at room temperature, washed in 0.2M Tris-buffered saline (TBS), blocked for endog-

enous peroxidase activity by incubation in 3% H_2O_2 for 5 min, washed in TBS, and then preincubated with 20% normal horse serum for 20 min before incubation overnight at 4°C with a 1 in 10 dilution of 5D3-F7, a monoclonal antibody to human MCP-1 [Peri et al., 1994]. Slides were then washed twice with TBS, incubated for 1 h at room temperature with a 1 in 200 dilution of biotin-conjugated sheep anti-mouse immunoglobulin (Silenus, Melbourne, Australia), followed by two washes in TBS and incubation for 1 h at room temperature with a 1 in 200 dilution of peroxidase conjugated streptavidin (Silenus). Chromagen solution containing 6 mg 3,3'-diaminobenzidine (BDH Chemicals, Poole, UK) in 10 ml of TBS/0.03% H_2O_2 was added and colour allowed to develop for 5 min before being stopped by washing in TBS. The slides were counterstained in hematoxylin, dehydrated in alcohol, and mounted in Depex. Preparations in which the primary antibody to MCP-1 was omitted served as negative controls. A positive control was provided by using imprinted cells from a human giant cell tumor of bone which contains cells which express chemokines including TGF-β1 [Zheng et al., 1994] and MCP-1 (unpublished observations).

Enzyme Histochemistry

Frozen arterial sections were examined for monocyte/macrophages by enzyme histochemical detection of non-specific esterase using α-naphthyl acetate as a substrate [Li et al., 1973; Bancroft, 1979].

RESULTS

Endothelial Denudation and Intimal Hyperplasia in Pig Iliac Artery

The balloon catheter injury resulted in endothelial denudation and damage to the media in all the arteries examined in this study. We wanted to demonstrate that the inflammatory response caused by this injury to the arterial wall would be subsequently followed by intimal hyperplasia. To confirm this, three pigs were sacrificed 21 days after balloon injury to the right iliac artery. In the uninjured contralateral artery, the internal elastic lamina and the overlying endothelium delineated the border of the lumen (Fig. 1A), whereas the injured artery displayed an obvious thickened intima with the internal elastic lamina at some distance from the periphery of the lumen (Fig. 1B). The presence of a thickened intima first became apparent at 14 days after balloon injury (not shown) and was a consistent finding following arterial denudation.

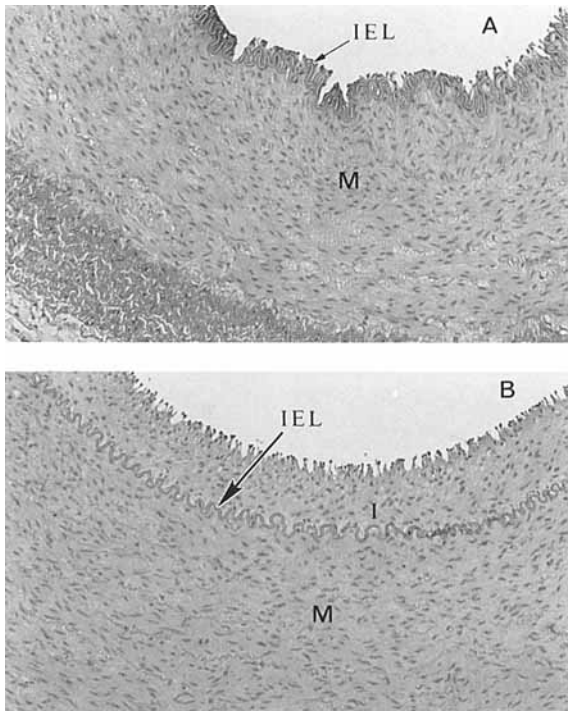

Fig. 1. Histological appearance of pig iliac arteries before and after balloon injury. **A:** Uninjured artery; **B:** artery 3 weeks after injury. Haematoxylin and eosin staining of paraformaldehyde-fixed artery. Original magnification: ×100. I: Intima, M: media, IEL: internal elastic lamina. Note that a thickened fibrocellular intima is apparent in pig iliac artery 3 weeks after balloon catheter denudation of the arterial endothelium.

Expression of the MCP-1 Gene in Pig Artery Following Balloon Injury

Monocyte/macrophages have been shown to be located in the vessel wall following mechanical injury to arteries from several species and emigration of these cells is believed to result from local production of the potent monocyte chemoattractant, JE/MCP-1 [Taubman et al., 1992]. Since we had previously observed significant phagocytic infiltration of pig arteries following balloon injury, we have studied the expression of the MCP-1 gene during arterial repair in the pig. The MCP-1 transcript, which was barely detectable in uninjured iliac artery, was soon elevated at 2 h and continued to increase to reach a maximum at 8 h after injury (Fig. 2).

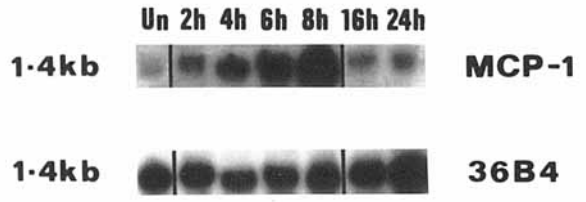

Fig. 2. Expression of the MCP-1 gene in pig iliac artery after injury. Total RNA was extracted from uninjured contralateral arteries and from iliac arteries at specified times after balloon catheter injury. Northern blots were hybridized with ^{32}P-labelled cDNA probes as indicated. 36B4 is human acidic ribosomal phosphoprotein and has been used as an internal reference to reflect RNA loading. Note that the MCP-1 transcript was rapidly elevated at 2 h and continued to increase to a maximum at 8 h before being sharply reduced at 16 h after balloon injury.

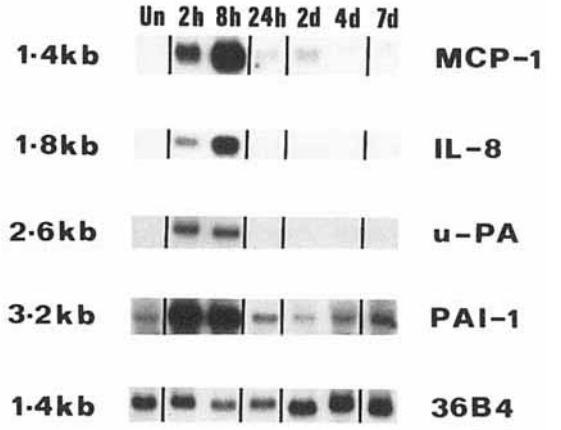

Fig. 3. Expression of MCP-1 and Il-8 genes in relation to expression of the immediate early genes u-PA and PAI-1 in pig iliac artery after balloon injury. Total RNA was extracted from uninjured iliac arteries and from contralaterals at specified times after balloon catheter injury. Aliquots were then subjected to Northern blot analysis and hybridized with ^{32}P-labelled cDNA probes as indicated. 36B4 mRNA has been used as an internal reference to reflect RNA loading. Note that all four transcripts were rapidly elevated in arteries at 2 h after injury and that MCP-1 and Il-8 mRNAs continued to increase sharply at 8 h whereas u-PA and PAI-1 mRNAs did not change as much.

The marked elevation in MCP-1 mRNA was not due to a generalized increase in mRNAs as part of the injury response because the ubiquitously expressed reference mRNA, 36B4 [Russel et al., 1993], was relatively constant during this period. Over the next 8 h, MCP-1 gene expression in damaged artery was markedly down-regulated, so that by 16–24 h after balloon injury, MCP-1 mRNA was only slightly raised.

Expression of the MCP-1 and Il-8 Genes in Relation to Immediate Early Genes u-PA and PAI-1

The production of JE/MCP-1 mRNA can also result from mitogenic stimuli [Taubman et al., 1992; Rollins et al., 1988] and be associated with cellular proliferation rather than merely reflecting a mechanism for recruitment of leukocytes. For this reason we have examined MCP-1 and Il-8 expressions in relation to growth state-regulated u-PA and PAI-1 gene expressions during arterial repair. Using a shorter exposure time to avoid overexposure of bands, we found that gene transcripts being studied were not detectable in uninjured pig iliac arteries with the exception of a very weak signal for the PAI-1 transcript (Fig. 3). At 2 h after balloon injury, all 4 mRNAs were highly elevated, and at 8 h, MCP-1 mRNA was further markedly increased while u-PA and PAI-1 mRNAs were only slightly elevated. Changes in IL-8 mRNA, like MCP-1 mRNA, were quite different from the growth-related gene expressions. The surge in chemokine gene expressions was short-lived so that by 24–48 h after injury the transcripts were markedly reduced again and they remained low for the remainder of the study. We obtained an estimate of the size of the continued increase in chemokine expression in arteries between 2 and 8 h after injury by using densitometry and normalizing to 36B4 mRNA. MCP-1 and Il-8 mRNAs were increased a further 6–8 fold during this period of arterial repair whereas u-PA and PAI-1 mRNAs were only modestly increased (Fig. 4).

Phagocyte Localization and Detection of MCP-1 Protein in Injured Arteries

Examination of pig iliac artery at 8 h after balloon injury using electron microscopy showed the attachment of a thrombus containing platelets (Fig. 5a). Although a previous study had commented that there were few phagocytes in rabbit aortae during the first 8 h after injury [Taubman et al., 1992], we demonstrated infiltration of substantial numbers of polymorphonuclear neutrophils and monocyte/macrophages into pig artery during this early period of repair (Fig. 5a–b). We were also able to observe and identify these phagocytes in injured arteries by using high magnification light microscopy but felt it was not appropriate to attempt to quantitate changes in leukocyte numbers within these arteries because of the small fields being exam-

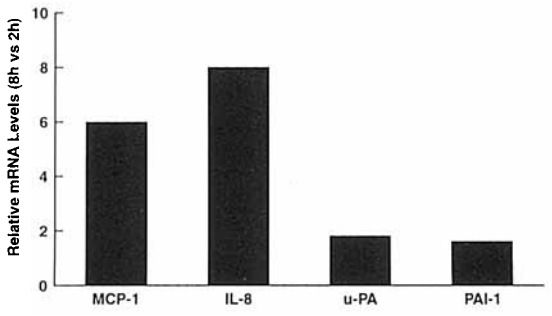

Fig. 4. Relative changes in gene expressions in pig iliac arteries during the first 8 h of repair after balloon injury. Bands in the autoradiogram shown in Figure 3 were scanned using a densitometer and signal intensities were normalized against the *ubiquitous* 36B4 transcript. The value for each transcript at 8 h was then divided by the value for that particular transcript at 2 h after injury to provide an indication of relative changes in gene expression during this period of arterial repair. Note that the MCP-1 and Il-8 gene transcripts were amplified 6-fold and 8-fold respectively at 8 h after injury, whereas u-PA and PAI-1 mRNAs were only increased 60–80%.

ined and the focal nature of the inflammatory response. However we felt we did observe several consistent qualitative changes. Early in the inflammatory response, at 2 h after injury, neutrophils were far more numerous than monocyte/macrophages. Later on, at 8 h, total phagocyte numbers appeared to peak in arteries and consisted of a mix of neutrophils and monocyte/macrophages. Over the next 8–16 h of the injury response phagocyte numbers in the vessel wall were rapidly reduced to low levels again.

In an effort to determine the cellular origin of MCP-1 expression in injured arteries, frozen sections were analyzed using immunohistochemistry. At 8 h after injury, when MCP-1 gene expression was maximal, we identified foci of cells within the arterial wall which demonstrated significant immunostaining for MCP-1 protein (Fig. 6A–B). These MCP-1-positive cells were rounded and had a central sometimes slightly indented nucleus. By careful examination of serial sections the vast majority of these cells displayed non-specific esterase activity (not shown) and were therefore monocyte/macrophages. Occasionally, we were able to identify a MCP-1-positive cell with a multi-lobed nucleus which was probably a polymorphonuclear neutrophil. We were however unable to find evidence of immunostaining for MCP-1 protein in SMC in injured arteries or within the extracellular matrix.

Taking into account our qualitative observations on phagocyte localization in the arterial wall together with our molecular data, we felt that monocyte/macrophages were the major source of MCP-1 expression in injured arteries, although a small contribution was also made by neutrophils.

DISCUSSION

In the present study, we have demonstrated that balloon catheter de-endothelialization of pig iliac artery resulted in an inflammatory response which included thrombus attachment, platelet deposition, and emigration of polymorphonuclear neutrophils and monocytes into the vessel wall. This was subsequently followed by arterial intimal hyperplasia some 2 weeks later. Our findings confirm an earlier study of balloon catheter injury to pig carotid artery where platelet-thrombus deposition preceded proliferation in the arterial intima by 7–14 days [Steele et al., 1985]. It is well documented that inflammation following acute tissue injury is characterized by platelet activation and migration of numerous neutrophils and somewhat fewer monocytes into the site of injury [Hurley et al., 1966; Ryan, 1967; Issekutz and Movat, 1980; Issekutz et al., 1981]. After migration, the monocytes differentiate into macrophages in the inflammatory foci [Naito, 1993]. In the context of arterial intimal hyperplasia, monocytes/macrophages are believed to be a source of growth factors which may act in concert with those produced by platelets, endothelial cells, and SMC themselves to initiate SMC proliferation and migration [Ross, 1993]. Monocyte/macrophages also exhibit the capacity to maintain the inflammatory response by synthesizing the chemokines MCP-1 and IL-8 [Yoshimura et al., 1989b; Colotta et al., 1992; Liebler et al., 1994], as well as being the likely mechanism by which the inflammation may be resolved [Savill et al., 1989; Henson and Riches, 1994]. Macrophages have yet another function in lipoprotein metabolism within the vessel wall and appear to be transformed into the foam cells observed in atherosclerotic plaques by excessive storage of cytoplasmic cholesterol [Roessner et al., 1993].

Since monocytes play an important role in several mechanisms which contribute to arterial pathology, we have studied the expression of MCP-1, a potent chemokine for monocytes, in injured arteries from pigs, a species which serves as a useful model of human arterial restenosis [Schwartz et al., 1990]. Previous investigation had examined MCP-1 (JE) expression in de-

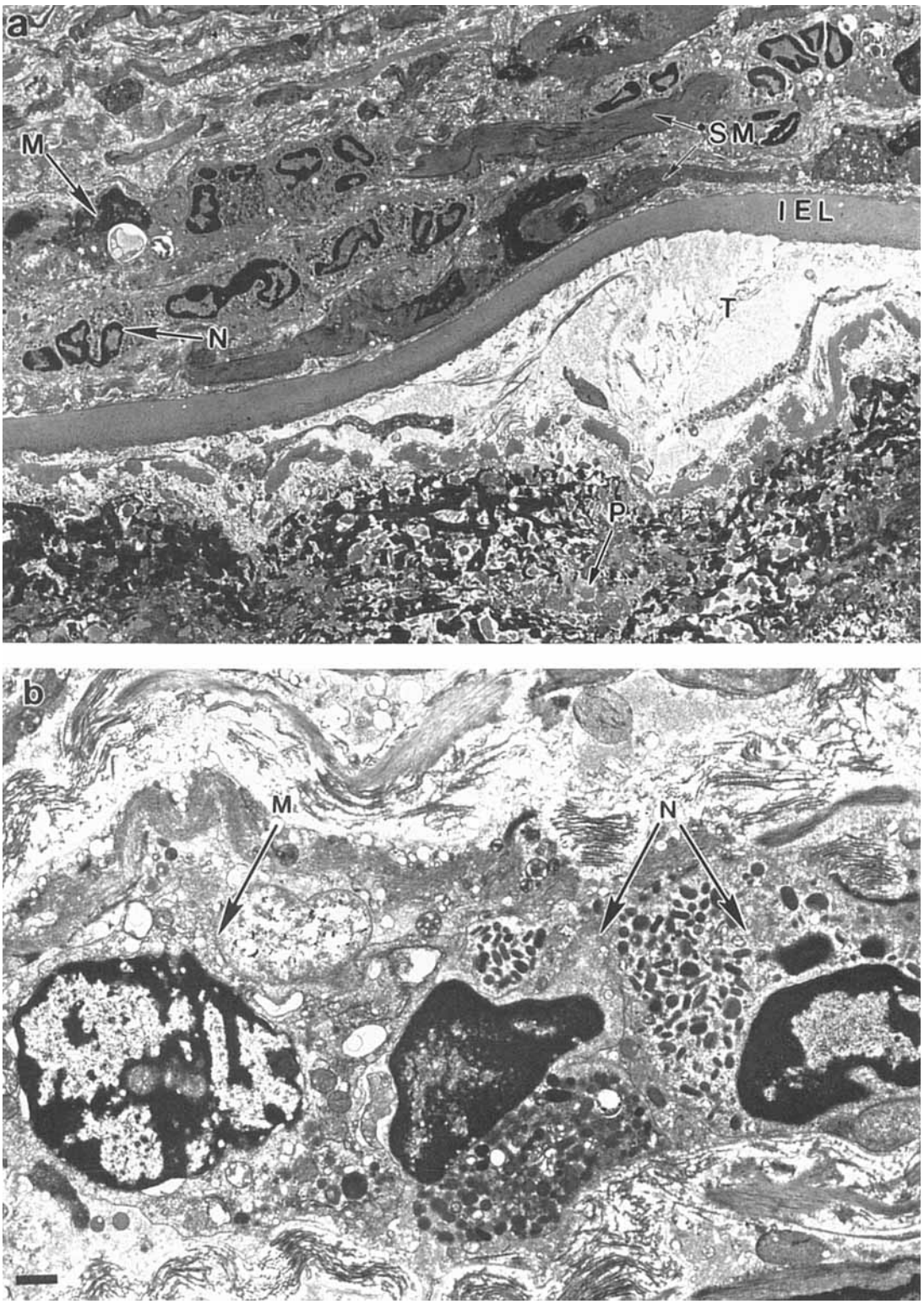

Fig. 5. Electron microscopy of the luminal edge of pig iliac artery at 8 h after injury. Ultrathin sections (50 nm) of glutaraldehyde-fixed artery were examined using standard electron microscopy techniques. **a:** Lower magnification: ×1500. The arterial wall is uppermost. M, Monocyte/macrophage; N, polymorphonuclear neutrophil; SM, smooth muscle cell; IEL, internal elastic lamina; T, thrombus; P, platelet. Note that phagocytes were located 1–2 cell layers within the arterial wall and included both monocyte/macrophages and polymorphonuclear neutrophils. A multi-lobed nucleus was evident within several neutrophils. The thrombus which was attached to the denuded artery contained platelets. **b:** Higher magnification: ×4500. The size bar is 1 μm. Note that the monocyte/macrophage had a large nucleus and typical phagocytic vacuoles and lysosomes. The two neutrophils pictured contained abundant characteristic cytoplasmic granules.

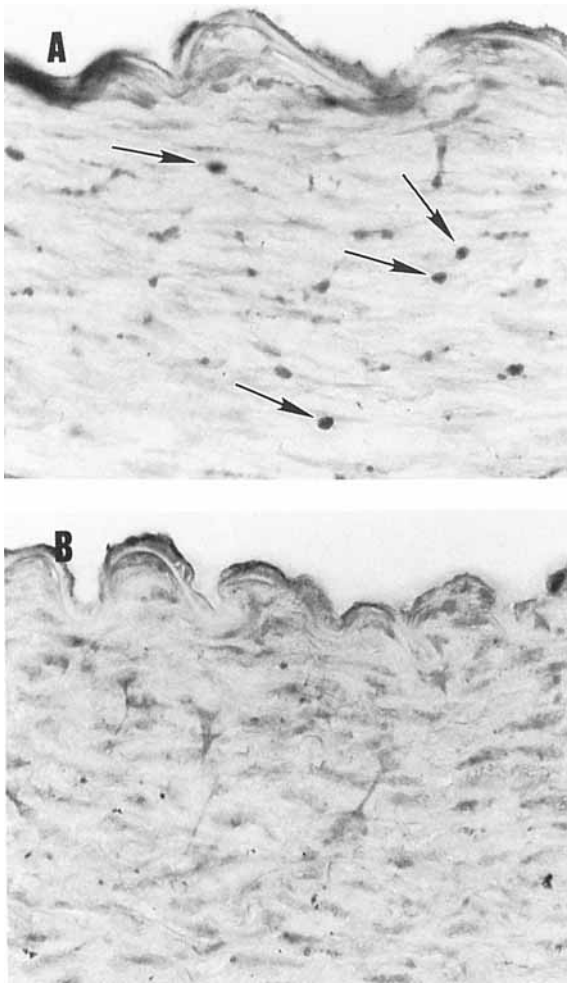

Fig. 6. Detection of MCP-1 protein-positive monocyte/macrophages in pig iliac artery at 8 h after injury. **A:** MCP-1 protein immunostaining of a transverse section of artery. **B:** Immunostaining of an adjacent serial section in the absence of the primary antibody. Frozen arterial sections were processed using a monoclonal antibody to human MCP-1 and standard immunohistochemical methodology followed by counterstaining to hematoxylin. Original magnification: ×400. The lumen of the artery is uppermost. Note the location of scattered MCP-1 protein-positive cells within the damaged arterial wall (A, arrowed) which were not seen in the absence of primary antibody to this chemokine (B).

endothelialized aortae from rabbits [Taubman et al., 1992], a species which, unlike the pig, does not develop spontaneous atherosclerosis. We found that MCP-1 mRNA was rapidly induced in pig arteries at 2 h after injury and this transcript was further greatly amplified at 8 h before rapidly returning to low levels at 16–48 h. This pattern of MCP-1 gene expression was quite different from that observed for rabbit aortae where JE mRNA had returned to baseline levels by 8 h after balloon injury [Taubman et al., 1992] and we felt this warranted further investigation.

Because the JE gene was originally isolated from mitogen-stimulated fibroblasts [Rollins et al., 1988; Schall, 1991], we next addressed the question as to whether the increase in MCP-1 gene expression that we observed could be growth-related. To do this, we compared expression of the MCP-1 gene to that of the u-PA and PAI-1 genes, the latter being two immediate early gene expressions associated with transition to a proliferative state [Grimaldi et al., 1986; Ryan and Higgins, 1993]. We demonstrated that the continued increase seen for MCP-1 in pig arteries for 8 h following balloon injury was not apparent for u-PA and PAI-1 mRNAs which were only slightly further increased after 2 h. These in vivo findings are in agreement with in vitro observations that these latter genes peaked at 2–3 h post stimulation and were maintained at a high level for another 3–5 h [Grimaldi et al., 1986; Ryan and Higgins, 1993]. The continued increase seen for MCP-1 in the present study was also not observed by us for TGF-β1 [Wysocki et al., 1996], which has been reported to be a delayed early gene expression during arterial repair [Miano et al., 1990]. We therefore concluded that the elevation in MCP-1 gene expression appeared to be independent to processes associated with proliferation in the vessel wall.

Knowing that monocytes and neutrophils are both capable of expressing the MCP-1 gene after stimulation [Yoshimura et al., 1989b; Colotta et al., 1992; Burn et al., 1994; Liebler et al., 1994], we examined injured pig arteries for the presence of infiltrating leukocytes. We found the infiltrating leukocytes were mainly neutrophils at 2 h after balloon injury but 6 h later phagocyte numbers were greatly increased and consisted of a mix of monocyte/macrophages and neutrophils. We also observed that foci of cells in injured arteries at 8 h showed significant immunostaining for MCP-1 and consisted predominantly of monocyte/macrophages. Bearing in mind that there were limits to the sensitivity of our detection technique, we were unable to find immunostaining for MCP-1 in SMC or in the surrounding extracellular matrix. The inability to detect MCP-1 expression in SMC in the presence of a positive signal for MCP-1 in macrophages has also been reported for atherosclerotic lesions in human and rabbit aortae [Yla-Herttuala et al., 1991]. We concluded from our

studies that infiltrated monocyte/macrophages were strongly expressing MCP-1 during the first 8 h of the arterial injury response. We are uncertain of the significance of our preliminary observation that Il-8 mRNA was also raised at 8 h after injury although this could reflect a relative increase in numbers of neutrophils as well as monocyte/macrophages at this point in the inflammatory response.

A temporal link has been reported between a peak in JE/MCP-1 expression and subsequent maximal macrophage infiltration in a recent study of a murine model of dermal wound repair, the lag period being some 2–3 days [DiPietro et al., 1995]. In the present study there was much less evidence of a similar temporal link. Rather we found that MCP-1 mRNA levels paralleled monocyte/macrophage numbers in pig arteries following injury. The fact that monocyte/macrophages were still strongly expressing MCP-1 when the vast majority of these cells had already entered the arterial wall suggested to us that there may be other functions being carried out by this protein in addition to action as a chemoattractant for monocytes. We speculate that one function may be desensitization of monocyte/macrophages to the chemoattractant gradient which initially attracted these immune cells into the damaged artery. Several investigations can be cited in support of this hypothesis. In vitro studies have demonstrated that monocytes undergo homologous desensitization in response to multiple challenges with MCP-1 [Charo et al., 1994]. Guinea pig resident (differentiated) peritoneal macrophages also were no longer attracted when exposed to MCP-1 [Yoshimura, 1993]. Once desensitized, the maintenance of a particular type of differentiated macrophage in situ may be dependent on the continued expression of MCP-1 and possibly pro-inflammatory cytokines whose production may be stimulated by MCP-1 [Jiang et al., 1992]. This group of cytokines may also have an input into determination of the length of the macrophage life-span since interleukin-1 has been shown to inhibit monocyte apoptosis [Mangan et al., 1993]. In this regard it is interesting that many of the longer-lived macrophages located in advanced atherosclerotic lesions strongly express MCP-1 [Yla-Herrttuala et al., 1991].

In summary, the results of our study demonstrate that the production of MCP-1 by monocyte/macrophages occurs as part of the inflammatory response following denudation of pig arteries. Our findings are consistent with the concept that monocyte/macrophages in situ may regulate the recruitment of more monocytes to injured artery by the induction of MCP-1 [Colotta et al., 1992; Cushing and Fogelman, 1992]. It appears likely that monocyte/macrophages which infiltrate damaged artery soon after the injury play an important role in fine-tuning the recruitment of additional monocytes and possibly also other immune cells. We also have preliminary data suggesting that some of the early-appearing, numerically-dominant, neutrophils may play a minor role in attracting circulating monocytes to the site of injury. This finding is further evidence of inter-communication between neutrophils and monocyte/macrophages during inflammation [Burn et al., 1994] and provides another mechanism for adjusting the mix of the cellular response. The current investigation presents data suggestive of functions for MCP-1 expression by monocyte/macrophages in addition to that of production of chemoattractant for monocytes. It is hoped that increasing knowledge of the receptors and signal transduction pathways activated by MCP-1 [Sozzani et al., 1995] will enable selective targeting of MCP-1 functions and therefore provide a means of controlling monocyte/macrophage activities which contribute to arterial pathology.

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