Identification of Macrophages in Intimal Thickening of Rat Carotid Arteries by Cytochemical Localization of Purine Nucleoside Phosphorylase

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Complete desquamation of the endothelium of the rat carotid artery by balloon catheter stripping resulted within 2 weeks in the formation of a large intimal thickening. After an enzyme cytochemical technique was applied to localize cytosolic purine nucleoside phosphorylase (PNP), light microscopical evaluation indicated that this intimal thickening in normocholesteremic rats was composed of 5.8% to 11.8% (mean 8.8%) PNP-positive cells. At the electron microscopic level, all these PNP-positive cells were identified as macrophages by the absence of a basement membrane and plasmalemmal vesicles and by the occurrence of specific intracytoplasmic granules. Additional evidence of the macrophage nature of the PNP-stained intimal cells was obtained by differential immunogold labeling of these cells with a monoclonal antibody against rat macrophages. Moreover, in hypercholesterolemic rats, only the cells stained for PNP transformed into foam cells (between 8.5% and 11.4% of all nucleated intimal cells; mean 9.6%). This study shows that PNP cytochemistry discriminates macrophages from modified smooth muscle cells in the rat carotid intimal thickening. It further suggests that the intimal thickening in normocholesterolemic rats originates not only from migration and proliferation of smooth muscle cells, but also from a considerable number of leukocyte-derived macrophages. Whether the latter cells are actively involved in the establishment of the intimal thickening, as has been suggested in dietary hypercholesterolemia, remains to be verified. (Arteriosclerosis 8:759–767, November/December 1988)

Adhesion and migration of monocytes into the intima of the large arteries of hypercholesterolemic animals is frequently described. It has been suggested that these cells transform into tissue macrophages and accumulate large amounts of lipids to produce foam cells. More recent immunocytochemical studies have shown that early fatty streaks are predominantly composed of monocyte-derived foamy macrophages. More advanced intimal lesions contain mostly smooth muscle cells (SMC) but also a considerable number of macrophages. Comparative results were obtained for human atherosclerotic plaques. Because of the obvious presence of monocyte-macrophages in the atherosclerotic arterial wall and the known diversity of secretory products playing putative roles in pathogenesis, the role of monocyte-macrophages in intimal plaque formation has been considered important.

In normocholesterolemic animals, only minor attention has been paid to the occurrence of macrophages in the intimal thickening induced by endothelial denudation, probably because platelet adhesion, endothelial regeneration, and SMC proliferation tended to be the main focus points. The presence of monocyte-like cells has only occasionally been reported. Yet, monocyte adhesion and intimal invasion were found to be relatively obvious in normal arterial blood vessels (reviewed by Joris et al.). In normal intimal masses of swine abdominal aorta, and in the intima of developing dog coronary collaterals. It is, therefore, possible that macrophages could also contribute to the establishment of these intimal thickenings. In the present study, we examined in detail the intimal thickening, which was induced by balloon catheter stripping of the rat carotid artery, for the presence of tissue macrophages. For this purpose, we used an enzyme cytochemical technique to localize the cytosolic enzyme purine nucleoside phosphorylase (PNP). This enzyme catalyzes the reaction of purine nucleoside + P → α-ribose-1-phosphate + purine base. PNP activity has been found in the circulating monocytes of different animal species, including the rat.

We therefore decided to use this technique to study tissue macrophages in the intimal thickening of rat carotid arteries. For this purpose, we used a new enzyme cytochemical technique to localize the cytosolic enzyme purine nucleoside phosphorylase (PNP). This enzyme catalyzes the reaction of purine nucleoside + P → α-ribose-1-phosphate + purine base. PNP activity has been found in the circulating monocytes of different animal species, including the rat.

Methods

Animals and Diet

Male Wistar rats from our own breeding colony were 5 to 6 months old and weighed 550 to 650 g. The animals...
received a normal standard stock diet (rat chow, Huybreaks, Arendonk, Belgium) and tap water ad libitum. Five additional rats were made hypercholesterolemic by a diet containing 4% cholesterol (Janssen Chimica, Geel, Belgium), 1% cholic acid (Serva, Heidelberg, FRG), and 0.5% 2-thiouracyl (Janssen Chimica) mixed with the standard stock diet. This diet was fed from 1 week before balloon catheterization until sacrifice.

**Plasma Cholesterol Analysis**

Blood samples of normo- and hypercholesterolemic rats were obtained at the time of sacrifice via a jugular catheter. The blood was anticoagulated with 77 mM EDTA (0.75 ml/10 ml) and the plasma was obtained by centrifugation at 900 g for 30 minutes. Levels of plasma cholesterol (mg/100 ml) were measured using a Hitachi 704 Analyzer (Hitachi, Tokyo, Japan).

**Experimental Procedure**

**Arterial Injury**

Balloon catheter injury of the left common carotid artery was performed as previously described. Briefly, after anesthesia of the animals with Thalamonal (i.m., 0.135 ml/100 g body weight) and sodium pentobarbital (i.p., 0.675 mg/100 g body weight), a midline incision was made in the neck region to expose the distal left common and external carotid arteries. A Fogarty 2F balloon catheter, which was introduced through the external carotid artery, was gradually inflated with saline and passed three times through the lumen of the common carotid to denude it of endothelium. After removal of the catheter, the external carotid was ligated and the wound was closed. Fourteen days after the arterial injury, the animals were again anesthetized with pentobarbital (6 mg/100 g body weight) and the vena jugularis was catheterized. After blood sampling as above, Evans blue dye (60 mg/kg) was injected to verify endothelial denudation after fixation. Ten minutes before and also during the perfusion procedure, continuous intravenous infusion with papaverine (Janssen Chimica; 0.6 mg/min of a solution of 30 mg/ml in normal saline) was initiated to dilate the carotid arteries. Perfusion was performed retrogradely via the abdominal aorta (perfusion pressure was 120 mm Hg). The blood was rinsed out with ice-cold Ringer’s solution before beginning perfusion fixation with the appropriate fixatives for cytochemistry and immunocytochemistry.

**Purine Nucleoside Phosphorylase Cytochemistry**

In five normocholesterolemic and five hypercholesterolemic rats, perfusion fixation was performed with 2% glutaraldehyde buffered to pH 7.4 with 0.1 M sodium cacodylate buffer for 5 minutes at 4°C. Small samples from the left and right common carotid arteries were excised. Some of these samples were processed for conventional morphological examination (further immersion fixation in 3% glutaraldehyde, postfixation in OsO4, uranyl impregnation, embedding). The others were immersed for 2 hours in cold sodium cacodylate buffer supplemented with 0.22 M sucrose. Frozen sections, which were 30 μm thick, were then prepared and incubated in the medium for PNP as described by Borgers and co-workers. The final concentration of the medium constituents was: 48 mM Tris-maleate buffer (pH 7.4), 3 mM lead nitrate, 4 mM α-D-ribose-1-phosphate, 10 mM hypoxanthine (solubilized by adding concentrated NaOH before addition to the medium), and 220 mM sucrose; the final pH of the medium was adjusted to pH 7.2 with HCl. The incubation proceeded for 15 or 30 minutes at 37°C during slow shaking. After incubation, the sections were briefly washed in 0.22 M sucrose, were postfixed in 2% OsO4 in Michaelis buffer (pH 7.4), were dehydrated in a graded series of ethanol, and were embedded in epon. Sections, which were 2 μm thick, were prepared and treated with 0.2% aqueous ammonium sulphide for light microscopic visualization of the end product of the cytochemical reaction. The resulting lead sulphide was blackish-brown. The nuclei and elastic tissue were counterstained for 30 seconds with methylene blue as described previously, or for 10 seconds with 0.1% toluidine blue. At the electron microscopic level, the reaction product of the cytochemical reaction is represented by the precipitated, highly electron-dense lead phosphate. The ultrathin sections, either unstained or stained with uranyl acetate and lead citrate, were examined.

PNP enzyme cytochemistry was also verified on rat resident peritoneal macrophages and on monocytes in buffy coats. The pellet containing the resident macrophages was gently stirred and subsequently fixed for 5 minutes in cold 2% glutaraldehyde in sodium cacodylate buffer (pH 7.4). After centrifugation (380 g), 0.1 ml bovine serum albumin and 5 drops of 25% glutaraldehyde were added, stirred, and again centrifuged. The solidified pellet was rinsed for 2 hours in 0.1 M cacodylate buffer containing 0.22 M sucrose (4°C). Buffy coat preparations were fixed for 1 hour in cold 2% glutaraldehyde buffered with 0.1 M sodium cacodylate and rinsed for 2 hours in the same buffer supplemented with sucrose. Frozen sections, which were 30 μm thick, were prepared from the macrophage pellet and theuffy coats and were processed for PNP cytochemistry as described above.

**Combined Immunocytochemistry and Purine Nucleoside Phosphorylase Cytochemistry**

To obtain evidence for the macrophage nature of the PNP-positive cells in the intima, in addition to the morphological and cytochemical characterization, immunocytochemical experiments were performed by using a monoclonal antibody directed against rat peritoneal macrophages. This monoclonal antibody, F369w, was produced in the laboratory of one of the co-authors (Marc L. Saint-Guillain) according to the method described for other monoclonal antibodies against cell surface antigens. This antibody was directed against a surface antigen associated with Fc receptor function and recognized all macrophage-like cells including the blood monocytes and polymorphonuclear neutrophils; it was not reactive with lymphocytes or any other cell type (M.P. Peltgen, et al. unpublished observation).

Initial experiments were performed on rat peritoneal macrophages. The macrophage pellets were fixed in cold
of all sectioned intimal cells also revealed another type of cell. Because of the presence of some intracytoplasmic inclusions, were negative. At the electron microscopic level, the cytochemical features were comparable to the light microscopic preparations. While a large number of intimal cells were nearly unreactive, others were intensely stained (Figure 2). The lead phosphate precipitate was confined to the cytosol excluding all membrane-bound organelles such as the nucleus, mitochondria, Golgi-apparatus, granules, and endoplasmic reticulum. Additionally, these PNP-positive intimal cells were characterized by the absence of plasmalemmal vesicles and basement membrane and by the presence of specific intracytoplasmic granules (Figures 3A and 3B). The readily identified, intimal SMC (plasmalemmal vesicles, myofilaments, and a surrounding basement membrane, these cells were probably macrophages. However, clear verification was sometimes difficult, especially when only small parts of the cells were sectioned. No signs of degeneration of intimal cells were found and apparently none of the macrophage-like cells had engulfed necrotized cellular material. In the de-endothelialized carotid artery of hypercholesterolemic rats, a prominent intimal thickening was also evident, although it was less elaborate than in the normocholesterolemic animals. A small proportion of intimal cells had transformed into foam cells. These were considered to be macrophages because of the lack of a basement membrane and plasmalemmal vesicles. The cells that could be established as SMC clearly carried fewer lipid globules.

**Purine Nucleoside Phosphorylase Cytochemistry**

Light microscopic investigation indicated that, under controlled conditions of fixation (5 minutes of perfusion and 30 minutes of incubation, at 37°C) in the balloon-stripped normocholesterolemic rat carotid, several intimal cells were strongly reactive for PNP, whereas others were devoid of activity (Figure 1A). The positive cells were distributed all over the intimal thickening without any preferential localization. Counting the number of nucleated cells (total number between 223 and 1021/carotid), revealed that 5.8%, 11.8%, 9.3%, 8.6%, and 8.3% (mean, 8.8%) of the intimal cells were PNP positive. The medial SMC were also stained but usually less intensely than the reactive intimal cells. In some samples, the SMC of the first medial layer (beneath the internal elastic lamina) were less reactive than the other medial SMC. Some adventitial structures also showed strong PNP activity. The normal (right carotid) endothelium and the proliferated (left carotid) endothelium were negative.

In the five hypercholesterolemic animals, the number of PNP-positive cells amounted to 9.5%, 9.5%, 9.3%, 11.4%, and 8.5% (mean 9.6%) of all nucleated intimal cells (totally counted number ranged between 295 and 405/carotid). Most of these PNP-positive cells within the intima were intensely stained foam cells (Figure 1B). The other intimal cells, which contained little or no lipid inclusions, were negative. At the electron microscopic level, the cytochemical features were comparable to the light microscopic preparations. While a large number of intimal cells were nearly unreactive, others were intensely stained (Figure 2). The lead phosphate precipitate was confined to the cytosol excluding all membrane-bound organelles such as the nucleus, mitochondria, Golgi-apparatus, granules, and endoplasmic reticulum. Additionally, these PNP-positive intimal cells were characterized by the absence of plasmalemmal vesicles and basement membrane and by the presence of specific intracytoplasmic granules (Figures 3A and 3B). The readily identified, intimal SMC (plasmalemmal vesicles, myofilaments, and basement membrane; see contrasted section in Figure 3A) and endothelial cells were unreactive or only weakly stained. This is in contrast to the apparent PNP activity present in the medial SMC (Figures 2 and 3C). The reaction product was also found in...
some adventitial mesenchymal cells. In the hypercholesterolemic animals, an intense staining of the foam cell cytosol was evident (Figure 4). These cells showed no plasmalemmal vesicles or basement membrane. The modified, intimal SMC contained a comparatively lower number of lipid inclusions and were barely stained.

The rat peritoneal macrophages also carried large amounts of reaction product within the cytosol. The other peritoneal cells, such as mast cells, eosinophils, and lymphocytes, were not stained. In the buffy coats, activity was seen in monocytes, neutrophils, blood platelets, and part of the lymphocytes.
Combined Purine Nucleoside Phosphorylase Cytochemistry and Immunocytochemistry

In the peritoneal preparations, the PNP-positive macrophages showed gold labeling on their outer surface (Figure 5). The gold particles were distributed all over the surface of the macrophages and tended to form clusters. The gold label was often displaced over a small distance from the plasma membrane, which was probably due to the freezing and thawing of the cryostat sections. Single gold particles were rarely observed at
Figure 4. Hypercholesterolemic rat. Transmission electron micrographs of intimal tissue. Uncontrasted sections. A. The intense cytoplasmic staining of the foam cell-macrophage (FC(M)) in contrast to the weak activity in the modified smooth muscle cells (MSMC). l=lipid globule. Bar=1 μm. B. Enlargement of the enclosed area in A. Presence and absence of plasmalemmal vesicles (v) in the MSMC and foam cell, respectively, is presented. A basement membrane cannot be identified due to omission of contrasting stain. Bar=1 μm.

the surface of mast cells, eosinophils, or lymphocytes. In the buffy coats, labeling was seen on the PNP-positive monocytes in particular and, to a lesser extent, on neutrophils; neither PNP-positive nor PNP-negative lymphocytes were labeled.

Omission of glutaraldehyde fixation after the immunocytochemical procedure resulted in PNP-staining of all intimal cells. This was due to the less pronounced inhibition of enzyme activity by the aldehyde fixation.²⁵ Yet the difference in activity observed between the cells verified as modified SMC and the macrophage-like cells was obvious. PNP activity was pronounced in the latter cell type, whereas the modified SMC were only moderately positive (Figures 6A and 6B). An additional distinction between cell types was evident; numerous gold particles were seen at the surface of the strongly PNP-reactive, macrophage-like cells only (Figure 6A) and not (or only sporadically) at the surface of the modified SMC (Figure 6B). Fixation with 2% glutaraldehyde after the immunocytochemical incubations resulted in a good subcellular preservation but in a less intense PNP staining of the vascular cells (Figure 6C). In these samples, the intimal modified SMC were not stained at all, the macrophage-like cells were moderately stained, and the medial SMC were stained weakly. Gold labeling was obvious only on the surface of the macrophage-like, PNP-stained intimal cells (Figure 6C). Membrane-associated gold particles were never observed on the medial SMC.

Figure 5. Transmission electron micrograph of a rat peritoneal macrophage after purine nucleoside phosphorylase (PNP) cytochemistry combined with immunocytochemistry. Dilutions: F₁B₄ monoclonal antibody (1/4), GAM IgG G10 (1/10). Contrasted section. Cytoplasmic PNP staining and immunogold labeling (arrows) of the outer surface of the pseudopodal extensions and some clusters of gold particles are seen. n=nucleus. Bar=0.2 μm.
Discussion

This study showed that in the balloon-stripped rat carotid artery controlled conditions of PNP cytochemistry clearly distinguished between two types of intimal cells: modified SMC with little enzyme activity and macrophages with strong activity. A relatively large number of intimal macrophages was apparent in the normocholesterolemic animals. The PNP-reactive macrophages only transformed into real foam cells in the hypercholesterolemic animals.

The possibility of distinguishing different intimal cells by the use of PNP cytochemistry was previously described in developing dog coronary collateral arteries. Under comparable experimental (cytochemical) conditions, the intimal modified SMC, as well as the endothelial cells of the rat carotid artery, were barely stained, whereas the intimal...
macrophages were strongly reactive and the normal medial SMC were moderately reactive. Earlier work has indicated that rat blood monocytes were intensely stained.21 This activity probably remained after the monocytes invaded the intimal thickening of the carotid artery and transformed into tissue macrophages. These PNP-reactive intimal cells were, indeed, macrophages because of their morphological criteria (absence of plasmalemmal vesicles and basement membrane, occurrence of specific intracytoplasmic granules); this was further proved by the differential immunogold labeling of the surface coat with the specific F6B4 antirat macrophage monoclonal antibody. Peritoneal macrophages and blood monocytes showed comparable cytochemical and immunocytochemical features. Moreover, only PNP-positive cells transformed into foam cells, whereas the intimal modified SMC contained a relatively small number of lipid inclusions. This difference in lipid accumulation, which occurs particularly in the early stage of intimal thickening, has often been described.1,2,4,5 This may result from a different metabolism or from a different avidity for lipid uptake between macrophages and SMC.17,26

The light microscopic evaluation verified the differential number of PNP-positive and PNP-negative intimal cells and showed a mean of about 9 PNP-positive cells per 100 intimal nucleated cells in five different carotid arteries of normo- and hypercholesterolemic rats. Although extensive quantification on a large number of rats was not performed, these results may indicate that not only in hypercholesterolemic animals but also in normocholesterolemic ones, the intimal thickening probably contains a significant number of macrophages. This corroborates the findings of Imai and colleagues17 in normal intimal cell masses in swine abdominal aortas and the obvious presence of macrophages in developing dog coronary collaterals, which are also characterized by intimal thickening formation.18 Whether these cells play a role in the establishment of intimal thickening (as has been suggested for hypercholesterolemia10,19) or whether the cells only invade and wait for further stimulation, remains to be determined. The PNP technique may be useful for further studies. PNP cytochemistry can also be used in other mammalian species, since the monocytes of all species investigated thus far have stained.21

It was recently proposed that a relatively large number of nondividing SMC contributes to injury-induced intimal thickening formation.27 This theory was derived from the fact that a substantial number of unlabeled cells was observed autoradiographically in the intima after continuous intraperitoneal infusion of 3H-thymidine. The observation that nearly 10% of the intimal cells in the balloon-striped rat carotids after 2 weeks of development are macrophages suggests that part of the quiescent SMC are, in fact, macrophages. Apart from the verification of intimal macrophages, additional information was obtained from our cytochemical study. First, in comparable conditions of fixation and incubation, the endothelial cells of dog coronary collateral arteries were clearly reactive for PNP, whereas the medial SMC were negative. The reverse situation was found in the rat carotid elastic artery. Whether this difference is related to a difference in species or anatomical location of the blood vessel remains to be determined. Second, an obvious difference in activity between the intimal modified SMC (synthetic phenotype) and the medial normal SMC (contractile phenotype) was observed. This may relate to a difference in the extent of the purine salvage between phenotypes, at least in the rat. This adds a new characteristic to the many differences between the SMC phenotypes already described (other enzyme activities, morphology, contractility, proliferative behavior, cytoskeletal protein content, and synthesis of extracellular material) between the SMC phenotypes.28,29,30

We conclude that PNP cytochemistry of the balloon-striped rat carotid artery makes identification and subsequent quantification of intimal macrophages quite feasible. We further conclude that in normocholesterolemic rats, macrophages contribute substantially to the composition of the intimal thickening.

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References


Index Terms: rat carotid artery • purine nucleoside phosphorylase • balloon catheter stripping • macrophages • intimal thickening
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