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The Origin of Subendothelial Cells in Developing Coronary Collaterals

A Cytochemical Approach*

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Summary. Using NPase as a specific and differential marker of endothelial cells and some blood-borne cells, we attempted to provide information on the origin of cells contributing to the formation of the intimal thickening occuring during coronary collateral growth in the dog. NPase reactive endothelial cells, polymorphonuclear and mononuclear blood cells and NPase unreactive smooth muscle cells and modified smooth muscle cells were seen in variable proportions in the subendothelial space during the early period of vascular growth and in the intimal thickening, formed a few weeks later. The medial smooth muscle cell appeared to be the main progenitor of cells present in this zone.

The possible transformation of endothelial and blood-formed cells into myointimal cells is discussed. The functional significance of NPase activity in these cells and the further outlook of NPase application as a marker enzyme in the process of atherosclerosis, in experimental hypertension and in various vascular injury processes has been outlined.

Introduction

The reaction of the blood vessel wall to various factors inducing vessel wall proliferation is rather stereotypic, i.e. formation of an intimal thickening, though this is not necessarily the earliest response. This is the case in spontaneous and experimental atherosclerosis, experimental hypertension, chemical, thermal and mechanical injury, experimentally induced vascular growth. One fundamental question, as to the origin of cells belonging to this intimal proliferative zone in still under debate. The currently promoted precursor cells are: medial smooth muscle cells (Thomas, Jones, Scott, Morrison, Goodale, Imai, 1963; Parker, Odland, 1966; Scott, Jones, Daoud, Zumbo, Coulston, Thomas, 1967; Wissler, 1967; Jarmolych, Daoud, Landau, Fritz, McElvene, 1968); blood-borne elements, such as monocytes and lymphocytes (Still, Marriott, 1964; Still, 1968); fibroblasts (Still, Marriott, 1964; Zollinger, 1967); and endothelial cells (Zollinger, 1967; Altschul, 1954; Haust, More, Movat, 1960; Kojimahara, Sekiya, Ooneda, 1971a–b).

The uniformity in vascular response has been confirmed in our previous studies, performed on growing collaterals, experimentally induced by chronic coronary artery occlusion (Schaper, 1971). These studies revealed that at different time intervals after induction of vessel growth, the collaterals went first through stages of injury and repair, characterized by endothelial hypertrophy, medial necrosis, perivascular inflammation and strong activation of adventitial mesenchymal cells and secondly through proliferative stages, characterized by a general thickening of the collateral wall and by the occurrence of an intimal thickening.

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These phenomena were particularly prominent in midzone pieces of the collateral. On the cellular level, we were faced with a multitude of morphologically different cell types involved in these processes.

Recent observations by Rubio, Wiedmeier and Berne (1972) in guinea pig hearts and by Borgers, Schaper, Schaper (1972) in dog hearts indicated that nucleoside phosphorylase (NPase) was localized in endothelial cells, in adventitial mesenchymal cells and in perivascular cells. Of special interest was the observation that in dog coronary arteries, the endothelial cytoplasm was thoroughly marked for enzymatic activity, whereas smooth muscle cells were completely devoid of it.

Nucleoside phosphorylase (EC 2.4.2.1.) is the enzyme which is responsible for the conversion of inosine into hypoxanthine: inosine + Pi $\rightleftharpoons$ hypoxanthine + ribose 1-phosphate.

Using rather high concentrations of hypoxanthine and ribose 1-phosphate in the incubating solution, the reaction is driven towards inosine. The liberated phosphate is captured by lead ions as in the classical Gomori reaction.

The topic of this paper was to study the cells involved in vessel wall proliferation and this in an experimental model which covers various progressive stages in vessel growth. The origin of subintimal cells, which proved to be difficult to elucidate by morphologic determination, was investigated by cytochemical means, i.e. speculating upon the divergence of NPase activity between different cell types constituting the vascular wall and the circulating blood elements.

Material and Methods

Adult mongrel dogs of either sex were used in these experiments. The development of collaterals was induced by progressive constriction of the left circumflex artery by means of an Ameroid constrictor (Litvak, Siderides, Vineberg, 1957; Schaper, Schaper, Xhonneux, Vandesteene, 1969; Schaper, 1971). Three, 8 and 12 weeks after the implantation of the constrictor, the animals were anesthetized and the hearts were fixed in situ by perfusion of the coronary arteries with distilled 2% glutaraldehyde, buffered with sodium cacodylate 0.1 M to pH 7.4 for 5 to 10 minutes. The excess fixative was rinsed away by a subsequent perfusion with the same buffer to which 0.22 M sucrose was added. Midzone segments of the epicardial collaterals connecting the anterior descending artery with the left circumflex artery, were rapidly excised and further washed for 2 to 22 hours in the buffer.

Forty micron thick frozen sections were prepared and subsequently incubated in the modified NPase medium of Rubio, Wiedmeier, Berne (1972) as described earlier (Borgers, Schaper, Schaper, 1972), The final composition of the medium was: tris-maleate buffer pH 7.2 60 mM; lead nitrate 3 mM; ribose 1-phosphate 4 mM; hypoxanthine 10 mM; sucrose 220 mM.

The incubation was performed at 37° C for 30 minutes. The following control experiments were done:

— incubation in a medium from which either ribose 1-phosphate, or hypoxanthine or both were omitted.

— incubation in a complete medium to which 10 mM NaF (to inhibit non-specific acid phosphatase) or 0.5 mM tetramisole (Van Belle, 1972) (to inhibit non-specific alkaline phosphatase) was added.

— incubation in a medium in which ribose 5-phosphate was substituted for ribose 1-phosphate in equimolar concentration.

After a short rinse in distilled water + sucrose, the incubated sections were postfixed in 1.5% OsO_4 in Michaelis buffer, dehydrated in graded series of ethanol at 4° C and embedded in epon.

Ultrathin sections were stained on the grid with uranyl acetate and lead nitrate before examination.

For light microscopy, 1 micron thick sections of the epon embedded pieces were prepared, the lead phosphate precipitate was converted to lead sulphide by incubating the sections in a 2% ammonium sulphide solution for 30 minutes.

These sections served as an indicator for the gross distribution of NPase activity and for exact topographical localization of the injured or proliferative zones. A total of 48 midzone pieces at 3 weeks; 52 pieces at 8 weeks and 18 pieces at 12 weeks, were examined.

Results

1. Normal Coronary Arteries

The distribution of NPase in normal coronary vessels as described earlier (Borgers, Schaper, Schaper, 1972) can be summarized as follows: the reaction product was exclusively localized in the cytoplasmic sap of all endothelial cells and advential mesenchymal cells (resting fibroblasts and fibrocytes). Examination of buffy coat preparations of circulating blood cells (same report) revealed that neutrophilic and basophilic polymorphs, monocytes, a small portion of the lymphocyte population and platelets were reactive for NPase; eosinophilic polymorphs, erythrocytes and the majority of small lymphocytes did not show any activity.

The subcellular localization of NPase activity, i.e. in the cytoplasmic sap and on the centrioles of reactive cells as seen in normal coronary arteries, remained the same in all the developing coronary collaterals, examined at different periods.

2. Early Stage of Collateral Development (3 Weeks after Implantation of the Constrictor)

As observed in earlier studies describing the morphologic features of the development (Schaper, 1971; Borgers, Schaper, Schaper, 1970), most midzones showed signs of damage to a varying extent. Endothelial cells were frequently hypertrophied, the elastic membrane was ruptured, the media looked disorganized with altered smooth muscle cells and the adventitia showed abundant active fibroblasts. At this stage, no real intimal thickening was observed.

The endothelial lining was constantly reactive for NPase. An important number of strongly reactive cells was seen in different topographical positions between the endothelium and the unreactive smooth muscle cells: (1) partially positioned between normally arranged endothelial cells; (2) just beneath the endothelial layer but still making close contact with the endothelium; (3) disseminated within the subendothelial space.

Most of the cells penetrating the subendothelial space could be identified as large endothelial cells by the presence of NPase activity, by the multiple pinocytic vesicles within the cell membranes and by the absence of basement membranes (cell coat which is characteristic for smooth muscle cells) (Fig. 1).

The appearance of the endothelium was rather peculiar in areas where these heavily labelled cells had entered the subendothelial space. The endothelial cells appeared flattened and met by slender cytoplasmic processes (Fig. 2).

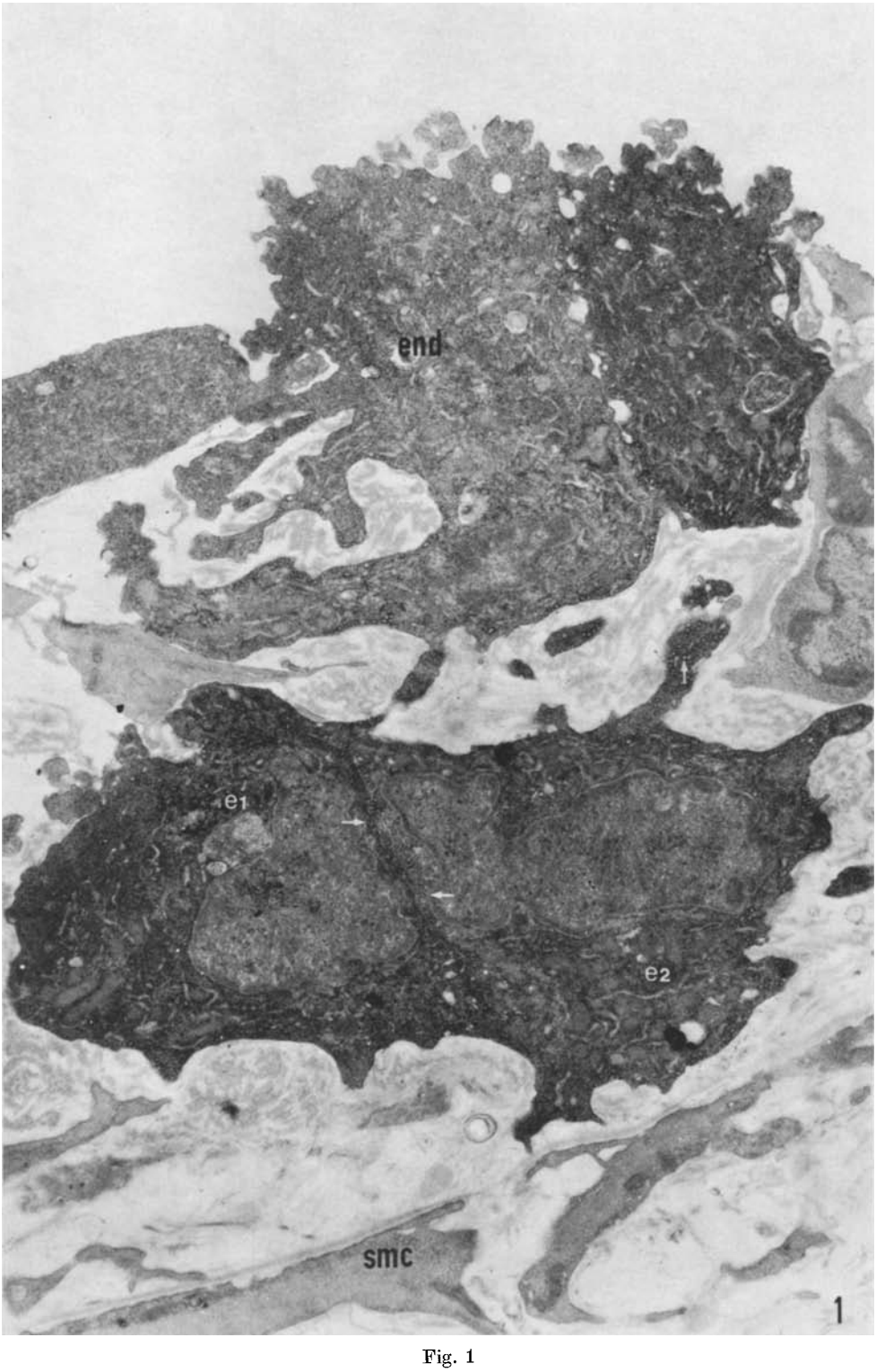


Fig. 1

Sometimes, clusters made up of 2 to 4 reactive endothelial cells closely linked together were present in the subendothelial space (Fig. 1). Besides these easily identifiable endothelial cells, other cells, equally rich in NPase activity, but lacking the other characteristics of endothelial cells (pinocytic vesicles) were regularly seen in this zone (Fig. 2). They closely resembled the mononuclear leukocytes examined in buffy coat preparations of circulating dog blood. A few very reactive neutrophilic polymorphs were observed in the subendothelial space.

The number of NPase positive cells varied from one midzone piece to another and between the individual collaterals on the order of 5 to 25 cells per cross-sectioned collateral wall.

A limited number of cells, closely resembling modified smooth muscle cells, unreactive for NPase, was seen intermingled with the strongly reactive cells.

Smooth muscle cells, healthy looking or in various stages of necrosis did not show any reactivity (Fig. 2).

The adventitial mesenchymal cells, very abundant at this stage of development, were completely devoid of reaction product (Fig. 3). The only activity which was observed in the adventitia was confined to the axonal cytoplasm of the nerves.

3. Proliferative Stage of Collateral Growth (8 Weeks after Implantation of the Constrictor)

The most prominent feature at this stage of development from the morphologic point of view was the presence of an intimal thickening. In the midzones, this layer comprised up to 50% of the total wall thickness. In general the intimal thickening appeared rather disorganized and was composed of a number of morphologically different cell types.

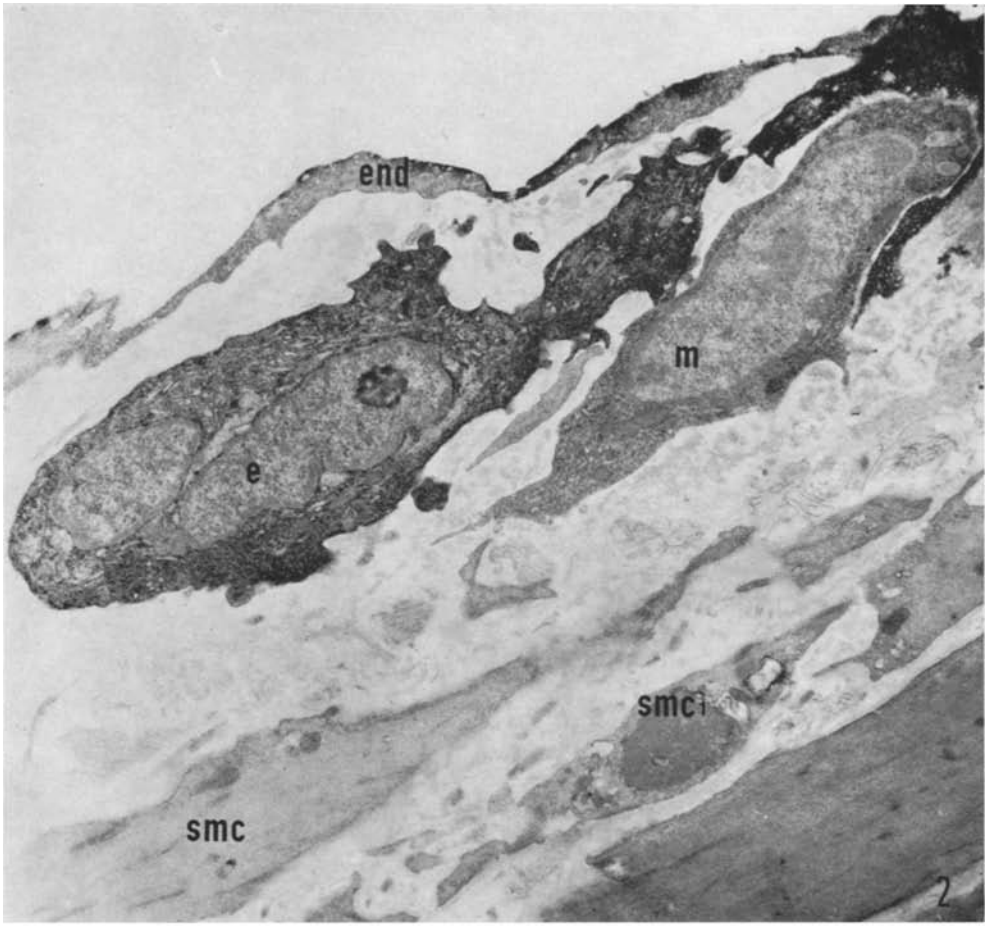
Enlarged endothelial cells, although not so abundant as in the earlier stage, were still prominent.

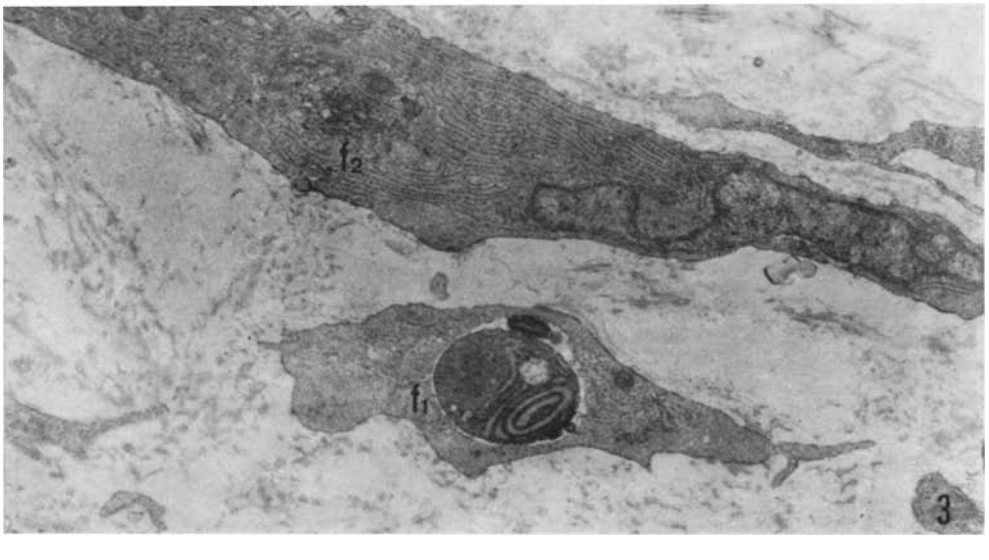
The number of NPase positive cells present in the intimal thickening varied between 8 and 21 per cross section through a collateral midzone.

Endothelial cells, filled with reaction product, were seen moving into the intimal thickening in progressive stages (Fig. 4a–b). Some hypertrophied cells of endothelial origin, positive for NPase, as seen in the 3 week group, were still prominent at this stage (Fig. 6). Clusters of endothelial cells were, however, no longer seen.

In those midzones presenting a highly developed thickening, the NPase positive cells were observed equally in the deeper part of the thickening, thus relatively far away from the endothelium (Fig. 5).

Fig. 1. Three weeks after constrictor implantation. The endothelium (*end*) consists of cells reactive in a variable degree. A cluster of two large endothelial cells (*e1* and *e2*), strongly positive for NPase is seen in the subendothelial space. The arrows point to pinocytic vesicles lining the plasma membranes. Smooth muscle cells (*smc*) are not reactive. (×8900). All micrographs represent cytochemical demonstrations on the distribution of NPase activity in growing collateral vessels of the dog heart





Figs. 2 and 3

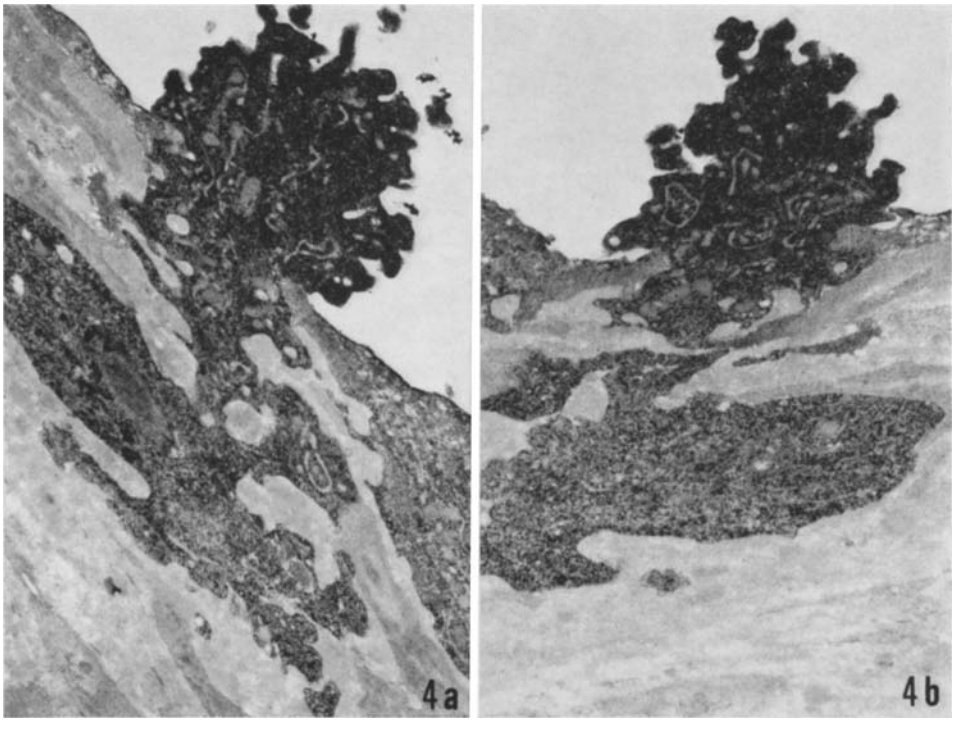


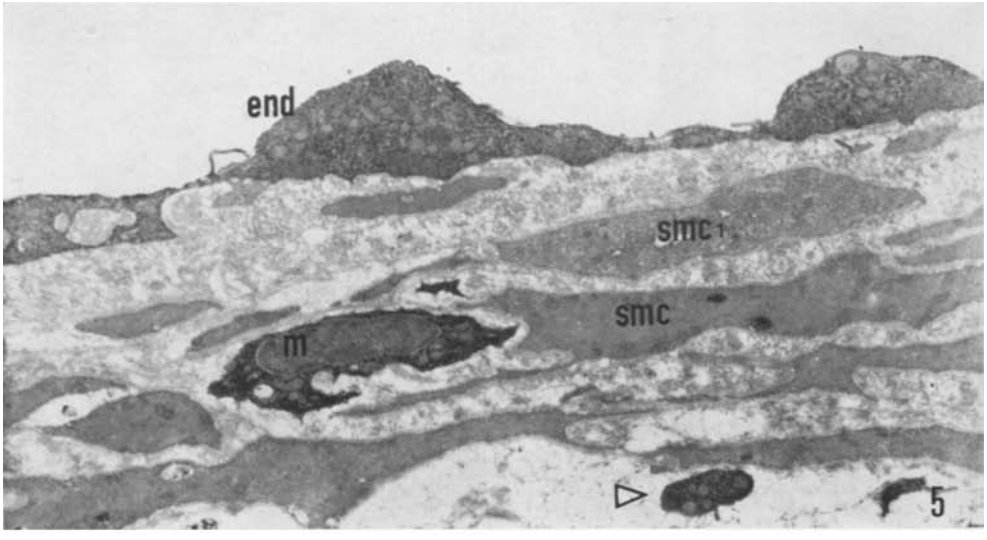
Fig. 4a and b. Eight weeks after constrictor implantation. Serial sections of a strongly reactive endothelial cell penetrating the intimal thickening. (×7800)

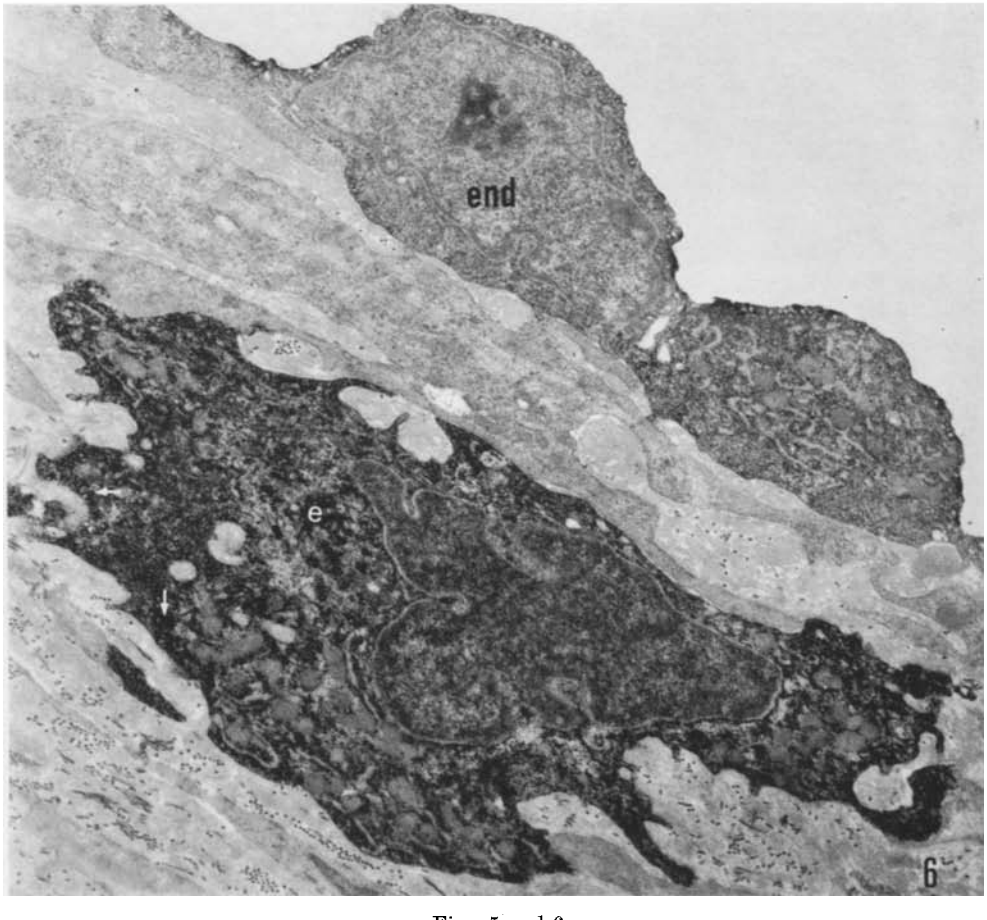
Again, certain heavily labeled cells, usually small in size, and not identifiable as endothelial cells by the lack of pinocytic vesicles, were present in this zone (Fig. 5).

The majority of cells occupying the intimal thickening were smooth muscle cells and modified smooth muscle cells, both types unreactive for NPase (Fig. 5). The circularly arranged smooth muscle cells of the media and the majority of adventitial mesenchymal cells did not present any reaction product. However, some elongated adventitial cells resembling the resting fibroblasts and fibrocytes of normal arteries showed some moderate activity.

Fig. 2. Three weeks after constrictor implantation. The endothelial lining (*end*) consists of slender cytoplasmic processes. A strongly labeled cell of endothelial origin (*e*) and a moderately reactive mononuclear leucocyte (*m*) inhabit the subendothelial space. Normal smooth muscle cells (*smc*) and a necrotic one (*smc1*) completely lack the precipitate. (×6340)

Fig. 3. Three weeks after constrictor implantation. Portions of two fibroblasts (*f1* and *f2*), not reactive for NPase activity, situated in the collateral adventitia. (×10500)





Figs. 5 and 6

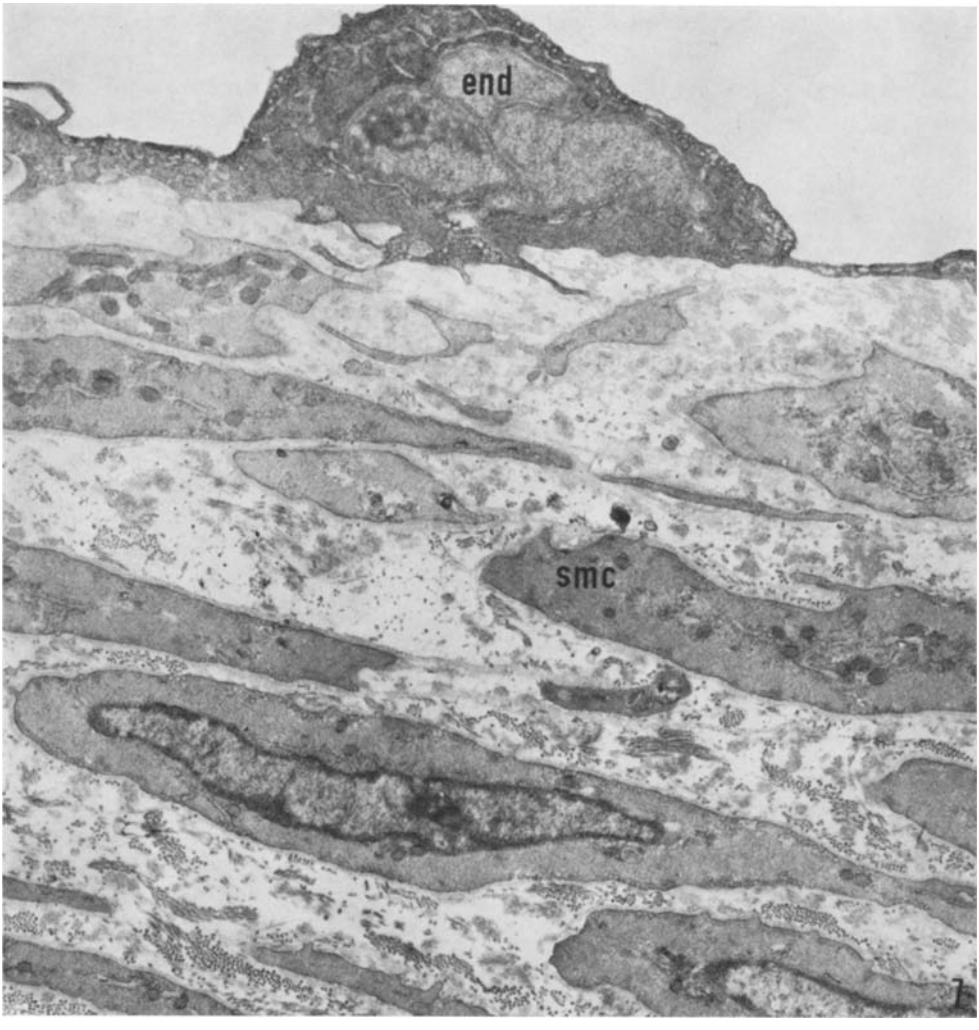


Fig. 7. Twelve weeks after constrictor implantation. NPase activity is exclusively confined to the endothelium (*end*). All cells in the intimal thickening are of the smooth muscular type (*smc*). (×9630)

Fig. 5. Eight weeks after constrictor implantation. Survey picture of the upper part of a collateral vessel wall. The endothelial cells (*end*) are invariably NPase positive. A strongly reactive mononuclear cell (*m*) is seen intermingled between non-reactive smooth muscle cells (*smc*) and a modified smooth muscle cell (*smc1*). A portion of a reactive cell (arrow) is seen in the deeper part of the intimal thickening. (×9910)

Fig. 6. Eight weeks after constrictor implantation. A very large cell of endothelial origin (*e*) (arrows point to pinocytic vesicles) is seen below an apparently normal endothelium. (×9910)

4. Advanced Proliferative Stage (12 Weeks after Initiation of Development)

Morphologically, the overall appearance of the collateral vessel resembled that of the 8 week group. In more detail, however, the intimal thickening appeared to be better organized and the cells occupying this area were all of the smooth muscular type.

The localization of NPase activity was confined exclusively to the endothelium and to the adventitial fibroblasts and fibrocytes. No activity was observed either in cells belonging to the intimal thickening or in medial smooth muscle cells (Fig. 7).

5. Control Experiments

Omission of either ribose 1-phosphate or hypoxanthine, or both from the incubating solution resulted in a complete loss of precipitate.

The addition of either NaF or tetramisole, on the other hand, did not influence the distribution pattern of NPase activity. Substitution of ribose 5-phosphate for ribose 1-phosphate in the complete medium, resulted in complete absence of reaction product.

Discussion

Using NPase as a marker enzyme of the endothelium and of some blood-formed elements, we tried to add some supplemental evidence for the participation of these cells in the formation of the intimal thickening occurring during coronary collateral development. That this problem is a matter of general interest in analogous models such as spontaneous and experimental atherosclerosis (Tho mas, Jones, Scott, Morrison, Goodale, Imai, 1963; Parker, Odland, 1966; Scott, Jones, Daoud, Zumbo, Coulston, Thomas, 1967; Haust, More, Ealis, 1962), experimental hypertension (Hackensellner, David, Uerlings, 1965; Still, 1967), vascular grafting (Rivkin, Friedman, Byers, 1963), wound healing (Raekallio, 1970), various kinds of vascular injury (Robertson, Moore, Merserau, 1959; Stehbens, 1965; Spaet, Lejnieks, 1967; Ts'ao, Spaet, 1967; Cotran, Remensnyder, 1968; Hoff, Gottlob, 1968; Björkerud, 1969) and vascular growth (Schoefl, 1963; Aloisi, Giacomin, Tessari, 1970) is clear from the enormous amount of work which has been done in this field.

The disagreement which exists among different groups of investigators on the topic of the origin of subintimal cells is easy to understand, since it is a matter which cannot be solved by pure morphologic determination. We think therefore that cytochemical support as outlined in this paper, could be of some help in elucidating this problem.

During the early stage of development of the collateral circulation, it was shown that morphologically different cell types inhabited the subendothelial space and that a few weeks later a subintimal thickening formed which was composed of a morphologically heterogenous cell population. After 12 weeks of development, the character of the intimal thickening had changed and almost all the cells belonging to this zone could be identified as smooth muscle cells.

For reasons of clarity, we felt that it would be easier to discuss separately the various cell types, currently proposed in the literature as precursors for the intimal cell.

1. Endothelial Cells

Most of the heavily labeled cells in the subendothelial space were of endothelial origin, this was clearly evidenced by the presence of numerous pinocytic vesicles (different from blood-borne cells) and by the lack of a basement membrane and the presence of strong NPase activity (different from smooth muscle cells and active mesenchymal cells). Subintimal cells of endothelial origin, recognized by these parameters are, however, always localized close to the endothelium.

The topographic localization of NPase labeled subendothelial cells at particular spots where the endothelial lining branches by thin cytoplasmic processes, strongly favors the hypothesis of the displacement of some endothelial cells into the subendothelial space.

Whether these diverted cells participate further in the development of the intimal thickening by mitotic division and transformation into myointimal cells, as present at 12 weeks, is not elucidated by our results. The fact that no degeneration and subsequent phagocytosis of NPase positive cells and cell dibris has been observed, together with the fact that intimal cells at 12 weeks completely lack NPase activity, strongly suggest that the former cells underwent transformation during which they lost NPase activity.

2. Smooth Muscle Cells

This cell type could easily be recognized morphologically among the cells inhabiting the disorganized subendothelial thickening. Neither the apparently normal looking cells, the degenerated and necrotic smooth muscle cells, nor the modified smooth muscle cells (fibroblast like cells) were found to be reactive for NPase. Modified smooth muscle cells were found intermingled with reactive cells in the intimal thickening at 8 weeks. The non-reactivity of these cells strongly suggests their medial smooth muscular origin. We are aware that no absolute proof exists for the fact that some cells may acquire NPase activity during transformation, our results, however, indicate that this is not the case for smooth muscle cells.

3. Adventitial Mesenchymal Cells

The very active mesenchymal cells of the adventitia, present 3 and 8 weeks after induction of collateral growth are hardly distinguishable from the enlarged endothelial cells on pure morphologic grounds, i.e. both cell types have a well developed GERL system and are actively endocytotic, and both cell types are not surrounded by a basement membrane. However, in contrast to the resting mesenchymal cells in the normal animals and the endothelial cells in normal and treated animals, the active fibroblasts do not possess NPase activity. Cells, corresponding to the morphological and cytochemical criteria of active mesenchymal cells, have never been observed in the intimal thickening.

4. Blood-Borne Cells

In previous investigations (Schaper, 1971; Borgers, 1971) it was clearly shown that some neutrophylic polymorphs and monocytes characterized mor-

phologically by their specific granules and by their nuclear shape (Shively, Feldt, Davis, 1969) and a number of unidentified mononuclear cells were found in the intimal thickening. Recent observations on NPase labeling of circulating blood cells (Borgers, Schaper, Schaper, 1972) revealed that neutrophils, monocytes and some lymphocytes were strongly reactive. Since quite a number of heavily labeled cells, morphologically easily distinguishable from the equally reactive displaced endothelial cells by the absence of pinocytic vesicles and by their size, were present in the subendothelial space at 3 weeks and in the intimal thickening at 8 weeks, we presume that these cells entered from the blood stream through the endothelial gaps. We presume also that the unidentified cells, encountered in the morphologic study, may correspond to diapedized lymphocytes. This presumption is based on the close resemblence on morphologic grounds and on NPase load between some intimal cells and the circulating lymphocytes. As for the fate of these diapedized leukocytes, we have no indication that they transform into myo-intimal cells later on, thus participating actively in the development of the collateral wall.

In conclusion we may say that these cytochemical data added the following information on the origin of intimal cells:

— medial smooth muscle cells participate as the main precursors of cells constituting the intimal thickening

— it is very unlikely that adventitial mesenchymal cells contribute to the intimal thickening formation

— endothelial cells and hematogenous cells penetrate in large numbers the subendothelial space and inhabit, at least in the early proliferative phase, the intimal thickening.

The usefulness of NPase as a marker of some specific cells will be further explored, more particularly in the research on the origin of fat-accumulating cells (foam cells) in the early and late stages of atherosclerosis and on the origin of cells initiating revascularization in occluded vessels.

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