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Connective Tissue Cells, Cell Proliferation and Synthesis of Extracellular Matrix - A Review [and Discussion]

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Connective tissue cells, cell proliferation and synthesis of extracellular matrix – a review

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[Plates 2 and 3]

The ubiquitous connective tissues contain a wide range of cells including fibroblasts, osteoblasts and chondroblasts. Recently it has been demonstrated that another principal cell of the connective tissue is the smooth muscle cell in several organ systems. These have been shown to be responsible for the synthesis of the connective tissue matrix components of the uterine myometrium and of the arterial system, including collagen, both elastic fibre proteins and glycosaminoglycan.

Microtubule inhibitors such as colchicine and vinblastine, and iron chelators such as α,α'-dipyridyl have been used to study their morphologic and chemical effects on collagen synthesis and secretion. Colchicine produces an increase in large Golgi-associated vacuoles, which sometimes contain material reminiscent of aggregates of collagen macromolecules. Vinblastine produces alterations in the endoplasmic reticulum cisternae similar to alterations seen in ascorbic acid deficiency, and α,α'-dipyridyl increases the frequency of regions in cells, interpretable as potential sites of communication of rough endoplasmic reticulum cisternae with the cell surface. Ferritin conjugated anti-procollagen sera were used to localize procollagen in cells and demonstrated procollagen not only in the cisternae of rough endoplasmic reticulum but in all of the elements of the Golgi complex as well.

The studies reported in this review have shown that in cell culture arterial smooth muscle will produce not only the microfibrillar protein of the elastic fibre but soluble and/or insoluble elastin as well. Recent studies on serum factors responsible for the proliferation of connective tissue cells have demonstrated that at least one of the principal factors responsible for fibroblast and/or smooth muscle cell proliferation in culture is derived from thrombocytes. Medium containing serum derived from cell-free plasma lacks most of this proliferative effect which can be reinstated when platelets are present during recalcification to form serum. This effect is due to the platelet release reaction as shown by combining supernatant factors derived from platelets exposed to purified thrombin to cell-free, plasma derived serum. Studies with macrophages have also suggested that phagocytic macrophages release factor(s) into a cell culture medium that may also participate in stimulating fibroblasts to proliferate *in vitro*. The means by which these factors stimulate fibroblast proliferation and connective tissue synthesis remains to be elucidated.

Introduction

The list of cells capable of the synthesis of the different components of the extracellular connective tissue matrix has greatly expanded in the last decade. Included in this list are the cells classically recognized as connective tissue cells, such as the fibroblast, osteoblast, chondroblast, and odontoblast, and more recently, the smooth muscle cell (Ross 1968*a*; Ross & Klebanoff 1971). Ectodermally derived cells, such as corneal epithelium, have also been shown actively to synthesize connective tissue proteins (Hay & Dodson 1973; Trelstad 1971).

The appropriate genetic information for the synthesis of these proteins is presumed to be present in all cells of a given mammalian species. In most cases, the genetic expression of this synthetic capacity has been efficiently repressed. However, Green & Goldberg (1965) have shown that many cell lines in culture, including cells such as HeLa, can synthesize small amounts of collagen as demonstrated by their ability to incorporate [^{3}H]proline into hydroxyproline-containing proteins.

The principal connective tissue components synthesized by these cells include: collagen (including basement membrane), both elastic fibre proteins (elastin and the microfibrillar glycoprotein) and the glycosaminoglycans (mucopolysaccharides). The amount and type of each constituent protein and glycosaminoglycan present varies from tissue to tissue (Grant & Prockop 1972), in different stages of development and in different pathological circumstances. The factors that control the genetic expression of the synthesis of each of these matrix components are not understood. However, this expression appears to be highly selective in some cells, as demonstrated by the high degree of specificity in the types of collagen molecules found in tissues such as cartilage, bone, and blood vessels (Miller & Matukas 1974).

Connective tissue cell proliferation is one of the principal reactions to injury. Understanding the nature of this response will be paramount if we are to control processes such as wound repair and atherosclerosis, both of which involve a hyperplastic response of connective tissue cells accompanied by increased formation of matrix components (Ross 1968*b*; Ross & Glomset 1973).

The circumstances that stimulate these cells to proliferate in response to different stimuli, such as those that occur during development, or during reactions to injury depend upon many cellular factors. These include cell surface receptors, hormones and specific extracellular substances, including components derived from cells, such as platelets and macrophages, which interact with the connective tissue cells and participate in this response. The importance of the amount and type of extracellular matrix components, as well as the different extracellular factors noted previously, upon feedback control, both in cell proliferation and in connective tissue synthesis, are only beginning to be understood. This paper will briefly review the present state of information concerning connective tissue cells, matrix formation and content and some of the factors recently shown to be important in the proliferation of these cells.

Connective tissue matrix constituents

Collagen

At least four different molecular types of collagen have been identified, based upon their amino acid chain composition and sequence. All of these proteins have been determined to be collagen, by using criteria of content of glycine, proline, hydroxyproline and hydroxylysine, as well as the characteristic triple stranded helical configuration of a large segment of the molecule (Grant & Prockop 1972; Miller & Matukas 1974).

Each collagen molecule consists of three strands, each designated as an α chain, coiled around one another in a superhelical fashion. The four molecular forms of collagen thus far discovered have been differentiated from each other based upon their combination of α chains and upon the different amino acid composition and sequence of the α chains in each of the tissues examined. The four types of collagen are listed in table 1.

The basement membrane collagens differ considerably from the other types of collagen in several ways. They are richer in hydroxyproline and hydroxylysine, and much of the hydroxy-

proline contains hydroxyl groups in the 3, rather than the 4 position of the proline ring, in contrast to the other types of collagen thus far identified (Kefalides 1970). In addition, basement membrane collagens contain much more carbohydrate, predominantly in the form of glucosyl-galactose dissacharides linked to hydroxylysine, in contrast to the interstitial collagens which contain either galactose of glucosylgalactose linked to hydroxylysine in much smaller, quantities (Grant & Prockop 1972).

TABLE 1

(After Miller & Matukas 1974)

chain	molecular type	tissue
$\alpha 1(I)$	$[\alpha 1(I)]_2\alpha 2$	bone, tendon, skin
α		
$\alpha 1(II)$	$[\alpha 1(II)]_3$	cartilage
$\alpha 1(III)$	$[\alpha 1(III)]_3$	foetal skin, arteries
$\alpha 1(IV)$	$[\alpha 1(IV)]_3$	basement membrane

The strand type is listed under 'chain' and the combination of chains is noted under 'molecular type'. In the case of skin, two of the strands are identical and one is different. In contrast, all three of the strands of cartilage collagen are identical and are homologous with the $\alpha 1$ strand in dermal collagen. The same is presumed to be true for arterial collagen, although thus far the evidence for the latter is based upon peptides analysed by cyanogen bromide cleavage (Miller, Epstein & Piez 1971; Trelstad 1974).

Elastic fibres

Elastic fibres contain two different proteins, each of which has strikingly different morphologic features as well as a different amino acid composition. The principal protein of the mature elastic fibre, elastin, provides the fibre with its elastomeric properties and is similar to collagen in that approximately one third of its amino acid residues are glycine. However, elastin differs from collagen in that it contains approximately 1% hydroxyproline, and no hydroxylysine, and is relatively poor in polar amino acids (Ross & Bornstein 1969). In addition this protein has two unique cross-linking amino acids, desmosine and isodesmosine (Partridge 1966) both of which are derived from four closely spaced lysine residues.

In contrast to elastin, the second protein of the elastic fibre, the microfibrillar protein, has a characteristic fibrillar morphology. In the electron microscope these fibrils appear to be 'tubular' and consist of microfibrils of approximately 11 nm diameter. This protein is rich in polar amino acids, relatively poor in neutral amino acids, contains no hydroxyproline, no hydroxylysine and no desmosines. A large number of sugar residues are present in this protein, including hexosamine, indicating that it is probably a glycoprotein (Ross & Bornstein 1969).

Glycosaminoglycans

The third principal component of the extracellular connective tissue matrix, the glycosaminoglycans, are widely distributed in many tissues, and consist principally of the chondroitin sulphates and hyaluronic acid. The different combinations of each of these glycosaminoglycans found in the various tissues undoubtedly provide them with their characteristic physical as well as chemical properties since the glycosaminoglycans provide the structural milieu in which the other fibrous proteins reside. For example, cartilage contains relatively large amounts of both chondroitin sulphate and collagen, whereas in contrast, tendons have very small amounts of chondroitin sulphate and relatively larger amounts of collagen (Grant & Prockop 1972).

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Under special circumstances arterial smooth muscle cells, to be described later in this report, synthesize small amounts of glycosaminoglycan but will form much larger amounts when appropriately stimulated. For example the lesions of atherosclerosis, which are focal proliferative lesions of smooth muscle cells located in the intima of the artery wall, are extraordinarily rich in glycosaminoglycan synthesized by these cells (Wight & Ross 1973).

It is not possible in a review of this type to cover adequately the extensive literature concerning the glycosaminoglycans (mucopolysaccharides). Most connective tissues contain varying amounts and types of these compounds which may be important in terms of feedback control, not only of connective tissue matrix synthesis, but in cell movement and proliferation as well. The presence of glycosaminoglycan, and cell surface glycoproteins, are undoubtedly important in cell–cell interactions and in reactions between the cell and its environment. The abilities of cells to receive and translate extracellular information that effect the control of intracellular protein synthesis and secretion, as well as control of RNA and DNA synthesis and cell proliferation, is a complex story that is beginning to unfold.

Collagen synthesis and secretion

In the last several years an intracellular precursor of the collagen molecule, procollagen, has been isolated which contains a rather large non-helical segment at the amino terminus of each α chain. This segment of the molecule differs from the helical portion in that it is relatively poor in hydroxyproline and proline, and contains cysteine in each of the three constituent α chains that presumably cross-links them to one another by forming interchain disulphide bonds (Bornstein *et al.* 1972; Müller, McGoodwin & Martin 1971; Bellamy & Bornstein 1971; Dehm, Jimenez, Olsen & Prockop 1972; Kerwar, Kohn, Lapiere & Weissbach 1972; Grant, Kefalides & Prockop 1972; Dehm & Prockop 1973). The cleavage of this nonhelical 'extra piece' by selective proteolysis after extracellular secretion, permits the aggregation of the collagen molecules into characteristic striated fibrils (Bornstein, Ehrlich & Wyke 1972; Layman & Ross 1973; Schofield & Prockop 1973). This extra segment on each of the procollagen molecules apparently confers solubility on them, and prevents intracellular fibril formation. These extensions of the molecule may also be important in the process of the alinement of molecules and in intracellular transport prior to secretion, all of which may be controlled by a discrete series of steps of enzymatic cleavage of different segments of the 'extra piece' (Miller & Matukas 1974; Bornstein 1974).

Until recently the intracellular modes of synthesis, transport and secretion of collagen has been a topic of controversy (Ross & Benditt 1965; Salpeter 1968; Revel & Hay 1963; Weinstock & Leblond 1974). Early studies using electron microscope autoradiography provided conflicting interpretations of the role of the Golgi complex in the secretion of collagen precursors. Studies by Ross & Benditt (1965) and by Salpeter (1968) suggested that there were two intracellular pathways for the synthesis and secretion of secretory proteins through connective tissue synthetic cells such as the fibroblast and the chondroblast. Their studies demonstrated that proteins labelled with [^{3}H]proline were localized both in the rough endoplasmic reticulum cisternae, and in the Golgi complex of these cells within 2 h after intraperitoneal administration of the isotope, and were subsequently found outside of the cells associated with collagen fibrils. Quantitative analysis of the concentration of autoradiographic grains to measure the kinetics of passage of isotope through the cells with time, suggested that two routes were

followed through the cells by labelled secretory proteins. One of these went from the rough endoplasmic reticulum to the Golgi complex vesicles and vacuoles before secretion. A second route that bypassed the Golgi complex was postulated for some of the secretory proteins, possibly collagen. In this case, the proteins that were synthesized in the rough reticulum cisternae were thought to be transported either by direct intermittant communication of the r.e.r. cisternae with the cell surface, or via vesicles that pinched off from the cisternae and migrated directly to the cell surface (Ross & Benditt 1965) (figure 1).

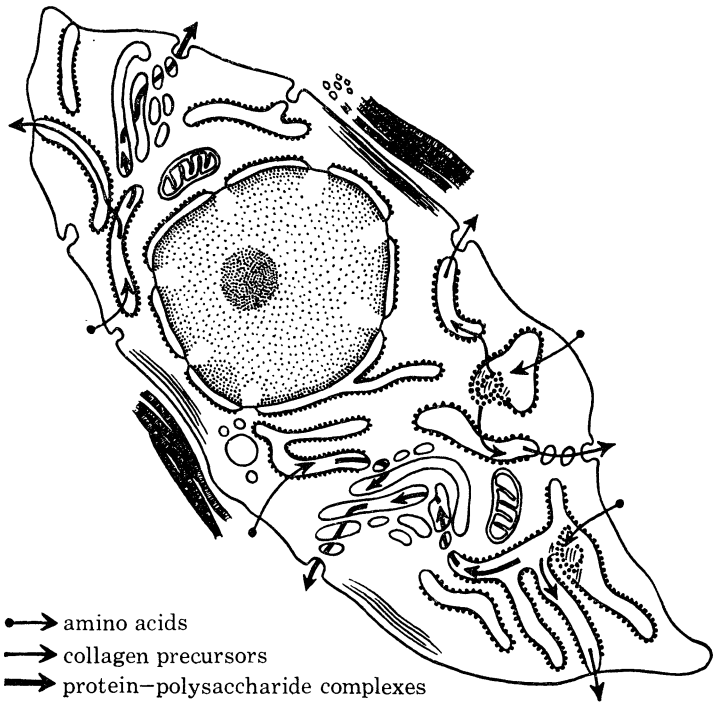

FIGURE 1. This figure represents an idealized diagram of a fibroblast. Two postulated pathways of amino acid incorporation and secretion into protein are shown by the three types of arrows. The arrow attached to the black dot represents the entrance of amino acids, presumably through the cell membrane, to the aggregates of ribosomes attached to the rough endoplasmic reticulum. There the amino acids will be incorporated into the various proteins that are sequestered in the cisternae of the rough endoplasmic reticulum. Dependent upon whether these proteins are collagen precursors, or proteins to be complexed with polysaccharide, they may follow at least two different routes through the cell. It is suggested that collagen precursors are secreted directly from the cisternae of the rough endoplasmic reticulum (small black arrow) either via direct intermittent cisternal communications with the plasma membrane or via the formation of vesicles that eventually fuse with the plasma membrane and release their material. It is proposed that both collagen and other proteins to be complexed with polysaccharide are also sequestered in the cisternae of the rough endoplasmic reticulum, and that both of these proteins separate by vesicle formation from the rough endoplasmic reticulum in regions adjacent to the saccules and vesicles of the Golgi complex. These vesicles are presumed to merge with the Golgi cisternae where they may be complexed with substances such as polysaccharide and are subsequently secreted from the cell, again by the process of vesicle formation, migration, and fusion with the plasma membrane.

In contrast, several other studies utilizing labelled amino acids suggested that all the proteins that were secreted by the cells, particularly in the case of the chondroblast and the odontoblast, passed through the Golgi complex. Evidence for the latter was originally presented by Revel & Hay (1963) and recently by Weinstock & Leblond (1974) in which labelled aggregates that appeared like collagen precursors were found in some of the large Golgi vacuoles in odontoblasts.

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The role of microtubules in the intracellular transport and secretion of collagen has been investigated by studying the effects of microtubule and metabolic inhibitors such as colchicine and vinblastine (Dehm & Prockop 1972; Diegelmann & Peterkofsky 1972; Ehrlich, Ross & Bornstein 1974). The effects of the iron chelator, α,α'-dipyridyl upon fibroblasts and osteoblasts were also investigated (Juva, Prockop, Cooper & Lash 1966). The results of studies such as these are somewhat difficult to analyse, because although the effects of these inhibitors are reasonably well documented, the other metabolic effects of these compounds are not well understood.

Using these inhibitors Ehrlich *et al.* (1974) examined the conversion of procollagen to collagen and its secretion by biochemical, light microscope autoradiography and electron microscopic analysis of embryonic rat calvaria osteoblasts and fibroblasts in culture. The secretion of procollagen, which was quantitated by light microscope autoradiography, was correlated with the extent of conversion of procollagen to collagen and with the rates of synthesis of both collagen and non-collagenous proteins by these cells.

The iron chelator, α,α'-dipyridyl, which inhibits peptidyl hydroxylation caused a marked intracellular retention of [^{3}H]proline as evidence by examination of the autoradiographs. Electron microscopic studies of these cultures demonstrated numerous regions in the osteoblasts in which the cisternae of the rough endoplasmic reticulum closely approached the plasma membrane and in one instance appeared to have fused (figure 2, plate 2). These observations may be related to a backup of material within the cisternae of the r.e.r. The Golgi complex of the dipyridyl treated cells are not changed in appearance.

Ehrlich *et al.* (1974) found that synthesis and secretion of procollagen were inhibited by colchicine, whereas colchicine did not effect the synthesis and secretion of non-collagenous proteins. Vinblastine also inhibited procollagen synthesis and secretion, but unlike colchicine, produced a generalized reduction in protein synthesis as well.

Cells exposed to colchicine contained an increased number of Golgi associated vacuoles although the appearance of the rough endoplasmic reticulum was not altered (figure 3, plate 2). In contrast, vinblastine produced a striking vacuolar change in the cisternae of the rough endoplasmic reticulum, similar to the alterations previously described in fibroblasts in ascorbic acid deficiency (Ross & Benditt 1965) (figure 4, plate 3).

Since dilation of the Golgi structures in the colchicine treated cells was associated with the intracellular retention of [^{3}H]proline labelled proteins, and with diminished synthesis and

Description of plate 2

Figure 2. This electron micrograph of an osteoblast treated with α,α'-dipyridyl demonstrates that the rough endoplasmic reticulum cisternae of this cell are unaltered in appearance. One of the more interesting features after the use of α,α'-dipyridyl was the appearance of numerous sites where the r.e.r. cisternae closely approach the cell surface and in one instance appear to have almost fused with the plasma membrane (arrows). Generally such sites are occasionally observed in untreated cells, hence the increased number in the presence of α,α'-dipyridyl is of interest. (Magn. $\times$ 11250.)

Figure 3. This electron micrograph demonstrates portions of several osteoblasts from chick calvaria grown in the presence of colchicine. The marked enlargement and prominence of the Golgi complex, as manifest by the increase in numbers of large Golgi associated vacuoles, is apparent. The rough endoplasmic reticulum in this cell is not altered, and demonstrates the usual three dimensional extensive canalicular appearance characteristic of osteoblasts. Several large Golgi vacuoles contain aggregates of filamentous structures (arrow). This appearance of increased numbers of large Golgi associated vacuoles was the most characteristic observation seen after colchicine treatment. (Magn. $\times$ 9000.)

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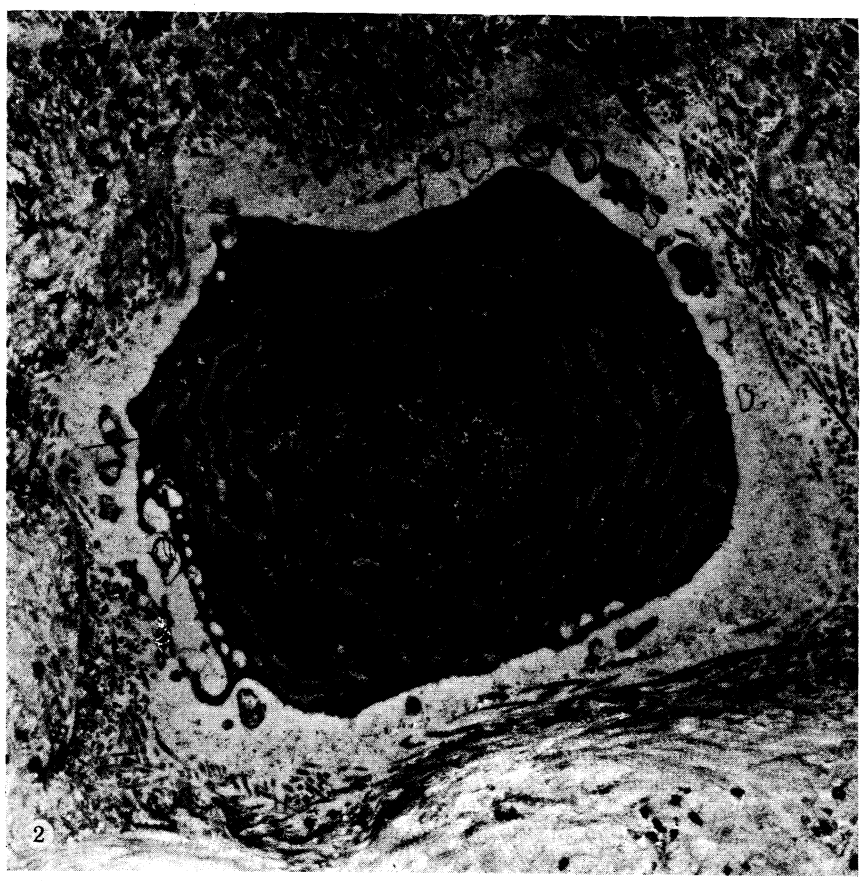

FIGURES 2 and 3. For description see opposite.

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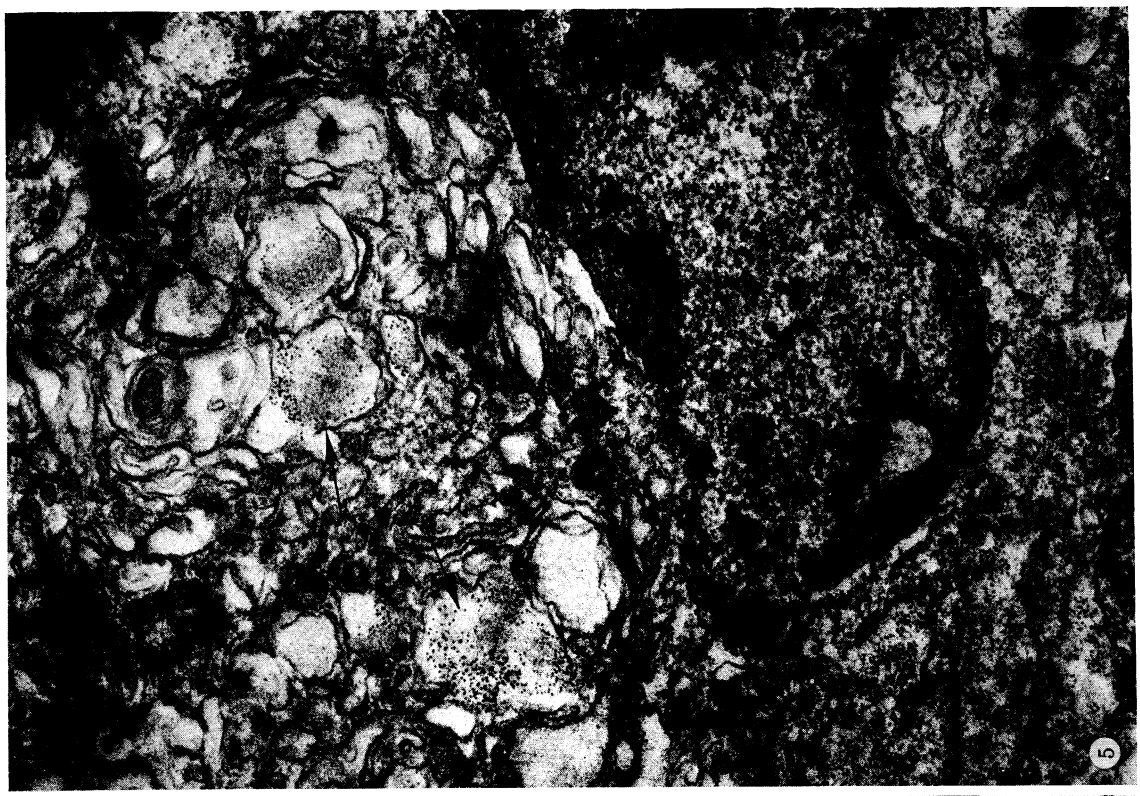

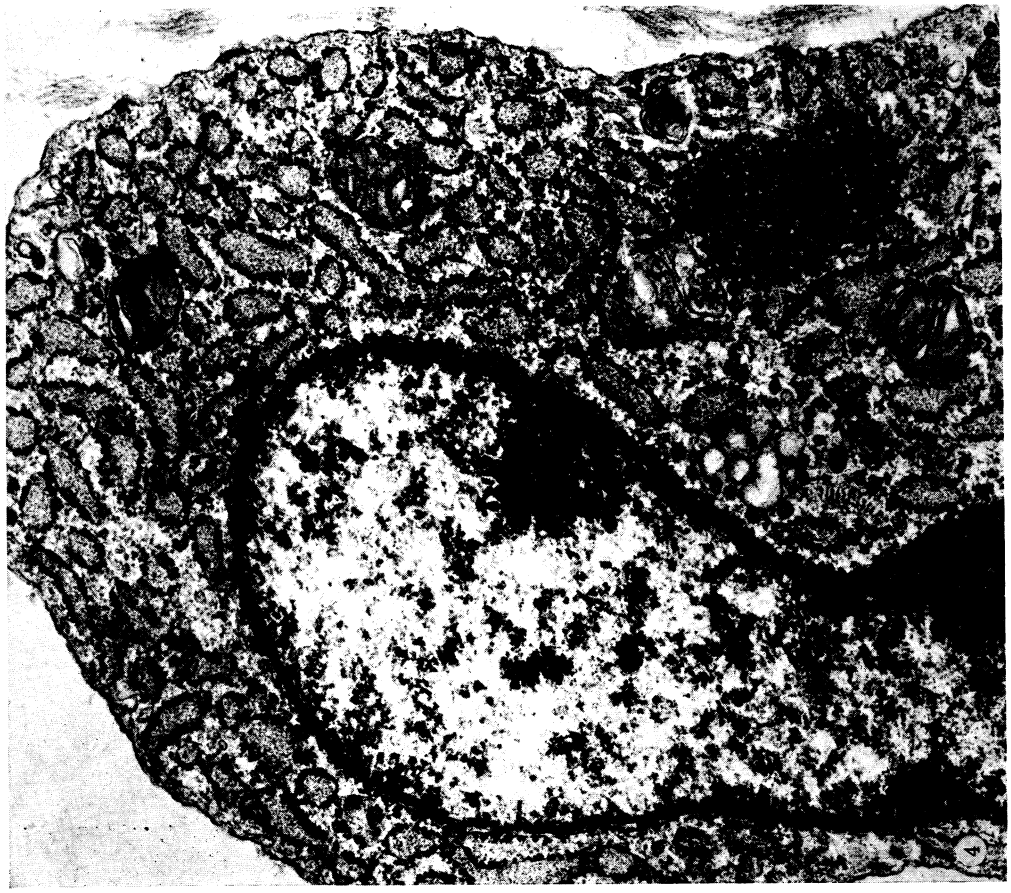

FIGURES 4 and 5. For description see opposite.

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transport of procollagen, it may be associated with an intracellular feedback inhibition of collagen synthesis. This was manifest by aggregates of filamentous structures in large Golgi associated vacuoles similar to those described by Weinstock & Leblond (1974) in odontoblasts which they suggested were aggregates of collagen molecules.

These investigations by Ehrlich *et al.* (1974), and more recent observations utilizing ferritin conjugated anti-procollagen, suggested that a considerable part of the procollagen is transported via the Golgi complex before secretion.

Nist *et al.* (1975) used anti-procollagen sera in a method developed by Olsen, Berg, Kishida & Prockop (1973) in which the localization of proline hydroxylase was studied in tendon fibroblasts. The studies of the localization of procollagen used four different ferritin conjugated anti-procollagen preparations, each of which was applied to tendon or corneal fibroblasts that had been appropriately prepared so that the antibodies could gain entrance to the different cell organelles. This was achieved by 'light fixation' of the isolated cells with formalin, followed by careful homogenization which was different and predetermined for each cell type. Dependent upon the degree of homogenization, the rough endoplasmic reticulum cisternae and elements of the Golgi complex were sufficiently disrupted so that individual organelle membranes were broken. With this method, the cells remained essentially intact leaving the organelles in the same relative positions found in the intact state. In earlier studies Olsen & Prockop (1974) also used this method to demonstrate the intracisternal localization of procollagen in the rough endoplasmic reticulum of isolated tendon fibroblasts. With this approach it is possible to demonstrate that when the membranes of the appropriate organelles are properly ruptured, ferritin-conjugated antibodies can gain entry, not only into the cisternae of the rough endoplasmic reticulum, but into all the elements of the Golgi complex as well (figure 5, plate 3) (Nist *et al.* 1975). Thus the importance of the Golgi complex in the intracellular packaging, transport, and secretion of procollagen as well as of other secretory proteins has been further confirmed by these studies.

Clearly, the role of the Golgi complex in this process is now well established. It probably plays a role in the glycosylation of hydroxylysine moieties in procollagen as well as in the addition of carbohydrates to proteins and in the sulphation of glycosaminoglycan (Miller & Matukas 1974). However, these observations do not rule out the possibility that some of the procollagen may bypass the Golgi complex, since it is not possible to apply kinetic analysis in this type of investigation.

Description of plate 3

Figure 4. This electron micrograph contains a part of a periosteal fibroblast treated with vinblastine. These cells contain numerous membranous aggregates sometimes associated with the Golgi complex and a striking alteration in the rough endoplasmic reticulum. The cisternae of the r.e.r. have a vacuolar appearance and have lost the three dimensional canalicular structure so characteristic of non-treated cells. (Magn. × 26250.)

Figure 5. This electron micrograph demonstrates part of a tendon fibroblast that had been prepared, as stated in the text (prefixation followed by controlled homogenization) and then exposed to ferritin conjugated antiprocollagen. The antibody is localized in large Golgi vacuoles (arrows) as well as in rough endoplasmic reticulum cisternae. (Magn. × 30000.)

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Elastic fibre synthesis and secretion

The demonstration that the elastic fibre contains two different structural proteins (Ross & Bornstein 1969), has led to further study of the synthesis and secretion of each of these proteins. At least three different connective tissue cells are known to synthesize elastic fibres. These are the fibroblasts of elastic ligaments such as ligamentum nuchae, fibroblasts of tendons, chondroblasts in elastic cartilage, and smooth muscle cells, principally in the walls of arteries.

The evidence for the synthesis of elastic fibres by arterial smooth muscle cells, as in other elastic tissues, has largely been the morphologic association of these cells with the elastic fibres and *in vivo* studies of the incorporation of [^{3}H]proline into both collagen and elastic fibres in the developing rat aorta by light and electron microscope autoradiography (Ross & Klebanoff 1971).

Recently, Narayanan, Sandberg, Ross & Layman (1974) have shown that arterial smooth muscle cells in culture are capable of synthesizing, not only the elastic fibre microfibrillar protein, as was earlier demonstrated by Ross (1971), but both soluble and insoluble elastin as well. In the presence of lathyrogens, such as β-aminoproprionitrile, Narayanan *et al.* were able to show that arterial smooth muscle cells in culture incorporate [^{3}H]lysine into a non-collagenous substance that acts as a lysyl oxidase substrate. This material had both electrophoretic and chromatographic properties similar to that of purified soluble elastin, a molecular mass of approximately 69 000, and was resistant to purified collagenase. When lathyrogens were omitted from the culture medium, these same cells were capable of incorporating [^{14}C]lysine into an extracellular protein that contained ^{14}C in both desmosine and isodesmosine, the cross-linking amino acids that are unique to insoluble elastin (Partridge 1966). The demonstration of the ability of these cells to form elastin in culture should now permit further studies of the organelles associated with the synthesis and secretion of this protein, and the factors that control these activities.

Factors affecting cell proliferation

Cell surface components probably control cell proliferation by their interaction with several extracellular factors including hormones (Van Haam & Cappel 1940). Some of the hormones known to be important in this phenomenon include somatomedin, insulin, and the prostaglandins together with mediators of the cyclic nucleotides, cyclic AMP and GMP. The intracellular levels of cyclic nucleotides appear to play a role in cell proliferation and the interrelationships between these and special cell surface receptors versus cytoplasmic components are not well understood.

Most studies of agents that affect cell proliferation have been performed in cell culture. Many of these investigations have focused on the role of serum factors in stimulating cell proliferation (Holley & Kiernan 1971; Clarke & Stoker 1971; Pierson & Temin 1972; Lieberman & Ove 1959). It has been known for a long time that serum contains components that will either stimulate or augment DNA synthesis and cell proliferation. However the source of these serum components and the number and type are as varied as are the experimental approaches. Holley and his colleagues (1971) have isolated serum factors in Sephadex-G 100 at pH 2 which can be separated into two factors, one of which stimulates cell proliferation versus one that stimulates cell migration in culture. These and other attempts to isolate serum active

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fractions have provided valuable information, however the source of these factors has not yet been elucidated.

Recently, Balk (1971) demonstrated that chicken serum derived from cell-free plasma lacked the proliferative effect seen in whole blood serum from the same original pool of blood. In his study, he observed that one of the factors partially responsible for this effect was the level of calcium. He suggested that cells such as thrombocytes might also be partially responsible for this effect.

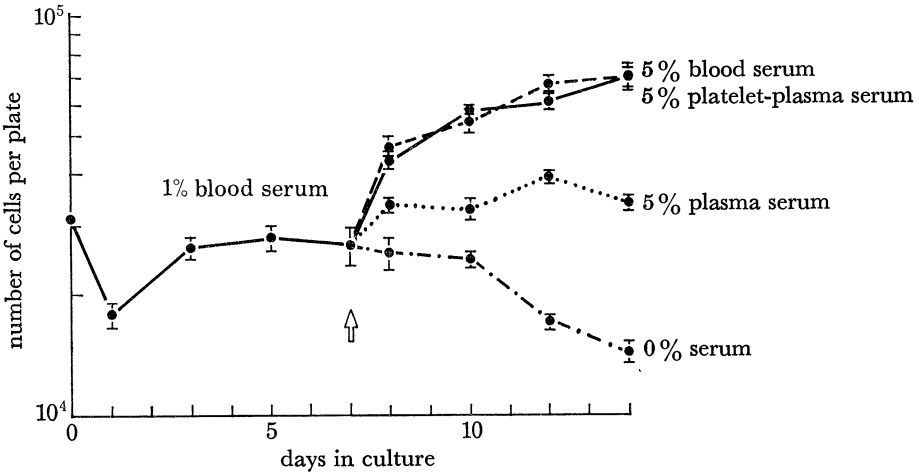

FIGURE 6. This graph shows the response of dermal fibroblasts to platelet factors present in blood serum. Equal numbers (3×10^4) of primate dermal fibroblasts were added to a large series of 35 mm petri dishes and incubated in medium containing 1% serum pooled from several *Macaca nemestrina*. After 7 days (arrow) the dishes were separated into four groups. One group was incubated in serum-free medium. The remaining groups were incubated in medium containing: 5% dialysed serum from whole blood containing 3.95×10^8 platelets per millilitre; 5% dialysed cell-free plasma serum which had been exposed, during the process of recalcification and serum formation, to an equivalent number of platelets derived from the same pool of blood; 5% dialysed cell-free plasma serum in which no platelets were present during the process of serum formation. This experiment demonstrates that 5% dialysed plasma serum has a much lower proliferative effect which can be restored by the addition of platelets at the time of recalcification of the cell-free plasma.

In a recent series of studies Ross, Glomset, Kariya & Harker (1974) have shown that one of the principal factors present in blood serum responsible for fibroblast and smooth muscle cell proliferation in culture, is, in fact, derived from thrombocytes. The experiments that demonstrated this were performed with blood derived from the pigtail monkey *Macaca nemestrina*. The effects of monkey serum factors were studied in culture on either aortic smooth muscle cells or dermal fibroblasts from the same species of primate. In these experiments, the blood was divided into several aliquots, one of which was drawn in the presence of citrate to prevent coagulation, and to permit all of the blood cells, including the platelets, to be removed from the blood by centrifugation. The plasma was then recalcified to form a platelet-poor plasma serum. The ability of this platelet-poor plasma serum to stimulate smooth muscle and fibroblast proliferation was then compared with that of the homologous whole blood serum (which stimulates maximal proliferation) and with a third aliquot of platelet-poor plasma serum that was recombined with platelets (at a number equivalent to the given volume of plasma) before serum formation by recalcification.

The results of such an experiment are shown in figure 6 and demonstrate that addition of platelets to platelet-poor plasma serum, at the time of recalcification returned the proliferative capacity that was present in whole blood serum, in contrast to platelet-poor plasma serum

(figure 6). To determine whether this platelet derived factor was released by physiological stimulation of the thrombocytes by thrombin or collagen, an aliquot of isolated pure platelets was separately exposed to purified bovine thrombin *in vitro*. The supernatant material derived from this stimulated platelet release reaction was then re-added to previously recalcified platelet-poor plasma serum. The platelet supernatant restored the proliferative activity of the plasma serum demonstrating that the proliferative factor was a result of the 'platelet release reaction'. These studies suggest that platelets play an important role in the proliferative effect produced by blood serum on cells *in vitro*. Equally important, platelets may play a role during coagulation following injury and repair, to stimulate fibroblast proliferation in healing wounds.

Platelets may also be important in the genesis of the early lesions of atherosclerosis. It is postulated that these lesions form as a result of chronic recurrent endothelial injury. Such injury could result in exposure of the subendothelial connective tissue, leading to local platelet aggregation and release of platelet factors that could combine with plasma constituents in the artery wall to stimulate the focal proliferation of arterial smooth muscle cells leading to the development of the preatherosclerotic lesion.

The results of the *in vitro* studies noted above suggest that it may be important to re-evaluate the factors present in foetal calf serum responsible for cell proliferation in most cell culture experiments. Since foetal calf serum undoubtedly contains platelet factors as well as other factors potentially responsible for cell proliferation, a re-analysis of these will be important for the better understanding of these *in vitro* phenomena.

Factors derived from other cells may be important in stimulating fibroblast proliferation as well. Experiments in which the macrophage has been suggested to be a source of mitogenic factors, has been pursued by Leibovich & Ross (unpublished observations). In a series of *in vitro* experiments with fibroblasts, and in wound healing experiments *in vivo* in which macrophage function has been impaired, they suggested that a combination of a monocytopenia and inhibition of phagocytosis, produced a delay in the fibroproliferative response (Leibovich & Ross 1975). In the *in vitro* experiments Leibovich & Ross suggested that the process of phagocytosis of inert particles such as latex, or of opsonized erythrocytes, resulted in the release into the cell culture medium of factors that stimulated fibroblast proliferation in culture. Thus, the role of the macrophage in inflammatory reactions such as wound repair may go beyond that of wound debridement, since inhibition of macrophage function *in vivo* leads to delayed fibroplasia whereas stimulation of phagocytosis appears to result in macrophage derived substances that stimulate fibroblast proliferation.

This review has attempted to relate the connective tissue cell with the matrix constituents that it forms and among which it lives. These constitute only one part of the environment in which the cell responds to external stimuli. Factors derived from circulating blood cells, particularly the thrombocyte, clearly interact with plasma components and the surface of these cells to stimulate the synthesis of RNA, DNA and protein. These factors appear to be particularly important in reactions to injury where the connective tissue is exposed to them in large quantities, thus mediating the response of the cells. Two examples of such a response to injury where platelets, plasma and connective tissue cells interact with a resultant fibroproliferative response are the healing wound and the developing lesions of atherosclerosis.

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The author would like to acknowledge collaboration in several of the studies presented in this review. Figures 2–4 were taken from work pursued jointly with Dr H. Paul Ehrlich and Dr Paul Bornstein (Ehrlich *et al.* 1974). Figure 5 was taken from work pursued with Dr Cynthia Nist, Dr Klaus von der Mark, Dr Paul Bornstein, Dr Elizabeth Hay, Dr Bjorn Olsen and Dr Peter Dehm (Nist *et al.* 1974) and figure 6 represents experiments performed with Dr John Glomset and Ms Beverly Kariya.

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Discussion

R. C. Tripathi (*Institute of Ophthalmology*, (*University of London*), *Judd Street, London WC*1 *H* 9*QS*). It is now well known that ultrastructurally elastic tissue consists of both amorphous and microfibrillar elements. Would Dr Ross kindly elaborate as to the differences in the chemical composition of these elements, and also whether the microfibrillar element has the same resilient properties as the amorphous component? In our ultrastructural studies of ocular elastic tissue in the sclera and in the trabecular meshwork of young and senile eyes (Tripathi 1969, 1974), we have observed that the microfibrillar element in the latter shows a marked periodicity ranging from 40–110 nm, and thus towards the smaller periodicity range it corresponds to the so-called small diameter collagen (Jakus 1961) and towards the larger periodicity

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range to the so-called curly collagen (Garron & Feeney 1959). Could this change possibly represent a transition of the microfibrillar element of the elastic tissue into collagen (Rodgers, Puchtler & Gropp 1967)? I would be further interested to know if Dr Ross has observed such changes in his own studies of non-ocular elastic tissue of the body.

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R. Ross. Dr Tripathi has asked about the differences in the chemical composition of two morphologic elements of the elastic fibre and about the elastomeric properties of the elastic fibre microfibrils versus the amorphous component, elastin. These two structural elements are clearly two different proteins. The microfibrils are a glycoprotein that is rich in polar amino acids, relatively poor in non-polar amino acids, contains no hydroxyproline, no hydroxylysine, no desmosines and is relatively rich in cystine (approximately 50 residues per 1000). The large number of cystine residues undoubtedly accounts for the relatively insoluble state of the polymerized microfibrillar protein. Thus the microfibrils cannot possibly be related to either collagen or elastin since they lack the proper amounts and/or types of specific amino acids which characterize them: namely, 30% glycine, and approximately 12% each of hydroxyproline and proline and hydroxylysine. There is no conceivable way in which the microfibrillar protein could represent a precursor of either collagen or elastin because of its strikingly different amino acid composition. It thus must represent a completely separate and distinct connective tissue glycoprotein.

We do have observations to suggest that there may be ionic interactions between the microfibrillar protein and soluble elastin that permits the microfibrillar proteins which form first closely adjacent to the extracellular surface, to act as a scaffolding between and around which the soluble elastin can be attracted and subsequently crosslinked (during desmosine formation) to form an insoluble complex that becomes morphologically related to the microfibrils. Such a reaction would permit the two proteins to form the elastic fibre, that has now been recognized to contain both of these components.

We agree with Dr Tripathi in his observations that the microfibrils are often beaded and tend to show a periodicity. We do not yet understand the significance of this periodicity nor the chemical basis for its appearance. We are in the process of isolating large amounts of this glycoprotein so that we can better understand its properties, its amino acid sequence and the reasons for its aggregation into fibrils.

The temptation to interpret the changes in dimensions and appearance of fibrillar aggregates in electron micrographs taken of tissues at different times during development as representing a sequence of events is very great. I would urge that caution be exercised in attempting this type of analysis, since it is virtually impossible to determine whether fibrils seen at one time period during development are related in any way at all to fibrils seen at another time.

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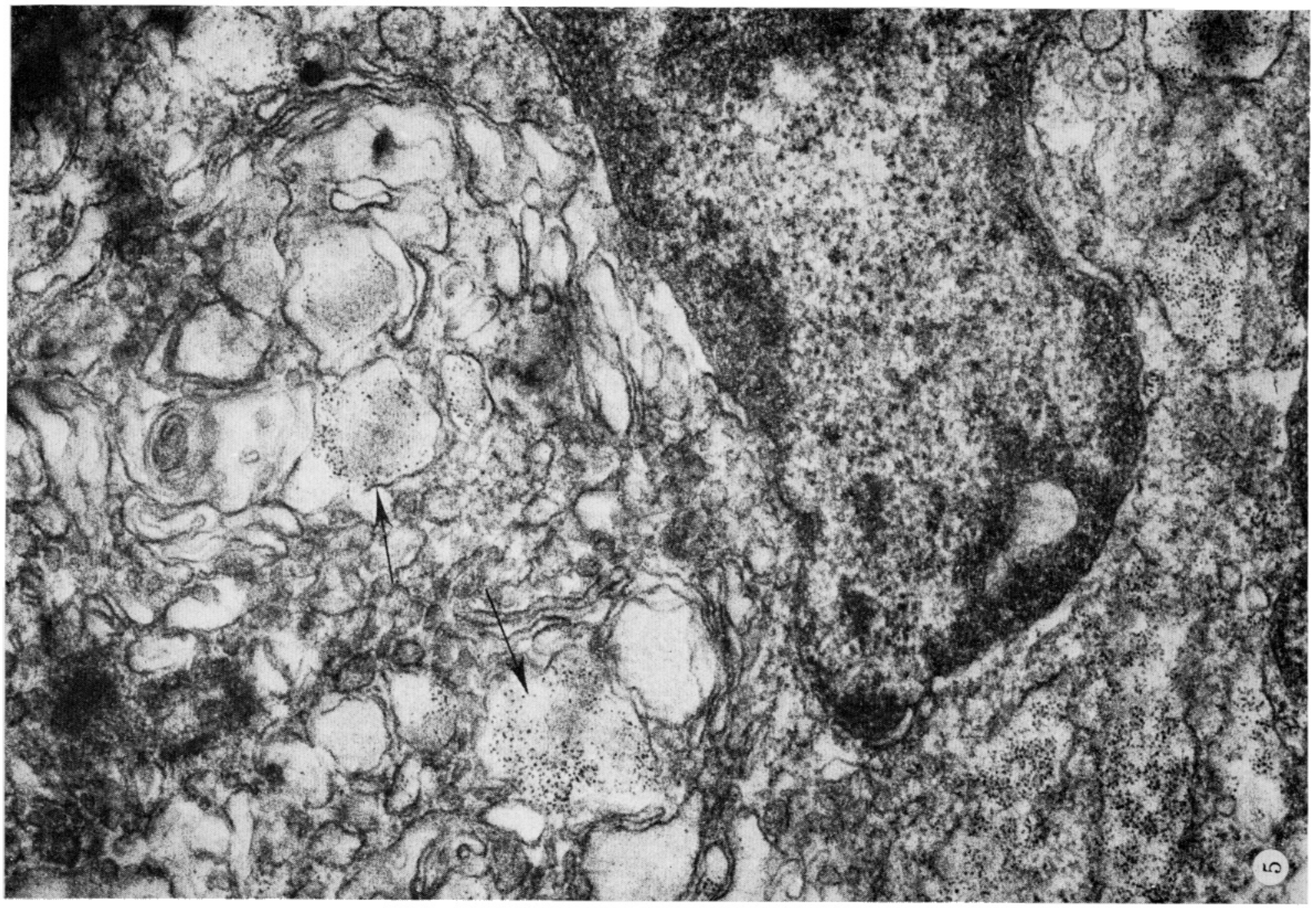
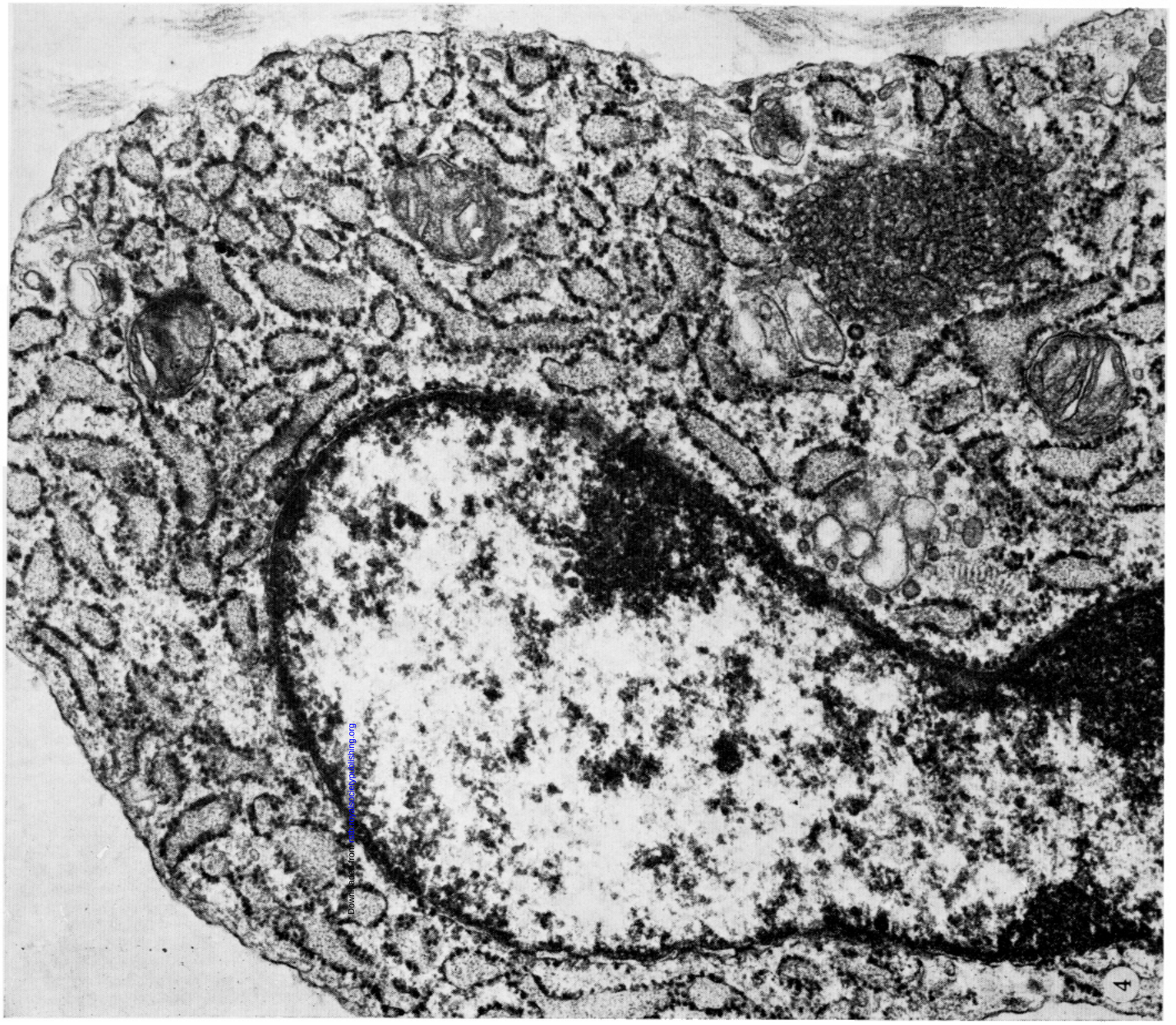
FIGURES 2 and 3. For description see opposite.





FIGURES 4 and 5. For description see opposite.