Immunocytochemical Localization of Procollagen and Fibronectin in Human Fibroblasts: Effects of the Monovalent Ionophore, Monensin

PHILIP W. LEDGER, NAOYUKI UCHIDA, and MARVIN L. TANZER

Department of Biochemistry, University of Connecticut Health Center, Farmington, Connecticut 06032

ABSTRACT   The monovalent ionophore monensin inhibits the secretion of both procollagen and fibronectin from human fibroblasts in culture. The distribution of these proteins in control and inhibited (5×10^{-7} M monensin) cells has been studied by immunofluorescence micros-copy. In control cells, both antigens are present throughout the cytoplasm and in specific deposits in a region adjacent to the nucleus, which we identify as a Golgi zone by electron microscopy. Treatment of cells with monensin causes intracellular accumulation of procollagen and fibronectin, initially in the juxta-nuclear region and also subsequently in peripheral regions. Electron microscope studies reveal that in such cells the juxta-nuclear Golgi zone becomes filled with a new population of smooth-membraned vacuoles and that normal Golgi complexes are not found. Immunocytochemically detected procollagen and fibronectin are localized in the region of these vacuoles, whereas more peripheral deposits correspond to the dilated cisternae of rough endoplasmic reticulum, which are also caused by monensin. Procollagen and fibronectin are often codistributed in these peripheral deposits. Accumulation of exportable proteins in Golgi-related vacuoles is consistent with previous analyses of the monensin effect. The subsequent development of dilated rough endoplasmic reticulum also containing accu-mulated proteins may indicate that there is an additional blockade at the exit from the endoplasmic reticulum, or that the synthesized proteins exceed the capacity of the Golgi compartment and that their accumulation extends into the endoplasmic reticulum.

Experimental manipulation of the synthesis and secretion of procollagen has been achieved by the use of agents, such as inhibitors of proline hydroxylation (12), local anesthetics (9) and, for fibronectin as well, an inhibitor of protein glycosyla-tion (18). Microtubule-disrupting drugs, such as colchicine, also inhibit secretion, apparently by impairing the transport of secretory vesicles to the cell surface (8). The use of such agents is accompanied by dramatic changes in cell morphology (7) and, in some systems, the inhibition of procollagen synthesis (8). We have demonstrated (39) that the monovalent ionophore monensin reduces the rate of release of procollagen and fibro-nectin from human fibroblasts without seriously disturbing the synthesis of these macromolecules. Moreover, secretion returns to normal upon removal of monensin. Subsequent studies with pulse labeling in conjunction with subcellular fractionations have shown that the passage of labeled procollagen and fibro-nectin through the cell is hindered at specific locations (38). Also, our studies of the effect of monensin on chondrocytes

have demonstrated the disturbance of Golgi-related sulfation (34).

The simultaneous inhibition of secretion of both procollagen and fibronectin in fibroblasts evokes further interest because of the known affinity of these molecules in vitro (10) and their association together in the extracellular matrix (40). Thus, the present investigation further characterizes the action of monen-sin on fibroblasts describing ultrastructural changes, the distri-bution of procollagen and fibronectin as determined by im-munofluorescence microscopy, and considers the data in terms of the secretion of these proteins and the inhibition of their secretion by monensin.

MATERIALS AND METHODS

Cell Culture

Human skin fibroblasts (strain CRL 1220, American Type Culture Collection, Rockville, Md.) were maintained in Dulbecco's modified Eagle's medium with

10% fetal calf serum (K.C. Biological, Inc., Lenexa, Kans.) and 50 μg/ml ascorbate. For immunofluorescence staining, cells were grown on glass cover slips and were not used before the third day after passage. For experimental incubations, cover slips bearing cells were rinsed three times in serum-free, ascorbate-supplemented medium and then incubated for 1 h or 7 h in that medium plus or minus 5 × 10⁻⁷ M monensin (provided by Dr. R. Hamill, Eli Lilly Co., Indianapolis, Ind.). In some experiments, cells were incubated in serum-free medium containing no ascorbate.

Antisera

Type I procollagen prepared from spent human fibroblast culture medium (6, 18) was used to raise antisera in New Zealand white rabbits following an established immunization schedule (25).

Plasma fibronectin (cold-insoluble globulin) was isolated from human plasma on a gelatin affinity column (10) using elution with 1.0 M sodium bromide in 0.02 M acetic acid. Preparations invariably contained a small proportion (<5%) of proteinaceous contaminants, so pure antigen was obtained by slicing the fibronectin band from preparative scale sodium dodecyl sulphate-polyacrylamide gels. These bands, which contained ~250 μg of fibronectin, were visualized on unstained gels (16), excised, triturated in calcium and magnesium-free Dulbecco's phosphate-buffered saline (PBS), and used to immunize Hartley guinea pigs. Also, rabbit antifibronectin was raised in New Zealand white rabbits. Some rabbit antifibronectin antisera, used in early experiments, were a gift of Dr. Deane Mosher, University of Wisconsin.

Specificity of antisera was monitored by immunoprecipitation. Logarithmically growing fibroblasts were labeled with L-[4,5-³H]leucine (20 μCi/ml) for 6 h. Cell layers were lysed in phosphate buffer at pH 11.0 containing 0.5% Nonidet P-40 (Particle Data Labs Ltd., Elmhurst, Ill.), 10 mM ethylenediamine tetraacetic acid, 1 mM phenylmethyl sulfonyl fluoride, and 5 mM N-ethyl maleimide. At this pH, solution of fibronectin is maximized (23). Homogenates were centrifuged, the supernates brought to pH 7.5, and 200-μl portions (containing labeled material from ~10⁴ cells) were immunoprecipitated with 10 μl of antiprocollagen or antifibronectin antisera following established double-antibody methods (28). Immunoprecipitates were run on 5-10% polyacrylamide gels in the presence of sodium dodecyl sulfate and processed for fluorography (3, 19).

Immunofluorescence Staining

After experimental incubation, cover slips bearing cells were rinsed in PBS and fixed in 2% paraformaldehyde in PBS (30 min at 21°C). After several rinses in PBS, cells were permeabilized by 30 min incubation in 0.05% Nonidet P-40 in PBS (20) and again rinsed in PBS. For exposure to antisera, 120 μl of antiserum diluted in PBS was placed on each cover slip, which was kept in a humid atmosphere. To effect double immunostaining, the sequence of incubations was the following: guinea pig antifibronectin, fluorescein-conjugated goat anti-guinea pig IgG (Cappell Laboratories, Inc., Cochranville, Penn.), rabbit antiprocollagen, and rhodamine conjugated goat anti-rabbit IgG (Cappel Laboratories, Inc.). Cover glasses were rinsed in PBS several times between antisera, and each incubation was for 30 min at 21°C. After preliminary titrations of primary and secondary antisera, optimal specific staining was obtained by using dilutions of 1:100, except for the rhodamine-labeled goat anti-rabbit IgG which was diluted 1:200. Controls included using the different primary antisera separately (with either their homologous or heterologous secondary antisera), nonimmune sera, and secondary (labeled) antisera alone. In the dilutions and sequence used, cross-reactions between different antisera did not occur. Stained cover glasses were mounted in glycerol-PBS (1:1 vol/vol), examined with a Zeiss RA microscope, and photographed on Kodak Tri-X film. Optimal exposure times were 30 s for both fluorescein and rhodamine when film was developed in Kodak Microdol X.

Electron Microscopy

For electron microscopy, cells were grown in 60-mm plastic petri dishes and, after experimental incubation as described above, were fixed in situ by a simulated glutaraldehyde/osmium tetroxide procedure modified from Hirsch and Fedorko (17). Plates were brought to room temperature, the medium poured off, and 1.0% glutaraldehyde in 0.1 M sodium cacodylate/HCl at pH 7.35 was added. Plates were then placed on ice and allowed to cool for 5 min, when prefixative was removed and the complete fixative was added. This was prepared by mixing ice-cold stock solutions to give a mixture containing 1.0% glutaraldehyde and 1.0% osmium tetroxide in 0.1 M cacodylate buffer. Fixation was for 1 h at 4°C. After this, plates were rinsed several times in buffer, stained en bloc with 1% aqueous uranyl acetate, rinsed, dehydrated in a graded series of ethanol, and embedded in araldite. Suitable areas were sawn out of the blocks and sectioned at a slight angle to the plane of growth of the cells, so as to include representative views of as many cells as possible. Thin sections were stained with lead citrate.

RESULTS

Specificity of Antisera

In immunoprecipitates of labeled cell homogenates (Fig. 1), fibronectin or procollagen were specifically precipitated by their respective antisera. As described by Yamada (44), some nonspecific radioactivity present in both immune and nonimmune immunoprecipitates could be attributed to labeled material carried with the dye front, at the front of the immunoglobulin light chain or, in some preparations, to myosin. The absence of cross-reaction between procollagen, fibronectin, and their heterologous antisera as well as the identical immunostaining obtained with antisera from different animals and different sources all support the validity of our observations.

Cell Morphology and Ultrastructure

The cells used in this study present a typical fibroblastic morphology. They contain a variety of dense or refractile cytoplasmic bodies which are often excluded from a specific region adjacent to the nucleus (Fig. 2 a). Variable in extent and containing an accumulation of Golgi complexes and other smooth-membraned structures (Figs. 3 a and 4 a), this region corresponds to the "Golgi zone" described by Goldberg and Green (15) in mouse fibroblasts. The Golgi complexes typically consist of several stacked cisternae, some with peripheral dilations (Fig. 4 a). The latter, plus separate, irregular vacuoles, contain presumed secretory products. Other cytoplasmic organelles, such as secondary lysosomes, mitochondria, and much of the endoplasmic reticulum, tend to be localized in more peripheral regions of the cells.

Treatment with monensin results in the formation of characteristic groups of refractile vacuoles which vary in extent between cells, but which always occur in the juxta-nuclear cytoplasm (Fig. 2 b). In electron micrographs (Figs. 3 b and 4 b), these vacuoles are seen predominantly in the Golgi zone.
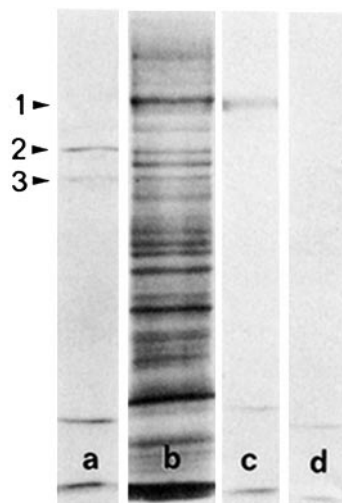

FIGURE 1 Immunoprecipitation of cell homogenates. Homogenates of cell layers labeled with [³H]leucine, as described in Materials and Methods, were incubated with rabbit or guinea pig antisera and precipitated with goat anti-rabbit IgG or goat anti-guinea pig IgG antisera, respectively. (a) Immunoprecipitate with rabbit antiprocollagen. (b) Cell homogenate. (c) Immunoprecipitate with guinea pig antifibronectin. (d) Immunoprecipitate using nonimmune rabbit serum. Arrows indicate the migration positions of (1) fibronectin, (2) procollagen α1, and (3) procollagen α2 (see Results).
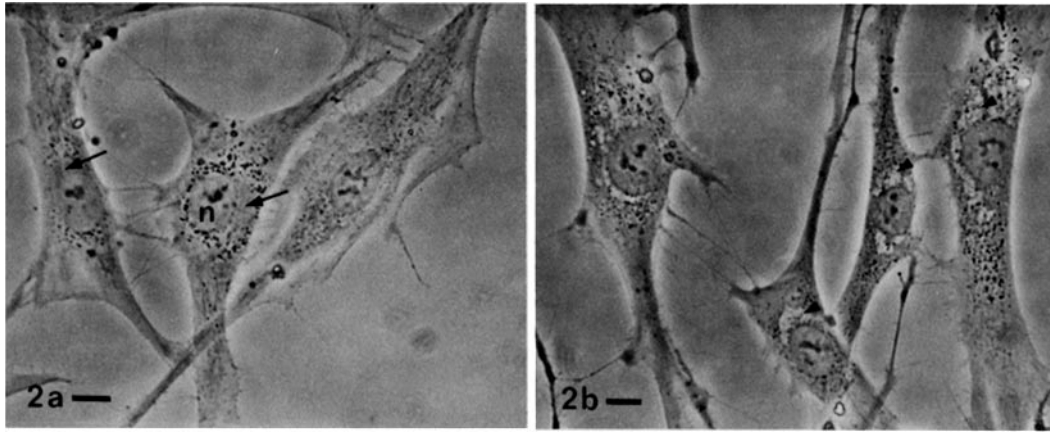
FIGURE 2 Phase microscopy of control and monensin-treated cells. Control cells (a) contain numerous inclusions around the nucleus (n). These are excluded from a specific Golgi zone (arrows). After 7 h of exposure to monensin (b), characteristic groups of refractile vesicles are formed (arrow heads). Bars, 10 µm. × 500.

Their faintly staining content is often condensed against the vacuolar membrane. Normal Golgi complexes are never encountered, but aberrant forms consisting of a few apposed, distended cisternae are seen, often closely applied to the nucleus (Fig. 4b).

Another result of monensin treatment is the appearance of distended cisternae of the rough endoplasmic reticulum, which contain granular, moderately electron-dense material (Fig. 3b). These cisternae occur predominantly toward the periphery of the cells. Monensin-treated cells also exhibit an increase in the electron density of the mitochondrial matrix.

Localization of Procollagen and Fibronectin

In control cells, immunofluorescent staining of intracellular procollagen reveals two distinct localizations (Fig. 5a). First, there is a general, reticular staining of relatively low intensity that becomes more sparse towards the edge of the cells. Second, adjacent to and spreading outwards from the nucleus is a more intensely staining network which, at higher magnifications (Fig. 5c), is seen to consist of discrete granules. Considerable variability in the extent of these two classes of deposits is usually encountered in different cells of a preparation. When examined at moderate magnifications (Fig. 5b), the staining pattern of cytoplasmic fibronectin only grossly resembles that of procollagen, particularly as the specific juxta-nuclear deposits are less easily differentiated from other cytoplasmic staining. However, at higher magnifications (Fig. 5c and d), groups of granules in the juxta-nuclear deposits can be discerned that stain for both procollagen and fibronectin. Staining of the reticular elements of the remaining intracellular deposits is not distinct enough to either support or negate codistribution.

Strands and sheets of extracellular fibronectin (identified as such by the omission of Nonidet P-40 permeabilization or by exposure of cells to trypsin before fixation [40]) can be seen on or between all cells (Fig. 5b and d). Procollagen contributes less to the pericellular matrix of cells at low cell density (Fig. 5a and c) and is generally less abundant than fibronectin until cells are confluent. Where extracellular procollagen is present, it codistributes with fibronectin; fibronectin, however, can occur without associated procollagen (Fig. 5c and d).

Our initial interest was in the intracellular localization of procollagen and fibronectin after 7 h of incubation in monensin, to complement our biochemical investigations (38, 39). At this time, the most striking change in procollagen localization

is the massive development of juxta-nuclear deposits, which increase in extent (Fig. 6a) and present a distinctly granular appearance (Fig. 6c). More peripheral cytoplasmic staining shows no increase in intensity, but its reticular appearance changes to one of more granular deposits. Intracellular fibronectin also accumulates in the juxta-nuclear region (Fig. 6b) and again discrete granules can be distinguished (Fig. 6d). As in control cells, groups of these granules stain for both antigens. The peripheral deposits stain far more strongly for fibronectin (Fig. 6b) than for procollagen, although both antigens apparently occur together in some of the deposits (Fig. 6c and d).

Cells were also examined after only 1 h of incubation in monensin. In this case, juxta-nuclear accumulations were already present (Fig. 6e) and again stained most strongly for procollagen. However, more peripheral cytoplasm showed only diffuse staining for procollagen and fibronectin, there being no indication of the granular deposits seen after 7 h.

To further check our ability to detect differences in immunofluorescent staining patterns, we compared ascorbate-deficient cells with control cells, both cultured in the absence of monensin. Confirming the results of Gay et al. (13), we observe that cells grown in ascorbate-free medium give a stronger reaction for procollagen (Fig. 7a). Specifically, the cytoplasmic staining becomes more extensive, more intense, and is more coarsely reticular.

DISCUSSION

Specificity of Antisera and the Staining Technique

Immunoprecipitation of labeled cell protein with our antisera indicates a complete absence of nonspecific binding (Fig. 1). In particular, there is no cross-reaction between procollagen, fibronectin, and their heterologous antisera. The identical immunostaining obtained with antisera from different animals and different sources further supports the validity of our observations.

Cell Ultrastructure and the Distribution of Procollagen and Fibronectin

Under control conditions, CRL 1220 fibroblasts are known to synthesize and secrete both procollagen and fibronectin (18, 39). Intracellular procollagen has been immunocytochemically
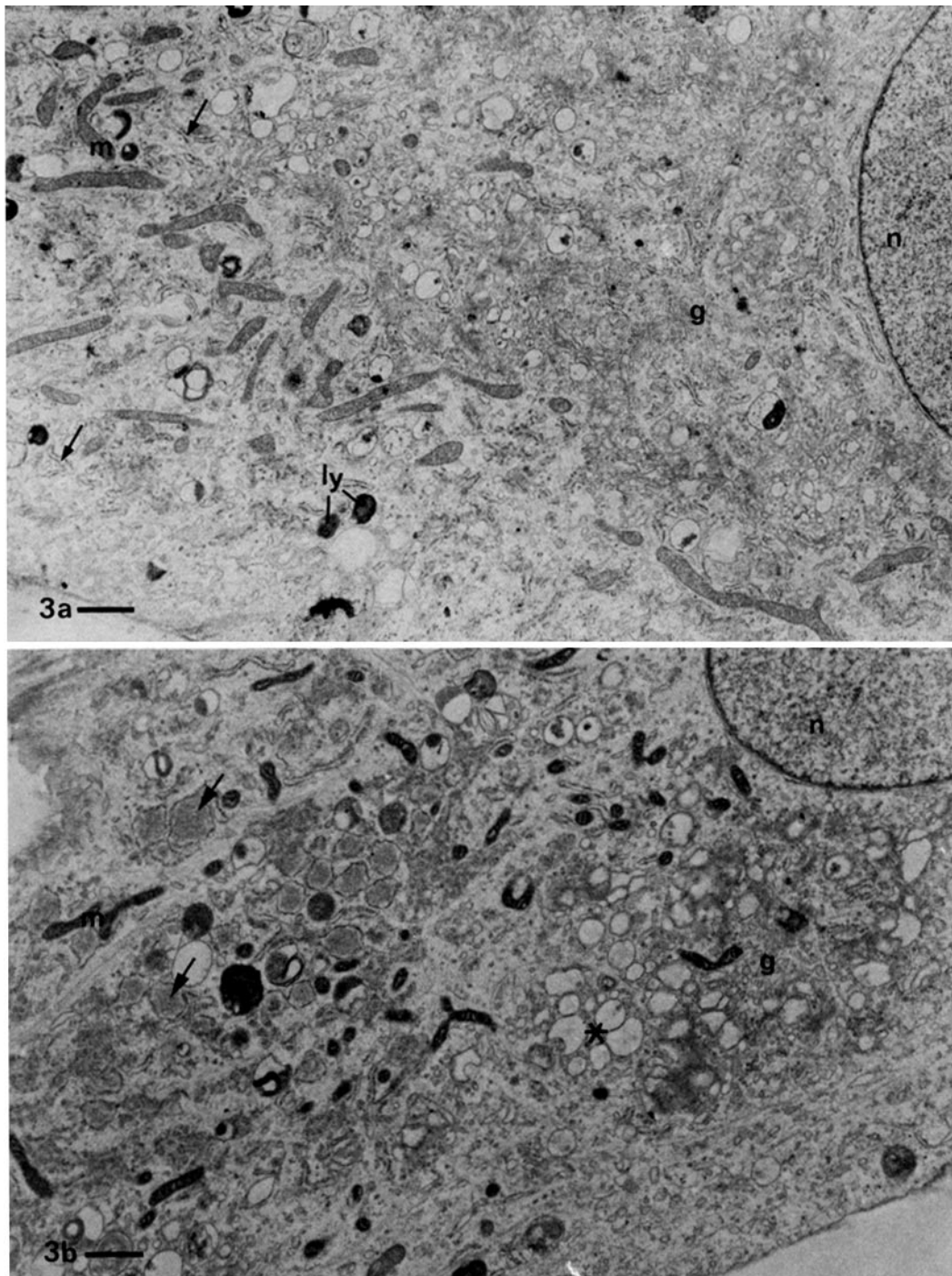
FIGURE 3 Fibroblast ultrastructure. In control cells (a), an accumulation of smooth-membraned structures adjacent to the nucleus (n) forms a distinct Golgi zone (g). Most secondary lysosomes (ly) and mitochondria (m) are found more peripherally, as is much of the endoplasmic reticulum, which is largely present as flattened cisternae (arrows). After 7 h of monensin treatment (b), the Golgi region (g) contains groups of smooth-membraned vacuoles (*), and much of the endoplasmic reticulum is present as expanded cisternae (large-headed arrows). Mitochondria (m) have a densely staining matrix. Bars, 1 μm. X 8,100.

detected in fibroblasts (40), and its augmentation after ascorbate deprivation noted (13). Procollagen is synthesized in the rough endoplasmic reticulum (26) and its presence there has been demonstrated by electron microscope immunostaining (21), so it is probably this organelle that is being stained in the general cytoplasmic deposits (i.e., other than the more distinct juxta-nuclear material). Such a location is consistent with the increased staining after ascorbate deprivation, which is known

to cause dilation of endoplasmic reticulum (29). As fibronectin is present in the same cytoplasmic areas, it also is probably localized in the endoplasmic reticulum, as Laurila et al. (20) considered.

At the stage of growth used in these investigations, CRL 1220 fibroblasts are found to contain a distinct, well-developed Golgi zone similar to that described in mouse fibroblasts subsequent to their log-growth phase (15). This zone contains
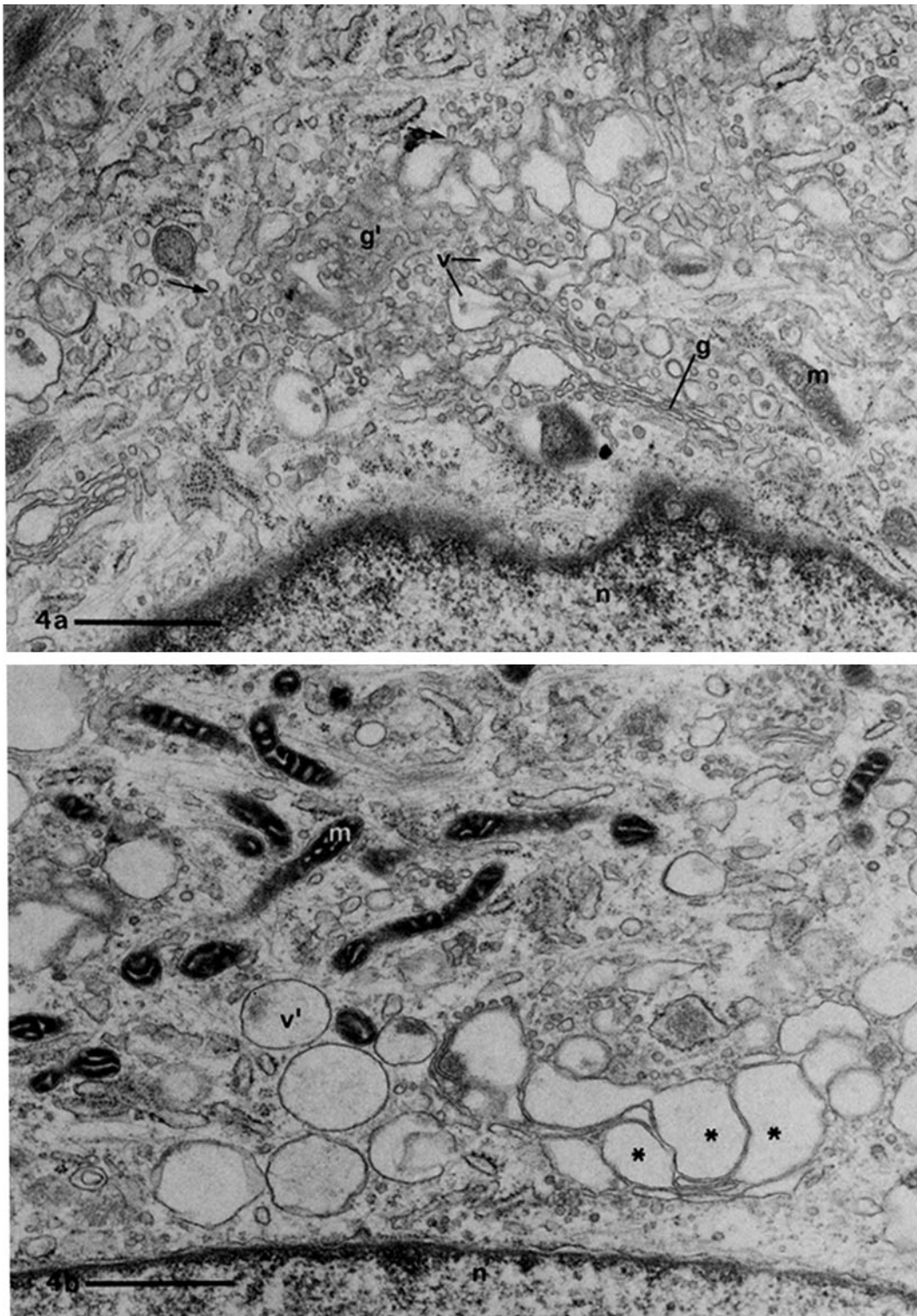
FIGURE 4   Detail of the Golgi region. In control cells (a), each Golgi apparatus (g) typically consists of several flattened cisternae with peripheral dilations. Where sectioned parallel to the plane of the cisternae (g'), a fenestrated construction is apparent. Numerous small vesicles are present, some attached to Golgi elements (arrows). Vacuoles (v) contain fibrillar bundles. After 7 h of monensin treatment (b), numerous large vacuoles are present, most containing diffusely staining material which is often condensed against the vacuolar membrane (v'). Only aberrant Golgi complexes are seen, consisting of groups of large, closely apposed vacuoles (*). Mitochondria (m) are electron dense. n, Nucleus. Bars, 1 μm. × 22,000.

several discrete Golgi complexes, and other smooth membraned structures. Some irregular vesicles and peripheral Golgi elements contain bundles of filaments which could represent parallel arrays of procollagen molecules, similar to those implicated in the secretion of procollagen in cultured fibroblasts, and other cell types in vivo (5, 37, 42). The immunofluorescently stained juxta-nuclear deposits in control cells can be related to the Golgi zone. Their reticular appearance is strik-
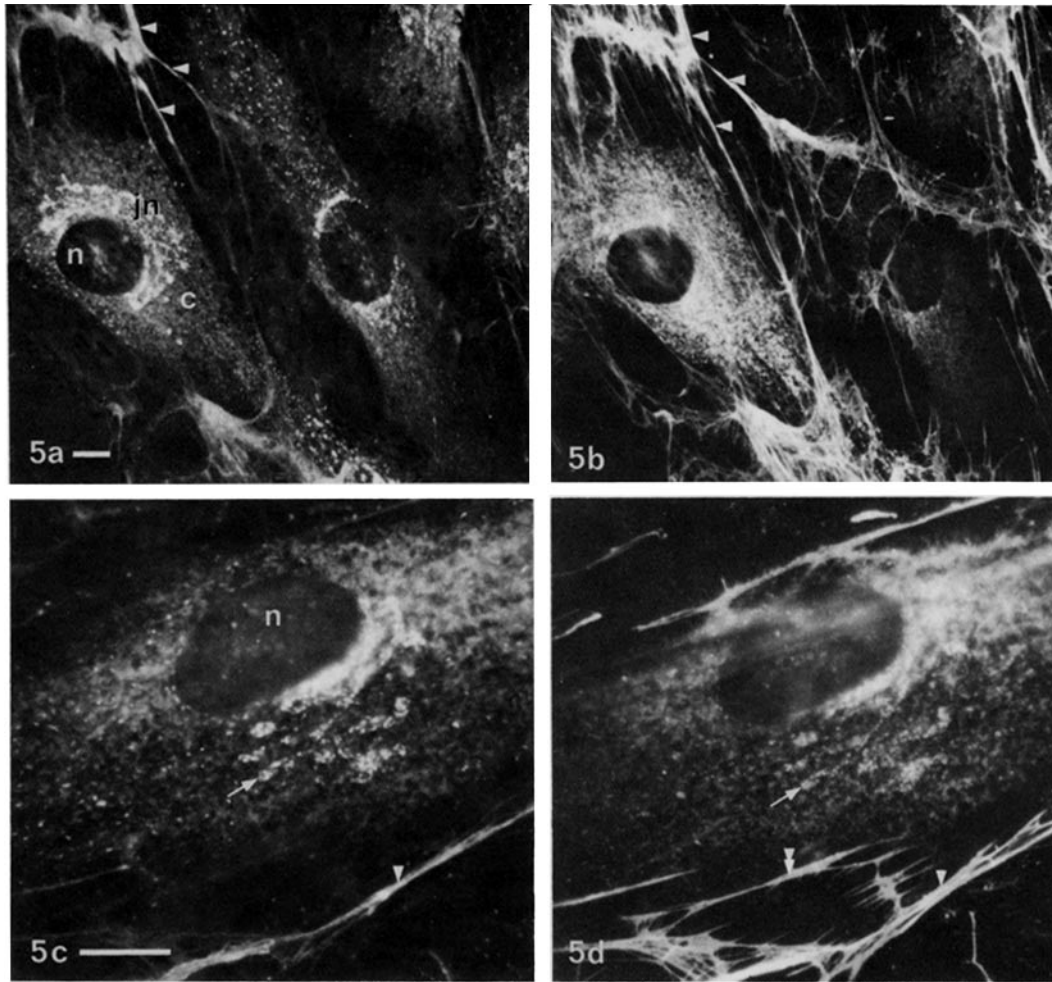
FIGURE 5   Simultaneous immunofluorescent localization of procollagen and fibronectin in control cells. Procollagen (*a*) is revealed in diffuse cytoplasmic deposits (*c*) and more intensely staining juxta-nuclear (*jn*) material and extracellular fibrils. Nuclei (*n*) are unstained, but extracellular material above or below the cell can be seen through them. The same cells also stain for fibronectin (*b*). The extent and intensity of staining for both antigens varies between cells. At higher magnifications, cytoplasmic deposits of procollagen (*c*) and fibronectin (*d*) appear reticular and the juxta-nuclear material, granular. Groups of granules stain for both procollagen and fibronectin (e.g., arrows, *c* and *d*). Extracellular material may stain for both antigens (single arrow heads in both sets of figures) or fibronectin alone (double arrow heads in *d*). Bars, 10 μm. *a* and *b*, × 500; *c* and *d*, × 1,200.

ingly similar to the juxta-nuclear network visualized by staining fibroblasts with certain fluorescently labeled lectins (41), this also being attributed to the Golgi apparatus. Size estimates of the individual immunofluorescently labeled granules can only be approximate, but at <0.5 μm and being distinctly punctate, they probably represent the secretory vesicles mentioned above or their structural precursors, the peripheral dilations of Golgi cisternae. Groups of these granules clearly contain both procollagen and fibronectin, but absolute codistribution within discrete vesicles cannot be claimed with certainty because of the limited resolution of fluorescence microscopy.

The distribution of cell surface and intercellular fibronectin described here is consistent with published accounts (33, 44), as is the general codistribution of extracellular fibronectin and procollagen (4, 40).

Ultrastructure and Antigen Distribution after Monensin Treatment

The most notable effects of monensin on fibroblast ultrastructure, namely the formation of numerous vesicles and

aberrant Golgi complexes in the Golgi zone, agree with the observations of Tartakoff and Vassalli (36). Similar effects have been noted in other cell types (30, 31, 35, 36) and/or using other ionophores (27, 32). Tartakoff and Vassalli (36) did, however, describe the normal appearance of the rough endoplasmic reticulum in several cell types after their exposure to 10^{-6} M monensin for 1 h. Our present observation of dilated cisternae presumably can be related to the longer incubation we used (7 h) albeit at a lower concentration of monensin (5 × 10^{-7} M).

After monensin treatment, the distribution of procollagen and fibronectin is modified. As expected from the biochemical data (39), there is a net accumulation of both antigens. First, there is a dramatic increase in the juxta-nuclear deposits. Because the predominant morphological change associated with monensin treatment is the appearance of numerous vesicles in the Golgi zone, we propose that these vesicles contain the procollagen and fibronectin found to accumulate in that region; no other organelles are so prominent and so distinctly located as to be candidates for this role. Even though these vesicles appear to contain little material, as measured by elec-
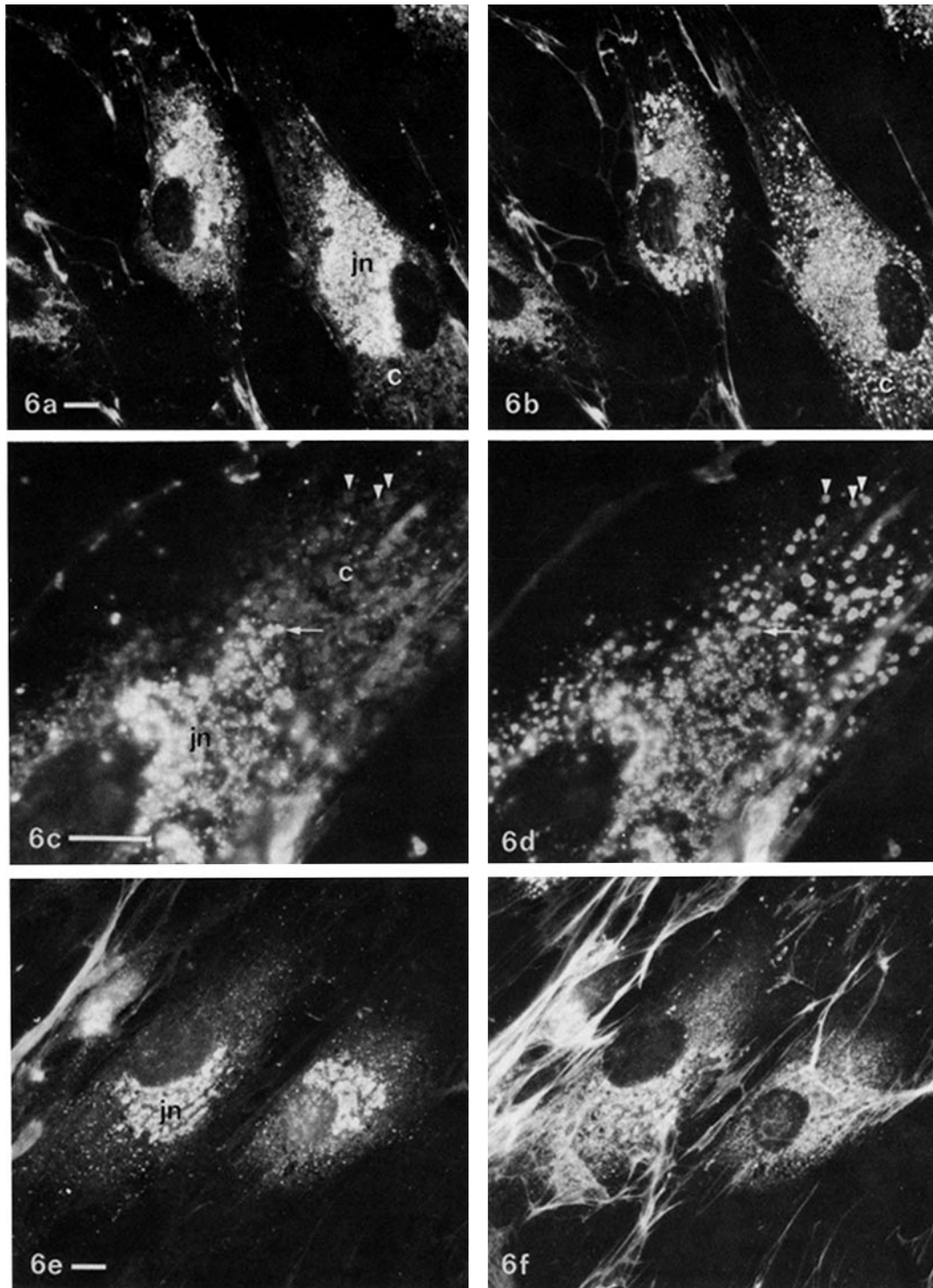
FIGURE 6 Simultaneous immunofluorescent localization of procollagen and fibronectin and monensin-treated cells. After 7 h of exposure to 5 × 10⁻⁷ M monensin, large accumulations of procollagen (a) and fibronectin (b) are seen in the juxta-nuclear region (jn). Other cytoplasmic deposits (c) are more granular and stain particularly heavily for fibronectin (b). At higher magnifications individual cytoplasmic deposits (c) can be discerned that stain for both procollagen and fibronectin (compare the deposits indicated by arrow heads in c and d, respectively). Groups of juxta-nuclear granules (arrows) also stain for both antigens. After only 1 h of incubation in monensin, juxta-nuclear deposits of procollagen are already evident (e), but peripheral cytoplasm does not exhibit the large deposits present at 7 h. f is stained for fibronectin. Bars, 10 μm. a, b, e and f, × 500; c and d, × 1,200.

tron density, the condensed appearance of that which they do contain and its adherance to the vesicle membrane (Fig. 3 b) has been noted in other ionophore-treated cells (32, 35).

The other major site of accumulation is in the peripheral deposits, which stain for both antigens, but most intensely for

fibronectin. These deposits develop only after extended exposure to monensin and can be related to the similarly located dilated cisternae of the rough endoplasmic reticulum. However, our present data cannot exclude the possibility that some of the accumulated procollagen is diverted into degradative or-
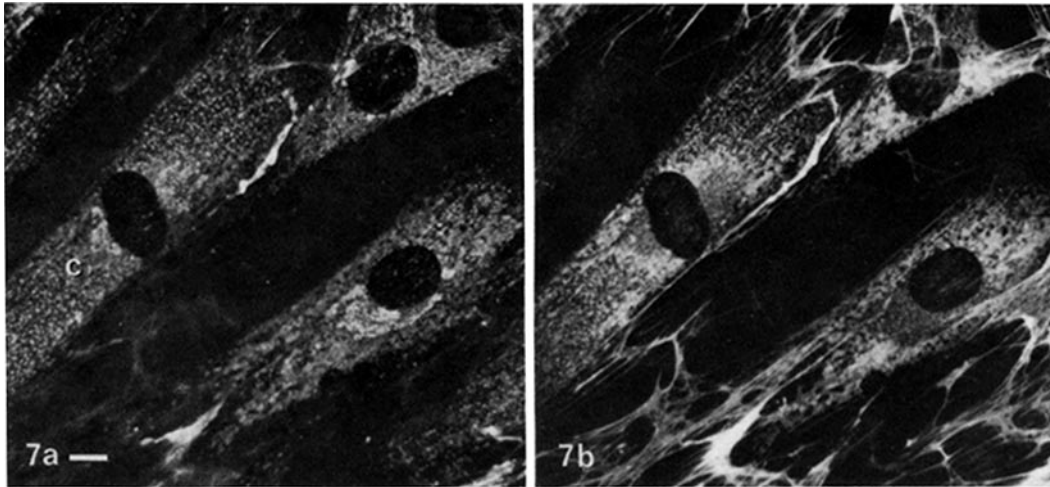
FIGURE 7 Effect of ascorbate deficiency on procollagen (*a*) and fibronectin (*b*) localization. Cytoplasmic procollagen (*c*) is more extensive and more coarsely granular than in control cells (e.g., Fig. 5 *a*). Bar, 10 µm. × 500.

ganelles, especially considering that normal cells degrade intracellular, newly synthesized procollagen (2).

The Secretory Pathway: Association of Procollagen and Fibronectin and the Site of the Monensin Blockade

We provide evidence that in control cells, fibronectin and procollagen are present in the same groups of juxta-nuclear deposits, although exact codistribution cannot be claimed. This also applies to juxta-nuclear material present in monensin-inhibited cells. However, in these same cells, the larger size of the peripheral deposits enables the resolution of discrete, coincident localizations of the two antigens (Fig. 6*c* and *d*). Although cross-reactivity of antisera was not observed in controls, we were aware of the possibility that apparent codistribution might have resulted from other artifacts, such as spectral leak-through between fluorochromes. However, the different relative intensities of the two antigens observed in ascorbate-deprived cells argues against this possibility. Also, staining different samples separately with the two antisera gave each of the characteristic distributions seen in double stained preparations. By immunostaining, Vaheri et al (40) had previously determined that fibronectin and procollagen can be synthesized by the same fibroblast, but they did not obtain the same degree of resolution presented here. According to Gay et al (13), types I and III (pro)collagen are also found in the same areas of cytoplasm in some cells, although in other cells they are contained in different areas of cytoplasm. Codistribution of secretory proteins along a secretory pathway (endoplasmic reticulum, Golgi, secretory elements) has been established in other cell types, including pancreatic cells (14) and eosinophilic leukocytes (1), and we intend to further investigate these details of the intracellular association and translocation of procollagen and fibronectin by exploiting the higher resolving power of electron-immunocytochemistry.

The involvement of the Golgi complex in secretory processes is well established (43) and specific evidence exists for the participation of this organelle in the secretion of procollagen (24). The absence of normal Golgi structures in monensin-treated fibroblasts, as well as other cell types (36), immediately suggests that the observed reduction of secretion results from the derangement of that organelle by a direct or indirect action

of monensin. Thus, in their study of guinea pig pancreas, Tartakoff and Vassalli (36) showed an accumulation of labeled material in a smooth-membraned fraction and placed the monensin blockade "at the point of exit of secretory product from the Golgi apparatus." The numerous smooth-membraned vesicles were, thus, considered as dilated Golgi elements. Our present observations support this interpretation.

Our use of extended incubations in monensin has implicated the eventual involvement of the rough endoplasmic reticulum in the intracellular accumulation of procollagen and fibronectin. Uchida et al. (38) have shown, by pulse labeling and subcellular fractionation procedures, that as well as inhibiting procollagen transit out of a Golgi fraction (a result consistent with accumulation in Golgi-related organelles), monensin also impedes the passage of procollagen and fibronectin from rough endoplasmic reticulum into that Golgi fraction. This is quite consistent with our present observations, but does not determine whether material in the rough endoplasmic reticulum is simply an "upstream" accumulation of protein which cannot be accommodated in the altered Golgi compartment or whether an additional monensin blockade exists at the exit of the rough endoplasmic reticulum. The latter possibility must be considered, as the effects of monovalent ionophores may well be multifarious. For example, Somlyo et al. (32) showed that the ionophore X537A can be incorporated into almost all parts of the cell, and Okhuma and Poole (22) have shown that nigericin induces an increase of lysosomal pH (in mouse macrophages).

In conclusion, we note that it is becoming increasingly apparent that the Golgi complex is a center of cellular membrane flow (11) and that its secretory activity should not be considered in isolation. Monensin and other ionophores promise to be of extreme value in the biochemical and morphological "dissection" of the multiple Golgi functions and in the study of the entire secretory pathway per se.

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