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An Axial Periodic Fibrillar Arrangement of Antigenic Determinants for Fibronectin and Procollagen on Ascorbate Treated Human Fibroblasts

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Fibronectin and collagens are major constituents of the cell matrix of fibroblasts. Fibronectin is a 220,000 dalton glycoprotein that mediates a variety of adhesive functions of cells examined in vitro. Fibronectin is secreted in a soluble form and interacts with collagen to form extracellular filaments. Fibronectin and procollagen type I were localized using the peroxidase anti-peroxidase method. Under standard culture conditions, fibronectin and procollagen were localized to non-periodic 10 nm extracellular fibrils, the cell membrane and plasma membrane vesicles. Ascorbate treatment of cells leads to a new larger fibril with a diameter of approximately 40 nm. Antibodies to fibronectin and procollagen I react to these native collagen fibrils with an axial periodicity of approximately 70 nm. Fibronectin is clearly associated with native collagen fibrils produced by ascorbate treated cells and there is an asymetric distribution or segregation of fibronectin on these collagen fibrils with a 70 nm axial repeat.

Key words: **human fibroblast, ascorbate, procollagen, fibronectin, axial periodicity, native collagen fibrils**

The cell matrix consists of highly structured glycoproteins, collagen and proteoglycans. Fibronectin is a 220,000 dalton glycoprotein which is secreted by numerous cultured cells in vitro and is associated with basal lamina in vivo [1–3]. Fibronectin is thought to be important for cell adhesion especially to collagen [4, and reviewed in 5]. Fibronectin and types I and III collagen and procollagen are major components of the cell matrix of connective tissue cells and occur largely as extracellular fibrils [6–9]. These matrix components also occur in a soluble form secreted into the medium and as a diffusely arranged membrane component [9, 10, and submitted]. Fibronectin interacts with collagen, more

Abbreviations: PBS, phosphate buffered saline; PBS–NSS, phosphate buffered saline + 1% normal sheep serum; PAP, peroxidase anti-peroxidase complex; RaFN, rabbit antibody to fibronectin; RaProcol I, rabbit antibody to procollagen type I; RaProcol III, rabbit antibody to procollagen type III.

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with type III than with type I [11, 12], and specific cyanogen bromide fragments of collagen are responsible for the interaction with fibronectin [12, 13], and specific sites on fibronectin interact with collagen [14]. Fibronectin is extensively disulfide bonded on the cell surface [15] and is able to be cross-linked to itself and collagen by transglutaminase [16, 17].

Collagen is secreted by fibroblasts cultured in vitro in the form of procollagen [18, 19], and one cell is capable of synthesizing multiple collagens, ie, types I and III [20]. Procollagen has been shown to be a component of the cell matrix by immunofluorescence and biochemical studies [6, 8]. Subsequent to synthesis multiple modifications of procollagen occur that lead to the final cross-linked fibrillar structure (reviewed in [21]). For example, procollagen peptidase cleaves non-helical segments from terminal components of the procollagen chain. Post translational processing also involves cross-linking following the action of lysyl hydroxylase and prolyl hydroxylase. A number of cofactors have been shown for the latter enzymes, including oxygen [22], iron [23], and ascorbic acid [24, 25].

There has been considerable interest in the possibility that posttranslational prolyl hydroxylation may regulate either procollagen synthesis or secretion. The activity of prolyl hydroxylase changes as a function of cell density or ascorbate treatment [25]. At confluence, when there is the largest accumulation of collagen [26], fibronectin, and fibrils, the activity of prolyl hydroxylase is highest. In certain cases prolyl hydroxylase may be a rate limiting factor in secretion of procollagen [21, 24, 25, 27].

Cultured fibroblasts are incapable of producing ascorbate, even when derived from species capable of synthesizing vitamin C in vivo and, therefore, synthesize under-hydroxylated collagen. The lack of vitamin C has been suggested by some to play a role in the altered synthesis and organization of collagen in cells cultured in vitro [24, 25]. Addition of ascorbate causes enhanced synthesis and secretion of collagen in vitro [24, 25]. It would therefore appear that there is a possible interrelationship among: cell density, collagen and fibronectin secretion, ascorbate treatment and fibrillogenesis. In this paper we report the results of ascorbate treatment on the evolution of the extracellular matrix using intra and extracellular immunocytochemical localization of fibronectin and procollagen I and III.

METHODS

Cell Culture

Human skin fibroblasts, passage (5–20) were grown in Dulbecco's minimal essential medium with 10% fetal calf serum (DMEM+S) on 60 mm Falcon tissue culture plates or Lab Tek® chamber slides as described [9]. For ascorbate treatment, upon reaching stationary phase, cells were treated with freshly prepared sodium ascorbate (50 μg/ml) (Sigma) in DMEM + S every other day for 7 days with controls receiving medium changes alone.

Antigen and Antibody Purification

Fibronectin was affinity purified from fresh frozen plasma by affinity chromatography on immobilized gelatin [28, 29] with antiserum prepared with affinity purification as described [30].

Procollagen Purification

Type I procollagen was purified from the skin of dermatosporactic calves as described [31, 32]. Briefly, the collagenous protein was extracted with 0.25M NaCl and Tris-HCl

0.05M pH 7.4 from skin homogenates. The type I procollagen was separated from type III procollagen by differential salt precipitation and DEAE cellulose chromatography as reported [19]. Purity of the antigen was demonstrated by amino acid analysis of the protein and by disc and slab gel electrophoresis before and after reduction with 20 mM dithiotreitol [33, 34]. When the gels were purposely overloaded the components chains migrated as $\alpha 1(I)$, $\alpha 2$, pro$\alpha 1(I)$, and pro$\alpha 2$ chains (Fig. 1). After pepsin digestion, even these chains migrated only as $\alpha 1(I)$ and $\alpha 2$ chains (Fig. 1). After collagenase treatment [35], these chains were completely destroyed, indicating their collagenous nature (not shown).

On 15% acrylamide gels, two peptides were isolated from the collagenase digests corresponding to the globular non collagenous peptides of pro$\alpha 1(I)$ and pro$\alpha 2$ chains (Fig. 2). Rabbits were immunized and antibody purified on a DE52 column and affinity chromatography was performed to remove any cross contaminating antibodies, and finally, specific absorption onto type specific procollagens was performed.

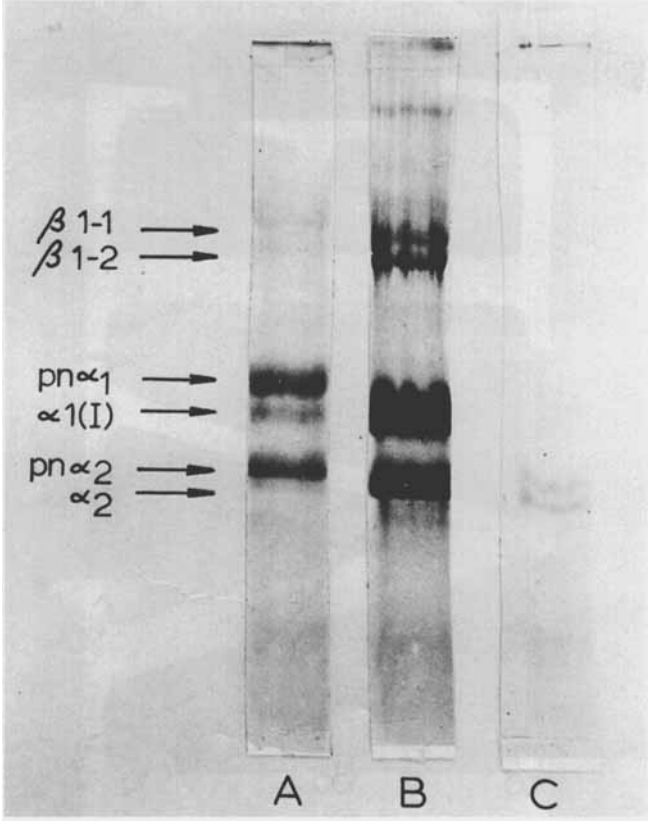


Fig. 1. 5% sodium dodecyl sulphate (SDS) acrylamide gel showing the component chains of type 1 pN collagen in the presence of 20 mM DTT. b. Similar to Figure 1a except pretreated with pepsin. c. 15% SDS gel; 2 bands are identified after bacterial collagenase treatment. On 5% gels no chains could be observed.

The review of Timpl et al [36] suggests that there are helical antigenic determinants of collagen which are restricted to triple helical structures, while there are also terminal propeptide determinants that are represented by the terminal propeptide extensions. Figure 2 shows immunoprecipitation data for type I procollagen antibody illustrating the specificity of this antibody to propeptide non-helical extensions of the pro I molecule. Other recent studies using these same antibodies in enzyme linked immunoassays show they lack cross reaction among fibronectin and the procollagen antibodies [37].

Light Microscopic Immunocytochemical Localization

Samples for light microscopy were grown in 60 mm culture dishes or Lab Tek chamber slides to the desired density and were then fixed with 0.2% glutaraldehyde for 20 minutes. To render the cells permeable to antibodies without completely disrupting architecture, fixed cells were first dehydrated and then rehydrated in a graded series of ethanol ranging from 10%–95%, method of DeMey et al [38]. Once cells were rehydrated they were washed with 0.05M glycine in phosphate buffered saline (PBS), and then incubated in PBS with 1% normal sheep serum (PBS–NSS). Cultures were incubated in antibodies (at dilutions determined by previous titration) for 30 minutes each with constant rotation. Primary antibodies included affinity purified RaFN (1/500), affinity purified RaProcol type I (1/50), affinity purified RaProcol type III (1/100), and RaFN with the antibodies adsorbed out with affinity purified fibronectin. The secondary (linking) antibody was goat antirabbit gamma globulin used at 1/500 dilution (Cappel). The detecting antibody com-

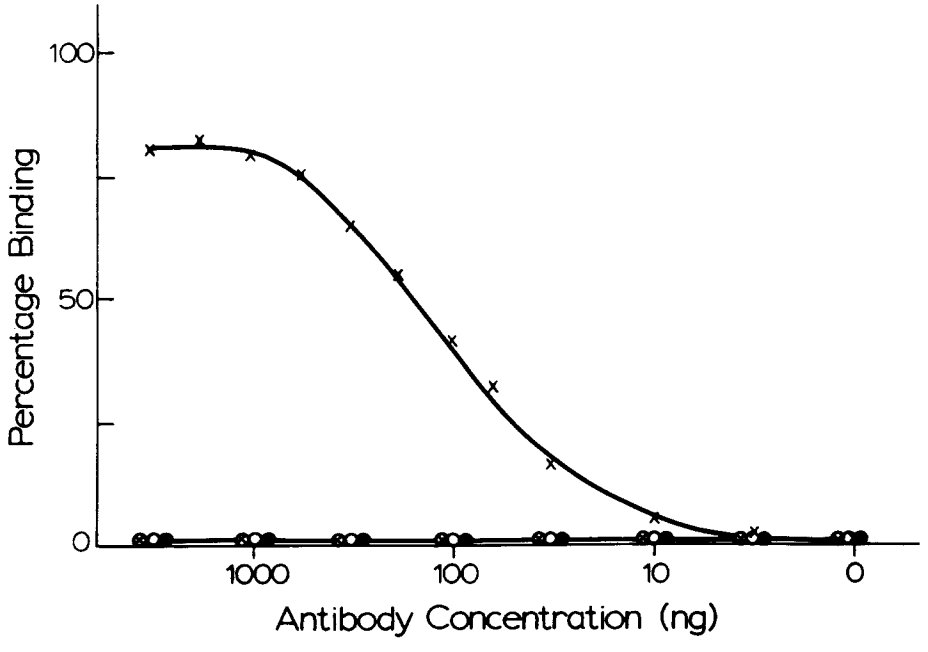


Fig. 2. Immunoprecipitation data as shown by the percent binding of C^{14} labeled protein vs. antibody concentration (ng). x–x, procollagen I; ⊗ – ⊗ , collagen I; ○–○, procollagen III; ●–●, collagen III.

plex peroxidase antiperoxidase (PAP) (Cappel) was used at a 1/1,000 dilution. Cultures were washed and agitated for 30 minutes before and between antibody steps with PBS–NSS. After PAP incubation cells were washed with PBS for 30 minutes and then reacted with 3–3′diaminobenzidine tetrahydrochloride (DAB) and 0.0025% H_2O_2 in PBS pH 7.6 for 6 minutes, rendering bound antibody brown via the complexed peroxidase. Cultures on Lab Tek slides were mounted in permount, while cultures in 60 mm dishes were viewed through buffer with an Olympus BH microscope and photographed with an automatic camera system.

Electron Microscopy

For extracellular immunocytochemical localization, cells were treated as described [9, 10]. Briefly, cells were grown to desired density and then fixed with 1% glutaraldehyde (Electron Microscopy Sciences) for 20 minutes at 37°C. Samples were washed with PBS, treated with 0.05M glycine (Sigma) in PBS and then rinsed with PBS + 1% normal sheep serum. Antibody incubations were done for 10 minutes with agitation following the protocol listed above. After PAP incubation samples were rinsed with PBS and developed with the DAB–H_2O_2 mixture for 6 minutes, at which time samples could be photographed for light microscopy. Samples were then post-fixed with 1% OsO_4 (Polyscience) in PBS for one half hour at room temperature and embedded in Epon 812 (Pelco). Sixty- to eighty-nm-thin sections were viewed and photographed without heavy metal counterstain with a Philips EM 300 electron microscope operated at 40 KV.

Cells for routine morphological examination were fixed with 1% glutaraldehyde and then 1% OsO_4, embedded and sectioned as described above. Thin sections were stained with uranyl acetate (aqueous saturated) (Sigma) for twenty minutes and then with Reynold's lead citrate for five minutes before viewing at 60 KV with a Philips EM 300.

RESULTS

Light Microscopic Localization on Subconfluent Fibroblasts

Immunocytochemical localization of fibronectin, type I procollagen, and type III procollagen after glutaraldehyde fixation and dehydration and rehydration in ethanol yields information on the dynamics of their intracellular synthesis, movement, and secretion as well as their involvement in the extracellular matrix. In subconfluent cultures of human fibroblasts viewed by bright field light microscopy, fibronectin is located in the periphery in cytoplasmic globules. At this stage a small amount of extracellular localization in the form of a matrix is seen (Fig. 3a).

Procollagen type I has quite a different intracellular localization compared to fibronectin in subconfluent cultures. Rather than being peripherally localized in the cytoplasm, procollagen type I is located in intensely reacting perinuclear granules which do not appear to stream all the way out to the cell margins. Essentially no extracellular distribution of type I procollagen is seen at this point (Fig. 3b).

Procollagen type III has an intracellular distribution quite similar to type I in low density cultures with the majority of staining being perinuclear. The staining is not as dense as it is for type I procollagen, and some reaction is also localized peripherally near the plasmalemma with a small amount of extracellular staining in the form of a matrix also present (Fig. 3c). This type of pattern has been shown using immunoferritin at the electron microscopic level, representing procollagen in the endoplasmic reticulum [21].

A fibronectin adsorption control which is free of staining demonstrates the specificity of the reaction (Fig. 3d).

Light Microscopy of Post-Confluent Fibroblasts

Cells cultured for one week beyond confluence develop a dense extracellular filamentous matrix of fibronectin (Fig. 4a), type I procollagen (Fig. 4b), and type III procollagen (Fig. 4c). Intracellular staining for fibronectin and type III collagen is diminished (Figs. 4a,c). Matrix formation for type I procollagen, although present, is not as heavily stained as for fibronectin and type III procollagen, suggesting a smaller amount of antigen present.

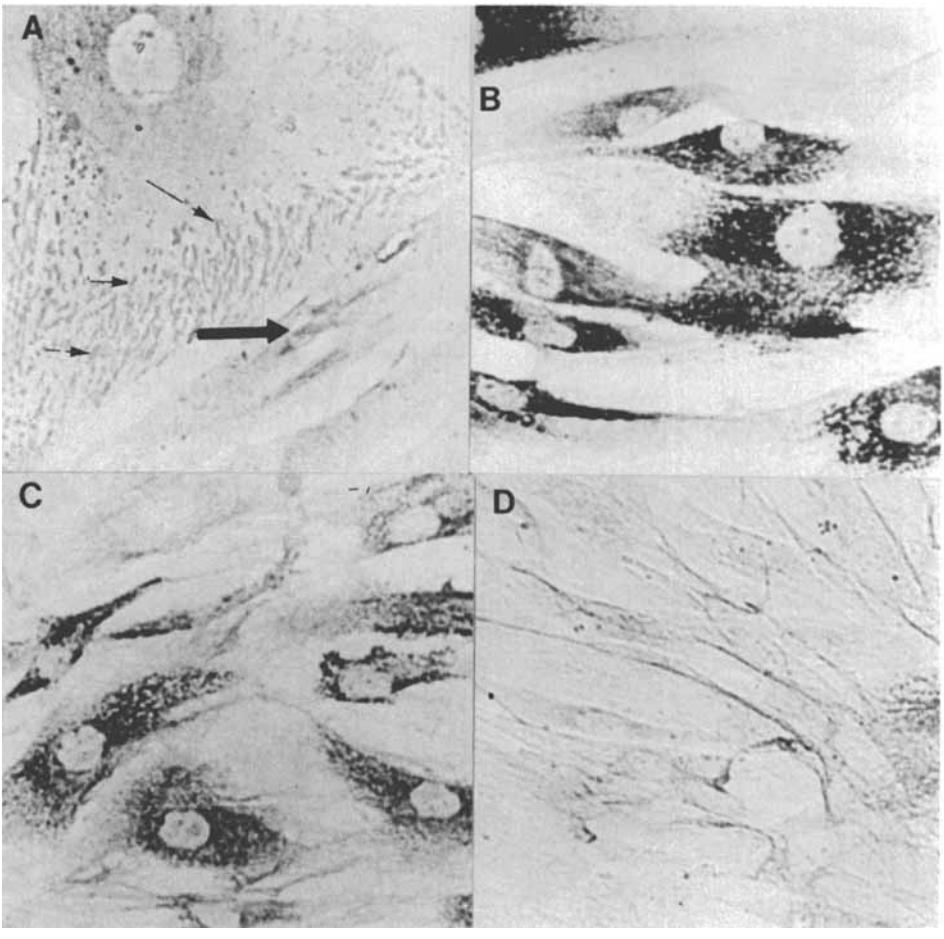


Fig. 3. Light microscopic localization (intra- and extracellular) of antigens on subconfluent fibroblasts. a. Fibronectin localization well spread low density cells. Intracellular fibronectin staining is seen in globules (small arrows). Some extracellular staining is seen forming a matrix between adjacent cells (large arrow). b. Type I procollagen localization in subconfluent cells reveals a dense packaging of the antigen in perinuclear granules. No extracellular matrix is visualized. c. Type III procollagen localization in subconfluent cultures reveals dense perinuclear staining as well as some extracellular matrix formation. d. Control in which antifibronectin is preadsorbed with fibronectin is free of reaction product.

Intracellular perinuclear granules remain heavily stained (Fig. 4b). Adsorption controls are negative (Fig. 4d).

Light Microscopy of Ascorbate Treated Post-Confluent Fibroblasts

Treatment of confluent cultures with 50 μg/ml ascorbate for one week results in no profound changes in the light microscopic localization pattern of either fibronectin (Fig. 5a) or type III procollagen (Fig. 5c). However, it appears that type I procollagen is transported from the perinuclear granules to the exterior of the cells where it is released into the medium and incorporated extracellulary (Fig. 5b). Adsorption controls are negative (Fig. 5d).

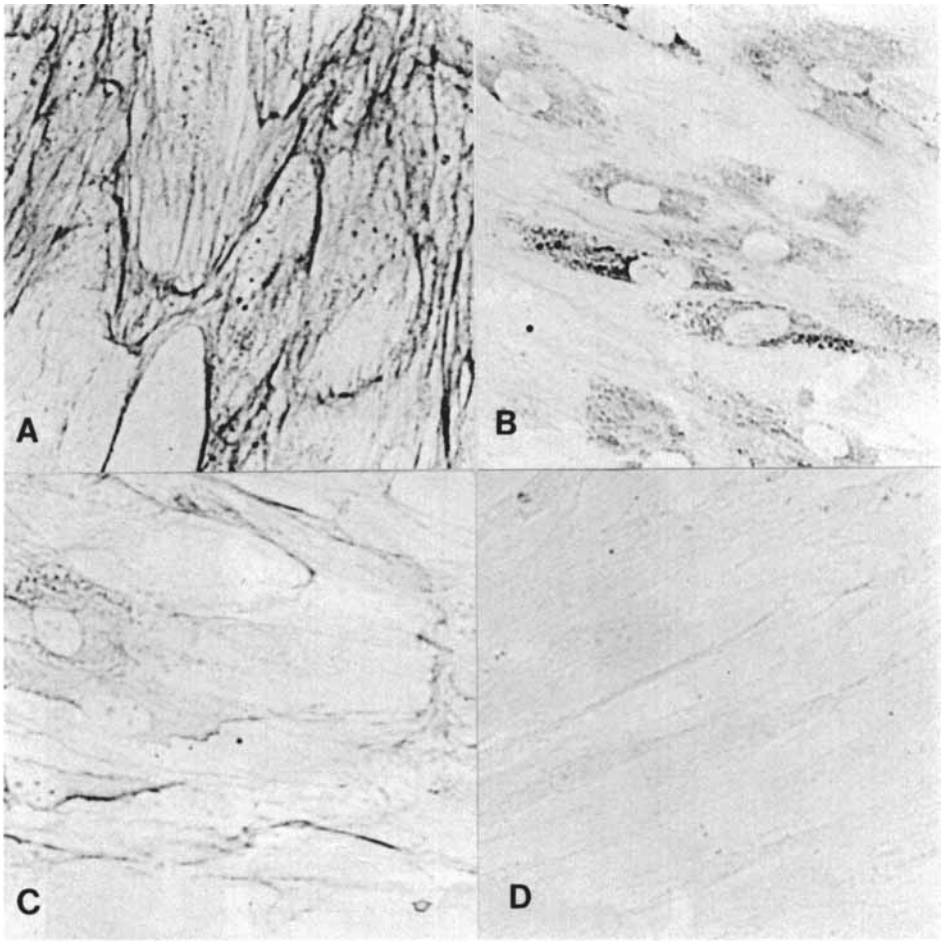


Fig. 4. Light microscopic localization (intra- and extracellular) of antigens in one week post-confluent human fibroblasts. a. Fibronectin forms a dense extracellular matrix which surrounds cells. Some intracellular staining is seen, but the majority of the antigen present is seen in the matrix. c. Type III procollagen is present throughout the cytoplasm of the cells as well as in the extracellular matrix.
d. Fibronectin adsorption control is free of staining.

Electron Microscopy of Post-Confluent Fibroblasts With and Without Ascorbate

Ultrastructural studies on cells grown with and without ascorbate and prepared for extracellular localization of fibronectin, type I procollagen, and type III procollagen reveal dramatic changes in the type of fibrils and the distribution of all three antigens on the fibrils.

In cultures which are one week post-confluent (no ascorbate treatment), the filaments are approximately 10–20 nm in diameter and all three antigens are localized in a non-periodic fashion on these filaments. In general all filaments are positive for all three antigens, suggesting that fibronectin (Fig. 6), type I procollagen (Fig. 7), and type III procollagen

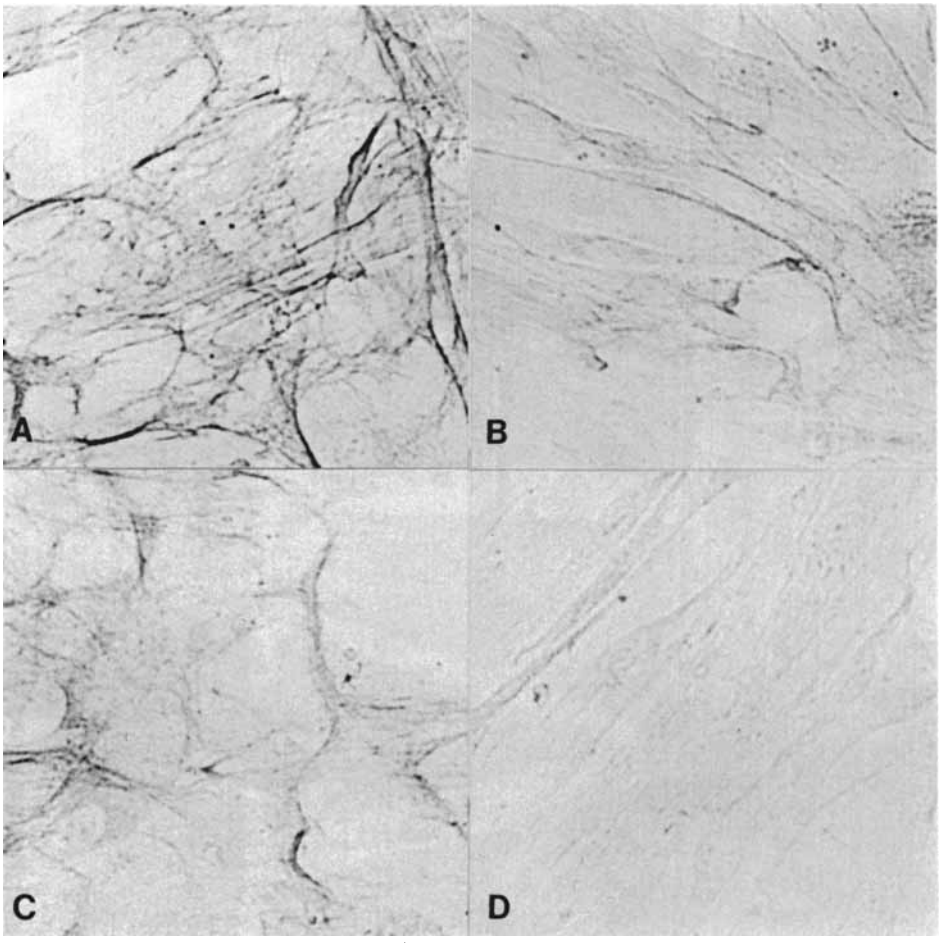


Fig. 5. Localizations of antigens (intra- and extracellular localization) in human fibroblasts treated with 50 μg/ml ascorbic acid for one week after confluence, light microscopic (× 350). a. Fibronectin localization reveals dense extracellular matrix with minimal intracellular staining (much as seen in Fig. 2a). b. Type I procollagen is present in an extracellular matrix and intracellular staining is greatly diminished as compared with non-ascorbate treated cultures (Fig. 2b). c. Type III procollagen is present in the extracellular matrix and intracellular staining has diminished in comparison with Fig. 2c. d. Fibronectin adsorption control is free of staining.

(Fig. 8) are all components of the nonperiodic filaments. Very frequently the filaments form at sites of apparent invagination of the cell, or where cells appear to overlap. Controls are free of reaction product (Fig. 9).

When similar cultures are not treated with antibodies, but are instead stained with uranyl acetate and lead citrate after sectioning, the filaments are nonperiodic and appear to range in diameter from 10–20 nm and course lengths of cells (Fig. 10). The extracellular filaments (EF) (Fig. 10) are often seen to bridge to the intracellular microfilaments (IMF), and such an association has been termed the fibronexus [39]. The lack of easily recognizable periodicity when sections are stained with heavy metals shows that the periodic peroxidase reaction produced in antibody studies is related to the periodicity of antibodies reacting along the fibrils (see below).

In cultures grown to confluence and then treated daily with 50 μg/ml ascorbate for one week, a new larger diameter fibril appears which is distinct from the nonperiodic thinner filaments. Antibodies to fibronectin, procollagens I and III react with an axial periodicity of approximately 70 nm repeat on these larger diameter fibrils (Figs. 11–13), and antibodies continue to react in a nonperiodic fashion to the smaller diameter (10–20 nm) filaments. Inserts on figures illustrate the periodicity of the antibody reaction product more clearly. Adsorption controls are free of peroxidase reaction product in ascorbate treated cells (Fig. 14).

Ascorbate treated cells which are not reacted with antibody but are counterstained with heavy metals also occasionally show 70 nm periodicity in some fibrils which are

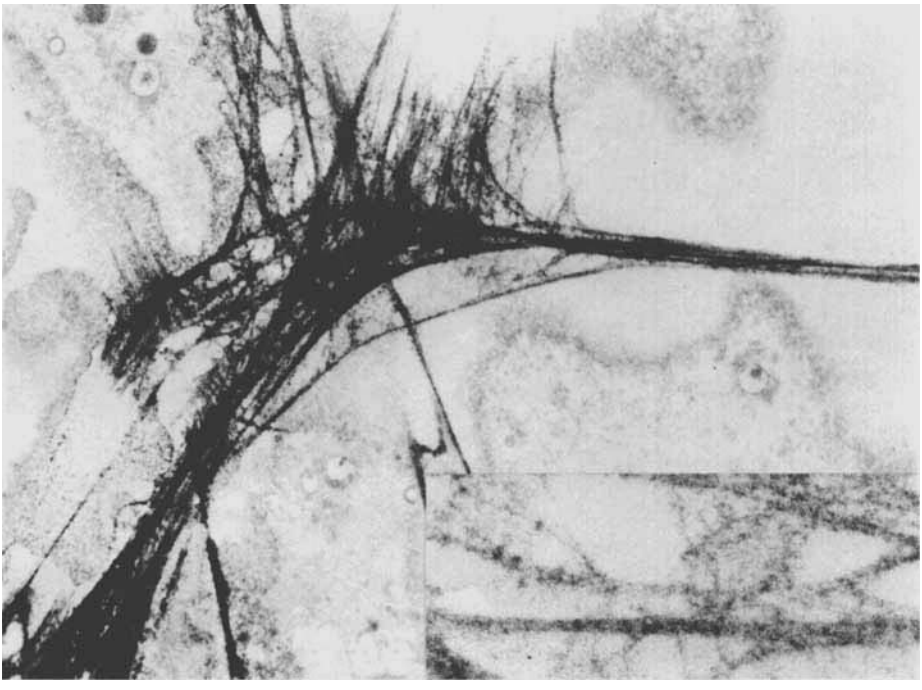


Fig. 6. Electron micrograph of extracellular fibronectin localization on one week post-confluent fibroblasts. The extracellular matrix, which stains for fibronectin, is composed of long filaments which course singly or from bundles (× 9,900). Inset is higher magnification (× 29,700).

40–50 nm in diameter (Fig. 15). In these cultures there is also apparent periodicity of extracellular material that cannot clearly be described as filamentous (Fig. 15). This most likely represents areas where fibrils are forming or are dipping in and out of the thin section. Adsorption of collagen antibodies with fibronectin yielded identical patterns, as seen with affinity purified collagen antibody alone, suggesting that the collagen antibody reactions were not due to any possibly contaminating fibronectin antibodies.

It appeared that these large diameter extracellular fibrils might essentially be primarily collagenous, upon which fibronectin and perhaps other components such as proteoglycans might associate. To test this hypothesis ascorbate treated cells were treated with 200 U/ml bacterial collagenase (Advance Biofactures Corporation) for three hours. After this treatment cells were reacted with procollagen I antibody – no staining was observed, and the extracellular material was mottled or had a sheltered appearance rather than fibrillar (Fig. 16). This extracellular material in collagenase treated cultures did react with antibodies to fibronectin, though the fibrillar nature of this material was now obviously destroyed (Fig. 17).

DISCUSSION

These studies have examined the development of the extracellular matrix and the effects of ascorbic acid on human skin fibroblasts cultured in vitro. The studies used

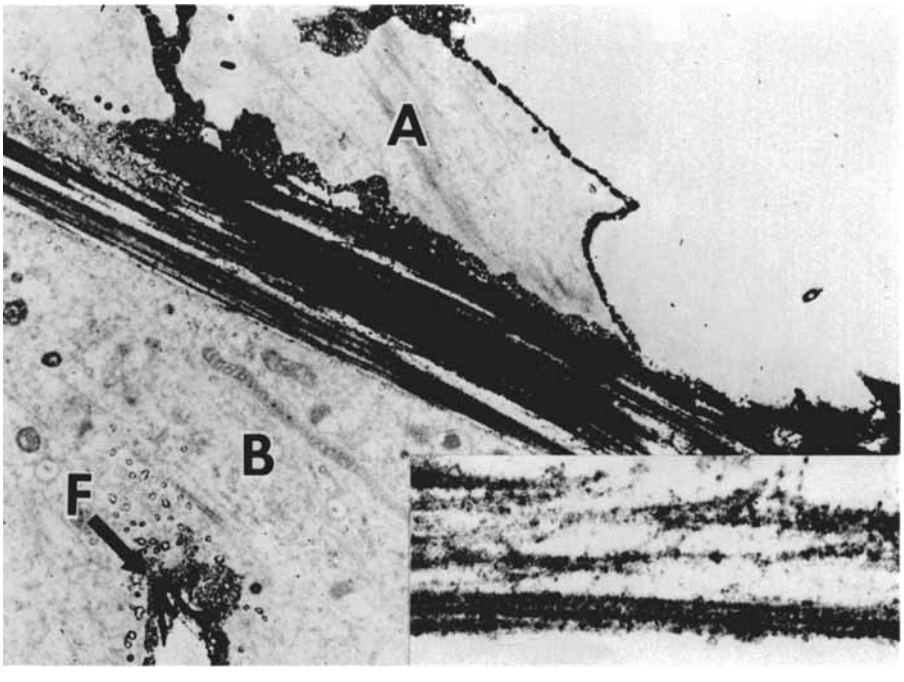


Fig. 7. Electron micrograph of extracellular Type I procollagen localization on one week post-confluent human fibroblasts. The extracellular matrix stains for Type I procollagen are much as seen in Fig. 4. Inset is higher magnification of area indicated by arrows, × 9,900, inset × 29,700. Note the diffuse stained membrane reaction; also fibrils coarsing at sites of cell (A) overlapping cell (B). Also note the beginning formation of fibrils at site of invagination (arrow) on cell (B) with beginning fibril (F).

immunocytochemical localization of fibronectin, procollagens I and III intra- and extracellularly at the light microscopic level, and localization of these same antigens extracellularly at the electron microscopic level. At low cell density very little if any cell matrix is seen. As cells increase contact with each other and more so at confluence, a dense extracellular matrix of fibronectin and procollagen I and III appears. There is a distinct change in the intracellular distribution of these proteins destined to be in the cell matrix as a function of density and ascorbate treatment. At low cell density there are small amounts of fibronectin within cells which appears to be moving out to the periphery of the cell in dense intracellular globules. An association of extracellular fibronectin and intracellular actin has been shown by others [39, 40]. As the matrix is being laid down extensively in confluent cultures, not very many cells have this dense globular intracellular fibronectin accumulation in comparison to lower density cells.

The intracellular localization of both procollagens I and III is quite distinct from that of fibronectin. In both low and higher density cells procollagens I and III are located in dense perinuclear areas probably analogous to the endoplasmic reticulum shown in earlier studies [41]. As cell density increases, the matrix forms and initially more procollagen III relative to procollagen I appears extracellularly on these cells. It almost appears that prior to matrix formation there is possibly a partial block in secretion and an intracellular accumulation of procollagen I and III occurs. As the procollagen I or III appears in the extracellular matrix there is correspondingly less of the particular procollagen type intracellularly. The apparent partial blockade of secretion is best evidenced by Figure 2b where

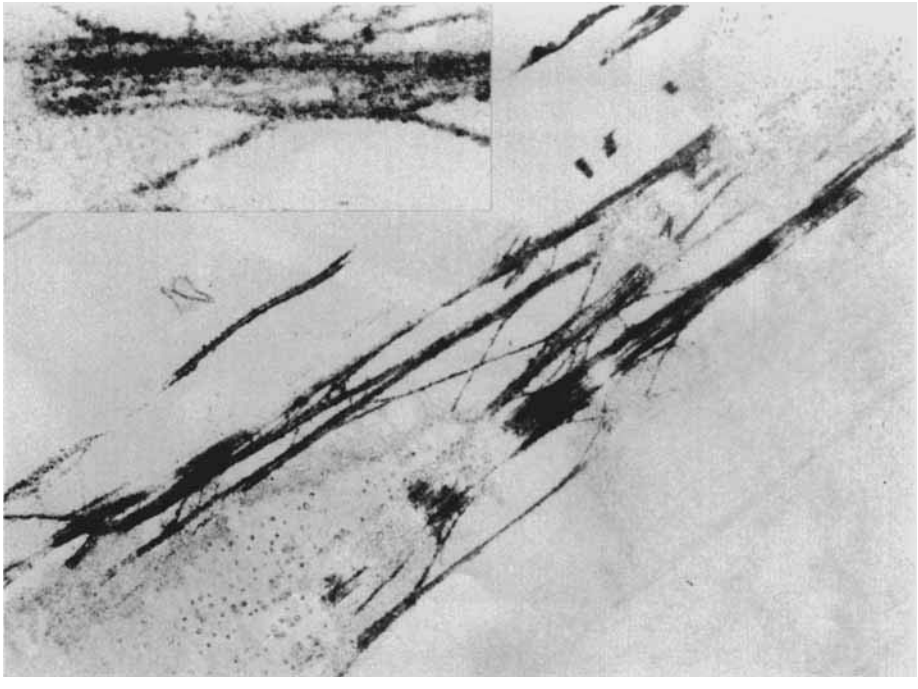


Fig. 8. Electron micrograph of extracellular Type III procollagen localization on one week post-confluent fibroblasts. Staining is very similar to that seen in Figures 4 and 5. Inset is higher magnification of area indicated by arrows, × 9,900, inset × 29,700.

high density confluent cultures are reacted with procollagen I antibody and significant intracellular localization is seen, while there is little procollagen I in the matrix at this point. At this same point in time, considerable fibronectin and procollagen III are seen in the matrix. The disparity between extracellular procollagen I and III could be due to more complete processing of procollagen I to collagen type I than procollagen III to collagen type III. Since the particular antibody, RaProcol I, we are using recognizes specific sites on the amino terminal extension of procollagen that are not present on collagen, less antibody reaction could be seen with a more complete processing of procollagen to collagen. This is a potential explanation for seeing less procollagen I relative to III in the extracellular matrix at this point.

Electron microscopic studies show that in confluent cultures nonperiodic fibrils are formed that were composed of fibronectin, procollagens I and III. These filaments are lacy by light microscopy, have a diameter of 10–20 nm, and appear to form at or near the cell surface sometimes at sites where there is an invagination of the cell. They are similar to fibrils demonstrated by routine heavy metal staining [42] of cultured cells shown by other investigators [26].

The effects of ascorbate are quite dramatic. Very low density logarythmically growing human cells are killed by ascorbate at a concentration (50 μg/ml) that has no effect on quiescent or near confluent cultures. At high cell density ascorbate appears to result in the movement of intracellular procollagen I (more so than III) out of cells and an enhanced matrix formation. This is most demonstrative when one compares procollagen I intracellularly at the light level in ascorbate versus control cultures. Most likely one could find cell densities where there is a more profound shift of procollagen III in the presence of ascorbate.

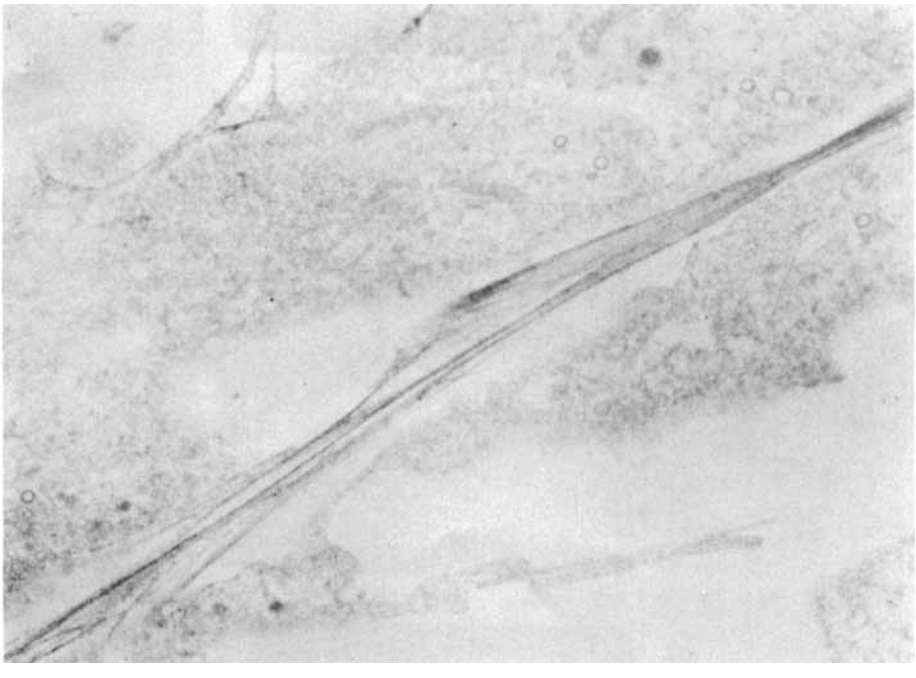


Fig. 9. Filaments are free of reaction product in electron micrograph of fibronectin adsorption control, × 9,900.

The effects of ascorbate are even more dramatic on the extracellular fibrils. Using heavy metal counterstaining, the periodic fibrils induced in ascorbate treated cultures are significantly larger in diameter (40–50 nm) than the nonperiodic thinner filaments (10–20 nm) seen in control cultures. Most surprisingly there is an unequivocal reaction of fibronectin, procollagen I and III antibodies in an axial periodic fashion with a repeat of approximately 70 nm on the 40–50 nm diameter fibrils in ascorbate treated cultures. This is precisely analogous to the axial periodicity seen in negative stained collagen fibrils (reviewed in [43]).*

We refer to these large diameter periodic fibrils as the definitive collagen fibril. This clearly is not artifact since no such reaction is observed when antibody is adsorbed with specific antigen and no such reaction is seen in control samples untreated with ascorbate. Additionally, if one were to argue we are simply adding more protein around the fibril which leads to the staining it should be noted that: 1) samples for immunocytochemical localization are not stained with heavy metals, only osmium tetroxide which leads to the perioxidase coupled antibody reaction product becoming electron dense and 2) there are

*Recently we have observed a periodic reaction of antibodies to helical determinants of collagen types I and III similar to that shown using the procollagen antibodies with specificities for the amino terminal extensions (Furcht, Wendelschafer-Crabb, Mosher, and Foidart, submitted).

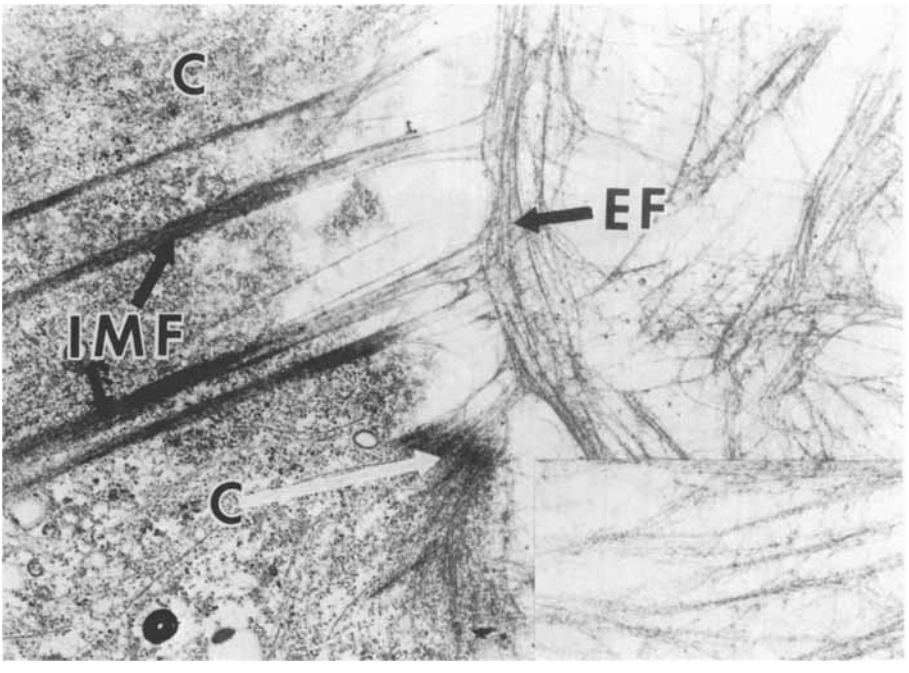


Fig. 10. Electron micrograph of heavy metal counterstained thin section of one week post-confluent fibroblasts reveals ultrastructural morphology of the filaments. The extracellular filaments (EF) range in diameter from 10–20 nm and often appear to juxtapose with intracellular microfilaments (IMF). The plasma membrane is not readily apparent because of the plane of sectioning, but one can see where the density of the cytoplasm (C) leaves off (white arrow). Inset is higher magnification of area indicated by small arrows, × 9,900, inset × 29,700.

routinely many more bands seen in positive heavy metal stained collagen fibrils formed from isolated components or in vivo [26, 42]. Based on this it is clear that the antibodies to fibronectin and procollagens I and III are reacting to antigenic determinants arranged with axial periodicity along the fibril. This apparent periodicity (approximately 70 nm) is reminiscent of the ¼ to ¾ staggered arrangement of independent collagen molecules within the microfibril (reviewed in [49]). Perhaps relative to this, studies have shown that there is a particular cyanogen bromide fragment, CB7, of the alpha$_1$ (I) chain that reacts with fibronectin [13]. Also, there is a characteristic proteolytic fragment of fibronectin with a molecular weight of 42,000 that binds collagen [14]. In sum, there is documented biochemical segregation of the fibronectin/collagen interaction, and we appear to be demonstrating a type of segregation of fibronectin and procollagen antigenic determinants along the definitive collagen fibril by immunocytochemical techniques. We have also observed that extended culture of fibroblasts for 4–8 weeks or more in the absence of ascorbate leads to the development of these large diameter collagen fibrils, which have an axial periodic arrangement of fibronectin and procollagen type I antigenic determinants along its length (Furcht, et al, submitted).

These large diameter (40 nm) fibrils appear to have a collagen backbone which holds them intact. Treatment of these fibrils with bacterial collagenase will destroy their integrity, yet when time studies are performed (not shown) one can show the fibronectin still present, though no longer fibrillar (Figs. 16, 17). Preliminary work to support the hypothesis that

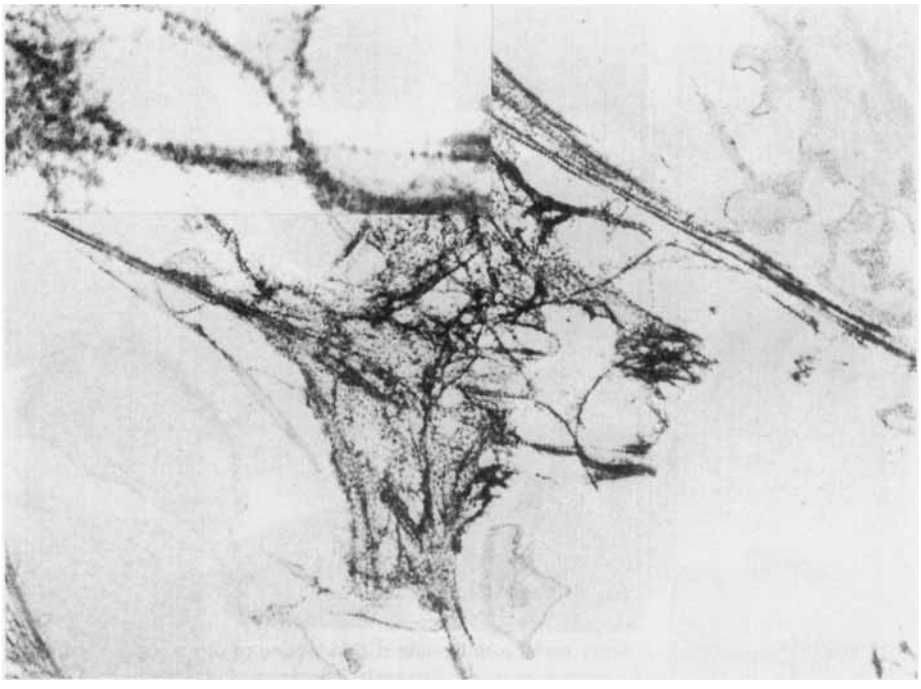


Fig. 11. Electron micrograph of fibronectin localization on human fibroblasts treated with 50 μg/ml ascorbate for one week after confluence. Periodic fibrils which bind to fibronectin antibodies develop after ascorbate treatment and comprise a large portion of the extracellular matrix. Inset is higher magnification of periodic fibril from area indicated by arrow, × 9,900, inset × 29,900.

these 40 nm fibrils have a collagenous backbone comes from data showing that trypsin can remove fibronectin yet leave the collagen intact (unpublished observation).

Many laboratories have examined collagen fibril formation in vitro from defined components and this has proven to be rather complex [44, 45]. Most recently studies from Trelstad's laboratory [45] have proposed the lateral association of thin microfibrils which ultimately lead an ordered packing of molecules.

Though many laboratories have studied in vitro collagen fibril formation with purified components, few have examined cells which may make collagen fibers. Recent work of Bruns et al has provided new insights into the process of cellular formation of collagen fibrils [46]. This study using human skin fibroblasts showed the presence of procollagen segment- long-spacing (SLS) crystallites and suggested these as the most primitive structural unit that then associates for collagen fibril formation. These SLS crystallites have a 67 nm axial repeat in negative stained preps, with a mean diameter of 5 and 10 nm. These procollagen SLS crystallites were observed to associate extracellularly in what is to be the extracellular collagen fibril [46]. These observations certainly lend support to our observation that procollagen is present in the extracellular matrix.

Questions arise as to whether the change in fibrils seen with ascorbate treatment is representative of a naturally occurring process. Early studies of Low [47] clearly show that nonperiodic fibrils (approximately 10 nm in diameter) are observed in extracellular spaces. Also, in examining wound healing in the cornea, Cintol et al [48] showed that with

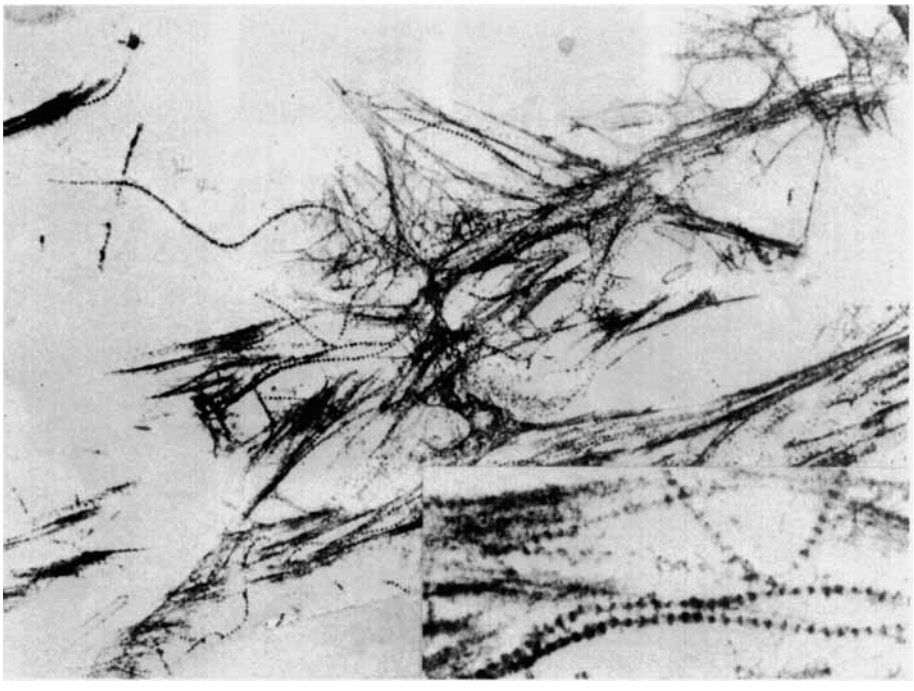


Fig. 12. Type I procollagen localized on ascorbate treated fibroblasts, visualized by electron microscopy. Many periodic fibrils are present in the matrix which stains for Type I procollagen. The periodic fibrils are thicker than filaments seen in non-ascorbate treated cultures and often curve throughout the matrix. Inset is higher magnification of periodic fibrils in area indicated by arrows, × 9,900, inset × 29,700.

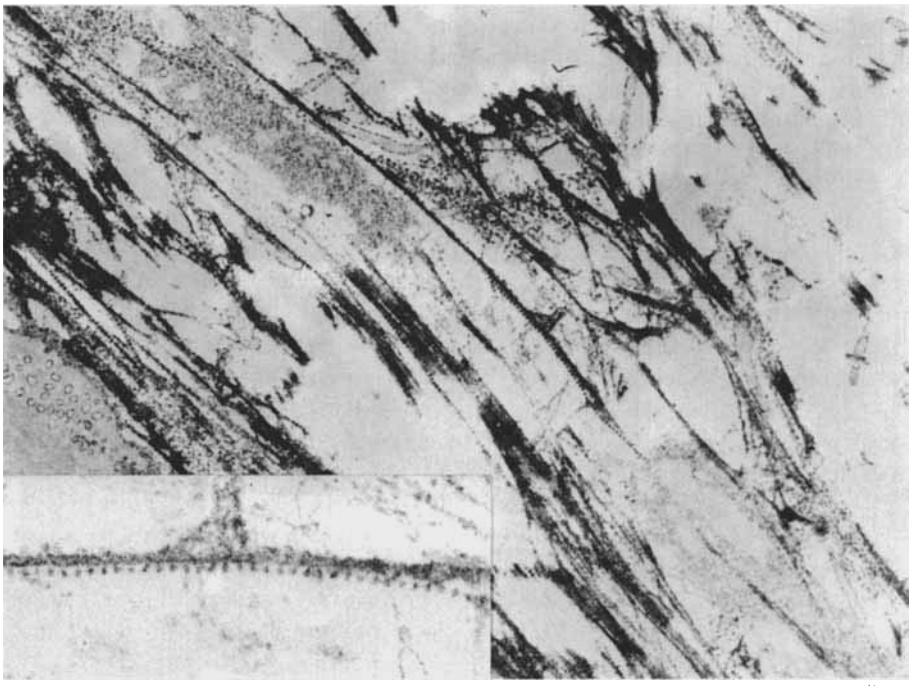


Fig. 13. Type III procollagen also develops periodic fibrils within matrix of ascorbate-treated fibroblasts. The localization pattern is indistinguishable from that seen in Figures 9 and 10. Inset is higher magnification of area indicated by arrows, × 9,900, inset × 29,700.

Fig. 14. A fibronectin adsorption control of ascorbate-treated fibroblasts is free of staining, × 9,900.

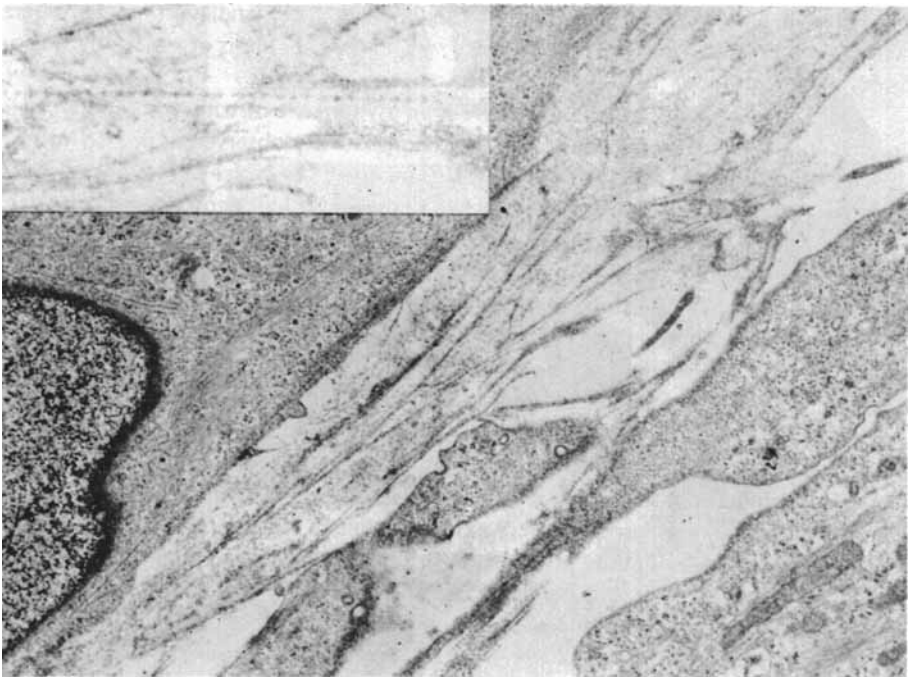
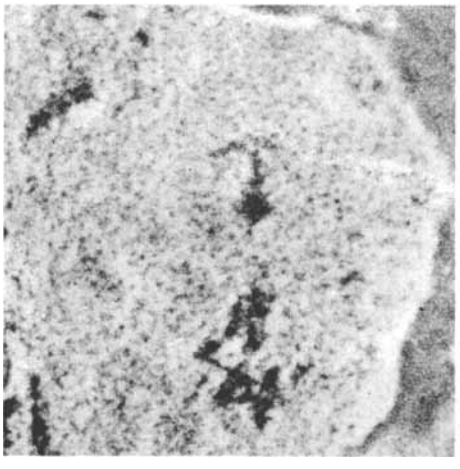
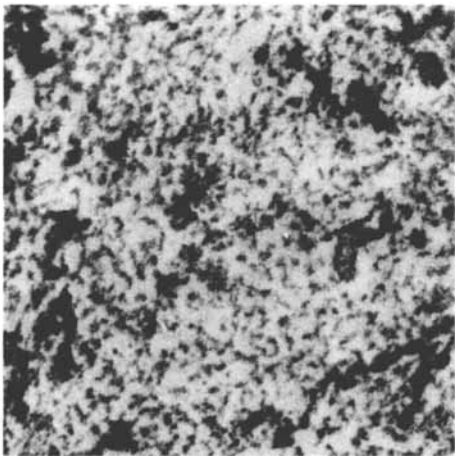


Fig. 15. Thin section of ascorbate-treated fibroblasts stained with heavy metals to reveal ultrastructural morphology. Periodic fibrils, seen in the matrix, are 40–50 nm thick and have the 60–70 nm axial periodicity characteristic of mature collagen. Inset is higher magnification of area indicated by arrows, × 9,900, inset × 29,700.

16





17

Fig. 16. Procollagen Type I localization on cells incubated with 50 μg/ml ascorbate every other day. Samples were then treated with collagenase (200 U/ml every 2 hrs.) and procollagen I localized. Note the absence of staining and destruction of fibrillar arrays (× 16,200).

Fig. 17. Fibronectin localization on cells treated as in Figure 16 (ascorbate treated, then collagenase). Fibronectin is localized in a stuttered appearance, and the collagen fibrils have been degraded by the collagenase (× 14,100).

repair, fibers tended to be of lesser diameter. Numerous other studies suggest the decreased conversion of procollagen to collagen in vitro, which presumably is occurring in the absence of ascorbate.

There is evidence to suggest that there is an asymmetric distribution of charge along collagen fibrils which may be responsible for the periodicity observed in negative staining preps of collagen fibers [49]. This is thought to be due to the axial stagger of collagen molecules within the fiber [43]. Furthermore, Kanwar and Farquar have shown that glomerular basement membranes have a periodic arrangement of negative charge which likely is due to the presence of glycosaminoglycans that have a 60 nm axial repeat and when removed enzymatically, the staining with cationized ferritin is removed [50, 51].

These studies suggest that precursor filaments are formed at early confluence which consist of nonperiodic filaments that are composed of at least fibronectin, procollagen III, and procollagen I. By inference these must all co-distribute, because when we react any of the affinity purified antibodies (fibronectin, procollagen I or III), we see that for any given antibody all of the fibrils seen ultrastructurally react positively. Since these are all specific reactions it would imply that there is co-distribution along the nonperiodic filaments. Under the influence of ascorbate or very extended time in culture (4–8 weeks) (Furcht et al, submitted) definitive 40 nm collagen and fibronectin fibrils are formed. Importantly, these definitive collagen fibrils have an asymmetric organization of fibronectin and procollagen types I and III, as evidenced by periodic reaction products of the antibodies.

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