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Simultaneous synthesis of types I and III collagen by fibroblasts in culture

(ascorbic acid/connective tissue/heritable diseases)

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ABSTRACT **Specific antibodies against types I and III collagens and procollagens were used to localize these proteins in cultured human cells. These studies indicate that the same cell makes both proteins. No type III procollagen synthesis was observed in cells from two patients with the Ehlers–Danlos type IV syndrome.**

When human skin fibroblasts in culture are supplemented with ascorbic acid, they synthesize collagen as 5–10% of the total protein (1). Most of this collagenous protein is found in the medium as procollagen although some collagen is deposited in fibrillar form in the cell layer (2–4).

Several chemically and genetically distinct collagens have been identified in the body. Type II collagen is found in hyaline cartilage (5) and type IV collagen in basement membranes (6). The tissue-specific localization of these collagens is consistent with the occurrence of their synthesis in distinct cell types. Types I and III are usually found in the same tissues—skin, blood vessels, intestines, etc.—but not in normal bone matrix, which contains only type I collagen (7–9). Previous studies (10, 11) have established that human fibroblasts, as well as a cloned strain of cells originating from calf skin, synthesize both type I and III collagens. This indicates that the progeny of a single cell have the ability to synthesize these proteins, but does not establish that the very same cell makes both proteins at the same time. In this study, we have attempted to determine if simultaneous production occurs, using antibodies that react specifically with these two proteins. In addition to cells from apparently normal individuals, we have examined certain cell strains from individuals with an inherited deficiency of type III collagen, the Ehlers–Danlos type IV syndrome (12).

MATERIALS AND METHODS

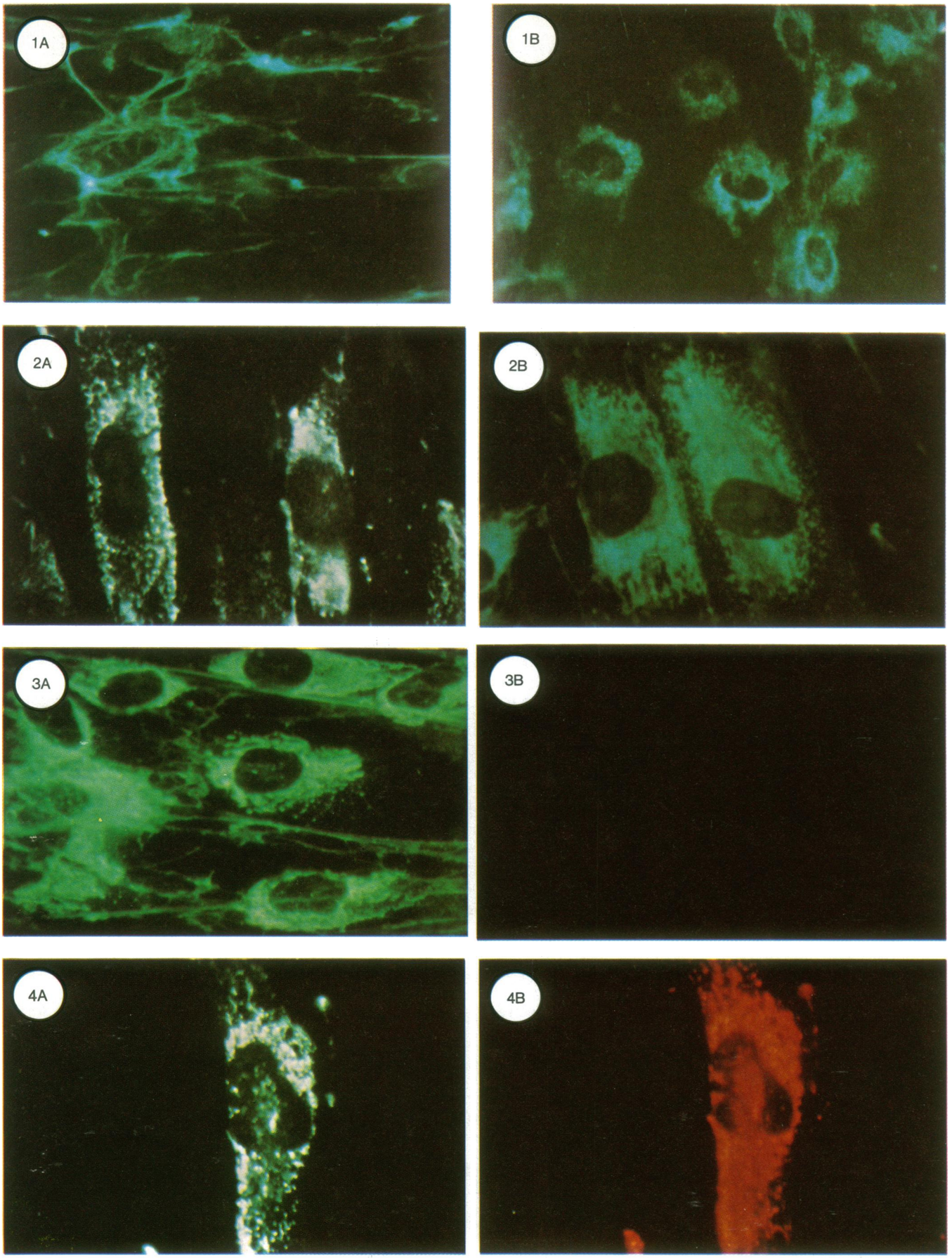
The cell strains studied were obtained from the American Type Culture Collection, Rockville, Md. These strains included three obtained from the skin of apparently normal individuals (CRL 1220, 1187, and 1106) (2, 3), two from the skin of patients with the Ehlers–Danlos type IV syndrome (CRL 1299 and 1145) (12), one from the skin of a patient with the Ehlers–Danlos type VII syndrome (CRL 1149) (13), and one from a patient with osteogenesis imperfecta (CRL 1262) (14). The culture conditions used here were the same as those described earlier (2, 3, 12–14).

In those studies where cells were to be exposed to various antibodies for immunofluorescent labeling, the cells in confluent monolayers were detached by exposure to trypsin and replated in the Dulbecco-Vogt modification of Eagle's medium containing 10% fetal calf serum in 35 × 10 mm Falcon plastic tissue culture dishes. About 6 hr later, the medium in each dish was replaced with fresh media which in some cases contained 50 μg/ml of newly dissolved ascorbic acid. These media were replaced every day. Peterkofsky (15) has shown that ascorbic acid rapidly decomposes in tissue culture media and that it is difficult to maintain maximal collagen synthesis unless ascorbic acid is frequently replenished.

At various times after plating the cells, dishes were taken for analysis, the media were removed, and the dishes were rinsed at least four times with 0.15 M NaCl, 0.05 M Tris·HCl, pH 7.4. In some studies, medium (3 ml) lacking fetal calf serum was added to the dishes. Some of the dishes received collagenase (Worthington CLSPA) at 50 μg per plate and all dishes were incubated at 37° in an incubator supplied with 5% CO_2/95% air for 1 hr. Subsequently, the media were removed from these dishes and the cells were washed with 0.15 M NaCl, 0.05 M Tris·HCl, pH 7.5, which contained 0.01 M ethylenediaminetetraacetic acid (EDTA) and 5 mM 2,2′-bipyridyl.

Preparation and Purification of Antibodies. Rabbit antibodies to bovine type I and III collagens and procollagens were prepared as described by Nowack *et al.* (16). These antibodies were rendered specific for the immunizing antigen by immunoadsorption (16). No significant crossreaction was observed between antibodies against type I or type III collagen proteins (16). The purified antibodies showed similar reactions with bovine and human tissue components when studied by immunofluorescence techniques. Antibodies to bovine type I collagen were also prepared in C57BL/10Sn mice as described by Hahn *et al.* (17). Type I collagen specific antibodies were purified from pooled mouse serum by immunoadsorption on native type I collagen.

Immunofluorescent Staining. The air-dried culture dishes were rinsed with acetone and allowed to dry. Purified antibody (20–50 μg of antibody per dish) dissolved in 0.15 M NaCl, 0.02 M sodium phosphate, pH 7.4, was added to the dishes and allowed to react for 2 hr at 20°. In some cases, antibodies to fibrinogen were added to the dishes to assess the nonspecific associations of reagents with the cells (16). Such control experiments indicated that the nonspecific association of label was negligible. Subsequently, the dishes were rinsed three times with 0.15 M NaCl, 0.02 M sodium phosphate, pH 7.4, and were layered with 1 ml of a 1/32 dilution of fluorescein-isothiocyanate-conjugated IgG prepared from goats immunized with rabbit IgG (Behring-Werke). In those studies where we simultaneously stained for two antigens, we first exposed the dishes to anti-type III collagen antibody from rabbits and then to the fluorescein-isothiocyanate-conjugated anti-rabbit IgG. Subsequently, the dishes were exposed to mouse antibodies to type I collagen, washed, and then reacted with rhodamine-conjugated antibodies specific for mouse IgG (Nordic-Pharmaceutics). Finally, all dishes were washed extensively to remove adventitiously associated reagents and sealed from the air with a solution of 90% (vol/vol) glycerol/10% saline under a cover slip. The localization of fluorescent stains on the dishes was observed in a Zeiss Universal overhead light fluorescence microscope and recorded by photography as described by Gay *et al.* (18).



FIGS. 1–4. *(Legend appears at bottom of the following page.)*

RESULTS

Ascorbic acid status and the localization of collagen types

In the experiments described in this portion of the paper, the cells in a single dish were exposed to a single one of the available antibodies. Estimates on the proportion of cells synthesizing types I and III procollagen were made by the examination of replicate dishes. When freshly plated cells were examined (not shown), little labeling with antiprocollagen or anticollagen antibody was observed. However, after 3 days in culture the situation was changed. Using antibody to type I collagen, one could observe in cultures receiving ascorbic acid that abundant material labeling with these antibodies was deposited outside of the cells (Fig. 1A). Intracellular labeling was also apparent (Figs. 1B and 4B). Essentially identical patterns were obtained with antibody to type III collagen. The label was associated with almost all cells and was located to a far greater degree outside than inside the cells. Using antibodies to procollagen to label the cells, we observed a different pattern (Fig. 2A and B). Here the great majority of the label was deposited in a perinuclear arrangement well within the borders of the cells. The label shows more granular than even distribution. With antibody to type I procollagen little label was observed outside of the cells (Fig. 2A), while with antibodies to type III procollagen the label was more evenly distributed between the cellular and extracellular compartments (Fig. 3A). A different pattern was observed when cells from an Ehlers–Danlos type VII patient were stained with antibodies to type I procollagen. Here antigenic material was also observed outside of the cells in a fibrillar array (not shown).

In comparison to cells grown with ascorbic acid, cells grown without ascorbic acid showed less extracellular reaction with antibodies to either type I or type III collagen (not shown). However, a much stronger reaction was observed in these cells with the antibodies to precursor forms of the collagens than in the cells which had received ascorbic acid (Fig. 2). Here the antigenic material accumulated with time until it occupied almost the entire cytoplasmic space. Using both the ascorbic-acid-supplemented and deprived cells, we estimated the proportion of labeled cells in the cultures. In general, more than 70% of the cells were labeled by antibodies to type I or type III procollagen. Similar results were obtained with two other strains of normal cells (ATCC nos. CRL 1220 and CRL 1187) and two strains (ATCC nos. CRL 1262 and CRL 1149) from patients with various inherited defects of connective tissue.

To confirm the apparent deposition of label in intra- or extracellular sites, cells grown with ascorbic acid were treated briefly with collagenase. The dishes were washed with phosphate-buffered saline containing both EDTA and 2,2′-bipyridyl after incubation with the enzyme, since collagenase has dual metal requirements (19). After this procedure had been followed we found that the material labeling with anticollagen antibodies outside of the cell was almost completely lost, while little difference was noted in the intensity of staining of intracellular antigens (compare Fig. 1A and B). However, if the chelating agents were omitted from the rinsing solution, the intracellular material was lost. Possibly some of the collagenase remained after rinsing and penetrated through the membranes of the air-dried cells during the staining procedures. These studies confirm the compartmentalization of collagen and collagen precursors that is apparent visually.

In addition to the expected extracellular and perinuclear localization of fluorescent label, we sometimes observed its deposition in the nucleus. An adventitious association would dry, procollagen in the cytoplasm above the nucleus is deposited on the nucleus.

Simultaneous localization of collagen types

Antibodies to type I collagen had been induced in mice, purified, and characterized for other studies by Hahn *et al.* (17). Since no crossreaction was observed with fluorescein-isothiocyanate- or rhodamine-labeled goat antibodies produced against rabbit or mouse gamma globulin, it was possible to obtain specific labeling and the simultaneous localization of collagens I and III in single cells. As shown in Fig. 4A and B, individual cells were observed to label with both antibodies. It was the common observation that most of the cells examined were labeled with both antibodies. In some of the cells examined, the localization of the individual labels was sufficiently discrete to indicate that separate areas of the cytoplasm contained the type I and type III procollagens. In other cells no difference in the deposition of the label was obvious.

Observations with Ehlers–Danlos type IV cells

Previous studies by Pope *et al.* (12) have shown that the tissues of these patients are deficient in type III collagen and that their cells do not synthesize type III collagen. Here we carried out immunofluorescence studies with cell strains obtained from two different Ehlers–Danlos type IV patients. Using antibodies to type I collagen or type I procollagen, we observed the normal distribution of these proteins in these cells. However, no reaction was obtained with antibodies to either type III collagen or type III procollagen (Fig. 3B). The cells were essentially unlabeled and this result was unaffected by ascorbic acid status. The extent of labeling observed in Fig. 3B, where antibodies to type III procollagen were applied to Ehlers–Danlos type IV cells, resembled that observed in various experiments where we assessed the level of nonspecific labeling.

DISCUSSION

Since collagen is synthesized in precursor form, it is possible to obtain antibodies that are directed toward determinants that are located either in the precursor-specific portions or in the collagen portion of the molecule. Additionally, each procollagen and collagen type is sufficiently distinct so that one can obtain specific antibodies against each (20). The specificity of the antibodies used here has been studied. The rabbit antibody to type I procollagen is directed toward a site in the amino-terminal, precursor-specific region of the pro-α1(I) chain (21). The antibody against procollagen type III is directed toward a site in the amino-terminal region of the pro-α1(III) chain (H. Nowack and R. Timpl, unpublished). The rabbit and the mouse antibodies to type I collagen are primarily directed toward a

FIGS. 1–4 (*on preceding page*). *Fig. 1.* Fetal human cells (CRL 1106) were cultured for 3 days with ascorbic acid and the cultures were stained with antibody to type I collagen (1A). The cells shown in 1B were incubated with collagenase. The exposure time of the photograph in Fig. 1B was 1.5 times that of 1A. ×400. *Fig. 2.* Fetal human cells in culture were stained with antibody to type I procollagen. The cells in 2A were grown in the presence of ascorbic acid and those in 2B in the absence of ascorbic acid. Twice the exposure time was used in photographing Fig. 2A as in 2B. ×480. *Fig. 3.* Fetal human cells in cultures with ascorbic acid were stained with antibody to type III procollagen (3A). Cells (CRL 1145) from a patient with the Ehlers–Danlos type IV syndrome showed no staining (3B). ×320. *Fig. 4.* Fetal human cells were double stained with rabbit antibody to type III procollagen (4A) and mouse antibody to type I collagen (4B). Cells were grown for 3 days in the absence of ascorbic acid. ×400.

site in the nonhelical, carboxyl-terminal portion of the α1(I) chain and to helical determinants of the collagen molecule, respectively. The rabbit antibodies to type III collagen are directed toward several sites in both helical and nonhelical portions of the molecule (20).

The specificity of these antibodies has been confirmed in immunofluorescence studies. For example, reticulin fibers in the spleen and liver react with antibodies to type III but not to type I collagen. The reverse is true of the interseptal fibers, which contain type I collagen (22, 23). None of the antibodies reacts with components in cartilaginous tissue or basement membrane (16). We believe the specificity of the antibodies used here is established and that it is possible to interpret the immunofluorescent labeling as showing the localization of specific molecules.

Our results indicate that most cells obtained in culture from normal skin synthesize both type I and type III procollagen. The greatest proportion of the type I procollagen was observed within the cell and the majority of the type I collagen was observed outside the cell. In part, this reflects the manner in which the cells were prepared for labeling with antibodies and also the solubility of collagen and procollagen. Human cells in culture do not convert procollagen to collagen efficiently and a substantial amount of intact procollagen accumulates in the medium and so is removed from the dish. On the other hand, the collagen formed by the cells accumulates outside of the cells because it is not soluble. The conversion of type III procollagen to type III collagen may differ from the metabolism of type I procollagen. Precursor forms of type III collagen are observed in reticulin fibers in normal tissue (16). Similarly, here we observed type III procollagen outside of the cell.

The greatest accumulation of precursor forms of the collagens was observed in the cells not receiving supplemental ascorbic acid. It is well known that the synthesis of collagen is reduced without ascorbic acid [see Barnes and Kodicek (24) for a recent review]. Current evidence indicates that the block is post-translational, a reduced ability to hydroxylate proline (25). Lacking hydroxyproline, the chains are not stable in helical form and are not secreted at a normal rate (26).

It should not always be assumed that the same cell makes both collagens in every tissue. Recent studies by Gay *et al.* (22) and Nowack *et al.* (16) suggest that types I and III collagen are located in separate fibers in various tissues and are not commingled at this level. However, in skin and aorta both types of fibers are found in the same areas (23, 27). Here one could readily imagine that a single cell produced both collagens. On the other hand, in liver and spleen these same authors have found that the type I and type III collagen fibers are in anatomically distinct locations. Here different populations of cells can be presumed to have synthesized them.

The Ehlers–Danlos syndrome type IV

There are a number of different forms of the Ehlers–Danlos syndrome which vary in severity, genetic transmission, and causal defects, as reviewed by McKusick and Martin (28). Most patients have loose hyperextensible joints and skins and are thought to have defects that directly or indirectly impair the crosslinking of type I collagen.

Patients with the type IV syndrome represent a distinctive group. They have fragile but not extensible skin. The condition is life threatening, since major arteries are fragile and susceptible to rupture. Previous studies have indicated that the tissue of these patients lack type III collagen and that their cells do not secrete significant amounts of these proteins (12). Since the same skin cells from other individuals make both type I and III collagens, it is unlikely that there is a lack of a specific cell population. Moreover, since no intracellular accumulation of type III procollagen was observed it is unlikely that we are dealing with a post-translational defect. Rather our studies indicate that the protein is not synthesized by these Ehlers–Danlos type IV strains and suggests that the defect is a transcriptional defect or a gene deletion.

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