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PRODUCTION OF BOTH INTERSTITIAL AND BASEMENT MEMBRANE PROCOLLAGENS BY FIBROBLASTIC WI-38 CELLS FROM HUMAN EMBRYONIC LUNG

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SUMMARY: The synthesis and secretion of type IV procollagen, in addition to that of procollagen types I and III, was detected in cells derived from human embryonic lung (WI-38) by immunofluorescence, metabolic labeling, immunoprecipitation, collagenase digestion and the characteristic polypeptide sizes of both intact procollagen type IV chains and their initial pepsin-resistant fragments as determined by polyacrylamide gel electrophoresis. Locally obtained human embryonic lung cells secreted the same procollagens, but neither embryonic nor adult human skin fibroblasts were found to secrete type IV procollagen in amounts detectable by the same methods.

INTRODUCTION

Collagen gene expression by human fibroblasts in culture usually conforms to a phenotype characterized by procollagen types I and III in a ratio depending on cell growth parameters (1,2). Both these interstitial collagens have been shown to be synthesized by the same cell simultaneously (3). No other molecular type of collagen has so far been demonstrated in cultures of fibroblasts derived from a variety of healthy organs, such as skin (4), gingiva (5) and synovium (6) and including embryonic lung parenchyma (7), a tissue rich in basement membranes, and known to contain four collagen types *in vivo* (8).

On the other hand, basement membrane collagen with characteristic chemical and immunological properties (for reviews, see 9, 10) has been shown to be synthesized by certain diploid non-fibroblastic cells (11,12,13) *in vitro*, as well as by certain tumor cells (14,15). In this report we show production of procollagen types I, III and IV by well characterized fibroblastic cells derived from human embryonic lung, WI-38 (16). Using the same methods, we cannot detect production of basement membrane procollagen (type IV) by skin fibroblasts.

MATERIALS AND METHODS

Cell culture and metabolic labeling. WI-38 lung fibroblasts (ATCC CCL 75), SV-40 virus-transformed VA-13/WI-38 cells (ATCC CCL 75.1),

Abbreviations used: $NaDodSO_4$ = sodium dodecyl sulphate; NaCl/Pi = 0.14 M sodium chloride, 0.01 M sodium phosphate, pH 7.4; $PhCH_2SO_2F$ = phenylmethylsulfonyl fluoride

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HT-1080 and RD sarcoma cells (ATCC CCL 121,136, respectively) as well as locally established human embryonic and adult lung and skin cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum, 100 U/ml of penicillin and 50 µg/ml of streptomycin. The cultures were routinely checked to detect mycoplasma contamination by DNA staining with negative results.

The HT-1080 sarcoma cells produce type IV procollagen as the only detectable collagenous component (Alitalo et al., submitted for publication), while the RD rhabdomyosarcoma cells produce both types III and IV procollagens (15).

For radioactive labeling, the cell cultures were incubated overnight with 10 µCi/ml of both [2-^{3}H]glycine (23 Ci/mmol; The Radiochemical Centre, Amersham, England) and/or L-[5-^{3}H]proline (14 Ci/mmol) in serum-free medium containing 0.05 % bovine serum albumin and antibiotics. Sodium ascorbate (30-50 µg/ml) and β-aminopropionitrile fumarate (50 µg/ml) were added to the labeling medium. Protein-bound radioactivity was determined by precipitation with 5 % trichloracetic acid.

Antibodies. Antibodies to collagens were a generous gift of Dr. Rupert Timpl, Max-Planck-Institut für Biochemie, Martinsried, FRG. Antibodies against human placental type IV collagen were purified from antisera raised in rabbits as described (17). The preparation of antiserum against human plasma fibronectin followed techniques described earlier (18). Antibodies against procollagen types I and III and to collagen type II (19) were those used in earlier studies on procollagens of sarcoma cells (14,15). The specificities of the antisera and lack of their cross-reactions between the various antigens have been established in these previous reports.

Immunofluorescence. Immunofluorescence of cell cultures fixed with paraformaldehyde and acetone as well as control experiments indicating specificity of the staining method were done as described (20). Unfixed frozen sections of tissue were treated with affinity-purified antibodies and stained like the cell layers. Fluorescence was observed with Leitz Dialux 20 fluorescence microscope and related to culture topography by use of phase-contrast optics.

Immunoprecipitation. Fibronectin was immunoprecipitated from the labeled culture medium in the presence of 1 mM $PhCH_2SO_2F$ by the double antibody method as described (21). Type IV collagen and procollagen types I and III were immunoprecipitated by adding per ml of sample 10-20 µg of specific antibodies diluted in NaCl/Pi containing 500 µg/ml of bovine serum albumin. After incubation for one hour at room temperature, a pretested amount of carrier rabbit serum and anti-rabbit IgG serum was added to precipitate all of the rabbit antibodies within an incubation overnight at +4°C. The precipitates were collected by centrifugation at 12.800 xg, washed several times with NaCl/Pi and dissolved in the electrophoresis sample buffer. The specificity of immunoprecipitation was controlled by using either nonimmune rabbit serum or antibodies raised against type II collagen. Background counts precipitated with these control reagents were low.

Isolation and analysis of secreted proteins. Culture medium was harvested by centrifugation and proteins were precipitated with $(NH_4)_2SO_4$ (176 or 281 mg/ml) overnight at +4°C in the presence of gelatin-carrier, protease inhibitors and 1 mM α,α'-dipyridyl (22). Precipitates collected by centrifugation were washed twice with 70 % ethanol and analyzed in polyacrylamide gel electrophoresis in $NaDodSO_4$ according to Laemmli (23) using fluorography (24) for the visualization of protein bands. Medium was digested with bacterial collagenase (form III, Advance Biofactures Corp., Lynbrook, NY) at 37°C for 60 min using 30 units/ml. For pepsin digestion, acetic acid was added into the medium to a final concentration of 0.5 M and pepsin (Sigma, 3 x crystallized, dissolved in 0.5 M acetic acid) was added to give a concentration of 100 µg/ml. Reaction was continued at 15°C for indicated periods of time. Pepsin digestion was stopped by neutralizing the acetic acid with an equimolar

amount of 1 M NaOH. Proteinase inhibitors were added and the polypeptides were precipitated with ammonium sulphate as above.

RESULTS

Cultures of WI-38 cells were labeled for periods of 6 h to 24 h with tritiated amino acids. Nearly linear incorporation of radioactivity into collagenase-senstitive acid-precipitable protein in the medium (about 45 % of radioactivity incorporated from [^{3}H]proline) was still taking place after an

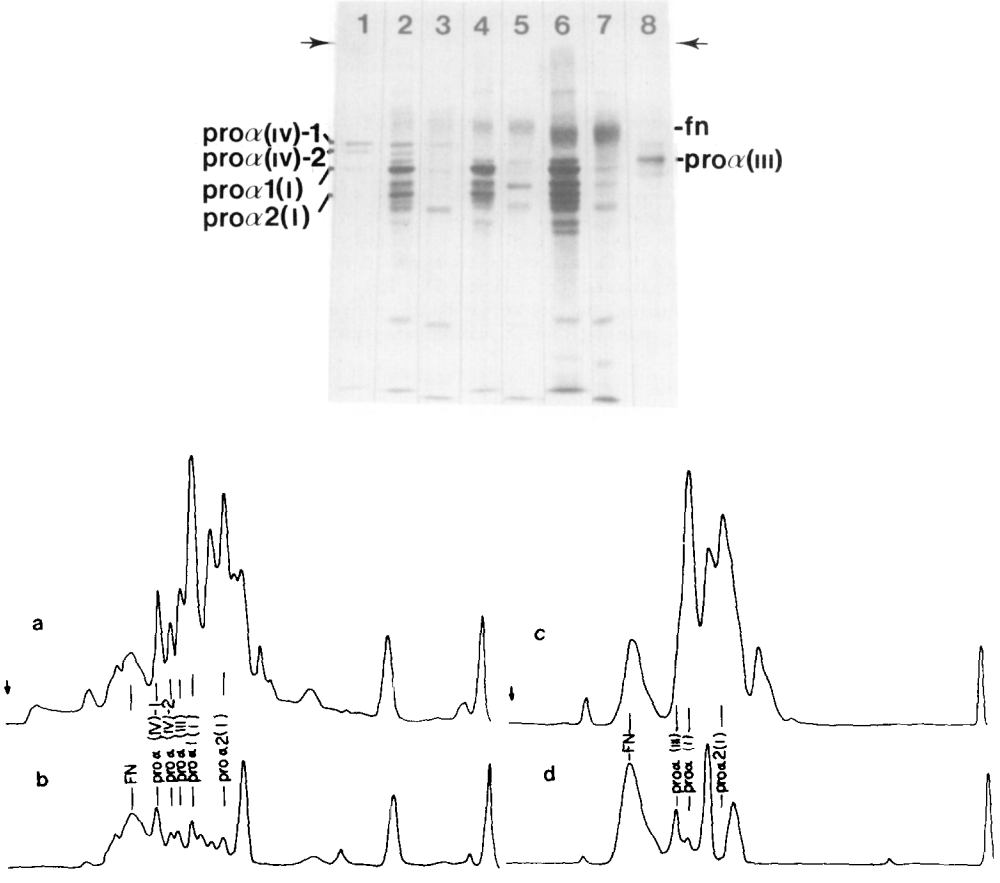


Figure 1. NaDodSO$_4$-polyacrylamide gel analysis under reducing conditions of [^{3}H]proline-labeled proteins in fibroblast culture media. Lanes 2 and 3 show proteins secreted by WI-38 cells precipitated with ammonium sulphate after an incubation with bacterial collagenase (3) or with buffer only (2). Similar collagenase digestion analysis for secreted proteins of human embryonic (lanes 5 and 4, respectively) and adult (lanes 7 and 6, respectively) skin fibroblasts are shown for comparison. Lanes 1 and 8 show secreted proteins of human HT-1080 and RD sarcoma cell cultures that identify the position of migration of procollagen proα(IV)-1 and proα(IV)-2 chains (lanes 1, 8) and procollagen type III pro α-chains (proα(III), lane 8; see text for further details). Proα1(I), proα2(I) and fn show migration of the procollagen type I chains and fibronectin. Scanning densitograms of similar material to those of lanes 2, 3, 4 and 5 are shown in a, b, c and d, respectively. Arrows indicate the top of the separating gel.

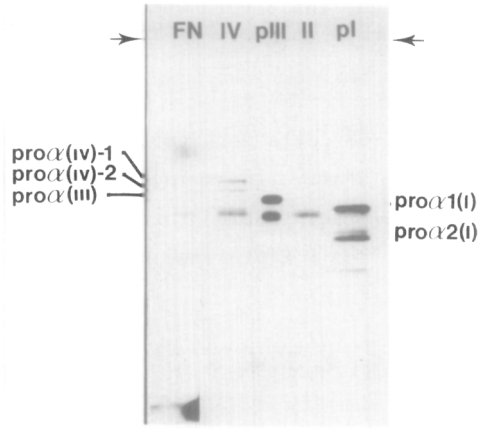
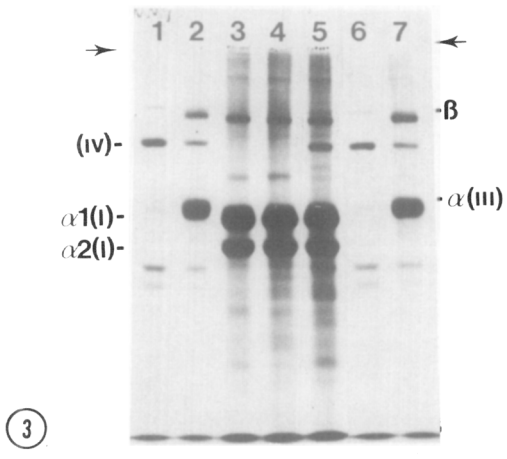


Figure 2. $NaDodSO_4$-polyacrylamide gel analysis of immunoprecipitated [^{3}H]-proline-labeled connective tissue glycoproteins in WI-38 cell culture medium. Lanes are marked according to antibodies used in the precipitations: FN, anti-fibronectin antiserum; IV, anti-type IV collagen; pIII, anti-procollagen type III; II, anti-collagen type II; pI, anti-procollagen type I antibodies. Other symbols are according to those in fig. 1. - The polypeptide at 170 kd (globular markers) precipitated by antibodies to type II collagen was seen to contaminate all immunoprecipitates (cf. lanes IV, FN). It was identified as the reduced subunit of α_2-macroglobulin known to be secreted in high amounts by WI-38 cells (33). $Alpha_2$-macroglobulin binds non-specifically to immune complexes (37).

incubation overnight. Analysis of radioactive polypeptides precipitated with ammonium sulphate (176 or 281 mg/ml) from culture medium labeled overnight by electrophoresis in 5 % polyacrylamide gels showed several (5 major) polypeptides (lane 2, fig. 1) that were sensitive to bacterial collagenase (lane 3). Three of these polypeptide chains were identified as subunits of procollagen types I (proα1(I), proα2(I)) and III (proα(III) in fig. 1) by their characteristic mobility as compared with procollagen markers and by immunoprecipitations (lanes pI and pIII in fig. 2).

The two collagenase-sensitive polypeptides migrating in between fibronectin and procollagen type III (lanes 2 and 3, fig. 1) were not seen in similarly prepared samples from skin fibroblast cultures (lanes 4 and 6, cf. densitometric scan of 4 shown in c, fig. 1), even after prolonged exposure of the gels to x-ray films, but they comigrated with procollagen type IV chains from sarcoma cell cultures (lanes 1 and 8). These bands were specifically immunoprecipitated with antibodies to human type IV collagen (lane IV, fig. 2; the chains are designated as proα(IV)-1 and proα(IV)-2 according to P. Bornstein). Further evidence for the presence of IV procollagen in WI-38 culture medium was the finding of characteristic initial pepsin- or chymotrypsin-resistant collagenase-sensitive fragments of type IV procollagen (12; cf. markers of sarcoma cells in fig. 3) in samples of labeled media treated with



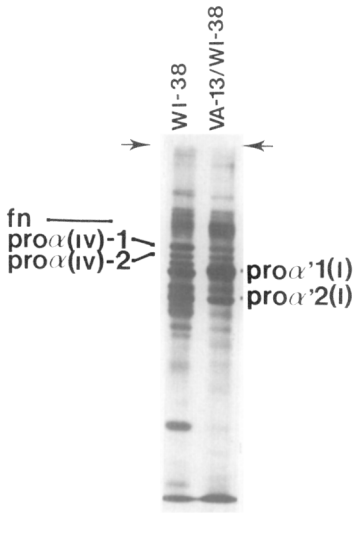


Figure 3. NaDodSO$_4$-polyacrylamide gel analysis of pepsin-resistant [^{3}H]-glycine-labeled polypeptides in culture media. - Culture medium of WI-38 cells (lane 5) as compared to human adult (lane 3) or embryonic (lane 4) skin fibroblasts. As markers, similarly treated media from human HT-1080 (lanes 1 and 6) and RD (lanes 2 and 7) sarcoma cell cultures (see the text) containing type III collagen (lanes 2 and 7, marked α(III)) and initial pepsin-resistant fragments of type IV collagen (lanes 1, 2, 6 and 7, marked (IV)) as well as pre-stained (38) type I collagen chains (lanes not shown, positions of migration marked α1(I), α2(I)) and β-components (ß) were used. Arrows indicate the top of the separating gel.

Figure 4. NaDodSO$_4$-polyacrylamide gel analysis under reducing conditions of [^{3}H]glycine and [^{3}H]proline-labeled proteins in WI-38 (left lane) and VA-13/WI-38 (right lane) cell culture media. Note that the pro (I) chains produced by VA-13/WI-38 cells (marked proα'(I)) migrate slower than those produced by WI-38 cells. Other symbols are as in fig. 1.

these proteases for 6 h at 15°C (shown for pepsin in fig. 3). In nonreducing conditions the type IV procollagen-derived fragment (marked (IV) in fig. 3) did not enter the 5 % separating gel (not shown).

In preliminary experiments on freshly seeded WI-38 cells and adult skin fibroblasts we found no difference in the pattern of procollagens labeled as compared to confluent cultures. The results did not differ whether young (7th) or old (24th) passages of either cells were used. In medium of WI-38 cells, we did not find obvious variability according to culture parameters in the basement membrane to interstitial collagen ratio (about 4 %) as assayed by radioactivity in the known polypeptide bands. Also, SV-40 virus-transformed WI-38 cells, VA-13/WI-38 (25), which probably secrete overhydroxylated form of type I procollagen in culture (26), appeared to retain their basement membrane procollagen production *in vitro* (fig. 4).

To locate type IV procollagen-secreting cells, WI-38 and control cell layers grown in the absence of sodium ascorbate were stained by double

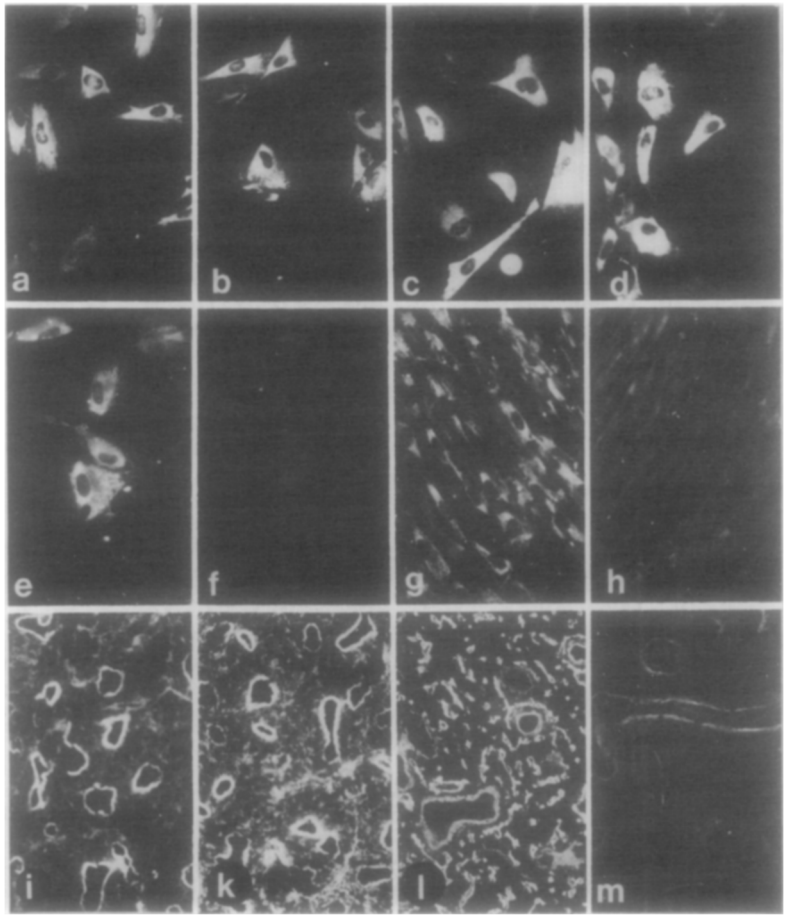


Figure 5. Immunofluorescence localization of collagenous antigens in fibroblast cultures (a-h) and in embryonic lung tissue (i-l). - One day after subculture (a-f) fixed WI-38 cells stained for (a) procollagen type I, (b) procollagen type III, (e) collagen type IV, (f) collagen type II. Human embryonic skin fibroblasts stained for (c) procollagen type I, (d) procollagen type III. Five days after subculture fixed WI-38 cells (g) and human embryonic skin fibroblasts (h) stained for collagen type IV. The exposure time for figs. a-h was 20 seconds. The prints were developed as to show cellular contours also when no fluorescence was present. (x 180). To illustrate specific patterns of distribution for the antigens used, frozen sections of a fetal (3 months of gestation; from a therapeutic abortion) female lung was stained with antibodies to (i) collagen type I, (k) procollagen type III, (l) collagen type IV, (m) collagen type II (x 50). Positive staining shown in (m) was obtained only after hyaluronidase (ovine testis; Serva, Heidelberg, FRG) treatment of the section (2 mg/ml in NaCO/Pi, 30 min at +21^{o}C) in accordance with the results of von der Mark et al. (39).

antibody immunofluorescence method for procollagen types I, III and collagen types IV and II. Most (over 80 %) lung cells showed intracellular, granular fluorescence both for procollagen types I (panel i, j, fig. 5) and III (k, l), as has previously been reported (3) and for collagen type IV (a, b, e, f), but not for collagen type II (c, d).

DISCUSSION

In this report we show production of basement membrane procollagen (type IV) in addition to interstitial procollagen (types I and III) *in vitro* by fibroblastic cells from human lung. Previously, synthesis, secretion and deposition of interstitial procollagens by the same cells has been documented in many contexts (20,27,28,29). As we in this report could verify the same pattern of procollagen type production for different strains of morphologically uniform fibroblastic cells from the lung as well as for the SV-40-transformed WI-38 cells, the data exclude the possibility that one here was dealing with a minor, selected clone or contaminating cells that would be present in the WI-38 population. In a more recent study (R. Myllylä, K. Alitalo, A. Vaheri and K. Kivirikko, in preparation) we also found a difference in total hydroxylysine, glycosylated hydroxylysine, and the collagen-specific post-translational enzyme activities between WI-38 and adult skin fibroblast cultures.

As the disulfide-reduced procollagen type IV polypeptides migrate close to the reduced β-components of type I collagen in polyacrylamide gels run according to Laemmli (23), they may earlier have been confused with the latter unless appropriate markers were used.

Bornstein and coworkers have also observed variable, small amounts of type IV procollagen being produced by cloned human fibroblasts obtained from second trimester amniocentesis aspirate (personal communication; see also discussion in ref. 12). The basement membrane procollagen production reported here seems to be a constitutional property of WI-38 cells. Whether culture parameters can modulate to any extent the amounts of the different collagen types in the lung and skin fibroblast culture systems, is currently under study.

Differential characteristics of fibroblastic cells derived from a variety of organs are poorly recognized in the literature, although both immunological (30) and enzymatic (31) criteria have been reported to distinguish between many of them. WI-38 cells are known to be exceptionally sensitive to growth of many viruses. Unlike skin fibroblasts they are also known to secrete high amounts of plasminogen activator (32) and of α_2-macroglobulin (33), properties shared by endothelial (34) and some epithelial (35,36) cells *in vitro*.

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