Collagen Gene Expression by Cultured Human Skin Fibroblasts

Abundant Steady-state Levels of Type VI Procollagen Messenger RNAs

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Abstract

Previous studies have suggested that procollagen types I and III are the major collagenous gene products of cultured human skin fibroblasts. In this study the expression of 10 different genes, encoding the subunit polypeptides for collagen types I–VI, by human skin fibroblasts in culture was analyzed by molecular hybridizations. Northern transfer analysis demonstrated the presence of specific mRNA transcripts for collagen types I, III, IV, V, and VI, but not for type II collagen. Quantitation of the abundance of these mRNAs by slot blot hybridizations revealed that type I, III, and VI procollagens were the major collagenous gene products of skin fibroblasts in culture. The mRNAs for type IV and V collagens represented only a small percentage of the total collagenous mRNA transcripts. Further analysis by in situ hybridization demonstrated that the majority of the cultured cells coexpressed the genes for type I, III, and VI procollagen pro-α chains. Further in situ hybridization analyses revealed the expression of type VI collagen genes in normal human skin. These data demonstrate that human skin fibroblast cultures can be used to study the transcriptional regulation of at least nine genetically distinct procollagen genes. The data further suggest that type VI collagen, in addition to types I and III, may be a major collagenous component of human skin.

Introduction

Fibroblasts, differentiated mesenchymal cells, are the major cell type responsible for the synthesis of collagen, the predominant extracellular matrix component in skin and other connective tissues. Previous studies have suggested that genetically distinct procollagens type I and III are the major collagenous gene products expressed by cultured dermal fibroblasts (1). These conclusions were initially based on biosynthetic studies using chromatographic and electrophoretic separation of type I and III collagen chains. Additional lines of evidence using biochemical techniques and immunodetection with specific antibodies have suggested that fibroblastic cells are capable of synthesizing other genetically distinct procollagens, including types IV, V, and VI (2–6). In accordance, the presence of specific mRNA transcript for the corresponding α-chains has been detected in skin fibroblasts by Northern transfer analysis (7–10). The results of these studies indicated that type IV collagen is a minor gene product in human skin fibroblast cultures (7, 8). The synthesis of collagens type V and VI, relative to types I and III, has not been quantitated, although the steady-state mRNA ratio for type I/VI collagen in a fetal skin fibroblast cell strain was shown to be ~ 5.3 (10).

A recent study has suggested that type VI collagen represents a major fraction of connective tissue collagens in a variety of tissues (11); however, the amount of type VI collagen in human skin was not determined. Type VI collagen is a heterotrimer consisting of α1(VI), α2(VI), and α3(VI) chains, which are synthesized as polypeptides with M_r of ~ 140, 140, and 260 kD, respectively (5, 12–14). These collagenous molecules contain unusually large globular domains at the ends of the polypeptides (5, 6). Type VI collagen molecules have been suggested to assemble into dimers and tetramers by lateral association before secretion (13). The assembly is stabilized by extensive disulfide bonding and this aggregation mechanism may be unique to type VI collagen (13). The genes coding for these polypeptides have been localized to human chromosomes 21 (α1 and α2 chains) and 2 (α3 chain) (15). Alterations in type VI collagen have also been implicated in heritable diseases (16, 17).

The present study examined the expression of 10 different procollagen genes, including the three subunits of type VI collagen, using three different mRNA hybridization techniques. First, the presence of specific mRNAs for distinct procollagen genes was analyzed by Northern transfer hybridizations with human sequence-specific cDNAs for types I–VI procollagen polypeptides. Second, the relative levels of expression of these genes were quantitated by slot blot hybridizations. Finally, in situ hybridizations were performed using cultured fibroblasts to address the issue of whether an individual cell has the capability of synthesizing several collagen types simultaneously, or whether evidence for heterogeneity with respect to collagen gene expression can be found within a defined population of normal diploid fibroblasts.

Methods

Cell cultures. Human skin fibroblast strains obtained from the American Type Culture Collection (Rockville, MD) or the Human Genetic Mutant Cell Repository (Camden, NJ) were cultured in DME supplemented with 2 mM glutamine, 50 μg/ml streptomycin, 200 U/ml penicillin G, 20 mM *N*-2-hydroxyethylpiperazine-2′-ethane sulfonic acid, pH 7.4, and 10% FCS. Total mRNA was isolated from the cell cultures in early visual confluency by extraction with guanidinium isothiocyanate, followed by cesium chloride density gradient centrifugation (18). Poly(A)$^+$RNA was isolated by oligo(dT) cellulose chromatography (19).

RNA hybridizations. For Northern transfer analysis RNA was electrophoresed on 1% agarose gels under denaturing conditions,

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transferred to nitrocellulose filters (20), and hybridized with cDNAs labeled by nick translation with $\alpha[^{32}P]$dCTP to a specific activity of at least 1×10^8 cpm/μg as described previously (7).

The relative abundance of the different collagenous mRNAs was determined by slot blot hybridizations. Total RNA was dotted onto nitrocellulose filters in varying concentrations and the filters were hybridized with radioactive cDNAs (7). The [^{32}P]cDNA-mRNA hybrids were visualized by autoradiography and the mRNA levels were quantitated by scanning densitometry of the autoradiograms. To allow comparison of the relative expression of genes coding for the genetically distinct collagen types, the densitometric units/microgram RNA dotted were corrected for the specific activities and sizes of the cDNAs, as well as for α-chain composition of the collagen molecules (21).

For in situ hybridization the cells were grown on acetylated object glasses (22), permeabilized, and fixed with 95% ethanol at −20°C for 15 min and 4% paraformaldehyde at room temperature for 15 min, after which the cells were pretreated to block charged groups as described (22, 23). The samples were hybridized in the presence of 50% formamide and 10% dextran sulfate for 50 h at 42°C using cDNAs labeled with [^{35}S]dATP by nick translation. After hybridization the samples were washed in solutions of decreasing ionic strength at increasing temperature, treated with S1-nuclease to degrade nonspecifically-bound single-stranded cDNA, washed, and dehydrated. The [^{35}S]cDNA-mRNA hybrids were detected using Kodak NTB 3 emulsion by exposing the samples for 5 d at 4°C. The samples were subsequently developed using Kodak D-19 developer (15°C, 120 s) and stained with hematoxylin to visualize the nuclei. The specimens were photographed using a Nikon optiphot microscope equipped with camera attachments (23).

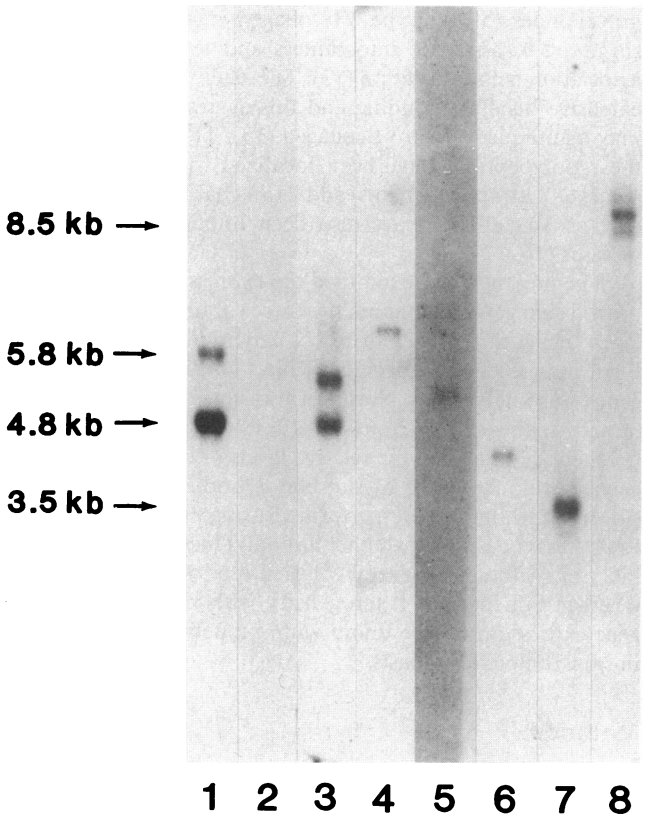

Figure 1. Northern transfer analysis of human skin fibroblasts mRNA. Poly(A)$^+$RNA was isolated from adult human skin fibroblast cultures and 1 μg RNA/lane was electrophoresed on a 1% agarose gel. The RNA was transferred to nitrocellulose filters, and parallel lanes were hybridized with α1(I), α1(II), α1(III), α2(IV), α2(V), α1(VI), α2(VI), and α3(VI) procollagen cDNAs in lanes *1–8*, respectively. The numbers on the left correspond to the sizes of the mRNA transcripts, in kilobases, of the α2(VI) (3.5 kb), and α3(VI) (8.5 kb) chains of type VI procollagen and the proα1(I) (4.8 and 5.8 kb) polymorphic transcripts of type I procollagen.

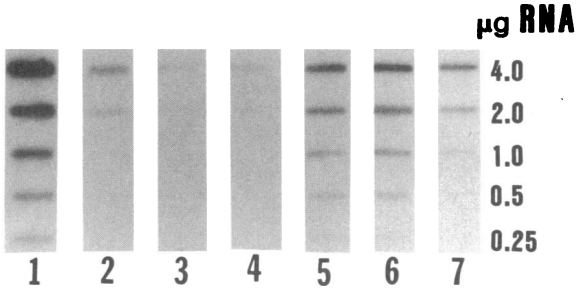

Figure 2. Slot blot hybridizations of RNA from a human skin fibroblast strain with different procollagen cDNAs. Total RNA was isolated from a fibroblast strain established from the skin of a 54-yr-old individual. Varying amounts of RNA, 4.0–0.25 μg, were dotted onto nitrocellulose filters and parallel filters were hybridized with α1(I) (lane *1*), α1(III) (lane *2*), α1(IV) (lane *3*), α2(V) (lane *4*), α1(VI) (lane *5*), α2(VI) (lane *6*), and α3(VI) (lane *7*) procollagen cDNAs. The filters were hybridized and washed in parallel (see Methods) and exposed to x-ray film for 24 h.

To demonstrate the expression of type VI collagen genes in vivo, specimens were obtained from the skin of a 19-wk-old fetus and from the apparently normal perilesional skin of a 52-yr-old female patient with type 1 neurofibromatosis. 5-μm frozen sections were pretreated and hybridized with a ^{35}S-labeled α1(VI) collagen cDNA as described previously in detail (23). Detection of [^{35}S]cDNA-mRNA hybrids was then performed as above.

cDNA probes. The following human sequence-specific cDNAs were used for the three different types of hybridizations: for type I collagen, a 1.8-kb proα1(I) (Hf 677) cDNA (24) and a 2.2-kb proα2(I) (Hf 32) cDNA (25); for type II collagen, a 0.525-kb proα1(II) (pCAR1) cDNA (26); for type III collagen, a 1.3-kb proα1(III) (Hf 934) cDNA (27); for type IV collagen, a 2.6-kb proα1(IV) (HT-21) cDNA (28) and a 1.7-kb proα2(IV) (HT-39) cDNA (29); for type V collagen, a 2.5-kb proα2(V) (Hf 511) cDNA (30); for type VI collagen, three different cDNAs, 2.1- (p18), 1.5- (p8), and 1.5- (p24) kb in size, coding for the proα1(VI), proα2(VI), and proα3(VI) chains, respectively (10).

Results and Discussion

To examine the expression of the different procollagen genes, poly(A$^+$)RNA isolated from adult human skin fibroblast cultures was subjected to Northern hybridizations with cDNAs corresponding to the pro-α chains of type I–VI procollagens. Autoradiograms of the Northern blots revealed the presence of characteristic mRNA transcripts corresponding to α1(I) (4.8 and 5.8 kb), α1(III) (5.4 and 4.8 kb), α2(IV) (6.7 kb), and α2(V) (5.2 kb), as well as the α1 (4.2 kb), α2 (3.5 kb), and α3 (8.5 kb) chains of type VI procollagen, when hybridized with the respective cDNAs (Fig. 1). Similarly, characteristic transcripts corresponding to the α2(I) (5.2, 4.6, and 4.2 kb) and α1(IV) (6.8 kb) procollagen polypeptides were detected (not shown). However, an α1(II) procollagen cDNA did not reveal any specific hybridization with the same RNA preparations even after extended exposure of the Northern filters to x-ray films (Fig. 1). These data demonstrate that human skin fibroblasts express the genes coding for the subunit polypeptides of type I, III, IV, V, and VI procollagens but not for type II procollagen.

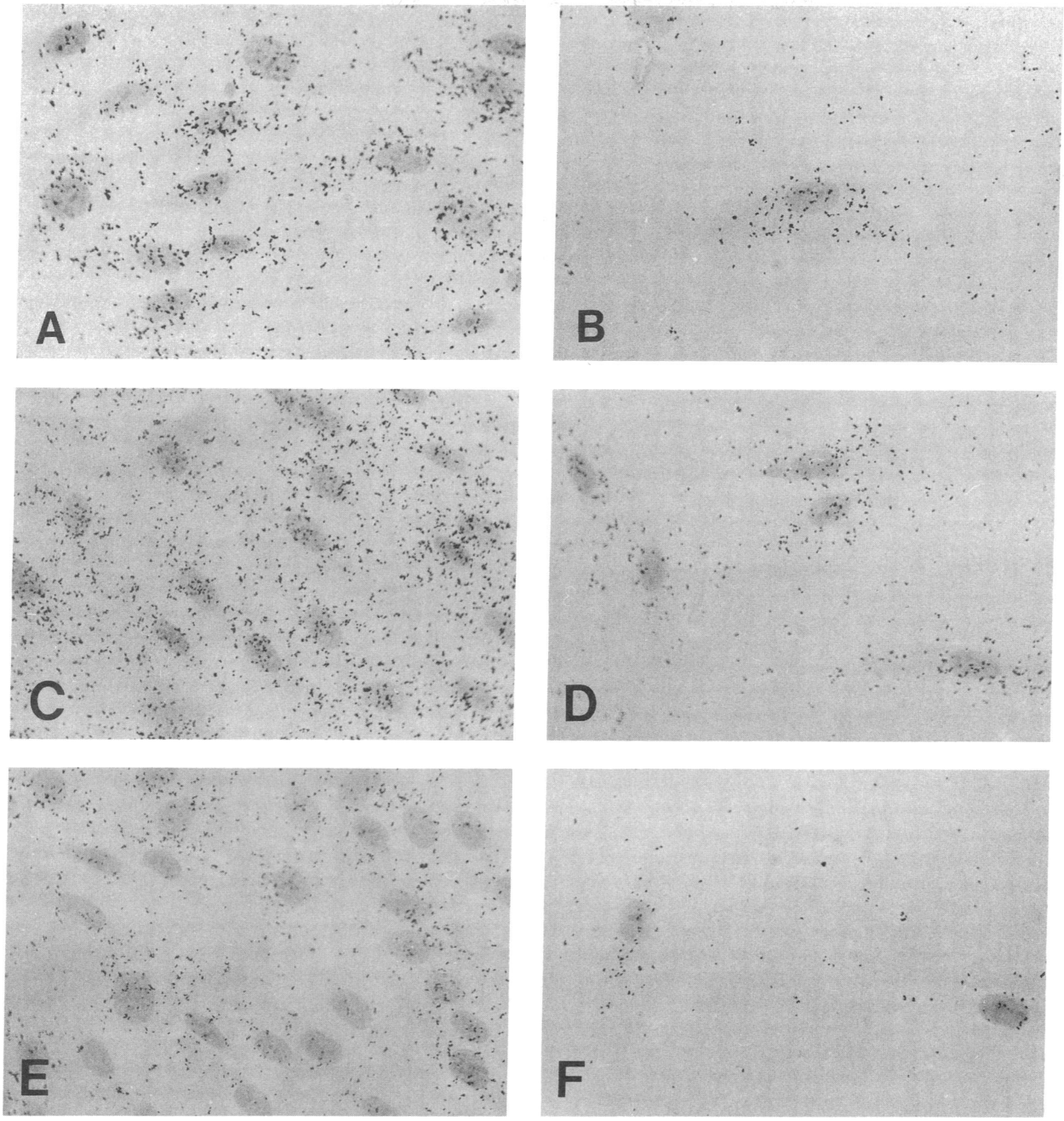

Figure 3. In situ hybridization of human skin fibroblast cultures in relatively high (*A, C,* and *E*) or low (*B, D,* and *F*) density. Hybridizations were performed with α1(I) (*A, B*), α1(III) (*C, D*), or α3(VI) (*E, F*) procollagen cDNAs.

Our initial impression, based on the intensity of the bands detected by Northern transfer analysis, was that the steady-state abundance of mRNAs for type I and VI collagens was the highest among the five genetically distinct types of collagens expressed in these cells. This observation was somewhat surprising because type VI collagen has been thought to account for a minor portion of total collagen in human dermis (31). To determine the relative expression of genes coding for the different procollagen pro-α chains, the ratios of the corresponding mRNA steady-state levels were quantitated by slot blot hybridizations. A representative set of blots is shown in Fig. 2. Since all the hybridizations were done in parallel using the same amount of RNA from the same preparation, and the exposure time of all blots was the same, these analyses allow a direct comparison of the relative abundance of the corresponding mRNAs (Fig. 2). The mRNA levels for type IV and V procollagens were found to represent < 1% of the total collagenous mRNAs. Examination of 15 separate control skin fibroblast strains indicated that the ratio of type I/VI procollagen mRNAs was 3.24±0.99 (mean±SD, n = 24). There was no apparent correlation between the age (20 fetal wk and 87 yr) or sex (4 male vs. 11 female) of the donors, or the passage number of the cell cultures (6–11 passages). The mRNA ratio of α1(VI), α2(VI), and α3(VI) chains was ~ 1:1:1, a ratio previously suggested by Northern transfer analysis (10). This stoichiometry also has been noted at the protein level (11–14).

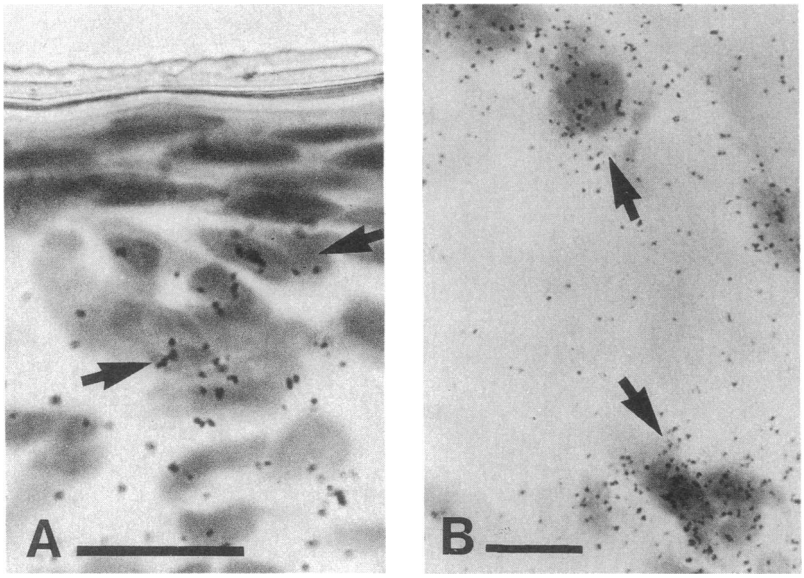

Figure 4. In situ hybridization of human skin with an α1(VI) procollagen cDNA. Frozen sections of normal human skin from a 19-wk-old fetus (*A*) or a 52-yr-old female (*B*) were hybridized under conditions described in Methods. After exposure the nuclei were visualized by counterstaining with hematoxylin. Note the presence of cytoplasmic grains representative of [^{35}S]cDNA-mRNA hybrids (*arrows*). The bars represent 20 μm.

Our data suggest that type VI collagen may be a more abundant connective tissue protein in human dermis than has been recognized earlier. This suggestion is supported by a recent study based on a new procedure for extraction of type VI collagen from other tissues (11). Using the improved method a tenfold higher yield of the protein was obtained from human placenta, bovine uterus, chicken gizzard, and whole mouse body, suggesting that type VI collagen is a major component of these tissues (11).

In situ hybridization was used to address the issue of whether an individual fibroblast was capable of coexpressing multiple procollagen genes, or whether evidence of heterogeneity with respect to collagen production could be observed within cultures of normal fibroblasts. This question was prompted by previous suggestions (31) that type VI collagen segregates into microfibrils that are clearly distinct by morphologic criteria from type I and III collagen-containing fibers. Hybridizations with α1(I) and α1(III) procollagen cDNAs revealed that essentially all cells present in fibroblast monolayer cultures uniformly expressed these genes both in high- and low-density cultures (Fig. 3). Similar observations were made with the α2(I) cDNA (not shown). Furthermore, the majority of the cells clearly expressed the α3(VI) gene of type VI procollagen (Fig. 3, *E* and *F*). Thus, on a statistical basis it is clear that a single fibroblast can coexpress at least four [α1(I), α2(I), α1(III), α3(VI)] and probably six (all subunits of collagen types I, III, and VI) different procollagen genes. These observations attest to the omnipotent characteristic of cultured human skin fibroblasts with respect to procollagen gene expression. These results also extend previous demonstrations of colocalization of type I and III procollagen epitopes on a single fibroblast cell as detected by immunocytochemistry (32). Thus, our results indicate that although type VI collagen is present in fibrils distinct from type I and III collagen fibers, all three collagen types are synthesized by the same cells. Therefore, the aggregation mechanisms unique to type VI collagen are apparently responsible for segregation of these collagen types to different fibrillar structures of the matrix (13, 31).

In situ hybridizations with type IV and V procollagen cDNAs were inconclusive. These findings are probably attributed to the low abundance of the corresponding mRNAs in these cells, as suggested by Northern transfer analysis. In situ hybridizations with the α1(II) procollagen cDNA yielded only a background signal, consistent with nonexpression of this gene.

To demonstrate the expression of type VI collagen genes in vivo, in situ hybridizations were also performed with sections of normal human skin. Direct evidence of the expression of α1(VI) collagen gene was obtained both in fetal and adult human dermis (Fig. 4). Thus, expression of type VI collagen genes in human skin fibroblast cultures appears to reflect the expression of these mRNAs in the relevant tissue.

In summary, the results of this study indicate that cultured human skin fibroblasts express the genes encoding type I, III, IV, V, and VI procollagens. The relatively high abundance of type VI procollagen mRNAs suggests this collagen may be a major connective tissue component of the skin. Thus, fibroblast cultures provide a convenient system to study aberrations in the expression of numerous procollagen genes in diseases, including those encoding type VI collagen.

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