

Expression of type XVI collagen in cultured skin fibroblasts is related to cell growth arrest

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Abstract The expression of type XVI collagen in various phases of cell growth in cultured skin fibroblasts was studied. A marked increase in type XVI collagen mRNA level was found in stationary phases of cell growth (non-adherent and confluent phases), whereas the expression of type I and III collagens was undetectable in the non-adherent phase but became greater in the confluent phase. When suspended cells were further cultured over 72 h (suspension arrest), mRNA level and gene transcription of type XVI collagen were time-dependently increased whereas those of type I collagen remained undetectable. When the confluent cells were further cultured for 72 h under the condition of serum deprivation (serum deprivation arrest), mRNA levels of both type XVI collagen and type I collagen were elevated. The level of type XVI collagen polypeptide in the culture media of suspension-arrested and serum deprivation-arrested cells paralleled the mRNA level of type XVI collagen. The results indicate that expression of type XVI collagen (a member of the fibril-associated collagens with interrupted triple helices), unlike interstitial collagens (type I collagen), is related to cell growth arrest brought about by two different growth inhibiting systems, suspension arrest and serum deprivation arrest.

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Key words: Type XVI collagen; Cell adhesion; Type I and III collagen; Skin fibroblast

1. Introduction

Collagen, a major constituent of connective tissue, is a large family of proteins with distinct tissue distributions and functions [1,2]. On the basis of their structure and function, the collagens are generally divided into two categories: the fibril-forming collagens and the non-fibril-forming collagens. The fibril-forming collagens such as type I, II, III, V and VI have long central triple-helical domains with Gly-X-Y repeating sequences and form highly organized fibrils in a quarter-staggered fashion. Type I and III collagens are major extracellular collagens in the skin (interstitial collagens). The latter classes of collagens are very heterogeneous in size and have imperfections in the Gly-X-Y repeating sequences as a common feature. Type IX, XII and XIV collagens form a sub-

group named the fibril-associated collagens with interrupted triple helices (FACIT) [3].

The gene of type XVI collagen has been isolated from human fibroblast and placenta cDNA libraries. The predicted polypeptide, designated $\alpha_1(\text{XVI})$, consists of 10 collagenous domains which are interspersed with 11 non-collagenous domains exhibiting several structural features characteristically seen in members of FACIT and cuticle collagens of nematodes [4,5]. However, no information on the physiological function of type XVI collagen has been available.

The proliferation of connective tissue cells including skin fibroblasts in culture is strongly dependent on attachment to a solid surface, which has been called anchorage dependence [6–8]. Attached cells spread, move out, and grow to colonize the surface. Upon adhesion, cells synthesize and deposit their own extracellular matrix components to spread and proliferate efficiently on the culture dishes. For example, it has been reported that adhesion of mouse 3T3 fibroblasts to plastic dishes results in the induction of growth-associated genes (*c-fos* and *c-myc*) and type I collagen gene, a major interstitial collagen [9,10]. To determine the functional role of type XVI collagen in cell adhesion and proliferation, we have studied the expression of type XVI collagen as well as type I and III collagens in the various phases of cell growth: non-adherent, attaching, spreading, proliferating and confluent phases.

2. Materials and methods

2.1. Fibroblast culture

Normal human skin fibroblasts were explanted from the skin obtained from the normal portion of benign skin tumors. The cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and sodium bicarbonate (3.7 g/l) in a humidified atmosphere of 5% CO₂ in air at 37°C. Cells of the third or fourth culture were used in this experiment. Non-adherent cells were obtained by trypsin treatment (0.05%) of 50% confluent cells (corresponding to day 3 in culture, see below) followed by suspension in DMEM containing 10% FBS for 10 min. To study the growth kinetics, the non-adherent cells were plated at a density of 2.2×10^4 cell/cm² in 35 or 100 mm diameter tissue culture dishes, and cultured in DMEM supplemented with 10% FBS for 6 h, 1, 3 or 8 days. At the end of the culture periods, the number and the shape of the cells were observed with an optical microscope. To bring cells to quiescence, the non-adherent cells obtained from day 3 in culture were suspended in 0.1% methylcellulose (4000 cP, Sigma, St. Louis, MO, USA) dissolved in DMEM containing 10% FBS at a density of 1.5×10^5 cells/ml [11] and cultured for 24, 48 or 72 h at 37°C (suspension arrest), or cultures of confluent cells on day 8 were placed under the condition of serum starvation (0.5% FBS) for 0, 24, 48 or 72 h (serum deprivation arrest).

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2.2. Ribonuclease protection and Northern blot assays

Ribonuclease protection assays were performed to quantitatively determine the steady-state level of specific $\alpha_1(\text{XVI})$ mRNA in dermal fibroblasts, because $\alpha_1(\text{XVI})$ mRNA signals were hardly detected by Northern blot assays. The fragment (*Bam*HI/*Pst*I, 604 bp) containing the N-terminal non-collagenous region of $\alpha_1(\text{XVI})$ collagen (NC11 domain) [4,5] was subcloned into pGEM vector (Promega, Madison, WI, USA). Antisense RNA probes were transcribed from the template sequences with T7 RNA polymerase and labeled with [α - 32 P]UTP (16 TBq/mmol, Amersham) according to the supplier's protocol (Promega). Full-length transcripts were purified from acrylamide (6% acrylamide/8 M urea) gel and used as probes. The probes were hybridized with sample RNAs isolated from cultured fibroblasts [12] at 42°C overnight, then digested with RNase A (10 U/ml) and RNase T₁ (2000 U/ml) (Ambion, Austin, TX, USA) at 37°C for 3 h. The protected RNA fragments were analyzed on sequence gel (6% acrylamide/8 M urea) and exposed to X-ray films [13]. Northern blot analysis was performed as previously described [5]. RNA was resolved on 1% agarose gel electrophoresis and blotted onto nylon membranes. The blots were hybridized with 32 P-labeled probes ($\sim 10^8$ cpm/ μ g DNA). Human pro- $\alpha_1(\text{I})$ (Hf 677) [14], pro- $\alpha_2(\text{I})$ (Hf32) [15], pro- $\alpha_1(\text{III})$ (Hf 934) [16] and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [17] cDNAs were labeled with [32 P]dCTP (110 TBq/mmol, Amersham). The filters were washed and exposed to X-ray film. The fluorograms were scanned with a densitometer.

2.3. Nuclear run-off assay

To determine the transcriptional activity of the $\alpha_1(\text{XVI})$ gene, a nuclear run-off assay was performed essentially as described previously [18]. Briefly, nuclei were prepared from suspension-arrested cells or actively proliferating cells (cells on day 3 in culture) according to the method of Greenberg et al. [19]. The nuclei were incubated with [α - 32 P]UTP (120 TBq/mmol, Amersham) for 20 min at 30°C. The samples were deproteinized, and the transcribed RNA was hybridized with cDNAs at the concentration of $\sim 10^6$ cpm/ml. One microgram of cDNAs, pro- $\alpha_2(\text{I})$ [16], $\alpha_1(\text{XVI})$ [4,5], GAPDH [17] and pBR322 DNA was dot-blotted onto nitrocellulose filters. After hybridization the filters were washed and subjected to autoradiography. Autoradiograms were scanned with a densitometer.

2.4. Detection of type XVI collagen polypeptide

Based on hydrophilicity characteristics [20], a sequence of 18 amino acid residues, TQPTRRVFPRGLPEEFAL, in the N-terminal non-triple-helical domain of $\alpha_1(\text{XVI})$ (NC11 domain) was selected for antibody production. The antibody against the synthetic $\alpha_1(\text{XVI})$ peptide was produced by intraperitoneal injection to rabbits and purified by protein A-TSK gel (Amersham). The peptide-specific antibody was eluted from the peptide-agarose affinity column with 0.1 M citrate buffer, pH 3.0 [21].

Fibroblasts (day 3 in culture) were trypsinized and cultured in 10% FBS-DMEM containing 0.1% methylcellulose for 48 h at 37°C, then cultured for 24 h (last 24 h during the 72 h culture) in fresh medium (10% FBS-DMEM containing 0.1% methylcellulose). Confluent cells (day 8 in culture) were cultured in serum-deprived DMEM for 48 h. Culture medium was replaced by fresh serum-deprived DMEM, then cells were cultured for an additional 24 h (last 24 h during the 72 h culture). For comparison, proliferating cells (day 3 in culture) were incubated for 24 h with fresh DMEM containing 10% FBS. The medium was harvested by centrifugation or aspiration, and mixed with protease inhibitor cocktails (1 mM ethylenediamine tetraacetic acid, *N*-ethylmaleimide and phenylmethylsulfonyl fluoride). The proteins in the media were precipitated with 30% saturated ammonium sulfate (176 mg/ml) and subjected to the analysis with 2–15% gradient sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions, then blotted onto nitrocellulose filters. The filters were stained with the affinity-purified antibody at 1/1000 dilution for 24 h. The blots were washed and incubated with second antibody (anti-rabbit immunoglobulins) at 1/1000 dilution for 1 h. Antigens were visualized by chemiluminescence (ECL, Amersham) [21]. DNA content in the cells was determined using fluorometric assays as previously described [22]. To compare the amount of $\alpha_1(\text{XVI})$ polypeptide in the proliferating, suspension-arrested and serum deprivation-arrested cells, the amount of the samples for SDS–PAGE was normalized to DNA content of the cell pellet.

3. Results

On optical microscopic observation, the cells of a round shape were attaching to the plates at 6 h in culture. The cells were spreading on day 1 in culture and had a skirt of cytoplasm and dark nuclei. On day 3, cells were exponentially growing and they reached confluent density on day 8. Determination of the $\alpha_1(\text{XVI})$ collagen mRNA level in the various phases of cell growth; non-adherent, attaching (6 h in culture), spreading (1 day in culture), proliferating (day 3 in culture) and confluent (day 8 in culture) phases revealed that the expression was highest in the conditions of non-adherent and confluent phases. In contrast, mRNA levels of $\alpha_1(\text{I})$, $\alpha_2(\text{I})$ and $\alpha_1(\text{III})$ collagens were not detected in the non-adherent phase but were elevated in the adhesive (attaching and spreading) phases reaching maximum level in the confluent phase (Fig. 1).

To further investigate $\alpha_1(\text{XVI})$ collagen expression in the stationary conditions, the non-adherent cells were incubated in 0.1% methylcellulose dissolved with 10% FBS–DMEM over

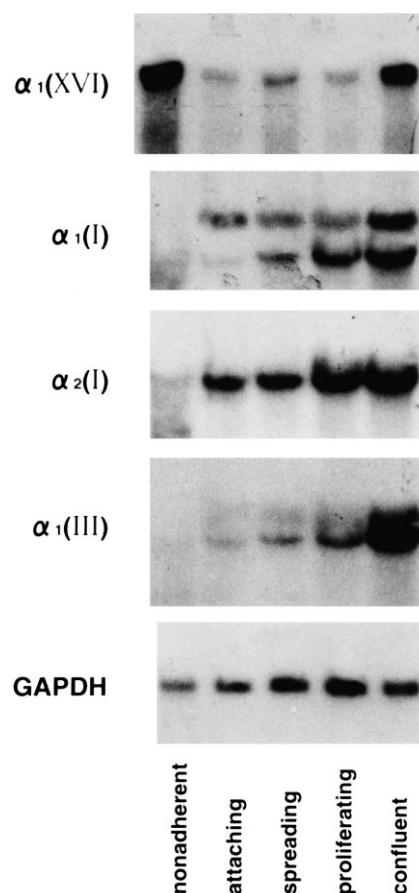


Fig. 1. Modulation of $\alpha_1(\text{XVI})$ collagen expression in the various phases of cell growth in cultured human skin fibroblasts. Cells grown to 50% confluent density were suspended in DMEM containing 10% FBS after trypsin treatment (non-adherent phase). The non-adherent cells were plated at a density of $2.2 \times 10^4/\text{cm}^2$ in petri dishes. Total RNA was extracted from the non-adherent cells or adherent cells at 6 h (attaching phase), on day 1 (spreading phase), day 3 (proliferating phase) or day 8 (confluent phase) in culture. The amount of mRNA for $\alpha_1(\text{XVI})$ was assayed by RNase protection assay and those of $\alpha_1(\text{I})$, $\alpha_2(\text{I})$, $\alpha_1(\text{III})$ and GAPDH by Northern blot analysis.

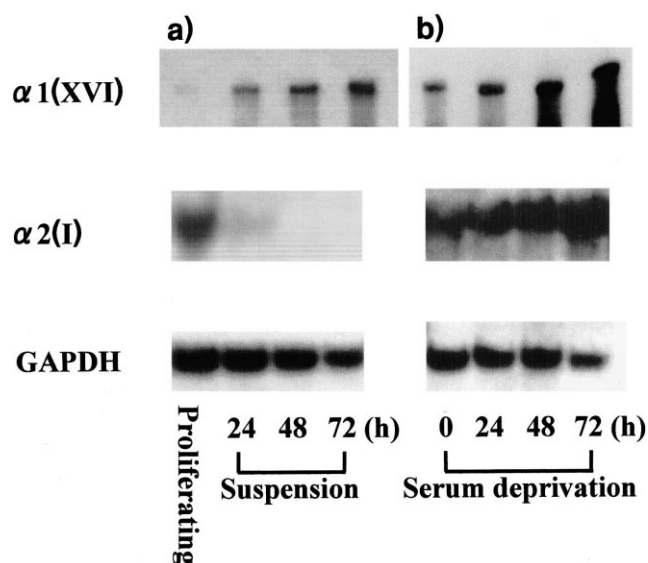


Fig. 2. Time-dependent increase in $\alpha_1(\text{XVI})$ expression in the growth-arrested conditions brought about by two different systems. Cells were grown to half-confluent density, then treated with trypsin. Non-adherent cells were suspended in 10% FBS–DMEM containing 0.1% methylcellulose for 24, 48 and 72 h (suspension arrest). For comparison, mRNA levels in the proliferating (50% confluent) cells were also studied (a). Cells were grown to a confluent density in DMEM supplemented with 10% FBS, then further cultured in serum-free DMEM for 0, 24, 48 or 72 h (serum deprivation arrest) (b). RNA was isolated from the cells, then $\alpha_1(\text{XVI})$, $\alpha_2(\text{I})$ and GAPDH mRNA levels were determined.

72 h (suspension-arrested condition), or confluent cells were incubated in serum-deprived DMEM for 72 h (serum-deprived condition). A time-dependent increase of $\alpha_1(\text{XVI})$ collagen mRNA during 72 h was found in both suspension-arrested and serum-deprived conditions (Fig. 2a,b). The type XVI collagen mRNA level in the suspension culture for 72 h

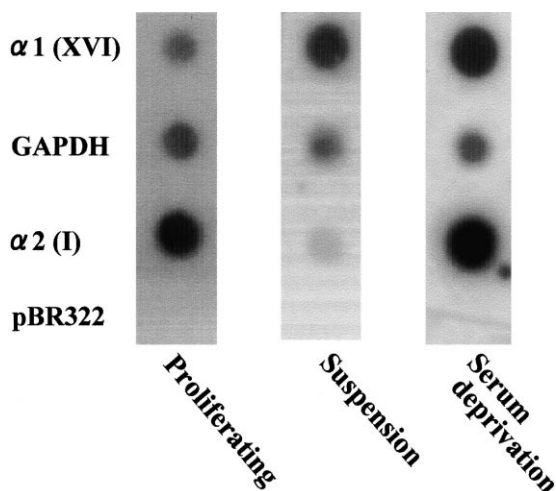


Fig. 3. Transcriptional activity of the $\alpha_1(\text{XVI})$ gene in the proliferating, suspension-arrested or serum-deprived cells in culture. Nuclei were isolated from actively proliferating cells (day 3 in culture), suspension-arrested cells (72 h in suspension culture) or serum deprivation-arrested confluent cells (72 h in serum-deprived culture). Messenger RNA precursors synthesized *in vitro* were labeled with [^{32}P]UTP, then hybridized with $\alpha_1(\text{XVI})$, GAPDH, pro- $\alpha_2(\text{I})$ cDNAs and pBR322 DNA. The filters were washed and subjected to autoradiography.

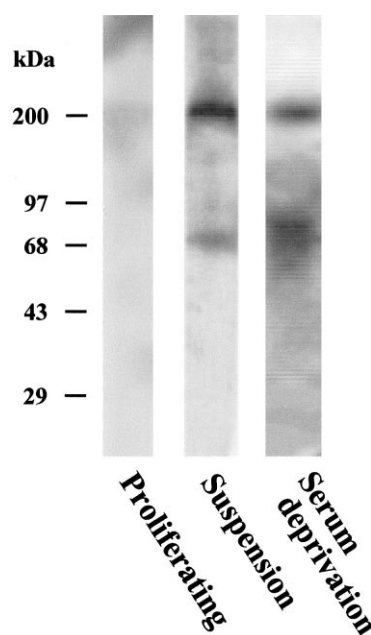


Fig. 4. Detection of type XVI collagen polypeptide in the media of proliferating, suspension-arrested or serum-deprived cells. Proliferating: proliferating cells (day 3 in culture) were cultured for 24 h in fresh 10% FBS–DMEM. Suspension: cells were suspended in 0.1% methylcellulose dissolved in DMEM containing 10% FBS for 72 h. During the last 24 h of the 72 h culture, cells were cultured with the same fresh medium. Serum-deprivation: cells at a confluent density were cultured in serum-deprived DMEM for 72 h. During the last 24 h of the 72 h culture, cells were cultured in fresh serum-deprived DMEM. Culture media obtained with these three different conditions were harvested. The proteins in the media were precipitated, resolved on 2–15% SDS–PAGE under reducing condition, then transferred to nitrocellulose. The filters were stained with affinity-purified anti- $\alpha_1(\text{XVI})$ antibody at 1/1000 dilution for 24 h, then with second antibody at 1/1000 dilution for 1 h. Antigens were detected with chemiluminescence. Molecular standard markers are indicated.

was found to be increased 11-fold compared with proliferating cells (Fig. 2a). In contrast, the $\alpha_2(\text{I})$ mRNA level, which was significant in the proliferating phase, became undetectable in the suspension-arrested condition during a 72 h incubation (Fig. 2a), whereas a time-dependent increase in $\alpha_2(\text{I})$ mRNA was observed in the serum-deprived condition (Fig. 2b).

To determine the mechanism of the increase in $\alpha_1(\text{XVI})$ mRNA in the suspension-arrested or serum deprivation-arrested cells, the transcription of the $\alpha_1(\text{XVI})$ gene in the proliferating, suspension-arrested and serum-deprived cells was compared. The transcriptional activities of the $\alpha_1(\text{XVI})$ gene in both suspension and serum-deprived cultures for 72 h were found to be greater (five-fold) than those in the actively proliferating cells (day 3 in culture). By contrast, transcription of the pro- $\alpha_2(\text{I})$ gene in the suspension-arrested cells was hardly detectable but became greater in the proliferating and serum-deprived cells (Fig. 3).

Major 210 kDa and minor 70 kDa polypeptides were detected in the culture medium of the cells which had been suspended for 72 h, whereas the polypeptides were hardly detected in the proliferating cells. In the serum-deprived cells for 72 h, 210 kDa and several 80–90 kDa polypeptides were seen (Fig. 4). The minor 70 kDa and 80–90 kDa polypeptides may be degraded products of the 210 kDa polypeptide, since

intact α_1 (XVI) collagen with a molecular weight of 210 kDa has been found to undergo proteolytic processing to several lower molecular weight polypeptides in the culture medium [23].

4. Discussion

We have demonstrated that type XVI collagen expression was elevated in the non-adherent cells and was suppressed in the attaching, spreading and proliferating conditions. This is in a sharp contrast to type I/III collagens, expression of which was undetectable in the non-adherent condition but enhanced in the subsequent adhesive and proliferative phases. This suggests that the expression of type XVI collagen may be anchorage-independent, while type I and III collagen expression is anchorage-dependent.

When the actively proliferating cells (3 days in culture) were dissociated from the culture dishes and maintained in the suspension culture for 72 h, type XVI collagen mRNA level was enhanced markedly (11-fold), but the degree of the increase in mRNA level was greater than the increase in transcriptional activity of the α_1 (XVI) collagen gene (five-fold), suggesting that the expression of type XVI collagen during cell dissociation was regulated at transcriptional and post-transcriptional levels.

Previous studies have demonstrated that the mRNA levels of type I and III collagens were increased when growth of fibroblasts was arrested at high cell density (density arrest), or by serum deprivation [24–26]. The increase in type I and III collagen expression has not been explained directly by the entry of cells into a quiescent state but by the alteration in the degree of cell–cell interaction with high cell density or by the alteration of cell–matrix interaction accompanying the loss of adhesive components (fibronectin or vitronectin) in serum, because type I and III collagen expression was found to be very low in the suspension arrest in which the cells enter the G0 phase within 48 h [9–11]. These previous observations on type I and III collagen suggest that the mechanism of increase in type XVI collagen in the growth arrest condition induced by serum deprivation may be different from that of type I collagen, although type XVI and type I collagen expressions were coordinately increased in the serum-deprived conditions.

The expression of type XVI collagen was shown to be markedly increased in two different growth inhibiting culture systems induced by suspension arrest and serum deprivation of confluent cells, indicating that type XVI collagen (a member of FACIT) expression, unlike type I and III collagens (members of interstitial collagens), is grossly related to the stationary phase of cell growth. In normal circumstances dermal fibroblasts are considered to be in the stationary phase of cell growth, but actively migrate and proliferate in inflamma-

tory or fibrotic conditions. Type XVI collagen may play an essential role in resting cells rather than proliferating cells by stabilizing the placement of fibroblasts in the dermal matrices.

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