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Downregulation of collagen synthesis in fibroblasts within three-dimensional collagen lattices involves transcriptional and posttranscriptional mechanisms

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Culturing human fibroblasts in a three-dimensional collagen matrix leads to a reduction of collagen I by more than 90%, both on the level of mRNA steady-state as well as protein. In order to differentiate changes in de novo transcription and posttranscriptional control, nuclear run on assays and pulse/chase experiments determining mRNA stability were used. Our results indicate that de novo transcription of the COL1A1 gene and pro-α1(I)collagen mRNA half-life are both decreased by 50% in fibroblasts grown in three-dimensional collagen lattices as compared to monolayer cultures. The extracellular matrix therefore elicits signals which are transduced from the cell surface to the inside of fibroblasts resulting in a specific reprogramming of transcriptional as well as posttranscriptional processes.

Gene expression; RNA stability; Extracellular matrix; Fibroblast

1. INTRODUCTION

Regulation of connective tissue metabolism plays an important role in a number of fibrotic processes and in wound healing [1–3]. Transcriptional as well as posttranscriptional control mechanisms have been identified [4–7], however, in most studies monolayer cultures were employed, and it remains unclear whether they represent the in vivo situation.

Culture of fibroblasts within a three-dimensional environment reconstituted mainly of collagen I fibrils was introduced about a decade ago [8] and has proven to be a valuable tool to study cell morphology [9], synthesis [10–12] and degradation of connective tissue components [11,13]. It also allows investigation of the responsiveness to growth factors [14–17], as well as the influence of drugs on fibroblast metabolism [18] under conditions which resemble the in vivo situation more closely than fibroblast monolayer cultures [8].

Beside others, the system is characterized by two prominent features, i.e. a strong induction of collagenase activity and a concomitant decrease in collagen synthesis. Both processes appear to be controlled on a pretranslational level [11–13], however, the mechanisms involved are not understood in detail.

This study focuses on the regulation of pro α1(I)collagen mRNA in fibroblasts grown in floating three-dimensional collagen lattices, and we provide evidence that the reduced steady-state level is partly due to reduced de novo transcription of the collagen I gene and in part to a reduction in pro α1(I)collagen mRNA stability.

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2. MATERIALS AND METHODS

2.1. *Cell culture*

Fibroblast cultures were established by outgrowth from skin biopsies of healthy human volunteers. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS), 50 μg/ml sodium ascorbate, 300 μg/ml glutamine, 100 u/ml penicillin, 100 u/ml streptomycin, and grown in the moist atmosphere of a CO_2 incubator (5% CO_2) at 37°C. Three-dimensional collagen lattice cultures were prepared as described [12,16] containing 10^7 cells per gel (150 mm diameter, 30 ml volume) and a concentration of collagen I of 1 mg/ml.

2.2. *Dot blot hybridization*

Total RNA was isolated from monolayer and collagen lattice cultures as described [12,16] using the guanidinium thiocyanate/CsCl method and applied in serial dilution (0.3–5 μg) to GeneScreen membranes (DuPont), fixed by UV crosslinking and hybridized to ^{32}P-labeled [19] cDNA sequences specific for pro α1(I)collagen (Hf 677) [20] and fibronectin (FN771) [21] in 5 × SSC (SSC is 0.15 M NaCl, 0.015 M Na-citrate), 50% formamide, 0.1% each of bovine serum albumin, ficoll, and polyvinylpyrrolidone, 0.1% Na-dodecylsulfate (SDS) and 100 μg/ml salmon sperm DNA at 42°C for 20 h. Filters were washed at a final stringency of 0.1 × SSC, 0.1% SDS at 65°C. Following autoradiography (Kodak X-Omat AR) at −80°C for 1–5 days, signal intensity was determined by densitometry. Values shown are representative of at least three repeated independent experiments.

2.3. *Preparation of nuclei*

The procedure for isolation of nuclei and run on assays was modified after Marzluff and Huang [22].

Monolayers. 2–5 × 10^7 cells were washed twice in cold phosphate

buffered saline (PBS), scraped off the cultures dishes, and pelleted (4°C, 1,000 × g).

Lattice cultures. 4–5 matrices corresponding to 5×10^7 cells which had contracted for 48 h were washed free of serum by gently swirling in PBS. Cells were liberated from the matrix by incubating each gel in 5 ml of collagenase solution (3 mg/ml CLS I, Worthington, and 1 mg/ml bovine serum albumin in DMEM without supplements) at 30°C for 10 min with vigorous shaking. The enzyme was inactivated by addition of 1 ml FCS, and the cells were then washed in cold PBS.

The cell pellets from monolayers or lattice cultures were suspended in 4.6 ml of buffer A (10 mM Tris-HCl, pH 8, 2.5 mM Mg-acetate, 0.5 mM dithiothreitol, 0.25% Triton X-100, 0.3 M sucrose) and homogenized in a glass dounce. Following filtration through miracloth (Calbiochem) to remove the bulk of cellular debris, the homogenate was mixed with an equal volume of buffer B (same as buffer A, containing 2.4 M sucrose) and layered on top of a cushion of buffer B. Separation of nuclei from cytoplasmic and membranous material was achieved by ultracentrifugation (1 h at 18,000 rpm and 4°C). Sedimented nuclei were briefly washed in cold PBS and resuspended in 100 μl of buffer C (50 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 0.1 mM EDTA, 40% glycerol). 40 μl aliquots were snapfrozen in liquid nitrogen and stored at −80°C. For standardization, an aliquot of each preparation was lysed in 10% SDS, and DNA content was assessed by absorption at 260 nm which ranged between 1.6–1.7 mg/ml [23]. In addition, each preparation was checked for purity and integrity by light microscopy.

2.4. *Nuclear run on*

40 μl of nuclei were incubated for 45 min at 27°C in 0.15 M KCl, 3 mM $MgCl_2$, 0.4 mM each of ATP, CTP, GTP and 1.85 MBq of [α-^{32}P]UTP (Amersham, specific activity 3,000 Ci/mmol). Transcription was terminated by incubation for 10 min at 27°C in the presence of 2 units DNase I (Promega, RQ1 quality) and 25 μg tRNA (*Saccharomyces cerevisiae*, Boehringer). Proteins were removed by adding 100 μg proteinase K and incubating for 30 min at 42°C in 10 mM Tris-HCl, pH 7.9, 10 mM EDTA, 0.5% SDS, followed by phenol extraction and Sephadex-G50 chromatography. 1×10^6 cpm were hybridized for 36 h to slot blotted cDNA sequences (0.2–0.5 μg of plasmid DNA/slot) coding for pro α1(I)collagen and fibronectin in 5 × SSC, 1% Na-laurylsarcosine at 65°C. Filters were washed twice each at 50°C in 3 × SSC, 0.5% Na-laurylsarcosine and 3 × SSC. Following autoradiography, hybridization signals were quantified by densitometry.

2.4. *Pulse/chase experiments and determination of mRNA stability*

The procedure used was modified according to Hämäläinen et al. [24]. Collagen lattices containing 10^7 cells in 30 ml were prepared in 150 mm bacteriologic dishes, and contraction proceeded for 7 h at 37°C. In parallel, monolayers were seeded at 3×10^6/100 mm culture dish and incubated for 7 h. Monolayers and gels were washed twice in PBS, lattices were transferred to 50 ml plastic tubes. For pulse-labeling, each culture was incubated for 1 h at 37°C in 5 ml serum-free DMEM with 15.4 MBq [5,6-^{3}H]uridine (DuPont). Pulse medium was removed by two washing steps in PBS, and replaced by chase-medium (DMEM, 10% FCS, 10 mM each uridine and cytidine, 5 μg/ml actinomycin D). Parallel monolayer and collagen lattice cultures were chased up to 12 h. At each time point of the chase period (0, 2, 4, 6, 8, 12 h) one set of cultures was lysed, and RNA was extracted. 20 μg of tritiated RNA was hybridized to dot blots (GeneScreen, DuPont) containing 0.2 μg of cDNA inserts coding for pro α1(I)collagen and fibronectin at 65°C in 5 × SSC, 1% Na-laurylsarcosine. Filters were washed at 50°C in 3 × SSC, 0.5% Na-laurylsarcosine, and in 3 × SSC. Unpaired single strands were removed with 10 μg/ml-RNase A in 2 × SSC at room temperature. Bound RNA was assessed by liquid scintillation counting, for which the hybridized excised dots were incubated in 1 ml Solvable (DuPont) for 30 min at 50°C, followed by the addition of 10 ml Aquasol-2 (DuPont) and 50 μl glacial acetic acid.

3. RESULTS

3.1. *Pro α1(I)collagen steady-state level is reduced in gel cultures*

Culture of human fibroblasts in a three-dimensional collagen lattice leads to a time-dependent depression of steady-state levels for pro α1(I)collagen mRNA. After 1 day, the pro α1(I)collagen mRNA has decreased to less than 50%, and after 2 days to about 5–10% of the value in monolayer cultures (Table I). In contrast, transcript levels for fibronectin, another abundantly synthesized extracellular matrix protein, remained unchanged (Table I). These results suggest a specific reprogramming of fibroblast biosynthetic capacities which is regulated on a pretranslational level when the cells are cultured in a three-dimensional environment.

3.2. *Decrease of de novo transcription of pro α1(I)collagen mRNA*

In order to clarify whether the reduced transcript levels could be ascribed to a decrease in newly synthesized mRNA, nuclei were isolated from fibroblasts in monolayer culture and after 48 h growth in lattice cultures. The nuclei were checked by light microscopy, and were found to be of similar morphology for both culture conditions. Identical amounts of nuclei, checked by determining the DNA content in lysed aliquots, were then applied in run on experiments (Fig. 1). In agreement with our results obtained from dot blot hybridization, relative de novo mRNA synthesis for fibronectin was identical in both culture systems (Fig. 1, Table I). In contrast, de novo synthesis of pro α1(I)collagen mRNA was reduced to about 50% relative to monolayer culture (Table I). This result thus indicates that transcriptional

Table I

	mRNA steady state level		mRNA synthesis	
	Monolayer culture	Collagen lattice	Monolayer culture	Collagen lattice
α1(I)Collagen	1.0 ± 0.09	0.1 ± 0.01	1.0 ± 0.2	0.5 ± 0.03
Fibronectin	1.0 ± 0.15	1.0 ± 0.15	1.0 ± 0.1	1.0 ± 0.1

Note. Relative mRNA steady state levels were determined by hybridizing dot blots containing 0.3–5 μg of total RNA from 48 h monolayer and lattice cultures with ^{32}P-labeled cDNA probes specific for pro α1(I)collagen and fibronectin [12]. Signal intensity was quantified densitometrically. Expression in monolayer cultures was set arbitrarily at 1.0. The values represent $\bar{x} \pm$ S.D. Relative de novo mRNA synthesis was quantified by densitometric scanning of the signals shown in Fig. 1. Values obtained from monolayer cultures were set as 1.0.

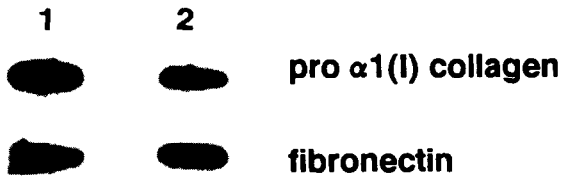

Fig. 1. De novo synthesis of pro α1(I)collagen (α_1I) and fibronectin mRNA in monolayer and three-dimensional lattice cultures. Fibroblasts were cultured as monolayers (lane 1) and in collagen lattices (lane 2) for 48 h, after which time nuclei were prepared and used for nuclear run on transcription. ^{32}P-labeled transcripts were hybridized to slot blotted cDNA sequences specific for pro α1(I)collagen and fibronectin.

control is obviously involved in the reduction of mRNA steady-state level, it is, however, not sufficient to explain the reduction in pro α1(I)collagen mRNA levels below 50%. Therefore, pulse/chase experiments using tritiated uridine were carried out in order to see whether the mRNA in gel cultures is less stable than in monolayers, and in this way accounts for the depressed steady-state levels.

3.3. *Decrease of pro α1(I)collagen mRNA stability*

For determining mRNA stability, total RNA was extracted from pulse-labeled fibroblasts grown in both culture conditions. Gel electrophoretic analysis indicated that the procedure used yielded intact RNA not only from monolayers, but also from lattice cultures (Fig. 2, inset).

Identical amounts of gradient-purified, tritiated RNA from fibroblasts cultured under both conditions were hybridized to dot blots containing immobilized cDNA sequences for pro α1(I)collagen and fibronectin. The results shown in Fig. 2a demonstrate different decay curves for pro α1(I)collagen mRNA in cells in monolayer culture compared to those kept in lattices. In contrast, the decay for fibronectin mRNA did not differ significantly under both conditions (Fig. 2b). Extrapolation of the curves indicated an approximate half-life of 2 h for fibronectin mRNA. Pro α1(I)collagen mRNA half-life was deduced to be 4.4 h in fibroblasts grown in monolayer cultures, whereas in collagen lattices it was found to be reduced to 2.1 h.

4. DISCUSSION

In the past, monolayer culture of fibroblasts has proven a valuable tool to study alterations in collagen gene expression. However, this system remains artificial with respect to the lack of interaction of the cell surface with a surrounding three-dimensional environment. It

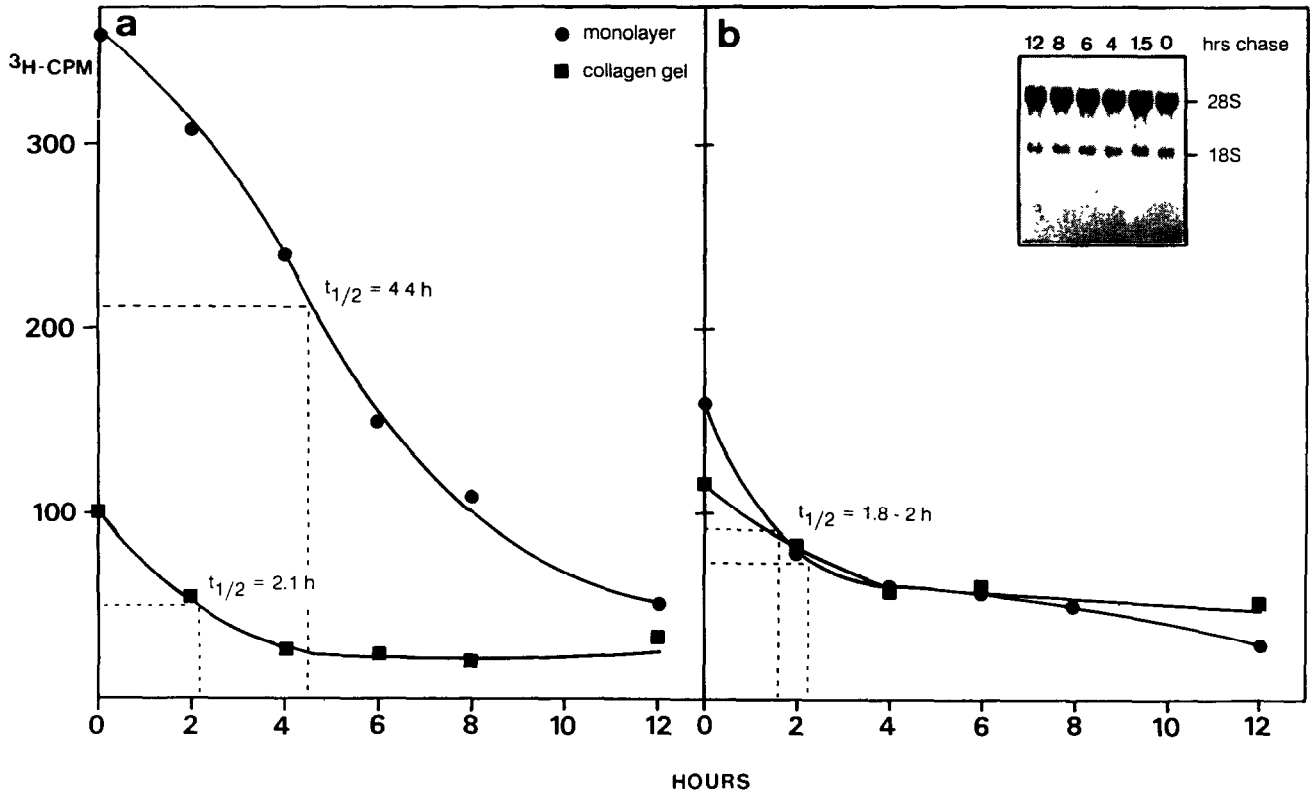

Fig. 2. mRNA decay for pro α1(I)collagen mRNA in monolayer and three-dimensional lattice cultures. Fibroblasts were maintained in monolayer and collagen lattice cultures for 7 h, pulsed for 1 h in the presence of [^{3}H]uridine, and chased for up to 12 h with medium containing an excess of unlabeled uridine and cytidine, and actinomycin D. Tritiated RNA was hybridized to dot blots of cDNA specific for pro α1(I)collagen (a) and fibronectin (b). cpm were determined by liquid scintillation counting. The inset shows a pherogram of RNA which was isolated from fibroblasts grown in lattice cultures, pulsed and chased for the indicated times, and liberated from the collagenous environment by collagenase.

has been shown that components of the extracellular matrix directly interact with cell surface receptors like integrins, and that this interaction leads to specific changes in gene expression [25]. Several of these receptors have been identified recently and were found to belong to the family of integrins [26]. So, contraction of floating collagen lattices depends on the expression of the $\alpha 2\beta 1$ VLA integrin on the surface of fibroblasts. Blocking the receptor by addition of neutralizing antibodies led to gels which could no longer contract [27,28].

This study corroborates previous work showing that the pro $\alpha 1$(I)collagen mRNA level is decreased in parallel to the protein level [12], and suggests pretranslational control. This reduction can be explained in part by diminished transcription of the gene. Analysis of the positive regulatory elements within the promoter of the $\alpha 1$(I)collagen gene described by Karsenty et al. [29] indicated no involvement in the downregulation of $\alpha 1$(I)collagen transcription (data not shown). Further studies are in progress to demonstrate whether negative regulatory elements as have been identified by Karsenty and de Crombrugghe [30] might play a role in those differentially regulatory mechanisms.

Further reduction of mRNA steady-state levels is attributed to posttranscriptional control. Pro $\alpha 1$(I)collagen mRNA in lattice cultures is only half as stable as in monolayers. Thus, in this system, less mRNA is initially transcribed, and in addition these molecules are less stable. In this way, the steady-state level is sharply reduced and corresponds to similarly depressed collagen protein levels.

Modulation of mRNA half-life has been described for many different gene products [31,32] including very short-lived species like cytokines and growth factors [33,34], but also very stable ones, like the maternal transcripts in maturing oocytes [35]. So far, none of the defined elements involved in transcript stability have been reported to reside in the pro $\alpha 1$(I)collagen mRNA, with the exception of a postulated structural foldback element located in the 3′ region [36]. Studies are in progress to identify nuclear or cytoplasmic proteins which by interaction with the 3′ end of this RNA species affect its stability (A. Määttä, personal communication).

Other groups have investigated the stability of pro $\alpha 1$(I)collagen mRNA in monolayer cultures of human [24] and mouse [37] fibroblasts who present half-lives of 8–10 h. These differences could be due to the culture conditions used. So it is established that decreased time of adherence is correlated with shortened half-life of pro $\alpha 1$(I)collagen mRNA [38].

It is not yet clear which integrin receptor is involved in mediating the signal leading to a decrease in collagen synthesis with both described mechanisms. Several integrin receptors have been shown to bind collagen, including $\alpha 1\beta 1$, $\alpha 2\beta 1$, and $\alpha 3\beta 1$ [39]. It also remains to be seen which signal transducing system is involved in transmitting the signal from the receptor to the cytoplasm and cell nucleus, and whether reduced transcription and decreased mRNA stability are due to the same signal transduction mechanism.

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