Protein synthesis in collagen lattice-cultured fibroblasts is controlled at the ribosomal level

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Abstract Fibroblasts cultivated in three-dimensional lattices exhibit a large decrease of protein synthesis, mainly through transcriptional control. However, no previous work was devoted to a potential ribosomal regulation. We evaluated ribosomal ribonucleic acid (RNA) in monolayer- and collagen lattice-cultured fibroblasts. After one week of culture, total RNA was 60% lower in lattice-cultured fibroblasts than in monolayer-cultured cells. The decrease was identical for 18 S and 28 S rRNA subfractions. The half-life of RNA was much shorter in collagen lattice-cultured fibroblasts than in monolayers. These results suggest that protein synthesis in lattice-cultured fibroblasts is partly regulated at the ribosomal level.

Key words: Fibroblast; Collagen lattice culture; Ribosomal RNA; Protein synthesis; Ribosomal control

1. Introduction

The use of three-dimensional models (lattices) for the culture of fibroblasts [1,2] has brought new insights into the knowledge of metabolic regulation of these cells. It was shown that fibroblast activity was strongly modulated by the presence of the extracellular matrix : when cultured in a lattice, cells stopped dividing or divided very slowly [3], their response to growth factors was decreased [4–6], and their protein syntheses were strongly inhibited [2,7]. These findings were of particular interest because these features look like those that characterize fibroblasts in vivo, when cells are surrounded by the extracellular matrix, as in the case of skin, and show a low metabolic activity, except in pathological situations such as fibrosis or wound healing. On the contrary, fibroblasts cultivated in monolayers exhibit a high metabolic activity. Thus, the use of lattice cultures in order to identify the mechanisms of regulation of fibroblast protein synthesis is of interest.

Previous studies have shown that the expression of various genes, particularly among those encoding proteins of the extracellular matrix, was inhibited at the pretranslational level when fibroblasts were cultivated in collagen lattices. This is the case particularly for the type I collagen gene [7]. However, the overall decrease of protein synthesis suggests that more general mechanisms are involved.

In this study, we checked the possibility of a regulation of biosynthetic activity of fibroblasts at the ribosomal level. We evaluated in a comparative way the amounts of ribosomal RNA (rRNA) in fibroblasts cultivated in monolayers and collagen lattices, and demonstrated that the decrease of rRNA quantities available could account for the low activity of protein synthesis in lattice-cultured fibroblasts.

2. Materials and methods

2.1. Materials

[5,6-^{3}H]Uridine (specific activity 1.4 TBq/mmol) was purchased from New England Nuclear (Paris). Reagents for molecular biology were bought from Sigma (St Louis, MO), and other reagents from Prolabo (Paris, France). α-Amanitin and bacterial collagenase were obtained from Boehringer Mannheim (Mannheim, Germany). Reagents for cell cultures and rabbit globin mRNA were purchased from Gibco-BRL (Cergy-Pontoise, France), and culture flasks and 24-well plates from Nunc (represented in France by Polylabo, Strasbourg).

2.2. Fibroblast cultures

Human dermal fibroblasts were explanted from adult skin biopsies and grown in monolayers according to routine techniques [2,6]. Cells from subcultures 3 to 10 were used in this study and seeded into monolayers and collagen lattices made of acid soluble calf skin collagen prepared in the laboratory, as previously described [6–8]. RNA studies were performed after 1 week of culture. As a general rule, collagen lattices had retracted to 1/10 of their initial diameter.

2.3. Study of the ribonucleic acids contained in cells

RNAs contained in the cells were measured according to two different protocols. In a first set of experiments, total RNAs were assayed with the orcinol reaction [9]. Fibroblasts were detached from monolayers by trypsinization (0.025%, m/v) 15 min at 37°C or liberated from collagen lattices by digestion of the lattice with 20 U purified bacterial collagenase (15 min at 37°C), counted with a Malassez device and collected by centrifugation. Then they were suspended in a convenient volume of distilled water and sonicated. 400 μl of solution were added to 600 μl of orcinol 0.02% (m/v) in concentrated HCl containing 3.7 mM $FeCl_3$, heated 20 min at 100°C and the absorbance at 665 nm measured. RNA content was calculated from a calibration curve using type III yeast RNA standards after deduction of the absorbance due to cellular DNA (as a general rule, DNA absorbance was 6 times lower than RNA absorbance for the same concentration). DNA was measured in parallel aliquots according to a previously described fluorometric method [8]. Results were expressed as pg of RNA per cell.

In a second set of experiments, RNAs were measured after extraction from cultured fibroblasts. Total RNA extractions were performed in collagen lattice and monolayer cultures of 18×10^6 fibroblasts from 4 different strains. Just before extraction, 200 ng of rabbit globin mRNA were added to the culture. They were used as internal standards, as described by Kim et al. [10], and their recovery was measured in final extracts by Northern blot analysis using a specific globin cDNA probe labelled with [^{32}P]-deoxycytidine by random priming [11].

For total RNA extraction, confluent monolayers or lattices were directly extracted in 4 M guanidinium isothiocyanate, 2.5 M sodium citrate buffer, pH 7.0, containing 0.5% sarcosyl and 0.1 M β-mercaptoethanol with turraxing for 20 s [12]. An equal volume of phenol and 0.2 volume of chloroform/isoamylic alcool (49/1, v/v) mixture were added in the presence of 0.3 M sodium acetate. The mixture was stirred, cooled on ice and centrifuged at $10{,}000 \times g$ for 20 min. The RNA contained in the aqueous phase was precipitated by addition of 2 volumes of ethanol and dissolved in water. Total RNA content was

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evaluated by measurement of absorbance at 260 nm, on the basis of one A_{260} unit corresponding to 40 μg/ml RNA [13]. The amount of RNA per cell was calculed on the basis of the number of cells counted in separate dishes and the yield of extraction.

Ribosomal 18S and 28S RNA subfractions were evaluated from these extracts which were submitted to a 1% agarose gel electrophoresis using a Mini Sub DNA Cell (BioRad, Ivry sur Seine, France). They were run simultaneously with commercial standards (Pharmacia). The fluorescence of every RNA band in the presence of ethidium bromide at 312 nm was recorded and computed using the Bioprofil software (Vilber-Lourmat, Marne-La-Vallée, France).

2.4. Pulse-chase experiments

Collagen lattices and monolayer cultures were prepared in 24-well plates, with 200,000 cells seeded per well, and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). This medium was discarded 24 h before the experiment, and replaced by a fresh medium of same composition. 1 h before labeling, 10 μg/ml α-amanitin, an inhibitor of type II RNA polymerases [14,15], were added in a half of the wells. A pulse of [^{3}H]-uridine at the concentration 370 kBq/ml was added to the cultures during 1 h at 37°C. At the end of the pulse period, labelled medium was removed, cultures washed twice with the chase medium, that is DMEM containing 10% FCS, 10 mM uridine, 10 mM cytidine and 0 or 10 μg/ml α-amanitin, depending on the series. Cells were then incubated with the chase medium during 1 to 23 h. At the end of these various times, the medium was discarded, and cells or lattices were washed twice with cold saline solution and incubated 10 min at 4°C with 1 ml of a cold 10% (v/v) trichloroacetic acid solution. Cells or lattices were then rinced 30 min at 4°C with a 3:1 (v/v) ethanol/ether mixture and dried. Treated cultures were solubilized with 0.5 M NaOH overnight at 4°C, neutralized with HCl and radioactivity counted. Total DNA content of the cultures and cell number were measured in parallel wells.

3. Results

The amount of total RNA content was estimated after 7 days of culture in 4 different strains of dermal fibroblasts. Total RNA content of lattice-cultured fibroblasts expressed on the basis of the pg concentration for one cell was found dramatically decreased in comparison with RNA content of monolayer-cultured fibroblasts, as measured by two different techniques in separate experiments (Table 1). RNA content of collagen lattice-cultured fibroblasts was from 2 to 3 times lower than that of monolayer cultured fibroblasts.

Ribosomal RNAs were submitted to agarose gel electrophoresis. In the 4 strains studied, both 18 S and 28 S subfractions were quantitatively lower in lattice-cultured fibroblasts than in monolayer-cultured fibroblasts (Fig. 1). They were quantified by the use of a calibration curve performed with dilutions of RNA solutions. A linear response was obtained in a zone corresponding to a deposition of 0 to 3 μg RNA. The decrease of 18S and 28S RNA subfractions was about 60% when fibroblasts were cultivated in collagen lattices. The ratio rRNA in monolayer/rRNA in collagen lattices was 0.39 ± 0.12 for 18 S subfraction and 0.38 ± 0.15 for 28 S subfraction (mean ± standard deviation), showing that 18 S and 28 S RNA amounts were parallelly decreased in collagen lattices.

Table 1
Effect of culture conditions on total RNA and ribosomal RNA content of fibroblasts

	Fibroblasts cultivated in		Mean ratio Collagen lattices/ Monolayers
	Monolayers	Collagen lattices	
Total RNA[a] (pg/cell)	29.0 ± 3.4	8.7 ± 0.9[d,***]	0.30
Total RNA[b] (pg/cell)	18.7 ± 4.3	8.5 ± 2.8[d,**]	0.45
18 S rRNA[c] (U/cell)	6.5 ± 2.3	2.4 ± 1.2[d,*]	0.37
28 S rRNA[c] (U/cell)	10.4 ± 2.8	4.0 ± 1.9[d,**]	0.38

All results were expressed as means of 4 determinations in different strains ± 1 standard deviation. For details, see text.
[a]RNA measured by orcinol technique; [b]RNA measured by A_{260} after extraction; [c]U = arbitrary fluorescence units; [d]Statistically significant difference from monolayers: *$P<0.05$; **$P<0.01$; ***$P<0.001$.

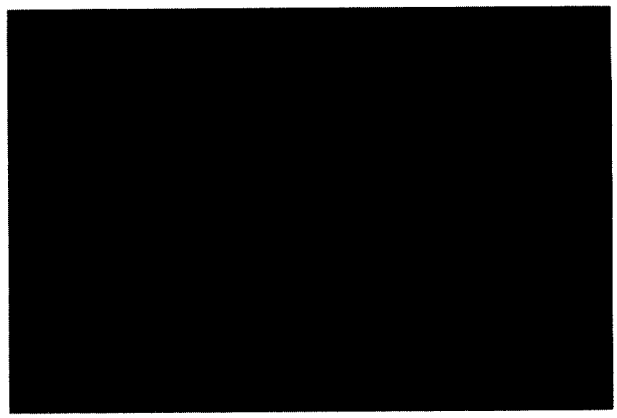

Fig. 1. Agarose gel electrophoresis of ribosomal RNA extracted from fibroblasts cultivated in monolayers and lattice cultures. RNA extracted from 300,000 dermal fibroblasts from 4 different strains (F1 to F4) cultivated in monolayers (M) or collagen (CL) were submitted to electrophoresis simultaneously with dilutions (S1 to S5, containing, respectively, 0.5, 1.0, 2.0, 3.0 and 4.0 μg total RNA) of RNA standards indicating the position of 18 S and 28 S subunits.

Pulse-chase experiments with tritiated uridine showed that RNA labeling was stable during at least 24 h in monolayers (Fig. 2A), whereas it was much shorter in collagen lattices, with a half life of about 4 h (Fig. 2B). In the presence of α-amanitin (Fig. 2C,D), tritiated uridine labeling was decreased to a significant level in monolayers (Fig. 2C), whereas it was only slighly modified in collagen lattices. In particular, the inital peak of labeling was not suppressed (Fig. 2D).

4. Discussion

The mechanisms controlling the activation level of metabolic activities in fibroblasts represent features of high interest, because their dysregulation is involved in physiopathological situations such as fibrosis or wound healing. The use of culture models brings some experimental data allowing to better understand these mechanisms of control. In particular, collagen lattices more or less reproduce the natural environment of fibroblasts, the extracellular matrix in which cells are dispersed. The major characteristic of this type of culture is the metabolic quiescence of cells, that is much more comparable to the in vivo situation than the artificial environment of monolayer cultures, where cells are cultivated in confluent layers, on plastic and without extracellular matrix.

From the data gained in this study, we hypothesize that the low activity of protein synthesis found in fibroblasts cultivated in collagen lattices is due to a general mechanism such as the reduction of the number of ribosomes. This hypothesis is conforted by the demonstration that total RNA is decreased. It is

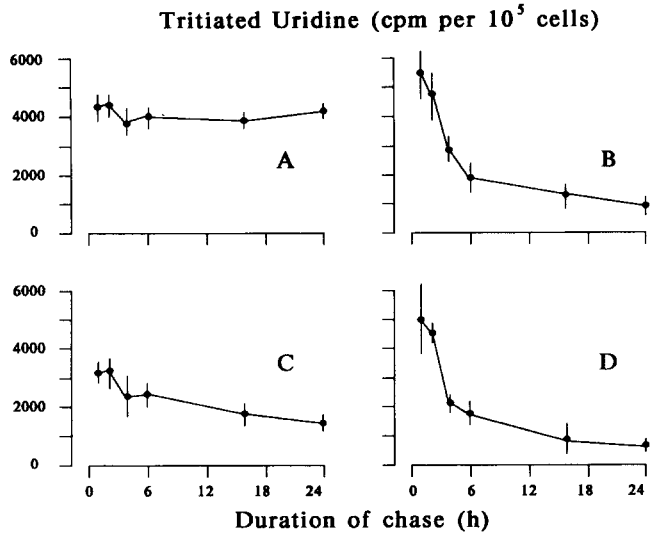

Fig. 2. Pulse-chase labeling with tritiated uridine of fibroblast cultivated in monolayers and collagen lattices. Tritiated uridine was added during a 1-h pulse to fibroblasts cultivated in monolayers (A,C) or collagen lattice cultures (B,D) in the absence (A,B) or presence (C,D) of 10 μg/ml α-amanitin. Tritiated uridine incorporated in RNA was measured after chase periods ranging from 0 to 24 h (for details, see text). The results represent the means of 4 determinations ± 1 standard deviation.

well known that rRNA constitute the bulk of cellular RNA and that any decrease of total RNA mainly depends on rRNA. The data obtained by different methods provide evidence that a major level of control of fibroblast activity is located at the ribosomal level, by triggering a dramatic decrease of rRNA synthesis and/or stability. The second hypothesis seems more convenient, because the initial peak of labeling is not lower in the case of collagen lattices. Furthermore, it is not inhibited by α-amanitin. This toxin, that preferentially inhibits biosynthesis of messenger RNAs by inhibition of type II RNA polymerase, was used in our culture conditions at concentrations known to exert the inhibiting effect and verified as non-toxic for our cells.

The interpretation of the data is still unclear. The decrease of rRNA level does not seem to be due to an inhibition of RNA polymerase I in fibroblasts cultivated in collagen lattices. The lowered half-life in this case may be related to a particular instability of these macromolecules, or to a trouble of association between newly synthesized rRNAs and ribosomal proteins. As it was previously shown that the syntheses of many proteins were down-regulated in collagen lattice-cultured fibroblasts, it may be hypothesized that the alteration of synthesis of one or several ribosomal protein could induce a trouble in the constitution of the ribosome and a degradation of rRNA.

Our study suggests that protein synthesis in collagen lattice-cultured fibroblasts is controlled by a post-transcriptional mechanism leading to a decrease in the number of ribosomes available for translation. This general mechanism does not rule out the involvement of other mechanisms already demonstrated, and particularly the transcriptional regulation of various genes [7], some of them being down-regulated and other up-regulated. However, this overall regulation of fibroblast metabolism should account for a great part of the modifications noticed in this type of cultures. Further studies should be devoted to the elucidation of the regulatory systems involved in this control of ribosomal activity, that could be affected in pathological situations.

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