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Modulation of Protein Synthesis by Extracellular Matrix: Potential Involvement of Two Nucleolar Proteins, Nucleolin and Fibrillarin

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Fibroblasts cultivated in a collagen matrix exhibit a large decrease in the synthesis of most proteins, depending on transcriptional and posttranscriptional controls. We have previously shown that ribosomal RNA content and half-life were decreased in collagen lattice cultures. Here, we cultivated human dermal fibroblasts in monolayers and in lattices and studied by competitive RT-PCR analysis the expression of the nucleolar proteins nucleolin and fibrillarin, two key factors in ribosome processing and association. Nucleolin expression was found increased, and fibrillarin expression decreased, in collagen-lattice vs monolayer-cultured fibroblasts, with some variability according to the strains (+25 to +250% and −40 to −60%, respectively). These data suggest that a possible trouble of the association between neosynthesized rRNA and nucleolar proteins is, at least partly, responsible for the inhibition of protein synthesis induced by the extracellular matrix.

The existence of major interactions between fibroblasts and their surrounding matrix, resulting in direct effects on cell metabolism, was demonstrated *in vitro* by the use of various types of three-dimensional culture models. In these cultures, cells are seeded in a network made of fibrillar proteins, which reconstitute an extracellular environment equivalent to the matrix surrounding cells *in vivo* (1-3). We and others demonstrated that fibroblasts cultivated in three-dimensional collagen lattices exhibit a low metabolic activity, characterized by a large decrease in the synthesis of various extracellular matrix proteins (3, 4). Various mechanisms involved in this regulation were evidenced. It was shown that transcriptional and post-transcriptional controls of the expression of different proteins occurred (5, 6) and that specific integrins were involved (7).

The demonstration of this type of regulation does not rule out the possibility of additional levels of control. We have found that ribosomal RNA (rRNA) content of collagen-lattice cultured fibroblasts was significantly lower than in monolayer cultures. This alteration was at least partly due to a decreased half-life of neosynthesized rRNA (8). In the present study, we hypothesized that these data might be explained by some disturbance of the processing and/or maturation of pre-rRNA, inducing a decrease in the number of ribosomes released to the cytoplasm. We have demonstrated experimentally that the expressions of two nucleolar proteins involved in the control of these processes are markedly altered in collagen-lattice versus monolayer-cultured fibroblasts.

MATERIALS AND METHODS

Materials. Reagents for cell cultures, Moloney-murine-leukemia-virus (M-MLV) reverse transcriptase and Taq DNA polymerase were purchased from Gibco-BRL (Cergy-Pontoise, France), and culture flasks from Nunc (represented in

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TABLE 1
Primers Pairs for Construction of Internal Standards (Competitors) and Amplification of Nucleolin, Fibrillarin, and GAPDH mRNAs

Protein gene studied	Amplified fragment	Size (bp)	Base sequence of the primers
Nucleolin	Competitor	570	Forward: 5′-GAT GCG AGA ACA CTT TTG GCT-3′ Reverse: 5′-TGG CAT CCT CCT CAC TGT TGG AGT TTT GGA TGG CTG GCT T-3′
	Target	700	Forward: 5′-GAT GCG AGA ACA CTT TTG GCT-3′ Reverse: 5′-TGG CAT CCT CCT CAC TGT TG-3′
Fibrillarin	Competitor	446	Forward: 5′-GGG GAA GAA TGT GAT GGT GG-3′ Reverse: 5′-ATG GCT CCA GGG TCA ACT GCC GGT ATT TGT GTG GGT GTC G-3′
	Target	659	Forward: 5′-GGG GAA GAA TGT GAT GGT GG-3′ Reverse: 5′-ATG GCT CAA GGG TCA ACT GC-3′
GAPDH	Competitor	376	Forward: 5′-ACC ACA GTC CAT GCC ATC AC-3′ Reverse: 5′-TCC ACC ACC CTG TTG CTG TAG TGG AGG AGT GGG TGT CGC-3′
	Target	452	Forward: 5′-ACC ACA GTC CAT GCC ATC AC-3′ Reverse: 5′-TCC ACC ACC CTG TTG CTG TA-3′

France by Polylabo, Strasbourg). Other reagents for molecular biology were bought from Sigma (St Louis, MO, USA) and reagents for biochemistry from Prolabo (Paris, France). Primers for PCR were synthesized by Eurogentec (Seraing, Belgium).

Fibroblast cultures. Human dermal fibroblasts were explanted from four different adult skin biopsies and grown in monolayers according to routine techniques (3, 9). Cells from subcultures 2 to 9 were used in this study and seeded in monolayers and in collagen lattices made from acid soluble rat tail tendon collagen prepared in the laboratory, as previously described (2, 5, 8). The ratio of the cell number to the collagen content was comprised between 0.7 and 0.9×10^6 cells/mg collagen. Collagen lattices were allowed to retract freely. Cell studies were performed after one week culture. At that time, collagen lattices had usually to 1/10 of their initial diameter.

RNA extraction and reverse transcription. Total cellular RNA was extracted from 20×10^6 fibroblasts cultivated in monolayers and collagen lattices in 4M guanidinium isothiocianate, 2.5 M sodium citrate buffer, pH 7.0, containing 0.5% sarcosyl and 0.1 M β-mercaptoethanol with turraxing for 20 s. An equal volume of phenol and 0.2 volume of choroform/isoamylic alcool (49/1, v/v) mixture were added in the presence of 0.3 volume sodium acetate, pH 4.0. The mixture was stirred, cooled on ice and centrifuged at 10,000 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 evaluated by A_{260} measurement and its integrity checked by 1% agarose electrophoresis.

cDNA was prepared from 5 μg of total cellular RNA by reverse transcription (RT) at 42°C for 45 min. The 100 μl reaction volume contained 1,000 units of M-MLV reverse transcriptase, 2.5 μM random hexamers, 0.8 mM dATP, dCTP, dGTP and dTTP, 10 units RNAsin, 10 mM dithiothreitol, 5 mM $MgCl_2$ and 50 mM KCl in 20 mM Tris-HCl buffer, pH 8.4. 2.5 μl of the RT reaction product was amplified in a 25 μl PCR mixture containing 0.2 μM forward and 0.2 μM reverse primers, 200 μM dATP, dCTP, dGTP and dTTP, 0.5 units of Taq DNA polymerase, 1.5 mM $MgCl_2$, 5% (v/v) DMSO and 50 mM KCl in the same buffer as above. The PCR reaction was performed in a Hybaid Omnigen thermocycler (Teddington, Middx, U.K.) by 30 cycles of denaturation at 95°C for 20s, primer annealing at 55°C for 30s and extension at 72°C for 30 s.

Primers for PCR were designed according to published sequences of cDNA of nucleolin (10), fibrillarin (11) and GAPDH (12).

Competitive PCR. Internal standards made of DNA fragments for nucleolin, fibrillarin and GAPDH were prepared in the laboratory by RT-PCR generation of slightly smaller product than the fragment amplified from extracted RNAs. For that purpose, composite primers were constructed as shown in Table 1. Internal DNA standards obtained with these primers were purified by Geneclean II kit (Bio 101 inc., La Jolla, CA, USA), quantified by A_{260} absorbance and used in competitive PCR experiments. The nature of the amplified fragments was confirmed by restriction enzyme digestion.

As a rule, series of seven dilutions of the internal standards (31 fg to 2 pg for nucleolin, 2 to 128 fg for fibrillarin,

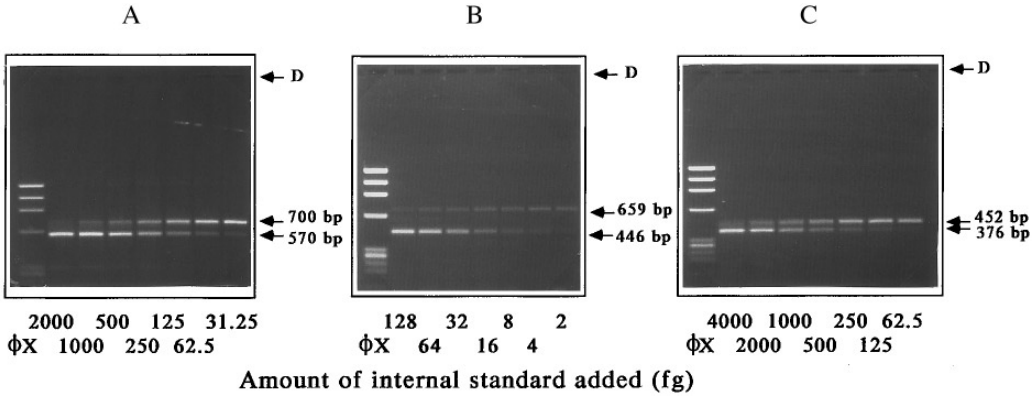

FIG. 1. Competitive RT-PCR quantitation of nucleolin (A) fibrillarin (B) and GADPH (C) mRNAs. 5 μl of the PCR mixture were deposited (D) in the gel and the molecular weights evaluated in comparison with ϕX-174-RF DNA Hae III Digest standard (ϕX).

62 fg to 4 pg for GAPDH) were performed and these various amounts of internal standards were added as competitors to a constant amount of cDNA obtained from cellular extracted RNAs.

PCR products were separated with a 2% agarose gel containing 1 μg/ml ethidium bromide. The fluorescence of the bands was evaluated by scanning of the gel at 312 nm and computed using the Bio-Profile software (Vilbert-Lourmat, Marne la Vallée, France). The ratio target/competitor was plotted against the amount of competitor added, and the amount of target fragments amplified from cellular RNAs calculated. The expressions of nucleolin and fibrillarin were evaluated as ratios to GAPDH expression and the differences tested by the Student's t-test.

RESULTS

Fig. 1 shows the results of typical competitive RT-PCR studies obtained for nucleolin, fibrillarin and GAPDH fragment amplification. In all cases, the range of internal standard dilutions was calculated in order to cover the equivalence zone between amplified target and competitor.

In 4 different strains of fibroblasts, the expression of nucleolin was found increased in collagen lattice-cultured fibroblasts as compared to monolayer-cultured fibroblasts (fig. 2A). The intensity of the increase varied according to the strains of fibroblasts, ranging from +25 to +250 %. The ratio nucleolin mRNA/GAPDH mRNA was 0.035 $\pm$ 0.005 (mean $\pm$ SD) in monolayers vs 0.067 $\pm$ 0.024 in collagen lattices ($t = 2.66$, $p < 0.05$).

By contrast, the expression of fibrillarin in lattice-cultured fibroblasts was constantly lower than that of monolayer-cultured fibroblasts (fig. 2B). The decrease was more constant from strain to strain than that of nucleolin : the values found in lattice cultured cells ranged from 40 to 60% of that found in monolayer. The ratio fibrillarin mRNA/GAPDH mRNA was 0.015 $\pm$ 0.003 in monolayers vs 0.007 $\pm$ 0.001 in collagen lattices. This difference was highly significant ($t = 5.33$, $p < 0.01$).

DISCUSSION

The metabolism of mesenchymal cells and particularly dermal fibroblasts is strongly regulated by the surrounding extracellular matrix. The behavior of fibroblasts cultivated *in vitro* on plastic or glass dishes (characterized by active divisions until confluency is reached, and by a high level of protein synthesis) is strikingly different from the quiescent state found *in vivo* : in physiological situations, these cells, often called fibrocytes, exhibit a low metabolic activity, unless pathological circumstances occur, such as inflammation, wound healing or

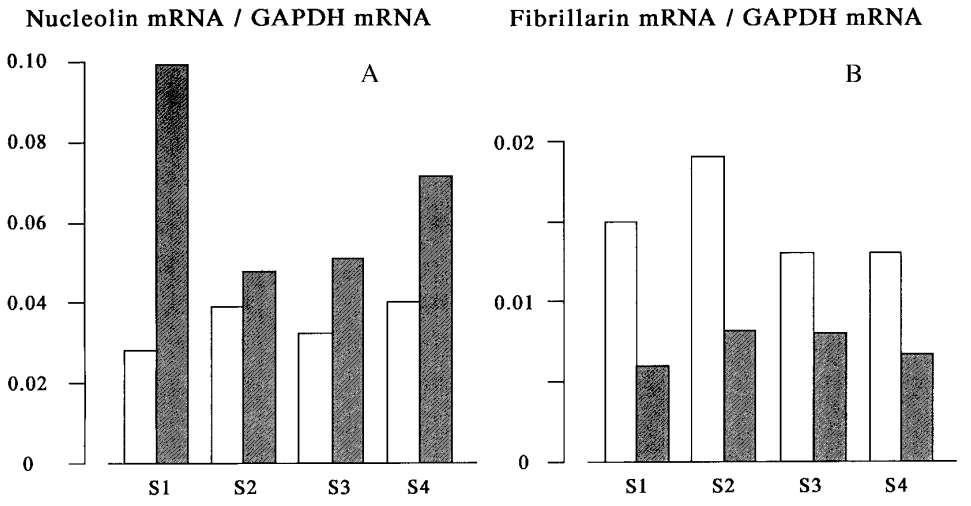

FIG. 2. Evaluation of nucleolin (A) and fibrillarin (B) gene expression in monolayer (open bars) and collagen lattice cultures (closed bars) of human dermal fibroblasts. Each set of bars represents the results obtained in individual fibroblast strains (S1 to S4), expressed as the ratio of nucleolin or fibrillarin mRNA to GAPDH mRNA.

fibrosis, where fibroblasts divide and synthesize large amounts of extracellular matrix macromolecules. In this regard, models of tridimensional collagen lattice cell cultures represent interesting attempts to reconstitute the *in vivo* conditions : in such cultures, as in physiological conditions, cells divide very slowly and synthesize very low amounts of most of the extracellular matrix proteins.

The mechanisms leading to this low metabolic activity are still uncompletely understood. The inhibition of gene transcription of various proteins of the extracellular matrix (e.g. : collagens) was demonstrated by our group (5) and others (6). Moreover, we have shown that additional post-transcriptional mechanisms were involved, depending on the nature and the physical characteristics of the extracellular matrix (5). It seems that this down-regulation of gene expression involves signalling mechanisms linked to particular integrins (7).

Additional mechanisms of regulation may be involved. We have previously shown that RNA content of fibroblasts cultivated in collagen lattices was much lower than in monolayer cultures, suggesting a decrease of the number of ribosomes available for protein synthesis (8). Simultaneously, we demonstrated that rRNA stability, but not synthesis, was decreased in these conditions, and hypothesized that some disturbance in rRNA processing might occur.

Pre-rRNA processing takes place in the nucleolus and is characterized by its association with many nucleolar proteins, some of them being structural proteins, others being associated in the maturation process (13). In this study, we investigated the expression of two nucleolar proteins, nucleolin and fibrillarin, in collagen-lattice versus monolayer-cultured fibroblasts, by competitive RT-PCR methods. Since the total RNA content of fibroblasts cultivated in collagen lattices is significantly lower than in monolayer cultures (8), the expression of results on a total RNA basis could be misleading. Therefore, we compared nucleolin and fibrillarin to GAPDH gene expression in order to give evidence for a particular effect of collagen lattice on nucleolar protein gene expressions.

We considered these two proteins as candidates for modulating the processes of rRNA maturation because of their characteristics. Nucleolin is a major nucleolar phosphoprotein that contains four consensus RNA binding domains (10,14) and is involved in ribosome biogenesis,

even if its exact role is still largely unknown (15). As a particular feature, small nucleolar (sno) RNA U20 is encoded in intron 11 of the nucleolin gene (16). Other protein coding genes share this property : L1 ribosomal protein gene contains two sno RNAs, U16 and U18, and their processing is alternative to splicing (17). In this paper, we demonstrate an increase of nucleolin gene expression in fibroblasts cultivated in collagen lattices. The interpretation of these data is not easy since an increase of nucleolin is generally reported in proliferating cells whereas fibroblast divide very slowly in collagen lattices. Many events may modulate the role of nucleolin : the translation of nucleolin pre-mRNA, the possible alternative processes of nucleolin and U20 formation, the autoproteolytic activity of nucleolin (13). Furthermore, nucleolin might be involved in many other regulatory processes. For example, nucleolin was shown to act as a transcriptional repressor of alpha-1 acid glycoprotein gene (18). A specific role on matrix protein expression cannot be excluded.

The second protein studied was fibrillarin, which is localized in dense fibrillar centers of nucleoli. It is associated with sno RNAs and functions in pre-rRNA processing, especially at the 5′ end (11,19). NOP1, the yeast fibrillarin, is required for pre-rRNA processing. NOP1 depletion impairs production of cytoplasmic ribosomes, affecting all steps of pre-rRNA processing (20). Sno RNAs, containing complementary sequences with pre-rRNA, are associated with fibrillarin (21). They are thought to be involved in the control of pre-rRNA folding during pre-ribosome assembly. In this study, we constantly found a major decrease of fibrillarin gene expression in fibroblasts cultivated in collagen lattices. This decrease was twice more intense than general down-regulation of protein synthesis, as demonstrated by the decrease of the ratio fibrillarin/GAPDH. Considering the presently known roles of fibrillarin, we propose that the decrease of fibrillarin gene expression observed in fibroblasts cultivated in collagen lattices impairs pre-rRNA processing and participates in the down-regulation of mature rRNA(8).

Taken together, our results demonstrate for the first time that extracellular matrix modulates the expression of nucleolar proteins. This alteration of nucleolar protein expression might affect the processes of pre-rRNA maturation and, in turn, significantly alter ribosome formation. These mechanisms could be, at least partly, responsible for the general decrease of fibroblast biosynthetic activity when cultivated in three-dimensional collagen lattices.

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