Effect of transforming growth factor β on fibroblasts in three-dimensional lattice cultures

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Human skin fibroblasts were cultivated in three-dimensional fibrin or collagen lattices, under retracting or non-retracting conditions, and the influence of transforming growth factor β (TGFβ) was tested. TGFβ stimulated the synthesis of non-collagen protein and of collagen in all the systems. However, only in non-retracting fibrin lattices did it restore a level of protein synthesis comparable to that found in monolayers. The effects of TGFβ greatly depended on the type of substratum and on the presence or absence of retraction.

Transforming growth factor β; Collagen synthesis; Fibroblast culture; Collagen lattice; Fibrin lattice

1. INTRODUCTION

Fibroblast metabolism appears to benefit from more physiological conditions when cultured in lattices than in monolayers [1,2]. However, fibroblasts embedded in a collagen matrix eventually become dormant, stop dividing [3], and make practically no more collagen [4]. Earlier [5], we proposed a culture system using a fibrin matrix instead of collagen. By varying the technical conditions we could obtain two systems of fibrin lattices, one capable of retraction like collagen lattices, and another that was prevented from retracting. In the latter, the cells remained non-dividing but retained the ability to synthesize proteins and collagen. There seems to be a link between retraction of lattices and inhibition of collagen synthesis. In order to better demonstrate this link, we have looked at the effect, in various types of lattice cultures, of transforming growth factor β (TGFβ). This peptide growth factor [6] has recently been shown to stimulate the synthesis of several components of connective tissue, such as collagens types I and III, fibronectin [7-10], and some glycosaminoglycans [11,12].

We found that the effects of TGFβ on the synthesis of collagen and of non-collagen protein depended greatly on the type of substratum and on the presence or absence of retraction.

2. MATERIALS AND METHODS

2.1. *Materials*

Chemicals were purchased from Prolabo (Paris, France), unless another source is mentioned. Ascorbic acid was purchased from Merck (Darmstadt, FRG), β-aminopropionitrile (fumarate) from Sigma (St. Louis, MO, USA), L-proline from Calbiochem (Meudon, France), L[U-^{14}C]-proline (sp. act. > 10.4 GBq/mmol) from New England Nuclear (Paris), Triton X-100 from Technicon Corp. (Tarrytown, NY, USA), and bacterial collagenase (CLSPA grade) from Worthington (Freehold, NJ, USA) purified in the laboratory according to the method of Peterkofsky and Diegelmann [13]. Sterile human fibrinogen was obtained from the 'Centre de Transfusion Sanguine' (Strasbourg, France), human thrombin from Ortho Diagnostic Systems (Raritan, NJ, USA), and TGFβ purified from human platelets from Calbiochem.

All reagents for cell culture were purchased from Gibco (Paisley, Scotland), plastic culture dishes from Flow (Irvine, Scotland) and flat-bottomed glass dishes, 50 mm in diameter, from SVIP (Paris).

2.2. *Fibroblast cultures*

Human skin fibroblasts, explanted from infant (obtained with parental consent), were grown in the usual way [14]. They were used between the 2nd and 10th passages.

Collagen lattices were prepared according to Bell's technique modified in our laboratory [15]; the number of cells was kept at 200 000 per dish. As a rule, these lattices retracted to 1/5 of their original diameter within 8 days. Non-retracting collagen lattices were obtained by inserting nylon dishes 48 mm in diameter (sold by Polylabo, Strasbourg, as nylon-sieve) within the lattice just after the formation of the network.

Fibrin lattices were prepared as previously described [5]. Non-retracting lattices were prepared in sterile plastic Petri dishes to which the fibrin adhered, thus preventing any retraction. Retracting fibrin lattices were prepared in siliconized glass Petri dishes.

We compared protein and collagen synthesis in all five culture systems at day 5 after seeding (Protein synthesis is linear from day 3 to day 8 in these systems [5]). The cultures were first preincubated at day 4, for 24 h, in Eagle's minimum essential medium (MEM) containing only 0.5% fetal calf serum (FCS). After this preincubation, the dishes were washed twice with 4 ml of fresh MEM and incubated for 24 h in a medium consisting of 50 $\mu g \cdot ml^{-1}$ β-aminopropionitrile, 2 $\mu Ci \cdot ml^{-1}$ [^{14}C]proline diluted in 23 $\mu g \cdot ml^{-1}$ carrier proline, and optimal concentrations of ascorbic acid (50 $\mu g \cdot ml^{-1}$ in collagen lattices, 10 $\mu g \cdot ml^{-1}$ in fibrin lattices), all dissolved in MEM devoid of FCS.

Amounts of TGFβ ranging from 1 to 20 $ng \cdot ml^{-1}$ were added. Control dishes without TGFβ were processed in parallel. Every concentration was tried in quadruplicate dishes for all types of cultures.

At the end of the incubation period, the medium was pooled with

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two 1-ml rinses of the cell + lattice layer, to constitute the 'supernatant fraction'. The cell-containing collagen lattices were solubilized by treatment with 2 ml of a 0.2% Triton X-100 solution and heated for 10 min at 100°C. The fraction was dialysed against distilled water, an aliquot counted and the proteins in another aliquot precipitated by addition of ammonium sulfate to 1.33 mol·l^{-1}. The precipitate was digested with bacterial collagenase [13]. The non-collagen proteins were precipitated in 90% ethanol. Supernatants were evaporated to dryness, the residues were dissolved in 1 ml of water and radioactivity was counted.

The cell-containing fibrin lattices were suspended in 2 ml of distilled water, sonicated (3 × 10 s), heated at 100°C for 10 min and dialysed against distilled water. Cells in monolayer cultures were detached by scraping in a 0.2% Triton X-100 solution (cell fraction). Then the suspensions were processed like the collagen lattices.

Radioactivity was measured in a Packard (Minaxi 4430 model) scintillation counter.

For the cell counts, lattices were digested with either collagenase or trypsin [5], and the liberated cells were collected by low-speed centrifugation and counted in a Neubauer counting device. Lattice retraction was measured over a black background with a ruler graduated in millimeters.

3. RESULTS

The number of viable cells remained constant from seeding to day 7 of culture (data not shown). All the radioactivity data were corrected for an identical initial number of 200 000 cells. TGFβ did not affect lattice retraction (data not shown).

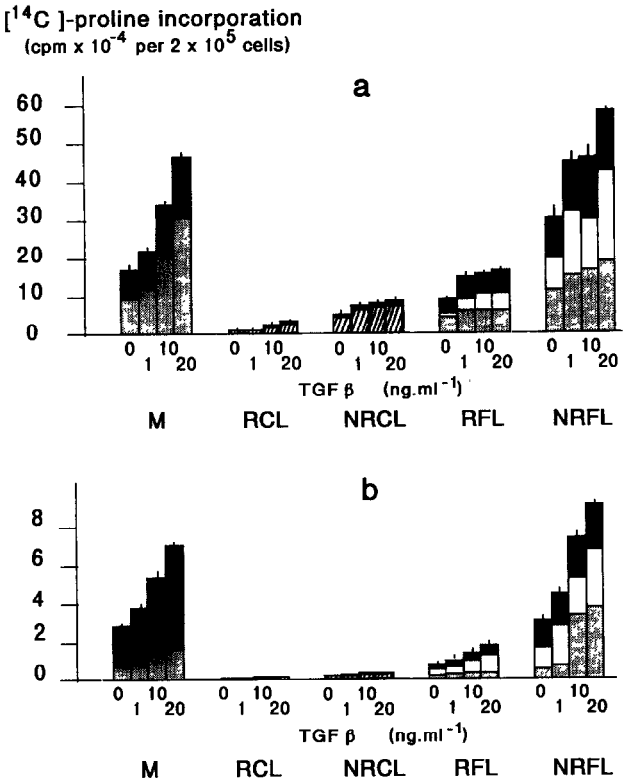

Fig.1. Synthesis (as judged from [^{14}C]proline incorporation) of (a) total proteins and (b) collagen by fibroblasts cultured with various concentrations of TGFβ in a (M) monolayer, (RCL) retracting collagen lattice, (NRCL) non-retracting collagen lattice, (RFL) retracting fibrin lattice, (NRFL) non-retracting fibrin lattice. The SD for each whole bar is represented at the top of the bar. The various fractions are represented as (▬) supernatant, (▭) lattice, (▩) cells, (▨) cells + lattice (when they could not be separated).

Table 1

Activation of collagen synthesis by TGFβ in various types of culture

Concentration of TGFβ (ng·ml^{-1})	Activation coefficient				
	Monolayer	RCL	NRCL	RFL	NRFL
1	1.3	1.3	1.2	1.5	1.4
10	1.8	1.6	1.6	1.9	1.7
20	2.5	2.1***	2.0***	2.5	2.2*

Activation coefficient, ratio of the radioactivity of collagen in TGFβ stimulated culture to the radioactivity of the corresponding control without TGFβ. RCL, retracting collagen lattice; NRCL, non-retracting collagen lattice; RFL, retracting fibrin lattice; NRFL, non-retracting fibrin lattice. Student's *t*-test was used to evaluate the statistical significance.

*** $P<0.001$ (in comparison with monolayer)

* $P<0.02$ (in comparison with monolayer).

The effects of TGFβ on the incorporation of [^{14}C]proline into total proteins and into collagen in the various culture systems are shown in fig.1. The amounts of protein (fig.1a) and of collagen (fig.1b) synthesized in non-retracting fibrin lattices were comparable to those found in monolayers. In both types of collagen lattice, synthesis of both substances was slight, particularly of collagen, and there was less synthesis in the retracting lattices than in the non-retracting ones. Total proteins increased faster in the insoluble fraction than in the medium. TGFβ concentrations 1 and 10 ng·ml^{-1} increased the amount of collagen synthesized in a manner roughly proportional to the basic level of synthesis in the corresponding non-treated culture (table I). For doses of 20 ng·ml^{-1} the activation was significantly lower in collagen lattices than in monolayer cultures.

4. DISCUSSION

In a previous paper [5], we demonstrated that fibrin lattices permit study of the fibroblast metabolism in physiological conditions. In the present experiments we compared the amounts of total proteins and collagen synthesized in five types of culture (monolayer confluent cells, retracting collagen lattice, non-retracting collagen lattice, retracting fibrin lattice, and non-retracting fibrin lattice), in the presence of 3 concentrations of TGFβ (1, 10, and 20 ng·ml^{-1}). For purposes of comparison, the non-dialysable radioactivity was taken as an index of total protein synthesis and the collagenase-digestible radioactivity was used as an index of collagen synthesis. The conditions of culture in collagen lattices did not permit evaluation of the specific activity of collagen or proteins. TGFβ is known to increase the biosynthesis of collagen, fibronectin and some other proteins in monolayer fibroblast cultures

[7–10] but its effects in the various types of lattices have not yet been characterized. TGFβ activated collagen synthesis in all the types of culture. Its effect was roughly proportional to the base level of synthesis in the same conditions of culture without TGFβ. Fibroblasts embedded in the collagen lattice remained dormant but were not dead as was shown by the trypan blue test, by their normal division after digestion of the lattice with collagenase and by the absence of any change in numbers from seeding to day 7 of lattice culture. We assume that being surrounded by collagen fibres inhibits fibroblasts not only from dividing but also from secreting new matrix. This inhibition could depend on some limitation of the diffusion of nutrients, oxygen, and labelled proline through the retracted, denser lattice. However, Nishiyama et al. [16] found that after complete retraction of lattices, fibrils occupied only 28 ± 5% of the plane of a cross section, demonstrating that large interstices remain for diffusion of small molecules. Several workers [3,4] have also demonstrated that low-molecular-weight substances can diffuse through lattices. Nishiyama et al. [16] recently demonstrated that $[^3H]H_2O$ equilibrates between the outside culture medium and the lattice within less than 30 min at 37°C and that a peptide factor such as EGF penetrates freely into the lattice.

The other factor inhibiting the cells seems to be the retraction of the matrix, because both types of matrix when prevented from retracting were less inhibiting to the point that collagen and protein synthesis were almost as great in non-retracting fibrin lattices as in monolayer cell cultures.

It appears that surrounding fibroblasts with collagen fibres powerfully inhibits matrix synthesis and that retraction of the matrix may be another inhibitory factor.

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