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Original article

Collagen biosynthesis in cell culture: Comparison of corneal keratocytes and skin fibroblasts Effect of rhamnose-rich oligo- and polysaccharides

Biosynthèse du collagène en culture cellulaire : comparaison entre kératocytes cornéens et fibroblastes cutanés Effet des oligo- et polysaccharides riches en rhamnose

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Abstract

Corneal keratocytes are often confounded with fibroblasts, although their matrix-synthetic phenotype is quite different as shown by the nature and relative amount of the different collagens and proteoglycans–glycosaminoglycans synthesized. In these experiments, we compared the concentration of collagens excreted in the culture medium by human corneal keratocytes and skin fibroblasts at three consecutives passages. Although the keratocytes excreted less collagen at earlier passages, they approached and reached fibroblasts at later passages. This can be taken as an indication of the progressive loss of a specific keratocyte phenotype with increasing passages (in vitro aging). The effect of rhamnose-rich oligo- and polysaccharides on collagen secretion also confirmed the subtle differences between these two cell–types, as well as the difference between saturation density of both cell types at confluence and the proportion of dead cells floating on top of the culture medium. These differences were also attenuated with passage number without disappearing completely. The significance of these findings will be discussed in the light of previous results in our and other laboratories on matrix-secreting phenotypes and aging.

Résumé

On confond souvent les kératocytes cornéens avec les fibroblastes cutanés, bien que leurs phénotypes, reflétant la synthèse de la matrice, soient différents : il suffit de regarder la nature et les quantités relatives des différents types de collagènes et protéoglycanes–glycosaminoglycanes synthétisés. Au cours des ces expériences, nous avons comparé les concentrations de collagènes excrétés dans le milieu de culture par les kératocytes cornéens et les fibroblastes de la peau, à trois passages consécutifs. Bien que les kératocytes excrètent moins de collagène aux passages précoces, ils se rapprochent, voire rejoignent les quantités de collagène synthétisées par les fibroblastes, aux passages plus tardifs. On peut considérer cela comme une indication de la perte progressive de spécificité du phénotype des kératocytes, au cours de la sénescence in vitro. L'effet des oligo- et polysaccharides riches en rhamnose, sur la sécrétion de collagène, confirme également les différences subtiles entre ces deux types cellulaires, à propos de la densité de saturation et de la proportion de cellules mortes, flottant à la surface du milieu de culture. Ces différences sont également atténuées au cours de passages sans disparaître complètement. La signification de ces découvertes sera discutée, en prenant compte les

Abbreviations: RROP-s, Rhamnose-rich oligo- and polysaccharides; ECM, Extracellular matrix; NHDF, Normal human dermal fibroblast; GAG, Glycosaminoglycans; PG, Proteoglycans

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résultats sur les phénotypes liés à la sécrétion des macromolécules de la matrice extracellulaire ainsi que sur le vieillissement, précédemment obtenus dans notre, et d'autres, laboratoires.

Keywords: Collagen; Fibroblasts; Keratocytes; Cornea; Skin; Aging; Connective tissues; Rhamnose-rich oligo- and polysaccharides

Mots clés : Collagène ; Fibroblastes ; Kératocytes ; Cornée ; Peau ; Vieillissement ; Tissus conjonctifs ; Oligo- et polysaccharides riches en rhamnose

1. Introduction

The structure and function of connective tissues, such as the skin or the cornea are largely dependent on the nature and relative amount of extracellular matrix (ECM) – macromolecules synthesized. This ''program'' of matrix biosynthesis is changing with cell-phenotype and also with age. The determination of these parameters, the nature and relative amount of ECM – macromolecules synthesized can be taken as a characteristic of cellular phenotype. Such determinations, carried out at successive passages can give some information on the age-dependent changes of cell-phenotype. We shall describe here such experiments carried out on human corneal keratocytes and skin fibroblasts at three successive passages based on the quantitative measure of total collagen excretion in the culture medium.

We also tested the action of rhamnose-rich oligo- and polysaccharides (RROP-s) previously shown to modulate matrix biosynthesis [1]

and collagen synthesis by these two cell types. Both RROP-s tested (RROP-1 and RROP-3) modulated differently the collagen synthetic activity of keratocytes and fibroblasts. These results also confirm the subtle differences between these two cell-phenotypes as far as their matrix synthetic activity is concerned.

2. Materials and methods

2.1. Cell culture

Human skin fibroblasts were obtained from Cambrex (Emerainville, France) (NHDF-Adult cryopreserved, product code CC-2511, lot number 4F1293) derived from the skin of a 39-year-old woman, according to the furnishers' informations.

Corneal keratocytes were obtained from corneal rings excised at transplantation from corneas kept at the French Eye Bank, as previously described [2].

Fibroblasts were cultivated in standard conditions: Dulbecco's modified Eagle culture medium (DMEM-glutamax, Invitrogen™) supplemented with 10% (v/v) foetal calf serum (FCS, Gibco®), antibiotics: penicillin (100 U/ml; Gibco®) and streptomycin (100 mg/ml; Gibco®), and an antifongic (0.25 µg/ml amphotericin B, Gibco®). This was designated as the complete culture medium.

Keratocytes were cultivated in a D-MEM/F-12, Dulbecco's modified Eagle culture medium, supplemented with 10% (v/v) foetal calf serum, antibiotics and antifongic (Penicillin 100 U/ml (Gibco®), Streptomycin 100 µg/ml and Fongisone).

These two kinds of cell were incubated in a temperature-controlled, humidified incubator with 5% CO_2 at 37 °C. Keratocytes were kept at the fifth passage in liquid nitrogen for one year. Fibroblast cultures were started freshly at the delivery of cells by Cambrex and kept also in liquid nitrogen. Fibroblasts and keratocytes were used at the 7th, 11th and 12th passages. In all experiments, cells were seeded at 50,000 cells per well, in a Nunc-plate of 12 wells. For every experiment, four to six parallel cultures were set up in order to reach significance.

All cells were grown in the complete culture medium, in 75 cm^2 surface ventilated culture flasks (Nunc) and subcultured by trypsinisation (0.05% trypsin, Gibco®). Culture medium was changed every two to three days. Both serial cultures of cells were used for the determination of the following parameters:

- time to reach confluency in the standard culture flasks of 75 cm^2;
- the total number of cells at saturation density determined after trypsinisation on the Coulter counter, at increasing passages [3];
- total amount of collagen synthesized and deposited by the cells after 72 h, using the colorimetric procedure with Picro-Sirius red staining [4,5]. This method has the advantage to estimate not only freshly synthesized collagen but also the total amount of collagen accumulated by the cells during the 72 h culture period. Shortly, cells were seeded in six well plates at 5.10^4 cells/well, after 72 h of culture, cells were washed twice with PBS then fixed for one hour with 1 ml of the Bouin's solution at room temperature. Following fixation, cells were washed twice with distilled water and then stained with Sirius-red solution (0.5 g Sirius red F3B Gurr BDH, 500 ml saturated aqueous solution of picric acid and a little solid picric acid to ensure saturation) for one hour under shaking at room temperature. Samples were then washed consecutively with distilled water and 0.01 M HCl to remove unbound dye. Then, bound dye was solubilised by incubation in 500 µl of 0.1 M NaOH for one hour under shaking. Samples were transferred in a spectrophotometer cuvette and absorbency read at 550 nm.

2.2. Rhamnose-rich oligo- and polysaccharides

All α-l-rhamnose-rich oligo- and polysaccharide preparations (RROP-s) were obtained from Solabia - BioEurope (Pantin, France). RROP-1 (commercial name Rhamnosoft®) is obtained from *Klebsiella pneumoniae* strains. The other polysaccharide of 5 kDa, RROP-3 (commercial name