IN VITRO SYNTHESIS AND DEGRADATION OF COLLAGEN BY CHICK CHONDROCYTES AND FIBROBLASTS

Michael DUCHÊNE, Sigrid WIEDEBUSCH, Klaus KÜHN and Peter K. MÜLLER
Max-Planck-Institut für Biochemie, 8033 Martinsried, FRG

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1. Introduction

Chondrocytes represent a biological system well suited to studying gene expression of eucaryotic cells. When these cells are kept in monolayer culture for several weeks, they change morphology from a polygonal to an elongated shape. They stop producing cartilage type II collagen and switch to synthesis of other types of collagen, mainly type I collagen, which is a major product of fibroblasts [1–4]. This process is more rapid when chondrocytes are maintained in the presence of 5-bromodeoxyuridine [5] or calvaria conditioned medium [6] or fibronectin [7]. Here, we have chosen a quantitative approach to analyze total synthesis and degradation of collagen by chondrocytes in parallel to their switch of collagen synthesis and we compared these parameters with those obtained from chicken fibroblasts. Our results show that chondrocytes retain at any stage cellular properties which are characteristically different from those of fibroblasts.

2. Materials and methods

2.1. *Cell culture*

Chondrocytes were liberated from sterna and fibroblasts from tendons of 16-day-old chick embryos by collagenase–trypsin treatment as in [3,8]. For long-term cultures, freshly liberated cells were plated onto 10 cm petri dishes at an initial density of 8×10^5 cells/dish. For incubation on day 2 and 3, 4×10^7 cells/dish were plated. Chondrocytes were maintained in Ham's F10 medium (supplemented with 10% fetal calf serum, 20 μM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml sodium ascorbate). Fibroblasts were grown in Dulbecco's modified Eagle's medium containing the same supplements as mentioned above. Cells were kept at 37°C in an atmosphere containing 5% CO_2. Medium was changed every other day. When cells reached confluence (in intervals of 7–9 days) they were trypsinized and passaged by a split of 1:2. Morphological evaluation of chondrocytes was based on counting cells in randomly selected unit fields and calculating the proportion of 'fibroblast-like cells' which were defined as being at least twice as long as wide.

2.2. *Radioactive labeling and chromatographic procedures*

Cells were kept in preincubation media (same as maintenance media however without streptomycin) for 24 h before labeling. Then cells were pulse-labeled for 24 h with 6 ml incubation medium (Dulbecco's modified Eagle's medium containing 5 μCi/ml L-[2,3-^{3}H]proline (NEN, Dreieichenhain), 150 μg/ml β-aminoproprionitrile, 50 μg/ml sodium ascorbate and 400 U/ml penicillin. For days 2 and 3 incubation cultures with high initial density (4×10^7 cells/dish) were used, for later incubations cultures with low initial density (8×10^5 cells/dish). In this way, cells were always in a state of confluence at the time of incubation. After the radioactive pulse, cell numbers were determined, the cell layer combined with the medium and homogenized using a glass homogenizer. EDTA (1 ml, 1%) and 0.5 ml of a 3 mg/ml phenylmethane sulfonyl fluoride and 3 mg/ml 4-chloromercuri-benzoic acid suspension were added as protease inhibitors. The resulting suspension was dialyzed twice against 250 ml 0.5% acidic acid.

For determination of the proportion of type I collagen in total collagenous protein, aliquots of the non-dialyzable material were subjected to limited pepsin digestion. Pepsin resistant (collagenous) material was separated on carboxymethyl cellulose as in

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[9]. The analysis was restricted to the measurement of radioactivity in the α1 and α2 peaks. The increase of α2 chain radioactivity was used to calculate the increasing proportion of type I collagen [9].

To measure total protein and collagen synthesis, aliquots of the non-dialyzable material were lyophilized, hydrolyzed with 6 M HCl and analyzed on a Beckman multichrom amino acid autoanalyzer equipped with a fraction collector. Total counts (hydroxyproline and proline) incorporated per 10^3 cells were taken as a measure for total protein synthesis. The radioactivity in the proline and hydroxyproline peaks were used to calculate the percentage of collagen with respect to total protein according to the formula:

$$\% \text{ Collagen} = \frac{2 \times \text{cpm Hyp}}{5 \times (\text{cpm Pro} - \text{cpm Hyp}) + 2 \times \text{cpm Hyp}} \times 100$$

For detailed discussion see [10].

Since hydroxyproline liberated by collagen degradation is not reincorporated [11], dialyzable hydroxyproline was used as a measure for collagen degradation. It was determined by subjecting aliquots of the acidic acid dialysates to amino acid analysis in the same way as the non-dialyzable material. The percentage of newly synthesized collagen degraded after the 24 h pulse was calculated as:

$$\% \text{ Degradation} = \frac{\text{Hyp (dialyzable)}}{\text{Hyp (non-dialyzable)} + \text{Hyp (dialyzable)}} \times 100$$

3. Results

3.1. *Parallel change in cell morphology and pattern of collagen synthesis in chondrocytes*

Freshly isolated chick embryo chondrocytes attach on plastic petri dishes in a spherical shape (fig.1A). After 2–3 days in culture they assume a polygonal morphology (fig.1B). With time in culture an increasing number of chondrocytes display a fibroblast-like elongated cell morphology (fig.1B,C) In two independent experiments we counted the number of fibroblast-like cells at various time intervals (fig.2). In the same cultures, the proportion of type I collagen in total collagen was determined. This proportion was found to increase parallel to the proportion of fibroblast-like cells (fig.2). After ~50 days in culture, type I collagen amounted to some 80% of total collagen synthesis and the proportion of fibroblast-like cells was in the same range.

3.2. *Rate of synthesis of collagen and total protein in chondrocytes and fibroblasts*

Chondrocytes and fibroblasts were maintained in culture for 7 weeks. In regular intervals cells were labeled with [^{3}H]proline and total non-dialyzable

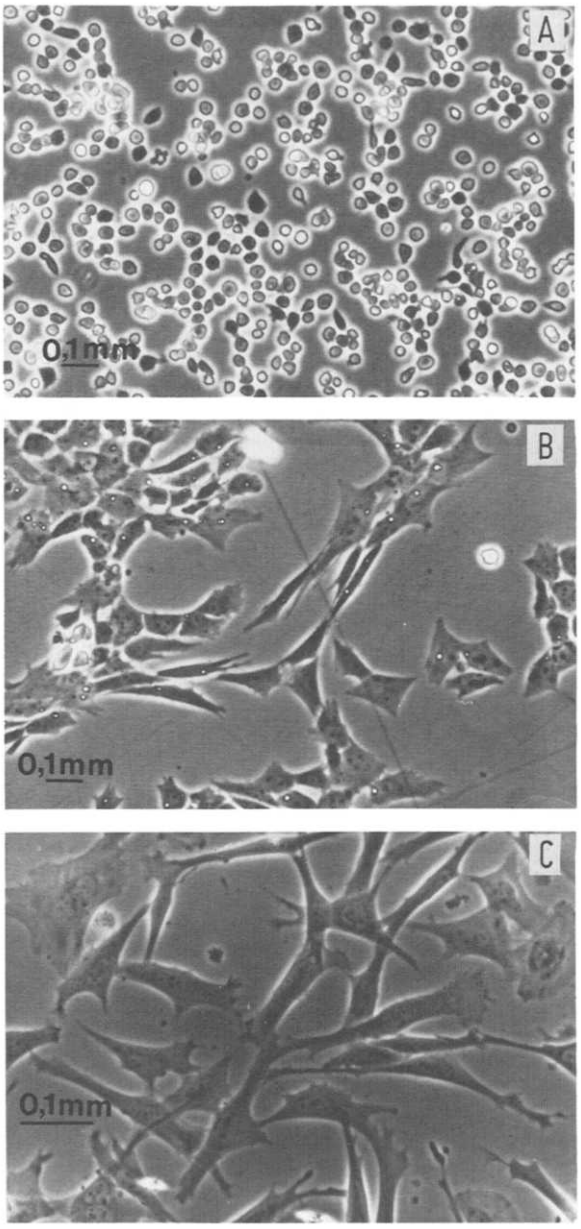

Fig.1. Chick embryo chondrocytes derived from sterna of 16-day-old embryos: (A) freshly attached cells (short-term culture), 1 day after plating; (B) cells after 10 days of culture; (C) cells after 50 days of culture (long-term culture, cells passaged 4 times).

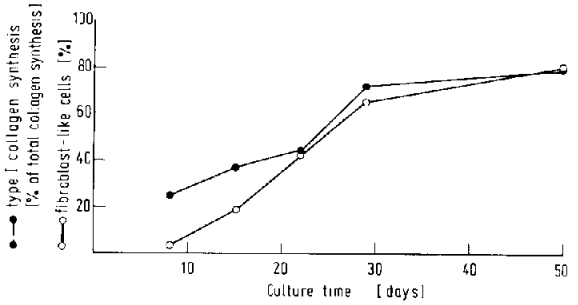

Fig.2. Change of morphology of chick embryo chondrocytes (% of fibroblast-like cells) compared to change in collagen synthesis (% of type I collagen, as determined by carboxymethylcellulose chromatography of radioactively labeled collagenous cell products). Values were derived from 2 separate experiments.

counts incorporated per 10^3 cells were determined as a measure for total protein synthesis. As seen in fig.3, proline incorporation was similar for short, intermediate and long-term culture. Chondrocytes incorporated slighty more proline than fibroblasts.

The relative rate of collagen synthesis was assayed by analysis of non-dialyzable cell products by their content of [^{3}H]hydroxyproline and proline. This rate

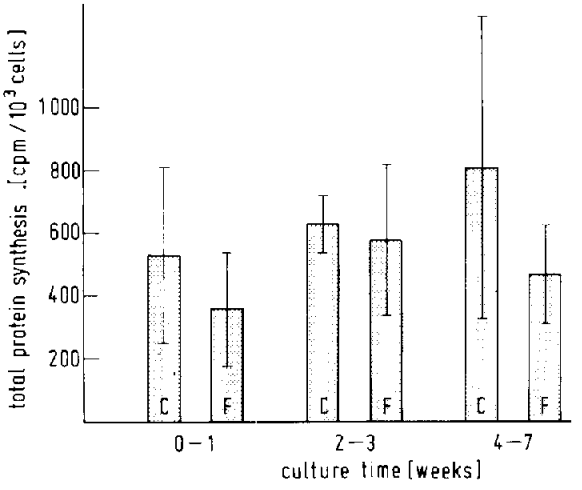

Fig.3. Total protein synthesis of chick embryo chondrocytes and fibroblasts during long term culture expressed as cpm incorporated per 10^3 cells into non-dialyzable materials. Values were derived from 6 independent determinations and summarized into 3 groups: 0–1 weeks, short culture time, differentiated chondrocytes; 2–3 weeks, intermediate culture time, chondrocytes changing phenotype; 4–7 weeks, long term culture, de-differentiated chondrocytes (F, fibroblasts; C, chondrocytes).

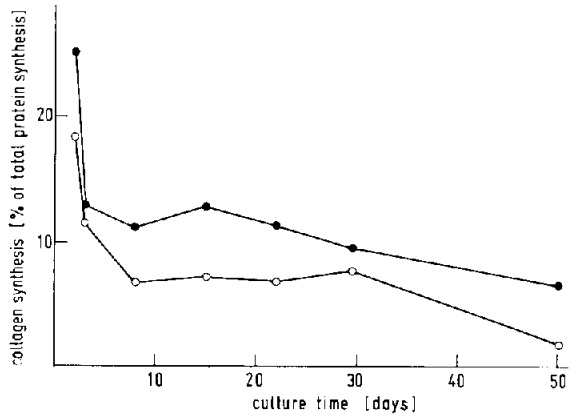

Fig.4. Percentage of collagen synthesis in chick embryo chondrocytes (●——●) and fibroblasts (○——○) during long term culture. Values were derived from 5 separate expt.

(fig.4) was always found to be higher in chondrocytes than in fibroblasts even after a rather complete switch to type I collagen synthesis. Initially collagen synthesis by both chondrocytes and fibroblasts was high, remained at an intermediate level for several weeks and then declined again.

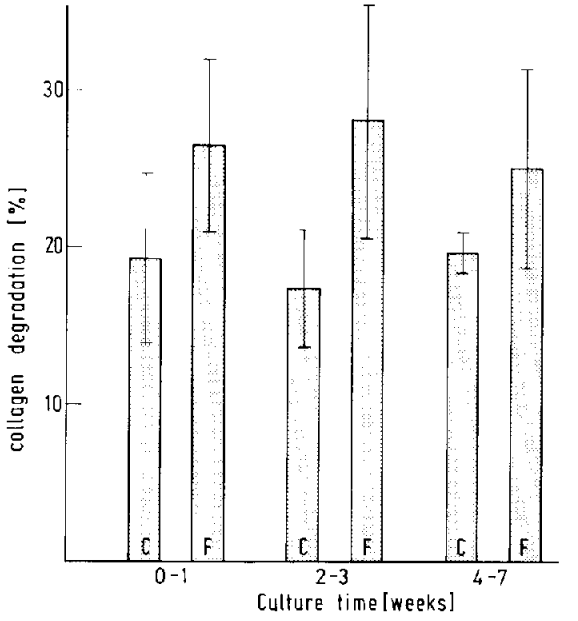

Fig.5. Percentage of newly synthesized collagen degraded to dialyzable material during a 24 h pulse in long term cultures of chick embryo chondrocytes and fibroblasts. Values were derived from 8 independent determinations (see also fig.3). (F, fibroblasts; C, chondrocytes).

3.3. *Collagen degradation in chondrocytes and fibroblasts*

The percentage of newly synthesized collagen degraded within a 24 h pulse was determined for chondrocytes and fibroblasts (fig.5). Degradation of collagen does not significantly depend on the culture time of cells. Fibroblasts, however, showed consistently higher degradation (29 ± 7%) than chondrocytes (18 ± 4%). The difference between the degradation values was tested by the Mann Whitney *U*-test [12] which showed that the difference was clearly significant (significance level <0.001).

4. Conclusions

In [13] using immunofluorescence techniques, we had shown that the cell shape of an individual chondrocyte does not unambiguously reflect the state of de-differentiation with respect to the proceeding switch of collagen synthesis from type II to type I collagen [13]. Here we analyzed mass cultures of de-differentiating chondrocytes and found that an increasing number of a fibroblast-like phenotype is closely paralleled by a growing proportion of type I collagen synthesis. Thus under these culture conditions the cell shape may serve as a useful marker for the initial evaluation of the presumptive state of the switch in collagen synthesis.

There is no obvious correlation between the relative rate of collagen synthesis and the types of collagen synthesized by chondrocytes as they are maintained in culture and de-differentiate (fig.2,4). This may indicate that the mechanisms required for control of amount and type of collagen are regulated independently from each other.

Depending on the animal species and the culture conditions the course of de-differentiation of chondrocytes may vary with respect to time and relative rate of collagen synthesis [3]. In general, calf chondrocytes seem to lose their cellular characteristics more rapidly and end up in a lower rate of collagen synthesis than chicken chondrocytes.

Incorporation of proline into total protein showed considerable variation for both fibroblasts and chondrocytes. In general, however, each of the 2 cell types seems to maintain its biosynthetic capacity during time in culture.

Analysis of the metabolic fate of collagen in both chondrocytes and fibroblasts provides evidence that the two cell types retain specific and distinguishable properties. The relative rate of collagen synthesis was always higher in chondrocytes than in fibroblasts while degradation of collagen was higher in fibroblasts. These differences were present in freshly isolated cells and in continuously cultured cells. Thus our study demonstrates that de-differentiating chondrocytes on one side approach a fibroblast-like state (cell morphology, pattern of collagen types) while they retain specific features of their own on the other side (overall synthesis and degradation of collagen).

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