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Influence of cell density on collagen biosynthesis in fibroblast cultures

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Characteristic features of collagen metabolism in human skin fibroblasts were studied in relation to cell density. Measuring peptide-bound hydroxyproline we found that collagen synthesis per cell decreased when cultures approached confluency. On the other hand, the relative rate of collagen synthesis (collagen/total protein) was higher in quiescent than in proliferating cultures. With increasing cell density the proportion of type III collagen in comparison with type I was found to be slightly increased. In addition, in low-density cultures $[\alpha 1(I)]_3$ collagen trimers were produced in considerable amounts, whereas they were no longer detected in cultures with a high cell density. Although hydroxylation of proline residues was normal in all cell stages, conversion of procollagen into collagen was found to depend strongly on the density at which the cells were investigated. Almost no cleavage of procollagen peptides was observed in rapidly growing cells, whereas highly confluent cell cultures converted most of the newly synthesized procollagen molecules.

Fibroblast cultures are commonly used to study the metabolic pathway of collagen in the normal (Layman *et al.*, 1971; Smith *et al.*, 1972; Pontz *et al.*, 1973; Lichtenstein *et al.*, 1975; Bienkowski *et al.*, 1978; Nusgens *et al.*, 1980) and the diseased state [for reviews, see Lapiere & Nusgens (1976) and Prockop *et al.* (1979)], and to analyse the influence of drugs and hormones thereon (Diegelman & Peterkovsky, 1972; Trash & Cunningham, 1973; Fodge & Rubin, 1975; Baum *et al.*, 1978; Uchida *et al.*, 1979; Clark *et al.*, 1980; Seidman & Castor, 1981). These sorts of studies are naturally based on a meticulous comparison of control cultures exposed to experimentally changing conditions. With such an experimental approach, meaningful results can be obtained only when the culture systems themselves are maintained in comparable states in order to minimize modulations caused by intrinsic factors.

Numerous reports have shown that the proliferation of fibroblasts, as well as their potential to synthesize collagen, depend upon the composition of the medium, the source of the serum, the addition of ascorbate and the pH of the environment (Jeffrey & Martin, 1966; Rubin, 1971, 1975; Peterkovsky, 1972; Paz & Gallop, 1975; Kao *et al.*, 1976; Schwarz & Bissel, 1977; Barnes & Sato, 1980; Breul *et al.*, 1980; MacKeehan & MacKeehan, 1980; Müller *et al.*, 1980; Booth & Uitto, 1981; Murad *et al.*, 1981; Tolstoshev *et al.*, 1981).

It is experimentally feasible to optimize these conditions and to standardize them (Booth *et al.*, 1980). It is more difficult, however, to control growth in such a way that cell density and extracellular matrix do not differ among the cell strains under investigation. As shown by previous reports, quantitative and qualitative changes in the pattern of collagen synthesis are probably caused by variations in cell density (Green & Goldberg, 1963; Priest & Davies, 1969; Peterkovsky, 1972; Steinberg, 1973, 1978; Paz & Gallop, 1975; Kamine & Rubin, 1977; Abe *et al.*, 1979; Kao & Berg, 1979; Risteli *et al.*, 1979; Schneider, 1979; Tolstoshev *et al.*, 1981), which is the basis for cell–cell communication and cell–matrix interaction. Since it is one of our prime interests to study metabolic changes of collagen disorders at the molecular level, we wanted to obtain more information about the cellular properties and biosynthetic capacities of normal human fibroblasts, which are often used as controls. Therefore several steps of collagen metabolism were

Abbreviations used: DME medium, Dulbecco's modified Eagle's medium; CM, carboxymethyl.

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determined in sparse, intermediate and dense cultures.

Experimental

Human skin fibroblast cultures

Skin biopsies obtained from human embryos at legal abortion and from healthy informed volunteer human donors were used to establish fibroblast cultures by outgrowth. Fibroblast subcultures were maintained as previously described (Aumailley *et al.*, 1980).

Cell seeding

For labelling experiments the cells were seeded in Petri dishes at the fifth to seventh passages. To obtain different cell densities, two procedures were used: either the cultures were started with different numbers of cells (10^4–10^6 cells/plate) and the pulse was carried out 4 days later, or the cultures were prepared by plating equal numbers of cells (10^5 cells/plate) and the pulse-label experiments were carried out after 4–11 days. In each experiment two Petri dishes were reserved to determine the cell numbers with a haemocytometer.

Protein labelling

Pulse-labelling of the cells *in vitro* was performed as previously reported (Krieg *et al.*, 1980) with two concentrations of L-[2,3-^{3}H]proline (15 μCi/ml or 300 μCi/ml).

Determination of total collagen and non-collagenous-protein synthesis and of collagen degradation

Total collagen and non-collagenous-protein synthesis and collagen degradation were determined as established previously, (Krieg *et al.*, 1978; Wiestner *et al.*, 1979).

Quantification of procollagens and measurement of the conversion of procollagen into collagen

Other samples of the labelled medium and of a 1 M-NaCl extract of the cell layer were separately precipitated with 20% (w/v) NaCl (Aumailley *et al.*, 1980). Phenylmethanesulphonyl fluoride (0.5 mM) and 4-(chloromercuri)benzoate (0.1 mM) were used to prevent proteolysis. The resulting pellets were redissolved in, and dialysed against, 0.2 M-NaCl/0.05 M-Tris/HCl (pH 7.4)/0.02 M-EDTA. The procollagen types I and III were separated by chromatography on DEAE-cellulose as described by Smith *et al.* (1972). The rate of conversion of procollagen into collagen was calculated on the assumption that mature collagen molecules elute with the buffer front (Pontz *et al.*, 1973).

Characterization of pepsin-treated collagenous material

Labelled medium together with the cell layer were dialysed against 0.02 M-sodium acetate/HCl, pH 1.8, and subsequently incubated with pepsin (0.1 mg/ml) for 6 h at 18°C. Part of the pepsin-treated material was precipitated by dialysis against 4.5 M-NaCl/0.05 M-Tris/HCl, pH 7.4. Another portion of the pepsin-treated material was submitted to fractional salt precipitation in, first, 2.7 M-NaCl/0.05 M-Tris/HCl, pH 7.4, and, secondly, in 4.5 M-NaCl/0.05 M-Tris/HCl, pH 7.4 (Krieg *et al.*, 1980). The precipitated material was analysed by chromatography on CM-cellulose as described by Piez *et al.* (1963), with minor modifications (Pontz *et al.*, 1973) and/or molecular-sieve chromatography on agarose A5m (Piez, 1968).

Quantification of type I and III collagens were done on the basis of CM-cellulose (Krieg & Müller, 1977) and of molecular-sieve chromatograms (Krieg *et al.*, 1981) respectively.

Hydroxylation of isolated α-chains

The collagen α-chains obtained by chromatographic procedures described above were freeze-dried and hydrolysed. Hydroxyproline and proline were separated with an automated amino acid analyser as reported previously (Krieg *et al.*, 1978).

Results

As judged by visual examination, and substantiated by cell counting, the extremes of cell densities ranged in our experiments from sparse cultures (2.5×10^3 cells/cm^2) to multilayer cultures (5×10^4 cells/cm^2).

Rate of collagen synthesis

The amounts of newly synthesized collagens were calculated by assuming that non-dialysable ^{3}H-labelled hydroxyproline is unique to collagen chains. Although the total amount of newly synthesized collagen per dish increased with cell density, the amount per cell decreased. As Table 1 shows, when the cell density increased from $(1.6–2.0) \times 10^5$ to $(2.2–3.0) \times 10^6$ cells per dish, collagen synthesis per cell decreased from 0.395 to 0.124 c.p.m./cell (seeding different numbers of cells) or from 0.419 to 0.078 c.p.m./cell (seeding equal numbers of cells) when radioactive precursor was supplied in low concentration (15 μCi/dish). A similar variation was observed when a high concentration (300 μCi/dish) of radioactive precursor was used (Table 1). Also, the way in which the cells were seeded (equal or different numbers of cells) did not significantly influence the synthesis of collagen.

The relative rate of collagen synthesis increased as cultures reached higher cell densities (Table 2). The

Table 1. *Collagen synthesis at different cell densities and with different amounts of labelling (15 μCi and 300 μCi/dish)*

Human skin fibroblasts at different densities (see under 'Cell seeding' in the Experimental section) were labelled for 24 h with [^{3}H]proline. Medium and cell layer were exhaustively dialysed against 0.5% acetic acid, freeze-dried and hydrolysed. Radioactivity of [^{3}H]proline and hydroxyproline was determined after amino acid separation. Collagen synthesis was calculated from analysis of non-dialysable hydroxyproline. Synthesis is expressed as c.p.m. of hydroxyproline produced per cell. The results represent the averages of duplicate determinations.

Cell density	Collagen synthesis (c.p.m./cell) Low labelling	High labelling
Seeding different number of cells		
1.6×10^5	0.395	5.556
1.5×10^6	0.158	2.149
1.9×10^6	0.179	1.892
3.0×10^6	0.124	1.086
Seeding equal number of cells		
2.0×10^5	0.419	3.880
6.0×10^5	0.157	1.056
8.0×10^5	0.155	1.801
2.2×10^6	0.078	0.793

Table 2. *Total collagen synthesis as a proportion of total protein synthesis at different cell densities.*

The experiments were carried out as described in Table 1. Collagen (in %) was calculated according to the formula:

$$\frac{2\,\text{Hyp} \times 100}{5(\text{Pro-Hyp}) + 2\text{Hyp}}$$

where Hyp is hydroxyproline. The formula was based on the assumption that collagen contains as much hydroxyproline as proline and that non-collagenous proteins contain five times less imino acids (proline and hydroxyproline) as collagen.

Cell density	Collagen (%)
9.0×10^4	5.1
1.6×10^5	3.3
2.0×10^5	3.1
3.0×10^5	3.1
6.9×10^5	3.6
8.3×10^5	5.1
1.8×10^6	12
2.3×10^6	8.7
4.8×10^6	14.2

stimulation of collagen synthesis over synthesis of total protein was about four times when cells at low density (10^5 cells/plate) were compared with those at high density (5×10^6 cells/plate).

Table 3. *Determination of percentage of type III collagen and pro-collagen relative to type I*

Human skin fibroblasts at different densities were labelled the same day for 24 h with [^{3}H]proline. Pepsin-treated proteins from the incubation medium as well as from the cell-layer extract were precipitated in 2.7 M-NaCl/0.05 M-Tris/HCl, pH 7.4. Half the precipitate was chromatographed on CM-cellulose and the other half on agarose A5m. The quantities of pro-(type III) collagen in relation to type I were calculated from chromatograms in Fig. 3.

Cell density	Type III collagen (%) CM-cellulose	Agarose A5m	Type III pro-collagen (%)
4.4×10^5	14	10	12
6.7×10^5	16	15	14
1.2×10^6	19	19	22
2.0×10^6	21	19	22

Degradation of collagen

A considerable proportion of the newly synthesized collagen (30%) was immediately degraded at any state of cell density. The results were similar for cultures seeded at different or at equal cell numbers.

Expression of genetically different collagen types

To investigate the genetic expression of collagen, media and cell layer were pooled and treated together with pepsin. One part of the pepsin-treated material was subsequently submitted to a differential salt precipitation in 2.7 M- and 4.5 M-NaCl, the other part was directly precipitated with 4.5 M-NaCl. In order to determine the ratio of collagen type I to type III, half the 2.7 M-NaCl precipitate was chromatographed on CM-cellulose and the other half on agarose A5m.

By using the two methods the proportion of type III collagen relative to type I collagen was found to increase from 14% in sparse cultures to 20% in very dense cultures (Table 3).

The pepsin-treated protein was directly precipitated with 4.5 M-NaCl and was chromatographed on CM-cellulose (chromatogram not shown) in order to determine the ratio between α1(I) and α2 chains (Table 4). In dense cultures the cells synthesized α1(I) and α2 chains in an expected ratio of 2.5 : 1–3.1 : 1 typical for type I collagen, whereas in sparse cultures the ratio was found to be 4.5 : 1. To identify this excess of α-chains, the pepsin-treated collagen from sparse cultures was subjected to fractional salt precipitation with 2.7 and 4.5 M-NaCl. By using molecular-sieve chromatography (Fig. 1) the material soluble in 2.7 M-NaCl and precipitated in 4.5 M-NaCl was found to consist of high-molecular-weight components and α-chains. The high-molecular-weight components contained less than 1% [^{3}H]hydroxyproline and were assumed to

Table 4. *Determination of the α1/α2 ratio at different cell densities*

Human skin fibroblasts at different densities were labelled the same day for 24h with [^{3}H]proline. Pepsin-treated proteins from incubation medium, together with the cell extract, were precipitated in 4.5M-NaCl/0.05M-Tris/HCl, pH7.4, and subsequently chromatographed on CM-cellulose. The α1/α2 ratio was calculated from the total radioactivity eluting as α1(I) chains and α2 chains.

10^6 × Cell density	α1/α2
0.1–0.5	4.5
0.6–1.0	3.7
1.1–1.5	3.4
1.6–2.0	2.5
2.1–3.0	3.1

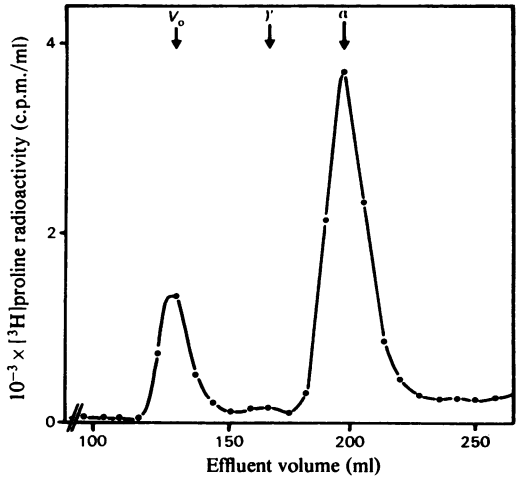

Fig. 1. *Molecular-sieve chromatography of 2.7M-NaCl-soluble proteins synthesized in human skin fibroblast cultures*

After a 24h pulse with [^{3}H]proline, the newly synthesized proteins from the incubation medium and the cell layer were pepsin-treated. Type I and III collagens were removed by precipitation with 2.7M-NaCl at neutral pH. The supernatant was further precipitated in 4.5M-NaCl/0.05M-Tris/HCl, pH7.4 and subsequently chromatographed on a column of agarose A5m (1.8cm × 120cm) equilibrated in 1M-$CaCl_2$/0.05M-Tris/HCl, pH7.4. V_0, γ and α denote respectively the void volume and the elution position of γ-components and α-chains as determined in a calibration run with lathyritic-rat skin collagen.

be non-collagenous proteins. The α-chains were found to elute as α1(I) chains when rechromatographed on CM-cellulose (Fig. 2). Similar results were obtained when the cell were seeded at equal or different numbers.

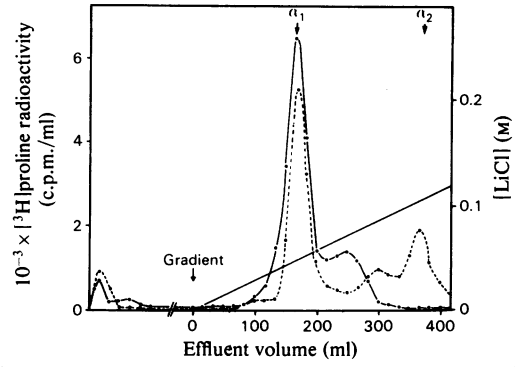

Fig. 2. *CM-cellulose chromatography of the α-chains recovered from molecular-sieve chromatography (Fig. 1)*

After chromatography on agarose A5m the material eluting as single α-chains was dialysed against dilute acetic acid, freeze-dried and redissolved in small volume of acetic acid. After further dialysis against 1M-urea/0.02M-potassium acetate, pH4.8, the α-chains were chromatographed (●——●) on a column (0.8cm × 10cm) of CM-cellulose equilibrated in 1M-urea/0.02M-potassium acetate, pH4.8 and eluted with a linear gradient (——) (0–0.14M) of LiCl (total vol. 500ml). The dotted line (●----●) represents the elution of 2.7M-NaCl-precipitated collagen as reference.

Processing of procollagens

Procollagens collected separately from the incubation media and from the cell layers were chromatographed on DEAE-cellulose (Fig. 3) where pro-(type I) and pro-(type III) collagen were distinctly eluted and quantified (Table 3). There was also a higher proportion of pro-(type III) collagen as the cells became confluent. In addition, the rate of conversion of procollagens into collagens was low (12%) in sparse cultures (7×10^3cells/cm^2) (Figs. 3*a* and 3*b*). In dense cultures (3×10^4cells/plate) the efficiency of conversion increased dramatically, as seen in the chromatograms (Figs. 3*c* and 3*d*), which show a high proportion of radioactivity (49%) eluting as mature type I collagen before the start of the gradient.

Hydroxylation of collagen

In isolated α-chains the ratio of hydroxyproline to proline was within the normal range (0.9) at any cell density studied.

Discussion

The various densities at which the cells were studied allow us to investigate collagen biosynthesis in proliferating and quiescent cells, and therefore to appreciate the influence of the proliferative activity of the cells on collagen metabolism.

Collagen is one major product of fibroblasts. Here

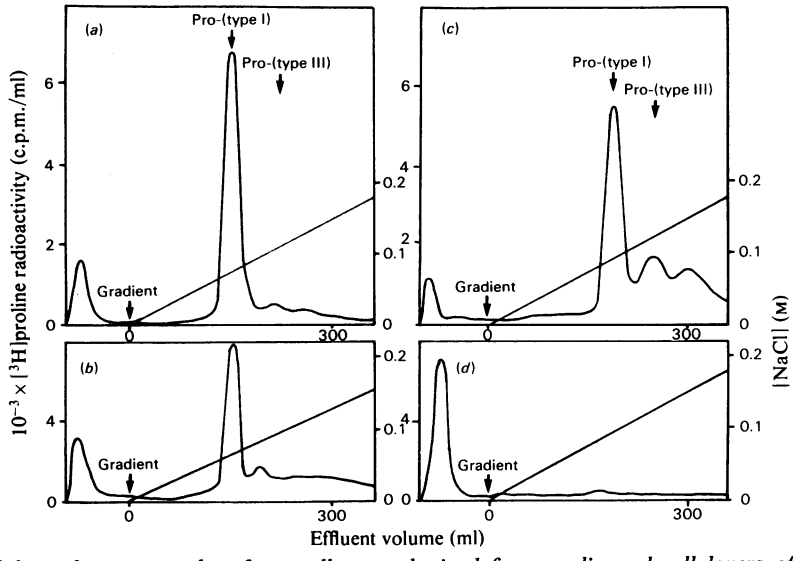

Fig. 3. *DEAE-cellulose chromatography of pro-collagens obtained from media and cell layers of sparse and dense cultures*

The cells were incubated for 24h with [^{3}H]proline. The pro-collagens were precipitated in 20% NaCl and subsequently chromatographed on a column (1.8cm × 8cm) of DEAE-cellulose equilibrated in 2M-urea/0.05M-Tris/HCl, pH 7.4. Elution was with a linear gradient (0–0.3M) of NaCl in a total volume of 600ml. Incubation medium (*a*) and cell layer (*b*) were from sparse cultures; incubation medium (*c*) and cell layer (*d*) were from dense cultures.

we gave a complete analysis of some quantitative aspects of its metabolic processing at different rates of cell proliferation.

On the basis of the cell unit, collagen synthesis was higher in the exponential phase of growth than in the quiescent state. These results agreed with previous data from Priest & Davies (1969), who showed that the rate of collagen synthesis expressed per unit of DNA was faster during the exponential phase of the culture than during the stationary phase. Such a variation in synthesis, dependent upon cell proliferation, is not a characteristic feature only for collagen but is a general phenomenon observed for the total bulk of proteins (Kaftory *et al.*, 1978). Nevertheless, as observed here and by Steinberg (1973, 1978), in non-growing cell cultures the total protein synthesis was more decreased than collagen synthesis; as a consequence the relative rate of synthesis of collagen, measured by the formation of labelled hydroxyproline, was less in exponentially growing than in stationary-phase cultures. However, measuring collagenase-sensitive proteins, Peterkovsky (1972) and Kamine & Rubin (1977) found in different fibroblast cultures that the rate of collagen synthesis remained at a constant fraction of overall protein synthesis, regardless of the growth rate of the cells. Since we found that hydroxylation of proline residues was the same at every cell density, this measurement might provide a reliable assessment of collagen synthesis. The discrepancy, therefore, is most likely due to the different cells that were investigated, or may be caused by other intrinsic differences of the biological systems studied.

Cell density also affected the expression of the main collagen types (I and III). In highly proliferating cell cultures, small amounts of type III procollagen and collagen were synthesized relative to type I collagen. In agreement with Abe *et al.* (1979), this amount increased in the stationary phase of the culture and reached values which are more similar to those found in skin *in vivo*.

Besides this variation, when the cells were highly proliferative they synthesized type I trimer, which was no longer produced in quiescent cells. Thus selection and synthesis of the right proportions of the different α-chains within one molecule were also modulated by cellular involvement in intracellular or environmental activities.

Cell density and, as a consequence, formation of an extracellular matrix, was also found to influence some steps in the processing of the collagen α-chains. The procollagens were converted into collagens at a much higher rate in stationary phase than in the exponential phase of the culture. Such data might explain the findings of Green & Goldberg (1963), who observed that collagen does not begin to accumulate in the cell layer until the period of rapid growth is completed.

It was already well known that culture conditions influence cellular properties, and the results reported here show that specific variations exist for collagen. Our data obtained with two experimental systems (seeding equal number of cells or different numbers) provide evidence for a direct influence of cell density on collagen metabolism that is not dependent on the conditions by which the various cell densities were reached. Therefore it is quite obvious that cell-culture systems can be used only for a comparative study of metabolic processing of collagen when such parameters as cell density or deposition of extracellular matrix are very precisely controlled.

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