

Changes in type of collagen synthesized as clones of chick chondrocytes grow and eventually lose division capacity

(stability of phenotype/collagen switching)

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Communicated by Earl P. Benditt, March 1, 1976

ABSTRACT Clones of embryonic chick chondrocytes have been isolated and collagen biosynthesis has been followed as the clones grow and eventually lose division capacity. Analysis of collagen type at each successive subculture until the time of cellular senescence has shown that a change in synthesis occurs from the cartilage-specific Type II collagen (chain composition $[\alpha 1(\text{II})]_3$) to a mixture of Type I collagen (chain composition $[\alpha 1(\text{I})]_2\alpha 2$) and the Type I trimer (chain composition $[\alpha 1(\text{I})]_3$). The results demonstrate unequivocally that the expression of the chick chondrocyte phenotype is unstable *in vitro*, and that previous experiments with mass cultures of chondrocytes cannot be accounted for by overgrowth of fibroblasts. Since similar morphological changes and a similar "switching" in collagen biosynthesis have been observed after growth of chondrocytes for a few days in 5-bromo-2'-deoxyuridine, it is proposed that growth in this analog accelerates those changes that eventually lead to cellular senescence.

Numerous studies have demonstrated that fibroblasts from a variety of sources have a limited life span when cultured *in vitro* (1-3). Relatively few studies have, however, investigated the morphology and biochemical behavior of other differentiated cell types when grown until cellular senescence ensues. In one such study, Chacko *et al.* (4) isolated clones of embryonic chick chondrocytes and followed the changes in morphology of these cells through several passages in culture. Initially, all cells of the clone displayed the characteristic polygonal morphology of chick chondrocytes in culture, and at higher densities accumulated metachromatic matrix typical of cartilage. After a few passages, however, numerous flattened, amoeboid cells began to appear in the cultures, until finally all cells displayed this morphology. The flattened cells no longer accumulated metachromatic matrix, and were said to have dedifferentiated (4, 5). At the time, comparison was made between the morphology of the flattened cells and cells grown for a short period of time in 5-bromo-2'-deoxyuridine (BrdUrd) (5).

Recently, we have shown that when chick chondrocytes are grown in BrdUrd for a few days, collagen biosynthesis will switch from the cartilage-specific Type II collagen (chain composition $[\alpha 1(\text{II})]_3$) to a mixture of Type I collagen (chain composition $[\alpha 1(\text{I})]_2\alpha 2$) and the Type I trimer (chain composition $[\alpha 1(\text{I})]_3$) (6). In this paper an analysis has been made of the changes that occur in the type of collagen biosynthesis as clones of chick chondrocytes are passaged until cellular senescence is reached and division potential is exhausted. The results demonstrate that the same "switching" in the type of collagen biosynthesis from Type II to Type I and the Type I trimer will eventually occur under these conditions.

MATERIALS AND METHODS

Materials. F-10 medium containing twice the usual concentrations of amino acids and pyruvate (F-10 2 $\times$), trypsin (2.5%), bovine-serum albumin (Fraction V), fetal calf serum,

Ca⁺⁺-, and Mg⁺⁺-free saline, and glutamine were obtained from the Grand Island Biological Co. The radioactive precursor [2-³H]glycine (6.9 Ci/mmol) was obtained from the New England Nuclear Corp. Carrier Type I and Type II chick collagens were prepared as described previously (6). Ascorbic acid, β -aminopropionitrile fumarate, and BrdUrd were purchased from the Sigma Chemical Co.

Cell Culture and Cloning Procedures. Chondrocytes isolated from the sterna of 13-day chick embryos were grown for 4-5 days without feeding in medium F-10 2 $\times$ plus 10% fetal calf serum (vol/vol) and 1% bovine-serum albumin (wt/vol) as described previously (7). To isolate clones, the cells selected as "floaters" (4) were first centrifuged from the medium and incubated for 10 min at 37° in Ca⁺⁺- and Mg⁺⁺-free saline containing 0.06% trypsin. After centrifugation, cells were resuspended in F-10 2 $\times$ and a finely-drawn glass micropipette was used to withdraw a single cell, which was placed within a drop of medium located at the center of a 60 mm tissue culture dish (Falcon Plastics) (4, 8). Several dishes were prepared and each dish was incubated at 37° for 4-6 hr until all cells attached. Medium (3 ml) was added to dishes and the morphology and growth characteristics of each clone were observed daily. Only those clones that grew rapidly and initially possessed the distinct polygonal morphology of chondrocytes were retained. By these criteria, successful clones were obtained from 20-30% of all single cells. Occasionally clones of fibroblasts were observed (5/208 chondrocyte clones) and these were also retained. After about 3 weeks, cells in the chondrocyte clones were enveloped with matrix and some of the cells began to float away from the central mass of chondrocytes and to form secondary colonies. At this time the cells were dissociated with 0.1% trypsin in Ca⁺⁺- and Mg⁺⁺-free saline for 45 min. At the first subculture cells were replated into two or more 60 mm tissue culture dishes at 50,000/ml, and at subsequent subcultures were replated into 100 mm dishes at 100,000/ml. When a high cell density was achieved after each subculture, the cells in one or more of the dishes were incubated for 24 hr with [2-³H]glycine (50-100 μ Ci/ml) in the presence of β -aminopropionitrile (100 μ g/ml) and ascorbic acid (50 μ g/ml) in order to label newly-synthesized collagen.

Isolation of Collagen from Cell Cultures. The procedures used to isolate the collagen from the combined cell layer and medium were similar to those described previously (6). Ammonium sulfate (25% wt/vol) was used to selectively precipitate collagen from the medium (9), and the combined cell layer and medium precipitate were then extracted with 10 ml of 0.5 M acetic acid at 4° for 24 hr in the presence of 1 mg/ml of carrier Type I collagen. The extracts were centrifuged at 10,000 $\times$ *g* for 20 min and the supernatant was adjusted to pH 7.0-7.5 by the addition of 2.0 M NaOH. Ammonium sulfate was added to a final concentration of 25 (wt/vol) and the precipitated collagen was redissolved in starting buffer for CM-cellulose

chromatography (see below), and dialyzed extensively against the same buffer.

To achieve a partial separation of Type I collagen from the Type I trimer by differential salt precipitation, the extraction of collagen in certain experiments was carried out in the presence of both Type I and Type II carrier collagens (1 mg/ml each) (6). After neutralization and subsequent precipitation with 25% ammonium sulfate the precipitate was redissolved in 10 ml of 1.0 M NaCl, 0.05 M Tris-HCl at pH 7.5. Type I collagen was then selectively precipitated by dialysis at 4° against 2.4 M NaCl, and Type II collagen was subsequently precipitated by dialysis against 0.01 M Na₂HPO₄. Each precipitate was dissolved in and dialyzed against the starting buffer for CM-cellulose chromatography.

Cyanogen Bromide (CNBr) Cleavage. Labeled and carrier collagen α chains isolated after CM-cellulose chromatography were mixed with an additional 10–50 mg of a known collagen α chain, and cleavage at methionyl residues was achieved in 3–5 ml of 70% formic acid after addition of 20–100 mg of CNBr as described previously (10).

CM-Cellulose Chromatography. After denaturation of native collagen molecules the chains were separated chromatographically on a 1.5 × 10 cm column of CM-cellulose (Whatman, CM-32) as described previously (6). Chromatography was performed at 42° and at a flow rate of 100 ml/hr in 0.02 M (Na⁺) sodium acetate buffer (pH 4.8) containing 1 M urea. Material was eluted with a linear gradient from 0.0 to 0.14 M NaCl over a total volume of 400 ml. CNBr peptides were chromatographed at 42° and at a flow rate of 100 ml/hr on an identical column. Elution was achieved in 0.01 M (Na⁺) sodium citrate buffer (pH 3.6) with a linear gradient from 0.0 to 0.17 M NaCl over a total volume of 1000 ml. In some experiments, CNBr peptides were separated on a 0.9 × 10 cm column, in which case the flow rate was reduced to 35 ml/hr and elution was achieved with a total gradient volume of 350 ml.

Molecular Sieve Chromatography. Molecular weights of collagen chains eluted from CM-cellulose were determined by rechromatography on a calibrated 1.5 × 155 cm column of agarose beads (Bio-Gel A-5m, 200–400 mesh, Bio-Rad Laboratories) eluted with 2.0 M guanidine-HCl, 0.05 M Tris-HCl at pH 7.5, as described previously (11).

Radioactivity Determinations. Aliquots (0.5–1.5 ml) of the fractions obtained during CM-cellulose and agarose chromatography were mixed with 5–15 ml of Aquasol (New England Nuclear) for counting in a liquid scintillation counter (model LS-233, Beckman Instruments).

RESULTS

Cell Morphology and Behavior During Growth of Clones.

During the period in culture, increasing numbers of flattened amoeboid cells appeared after each subculture. Similar observations have previously been made on the morphology of chick chondrocyte clones (4, 8, 12), and we believe the flattened cells to be equivalent to the "giant" cells described by Coon (12) or the "dedifferentiated, fibroblastic" cells described by Chacko *et al.* (4). Unlike the previous experiments, the cells were not continuously subcultured every few days, but were allowed to grow until high densities were achieved. Perhaps as a consequence of this, the cells could be passaged only an average of four times, and the total life span of the clones was reduced to 7–9 weeks after initiation. It was a common observation that clones that grew most rapidly also synthesized the greatest amount of metachromatic matrix and formed secondary colonies earlier.

Table 1. $\alpha 1:\alpha 2$ chain ratios of collagen synthesized by a clone of chondrocytes and a clone of fibroblasts at each successive subculture

	Chondrocyte clone	Fibroblast clone
1st subculture	26.4	2.0
2nd subculture	8.4	2.1
3rd subculture	5.5	2.3
4th subculture	2.9	2.5

For the initial population of cells, "floaters", the $\alpha 1:\alpha 2$ chain ratio was > 50.

Clones of authentic fibroblasts have previously been obtained from chondrocyte cultures (12, 13), and these cells probably arise from contamination by perichondrial tissue during the initial dissection of the cartilage rudiments. In our experiments the cells of such clones never displayed a polygonal morphology or accumulated metachromatic matrix, and initially showed the characteristic spindle shape of fibroblasts with prominent ruffled membranes at low densities. With each passage such cells became increasingly flattened until their morphology was similar if not identical to that eventually observed for chondrocyte clones.

Collagen Chain Ratios. Table 1 shows the $\alpha 1:\alpha 2$ chain ratios of collagen synthesized by representative chondrocyte and fibroblast clones as a function of time in culture. The chain ratios were calculated as the amount of radioactivity coincident with $\alpha 1$ and $\alpha 2$ following denaturation of the combined medium and cell layer collagen and chromatography on CM-cellulose. Radioactivity in the form of [2-³H]glycine was supplied to the cultures at the high cell densities achieved at the end of each subculture period.

For the initial population of chondrocytes ("floaters") the synthesis of only $\alpha 1$ -like chains could be detected. These chains have previously been characterized as $\alpha 1(\text{II})$ chains by comparing the CM-cellulose elution pattern of CNBr peptides derived from the newly synthesized chains with the CNBr peptides from authentic $\alpha 1(\text{II})$ chains present as carrier (6). These data emphasize how few fibroblasts are present in the initial population of "floaters", an observation further reinforced by the rare isolation of an authentic fibroblast clone. At each subculture for the chondrocyte clone there was a progressive decrease in the $\alpha 1:\alpha 2$ ratio until at the fourth subculture the ratio approached but never quite achieved a value of 2.0. As will be shown below, this is due to the synthesis at cellular senescence of both Type I collagen and the Type I trimer, accompanied by the apparent cessation in the synthesis of $\alpha 1(\text{II})$ chains.

For the fibroblast clone the $\alpha 1:\alpha 2$ ratio was initially 2.0, as would be expected for exclusive synthesis of Type I collagen, and a slight, but possibly not significant, increase in the ratio was noted with each subculture.

Analysis of Collagen Synthesized by Chondrocyte Clones at Cellular Senescence. Fig. 1 shows the separation by CM-cellulose chromatography of the radioactively labeled collagen chains isolated from a representative chondrocyte clone at the fourth and final subculture. The radioactive peaks coincide closely with the absorbance peaks of the $\alpha 1(\text{I})$ and $\alpha 2$ chains of the carrier Type I collagen. Molecular weight determinations by agarose chromatography of the two radioactive peaks showed the presence of material chromatographing only as α chains. In this experiment the $\alpha 1:\alpha 2$ ratio for the newly synthesized collagen was determined to be 3.1.

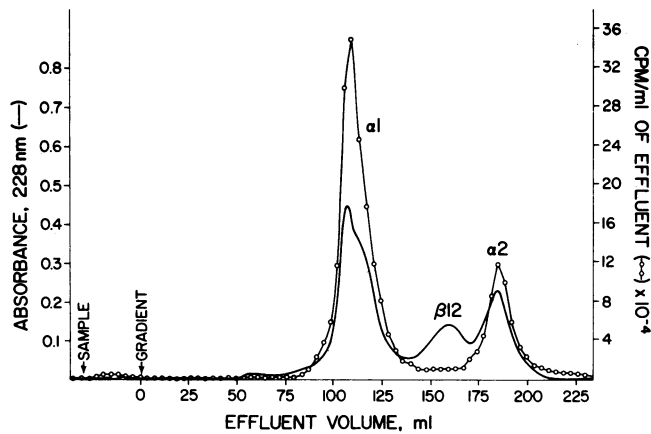


FIG. 1. Carboxymethyl cellulose chromatogram of collagen isolated from the combined cell layer and medium of a clone of chick chondrocytes at the fourth and final subculture. The cells were grown to confluency and then labeled for 24 hr with $[^3\text{H}]$ glycine in the presence of ascorbic acid and β -aminopropionitrile.

To characterize further the labeled collagen chains isolated in the previous experiment, the CNBr cleavage products of the $\alpha 1$ and $\alpha 2$ chains were examined. Each peak shown in Fig. 1 was desalted, lyophilized, and cleaved with CNBr in the presence of an additional 50 mg of either $\alpha 1(\text{I})$ or $\alpha 2$ chains as carrier. Fig. 2A shows the elution profiles of the labeled and carrier CNBr peptides of the $\alpha 1(\text{I})$ chain when chromatographed on CM-cellulose. Good agreement can be observed between the radioactivity pattern and the absorbance tracing from the authentic $\alpha 1(\text{I})$ CNBr peptides. Fig. 2B shows the close correspondence between the elution profile of the labeled $\alpha 2$ chain and carrier CNBr peptides of the $\alpha 2$ chain. For comparison, Fig. 2C illustrates the elution profile of CNBr peptides derived from $\alpha 1(\text{II})$ isolated from chick sternal cartilage.

The above experiments demonstrate that the collagen chains synthesized by a clone of chondrocytes at cellular senescence are largely $\alpha 1(\text{I})$ and $\alpha 2$ chains. Consistently, however, the $\alpha 1(\text{I})$: $\alpha 2$ chain ratio for the chains synthesized by such cultures was greater than 2.0, and this suggested that the additional $\alpha 1(\text{I})$

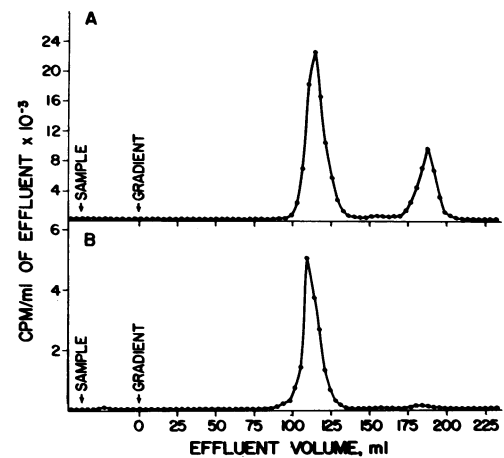


FIG. 3. Carboxymethyl cellulose chromatograms showing the distribution of radioactivity after differential salt precipitation of the collagen isolated from a chondrocyte clone at the fourth subculture and labeled with $[^3\text{H}]$ glycine. (A) The precipitate obtained at 2.4 M NaCl, 0.05 M Tris-HCl at pH 7.5. (B) The subsequent precipitate at 0.01 M Na_2HPO_4 .

chains might be accounted for by the synthesis of the Type I trimer (chain composition $[\alpha 1(\text{I})]_3$) as previously demonstrated for chondrocytes grown in the presence of BrdUrd (6). An attempt was therefore made to separate Type I collagen from the Type I trimer by differential salt precipitation in the presence of Type I and Type II carrier collagens (6). Fig. 3A shows the CM-cellulose elution pattern of the radioactively labeled collagen precipitated by dialysis against 2.4 M NaCl, and Fig. 3B shows the elution pattern of collagen subsequently precipitated by dialysis against 0.01 M Na_2HPO_4 .

Separation of two collagen types has clearly been achieved, one with an $\alpha 1$: $\alpha 2$ chain ratio close to 2.0 (Fig. 3A), and the second comprised almost entirely of $\alpha 1$ chains (Fig. 3B). To demonstrate directly that the 0.01 M Na_2HPO_4 precipitate consisted of $\alpha 1(\text{I})$ chains, the labeled chains together with the carrier $\alpha 1(\text{II})$ chains were cleaved with CNBr in the presence of an additional 10 mg of $\alpha 1(\text{II})$ chains. The resulting peptides

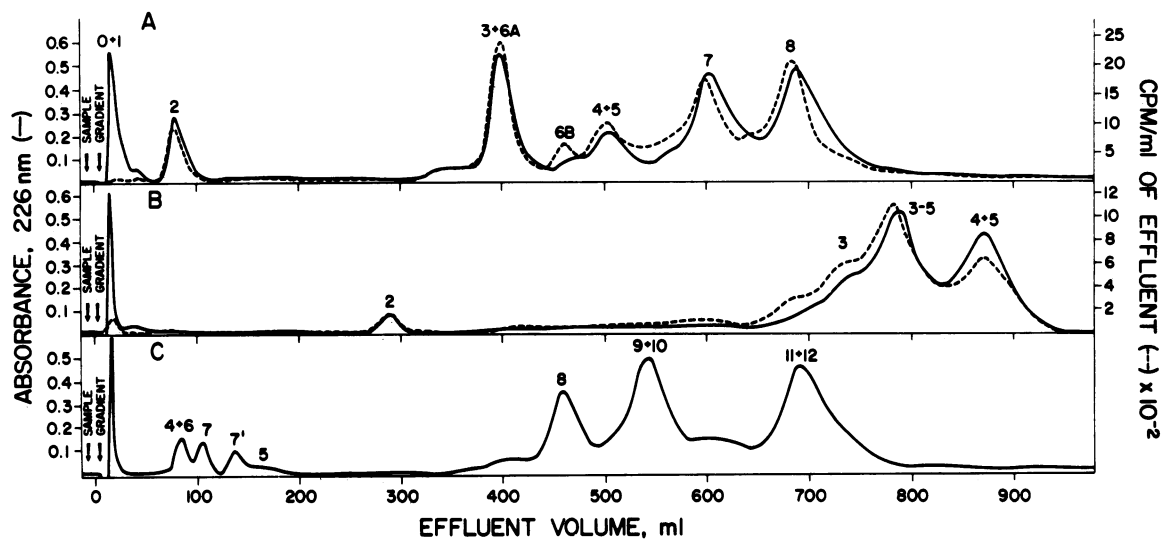


FIG. 2. (A) Carboxymethyl cellulose elution pattern of the CNBr peptides derived from the $\alpha 1$ chain isolated from the chondrocyte clone at the fourth subculture and labeled with $[^3\text{H}]$ glycine. Carrier was CNBr peptides from $\alpha 1(\text{I})$ chains. Numbers identify the peaks. (B) Carboxymethyl cellulose elution pattern of the CNBr peptides derived from the $\alpha 2$ chain isolated from the chondrocyte clone at the fourth subculture and labeled with $[^3\text{H}]$ glycine. Carrier was CNBr peptides from $\alpha 2$ chains. (C) Carboxymethyl cellulose pattern of the CNBr peptides derived from $[\alpha 1(\text{II})]_3$ collagen isolated from chick sternum.

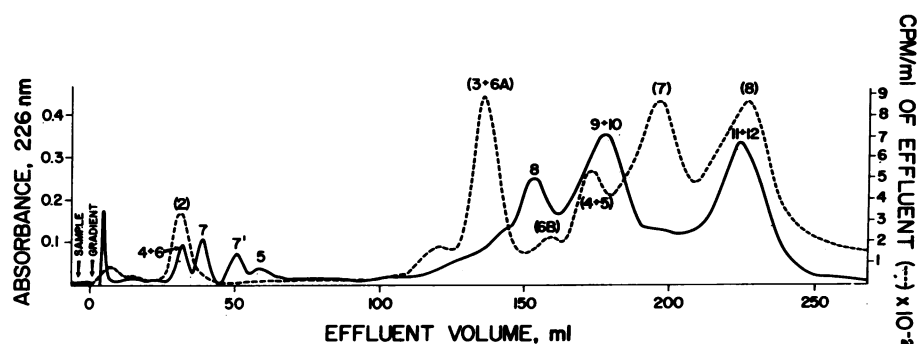


FIG. 4. Carboxymethyl cellulose elution pattern of the labeled CNBr peptides derived from the $\alpha 1$ chain isolated after precipitation of the collagen at 0.01 M Na_2HPO_4 (Fig. 3B). Carrier was $\alpha 1(\text{II})$ CNBr peptides.

were chromatographed on CM-cellulose using the smaller, 0.9×10 cm, column and the elution conditions described in *Materials and Methods*. Fig. 4 shows the lack of correspondence between the radioactivity profile and the carrier $\alpha 1(\text{II})$ chains. Comparison of the radioactivity elution profile with Fig. 2A shows that the major peaks of radioactivity are clearly derived from the CNBr peptides of an $\alpha 1(\text{I})$ chain. The labeled collagen isolated in the 0.01 M Na_2HPO_4 may therefore be identified as the Type I trimer, chain composition $[\alpha 1(\text{I})]_3$.

The results presented thus far demonstrate biochemically a close similarity for the changes in collagen biosynthesis after growing chondrocytes in BrdUrd and allowing them to reach cellular senescence. In both conditions collagen biosynthesis switches from Type II to a mixture of Type I and Type I trimer. BrdUrd therefore appears to accelerate both the morphological and biochemical changes that will eventually occur as a chondrocyte clone ages in culture. To demonstrate this directly, an additional experiment was carried out in which the cells derived from a chondrocyte clone were divided into two dishes at the first subculture. One dish was grown in BrdUrd for 5 days while

the other remained as a control. Fig. 5 shows the CM-cellulose elution profiles of the radioactively labeled collagen isolated from both dishes after exposure to $[2\text{-}^3\text{H}]\text{glycine}$ between the fifth and sixth days. For the control culture (Fig. 5A) most of the cells still displayed the chondrocyte morphology, and collagen biosynthesis consisted almost entirely of $\alpha 1$ -like chains. After growth in BrdUrd, however, most of the cells displayed the flattened, amoeboid morphology, and a distinct $\alpha 2$ peak was present, with an $\alpha 1:\alpha 2$ chain ratio of 3.4. Although the small amounts of incorporated radioactivity precluded further analysis at the level of the CNBr peptides, it would appear from the $\alpha 1:\alpha 2$ chain ratios that growth in BrdUrd has accelerated the changes in the type of collagen biosynthesis that will eventually occur. As shown in Table 1, an $\alpha 1:\alpha 2$ chain ratio of 3.4 in chondrocyte clones is not reached until the third or fourth subculture in the absence of BrdUrd.

DISCUSSION

The data presented in this paper demonstrate the changes in the type of collagen biosynthesis that will occur during growth of a clone of chondrocytes through several subcultures, and prove unequivocally that the chick chondrocyte phenotype is unstable *in vitro*. Such an analysis negates any possible interpretation of earlier experiments as being due to overgrowth by fibroblasts (14). Indeed, it has been possible to isolate a small number of fibroblast clones after the selection of "floaters", and to identify them both by their morphology and initial synthesis of Type I collagen. Such cells do not grow rapidly, and our estimates of contamination by fibroblasts are about 1–2% of the initial population of "floaters". This would agree with earlier estimates (12).

The embryonic chick chondrocyte apparently possesses an inherent and latent program of differentiation that involves "collagen switching", and by growing these cells *in vitro* it is possible to potentiate this program. Similar changes in the type of collagen biosynthesis have also been observed for mass cultures of rabbit articular chondrocytes (15) and with cultures of human embryonic chondrocytes obtained from fetal bone rudiments*. Reports have also appeared that show that during incubation of slices of bovine articular cartilage in the presence of either broken rat liver lysosomes (16) or a variety of lysosomal enzymes (17), synthesis of some Type I collagen occurs. It therefore seems likely that the ability to undergo "collagen switching" will be shared by all chondrocytes of hyaline cartilage regardless of origin or species.

As demonstrated in our previous paper (6) as well as the present results, "collagen switching" in chondrocyte cultures involves the synthesis of Type I collagen and the Type I trimer.

* R. Mayne, M. S. Vail, and E. J. Miller, unpublished observations.

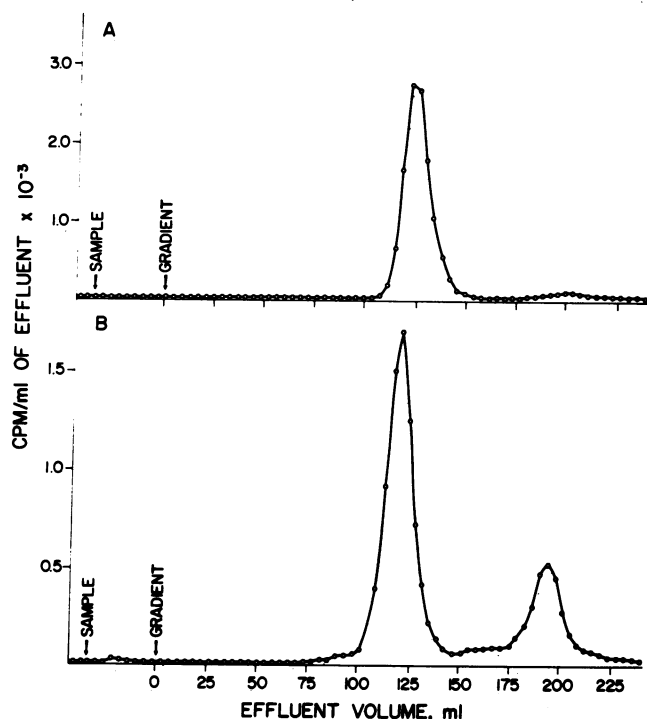


FIG. 5. Carboxymethyl cellulose elution patterns of the collagen isolated from a chondrocyte clone at the first subculture. The cells were grown for 5 days and then labeled for 24 hr with $[^3\text{H}]\text{glycine}$. (A) Control cells. (B) Cells grown in BrdUrd.

However, the significance of synthesis of the latter collagen by chondrocyte cultures is still unclear. In the present series of experiments we were unable to demonstrate convincingly that the synthesis of the Type I trimer occurs in the few fibroblast clones that were isolated and grown to cellular senescence. To clarify this point, more extensive experiments will have to be carried out with mass cultures of fibroblasts either grown for a short period in BrdUrd or continuously subcultured until cellular senescence is reached. Such experiments will demonstrate whether or not the ability to synthesize the Type I trimer is unique to chondrocyte populations, or also occurs with other cell types such as fibroblast populations.

Hayflick (2) proposed that the finite lifetime of normal human diploid fibroblasts *in vitro* may represent a manifestation of aging at the cellular level, such cultures usually being capable of undergoing 50 population doublings and surviving for about 1 year. Cultures of chick fibroblasts also have a finite lifespan *in vitro* and will apparently survive for only 2 months and undergo between 15–35 population doublings (2, 3, 18, 19). Although the present experiments were not originally intended to study senescence *in vitro*, it is interesting to note that the clones of chondrocytes and fibroblasts usually survived for about 2 months, and underwent an average of about 30 population doublings. Extrapolation of data obtained from studying the limited lifespan of cells in tissue culture to normal aging is clearly controversial (for reviews see 2, 3, 20). Nevertheless, our experiments would predict that, as hyaline cartilage ages, *in vivo* synthesis of Type I collagen might be detected. Indeed, recent reports indicating that human osteoarthritic articular cartilage synthesizes some Type I collagen (21) and the epiphyseal plate cartilage of calves (22) contains some Type I collagen would support such a theory. Some caution must, however, be placed on this interpretation, since in recent experiments (R. Mayne, M. S. Vail, and E. J. Miller, manuscript in preparation) we have demonstrated “collagen switching” to Type I and the Type I trimer after growth of chick chondrocytes for a few days in dialyzed embryo extract. In this condition the cells grow well, do not flatten, and will quickly become motile fibroblastic-appearing cells that at high densities form a multilayer. It thus appears that “collagen switching” may also result from the presence of factors other than those directly leading to cellular senescence.

As pointed out previously (5), the morphology of chondrocytes after a short period of growth in BrdUrd is very similar to that observed when a clone of chondrocytes reaches cellular senescence. The data presented in this paper, as well as our previous results (6), show that collagen biosynthesis will undergo

similar alterations in both conditions. Thus, at the time of excessive cell flattening, synthesis of only Type I and the Type I trimer is detected. It would appear that growth in BrdUrd accelerates both the morphological and biochemical changes that will eventually occur at cellular senescence, and this is demonstrated by the results presented in Fig. 5. If this proposal is correct then it would argue for a relatively nonspecific action of BrdUrd, possibly involving random substitution of thymidine bases by BrdUrd. Such substitution might result in the acceleration of macromolecular changes leading to “error accumulation” the underlying molecular basis and importance of which in studies of cellular senescence still remain unclear (20).

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