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Phenotypic Differences in Subclones and Long-Term Cultures of Clonally Derived Rat Bone Cell Lines

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Previous studies with clonally derived populations of cells have shown that cells released from embryonic rat calvaria by enzymatic digestion are heterogeneous with respect to their hormone responsiveness, morphology, and production of matrix components [Aubin JE et al; J. Cell Biol 92:452, 1982].Several of these clonal populations have been used to study the effects of long-term culture and inter- and intraclonal cell heterogeneity. During continuous subculture, marked changes in collagen synthesis were observed in two clonal populations. Both of these clones were originally responsive to parathyroid hormone (PTH) and synthesized primarily type I collagen with small amounts of type III and V collagens, although one clone (RCJ 3.2) had a fibroblastic morphology whereas the second clone (RCB 2.2) displayed a more polygonal shape. Following routine subculture over 3 yr, clone RCB 2.2 was found to synthesize exclusively α1(I)-trimer and not other interstitial collagens. When the same cells were maintained at confluence for 1–2 wk, however, they also synthesized type III collagen. Whereas RCJ 3.2 did not show such dramatic changes in collagen synthesis after long-term subculture, two subclones derived from RCJ 3.2 were found to synthesize almost exclusively either type III collagen (RCJ 3.2.4.1) or type V collagen (RCJ 3.2.4.4). Immunocytochemical staining indicated that both subpopulations also produced type IV collagen, laminin, and basement membrane proteoglycan, proteins that are typically synthesized by epithelial cells. The differences in collagen expression by the various clonal cell populations were accompanied by qualitative and quantitative differences in other secreted proteins and differences in cell morphology. The results demonstrate both the inter- and intraclonal heterogeneity of connective tissue cells and their diverse potentiality with respect to extracellular matrix synthesis.

Key words: collagens, extracellular matrix, bone cells, cell clones

Studies in vitro on gene expression by connective tissue cells have until recently been carried out by the use of tumorigenic cells or permanent cell lines. Whereas tumor cell lines frequently retain a relatively stable phenotype, "normal" cells, even

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as continuous cell lines, tend to change phenotype quite rapidly [1,2]. Since mass populations of connective tissue cells from individual tissues have been shown to be heterogeneous, changes in phenotype in such populations can, in part, be explained by differential growth rates of different cell populations expressing different phenotypes. However, studies on chrondrocytes in culture have shown the importance of the in vitro environment in maintaining a differentiated phenotype [3,4]. Loss of the differentiated phenotype by chondrocytes is generally accompanied by the acquisition of a phenotype more characteristic of fibroblasts.

Although unambiguous phenotypic markers have not been established for osteoblasts, various populations of isolated bone cells including a clonal cell line (MC 3T3 E1) from mouse calvaria [5] have been shown to produce a bone-like tissue in culture [6,7]. Recently, enzymatically released mixed primary rat calvaria cells have been found to produce mineralized nodules which have the characteristic of bone in long-term in vitro culture, in the presence of ascorbic acid and organic phosphate [8,9]. Normal bone cell populations maintained in culture, however, usually lose their osteoblastic properties and express a more fibroblastic phenotype as indicated by an increase in synthesis of type III collagen, loss of PTH responsiveness, and an increasing response to prostaglandin E_2 (PGE_2) (Aubin JE, Heersche JNM, Sodek J, unpublished observations).

In previous studies we have described properties of clonal populations of bone cells isolated from fetal rat calvaria [10]. A number of these clonal populations have long surpassed expected maximal population doubling levels and have become continuous cell lines, as commonly occurs in rodent cells [11]. Because the combination of ascorbic acid and organic phosphate allow bone cells to maintain cell viability at much higher densities, stimulate matrix production, and permit the expression of the osteoblast phenotype [6,9,12], we have maintained several of the clonal continuous cell lines in the presence of these factors to study both the effect of long-term culture on the phenotype of these cells and subclone heterogeneity that exists within the clonally derived populations.

MATERIALS AND METHODS

Cell Culture

The original bone cell clones used in these studies, RCB 2.2 and RCJ 3.2, were isolated by limiting dilution in 1980. The clonal populations were subsequently maintained in T-75 flasks by routine subculture with seeding densities of 2×10^2–2×10^3 cells/cm^2. The cells were grown in α minimal essential medium (αMEM) containing 15% v/v fetal bovine serum (FBS) and the following antibiotics: penicillin G, 100 μg/ml; gentamycin, 50 μg/ml; and amphotericin B, 0.3 μg/ml. RCJ 3.2 was subcloned in April, 1981, from which a number of subpopulations were isolated, including RCJ 3.2.4. Following routine subculture, RCJ 3.2.4 was recloned in November, 1981, generating second-step subclones RCJ 3.2.4.1 and RCJ 3.2.4.4.

For morphological examinations and radiolabeling experiments, cells were plated in 35 mm tissue culture dishes at a density of $1–2 \times 10^4/cm^2$ and grown in the same medium as above but supplemented with 50 μg/ml ascorbic acid and 10 mM sodium β-glycerophosphate. Culture medium was changed on day 1 after plating and every 2–3 days subsequently. At the plating density used, cells reached confluence by day 3–4. Cells could be maintained with medium changes every 2–3 days for up to

45 days at 37°C in a humidified atmosphere consisting of 5% CO_2/95% air. Cells were examined periodically by phase-contrast microscopy and photographed on Kodak Panatomic-X film.

Metabolic Labeling

To examine protein synthesis, cells were labeled at three time points: at day 3, when cells were approaching confluence; at day 9, when cells had formed distinct multilayers; and at day 16, when individual cells in multilayers were obscured by the deposition of extracellular matrix. Triplicate 35 mm dishes of each clone and subclone at each of the three time points were washed three times by incubating each with 2.0 ml Dulbecco's minimal essential medium (DMEM) deficient in methionine and containing 1% v/v dialyzed FBS for 15 min at 37°C. The cells were then pulse-labeled for 30 min at 37°C in 1 ml of the same medium but containing 50 μCi [^{35}S] methionine (SA > 800 Ci/mmol, New England Nuclear Corp, Lachine, Quebec, Canada) and 50 μg/ml ascorbic acid. Following the pulse period, the cells were washed twice with complete aMEM containing 1% dialyzed FBS and incubated in 1.0 ml of this medium for 4 hr. The media from the 4-hr chase period were collected, proteolytic enzyme inhibitors (10 μM phenylmethylsulfonyl fluoride; 0.2 mM EDTA; 50 μM benzamidine) were added, and the media were dialyzed against three changes of water. The cells were washed with ice-cold phosphate buffered saline (PBS) until free [^{35}S] methionine was removed and then extracted with 1 ml 0.5 N NH_4OH, leaving extracellular matrix [13], which was scraped from the dish with a rubber policeman and collected in 1 ml of ice-cold phosphate buffered saline PBS. The cellular fraction was sonicated on ice for 20 sec at 17 joules/sec with a Branson 185 Sonifier. Aliquots from the three compartments, cells, matrix, and medium, were analyzed for total protein synthesis by scintillation counting.

Analysis of Protein Synthesis

Aliquots of radiolabeled proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 7.5% gels or 5–20% gradient gels in the discontinuous buffer system of Laemmli [14]. Samples were freeze-dried, redissolved in sample buffer containing 75 μg dithiothreitol, and heated at 56°C for 20 min before loading. Following electrophoresis, the gels were processed for fluorography as described by Bonner and Laskey [15], and the dried gels were exposed to Kodak SB-5 x-ray film for 2–10 days at −70°C. The following: M_r markers—myosin, 212,000; β-galactosidase, 116,000; phosphorylase b, 92,500; bovine serum albumin, 66,000; ovalbumin, 43,000; carbonic anhydrase, 31,000; trypsin inhibitor, 22,100; lactalbumin, 14,400—were radiolabeled by reductive methylation with [^{14}C] formaldehyde and used as standards for SDS-PAGE.

Analysis of Collagen Synthesis

To quantitate collagen synthesis, aliquots of dialyzed media samples were digested with 25 μg of highly purified bacterial collagenase [16] in 0.05 M Tris HCl buffer, pH 7.5, containing 5 mM $CaCl_2$ and 0.5 mM N-ethylmaleimide for 2 hr at 37°C. Noncollagenous proteins were precipitated with ice-cold 7% w/v trichloroacetic acid and removed by centrifugation at 10,000 g on an Eppendorf Microfuge. The pellet was washed twice and then extracted with 500 μl of 7% w/v trichloroacetic acid at 90°C for 20 min to check the efficiency of collagenase digestion. Samples of

the pellet, solubilized in 200 μl of 70% v/v formic acid (noncollagenous protein), the hot trichloroacetic acid (nondigested collagen), and the supernatant (collagen), were analyzed for radioactivity.

To determine collagen types, aliquots of media were digested with 0.1 mg/ml Worthington pepsin (3× crystallized) at pH 2.3 (0.5 N acetic acid-HCl) for 4.5 hr at 15°C. Following digestion, the pepsin-resistant collagen was freeze-dried and the constituent α chains analyzed by SDS-PAGE with delayed reduction [17] and compared with purified standards. The fluorographic processing of the gels and subsequent quantitation of collagens was carried out as described previously [18].

Immunocytochemistry

The preparation and purification of antibodies to collagen types I and III have been described previously [19]; rabbit and sheep antisera to type IV and type V collagens, laminin, and the basement membrane proteoglycan were kindly provided by Drs. Hynda K. Kleinman, Gary Grotendorst, John R. Hassell, and George R. Martin of the National Institute of Dental Research (Bethesda, MD). For immunocytochemistry, cells were sparsely plated on coverslips [20] and after 2 days of culture were washed with PBS and fixed with periodate-lysine-paraformaldehyde for 10 min at 22°C followed by immersion in methanol at −20°C for 7 min. After washing in cold PBS, the cells were immunostained as previously described [21]. Briefly, cells were incubated for 30 min with appropriately diluted antibodies (dilutions: rabbit antiserum to type IV (1:40), and type V (1:20) collagen, and to laminin (1:40); sheep antibodies to type I (1:20) and type III (1:40) collagens). After washing with 0.05% v/v Tween 20 in TBS (TBS-Tween), the cells were incubated for 30 min with the appropriate affinity-purified $F(ab')_2$ fragments of immunoglobulins (IgG) conjugated with fluorescein isothiocyanate (FITC) (Cappel Laboratories, West Chester, PA) diluted 1:40 with TBS. After washing, specimens were mounted in Moviol 4-88 (Hoechst, Willowdale, Ontario, Canada) containing antibleach [22] and were observed and photographed on a Zeiss Photomicroscope III equipped for epifluorescence.

Immunoprecipitation

Aliquots (approx. 300,000 dpm) of [^{35}S] methionine-labeled culture medium were immunoprecipitated [23] with specific antisera raised in rabbits against type IV collagen and laminin by methodology previously described [24]. Samples from the immunoprecipitation were analyzed by SDS-PAGE and fluorography. Some samples were digested with bacterial collagenase, as described in the collagen synthesis assay.

RESULTS

When clone RCB 2.2 was first isolated, the cells were shown to have a fibroblastic morphology, to respond to PTH but not PGE_2 with an increase in intracellular cAMP, and to synthesize primarily type I collagen (97%) with small amounts of type III (3–4%) and type V (0.1%) collagens [10]. During 3 yr of routine subculture, these cells became more cuboidal in shape, lost their responsiveness to PTH, and after 2 yr synthesized primarily α1(I)-trimer collagen [25]. In contrast, clone RCJ 3.2, when originally isolated, was polygonal in shape, responded to both PTH and PGE_2, and also synthesized primarily type I collagen (79%), with type III

(17%) and type V (4%) collagens representing a greater proportion of the total collagen than in RCB 2.2.

Routine subculture showed clone RCB 2.2 to have a population doubling time (PDT) of ~15 hr and a saturation density of ~2 × 10^5 cells/cm^2 at the time examined. Subclone RCJ 3.2.4 had a PDT of ~13 hr and a saturation density of 1.1 × 10^5 cells/cm^2, and both second-step subclones RCJ 3.2.4.1 and 3.2.4.4 had PDTs of ~23 hr and saturation densities of 1.1 × 10^5 cells/cm^2.

Cells plated and maintained in the presence of ascorbic acid and organic phosphate for morphological examination were confluent by day 3; they were densely packed in multilayers by day 8, and by day 16, an extensive extracellular matrix had formed, which obscured individual cells and gave a tissue-like translucency. Cells of the parent clone, RCJ 3.2, (Fig. 1a–c) were bipolar with thick lamellipodia evident 24 hr after plating. These cells, which adopted a more spindle-shaped morphology at confluency (Fig. 1a), were cuboidal when densely packed and multilayered (Fig. 1b), but were not discernible at later periods because of the secreted matrix (Fig. 1c). RCJ 3.2 formed a number of macroscopic nodules by 16 days in culture; however, these nodules did not mineralize in the presence of the organic phosphate as found with primary cultures of calvarial cells [9]. Subclone RCJ 3.2.4 cells exhibited several filipodia 24 hr after plating, were bipolar at confluency (Fig. 1d), and multilayered in a manner characteristic of many fibroblast-like cells in that successive multilayers of cells were frequently arranged in a perpendicular manner (Fig. 1e,f). These cells were usually visible within the extracellular matrix and failed to develop nodules. RCJ 3.2.4.1 cells (Fig. 1g–i) had a more variable morphology and contained both polygonal and spindle-shaped cells. After multilayering, these cells formed a dense matrix that obscured most of the cells. Nodules did not develop in these cultures. RCJ 3.2.4.4 cells (Fig. 1j–l) exhibited heterogeneous morphologies before multilayering, similar to RCJ 3.2.4.1, but multilayered cells were polygonal to cuboidal and formed a dense extracellular matrix similar to clone RCJ 3.2. These cells also formed macroscopic nodules that failed to mineralize. Clone RCB 2.2 cells were typically more polygonal and developed a very extensive extracellular matrix in long-term culture.

Three time points were selected to study the protein synthesis profiles of the different populations: (1) as populations approached confluency (3 days), (2) populations with extensive multilayering (9 days), and (c) populations with extensive matrix formation (16 days). After pulse-labeling with [^{35}S] methionine, the radioactivity of proteins in the 4-hr chase medium (secreted proteins) in the cells (extracted with ammonium hydroxide) and in the cell matrix (material remaining after extracting with ammonium hydroxide) was determined. As shown in Figure 2, the amount of radiolabeled protein in the media was similar at the 3-day time point for each clone and subclone. In each case, the total radioactivity was greater at the 9- and 16-day time points, reflecting increased cell density; however, when radioactivity was normalized to calculated cell densities, [^{35}S] methionine incorporation was, in fact, progressively reduced at the 9- and 16-day time points. Notably, the amplitude of the changes in protein synthesis varied between the populations.

Radiolabeling of the cellular proteins was more variable than that of the secreted proteins, but the trend of increasing amounts of radiolabeled proteins with time in culture was the same. The amount of radioactivity associated with cell matrix proteins was low (approximately 1–5% of the total labeled proteins) and did not show consistent differences between cell populations or culture times (results not shown).

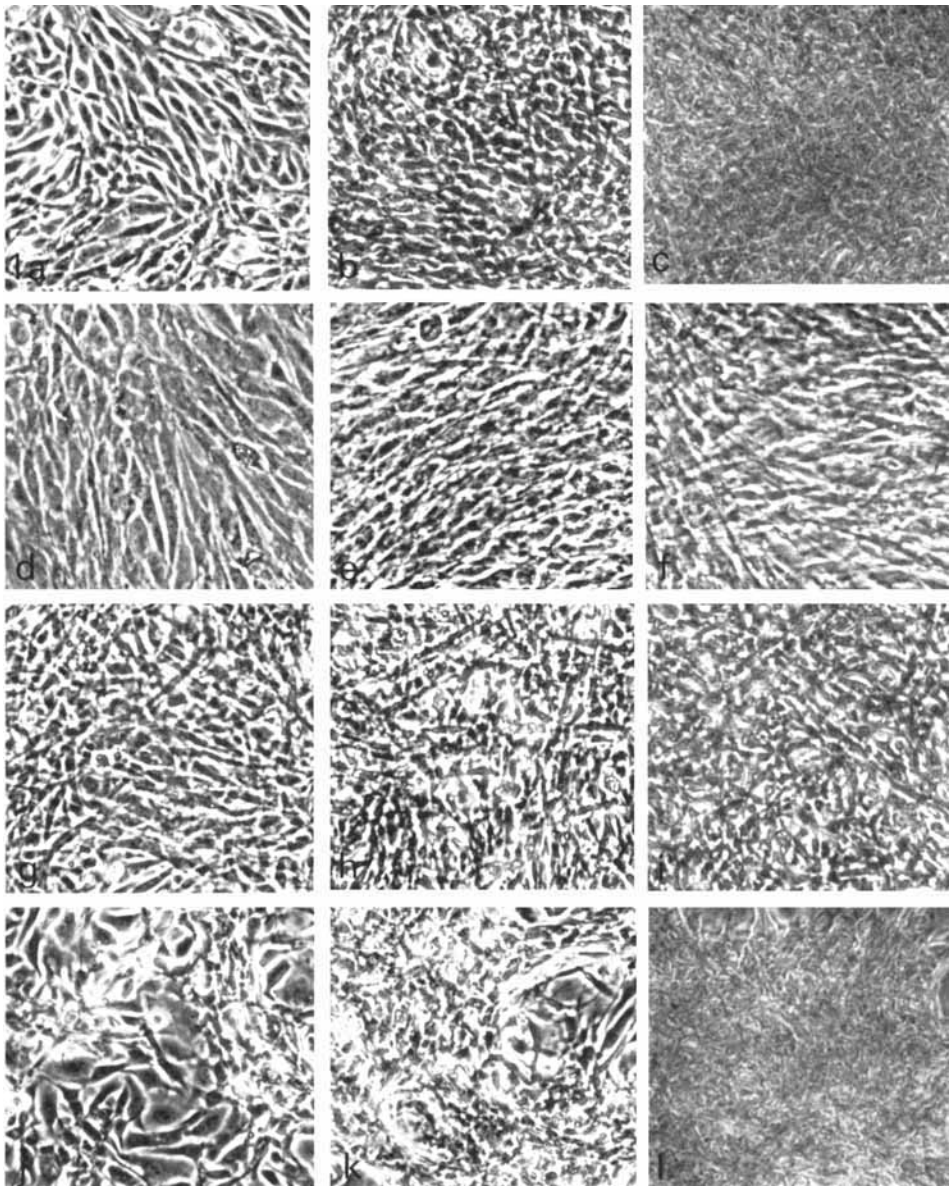


Fig. 1. Phase-contrast photomicrographs of parent clone RCJ 3.2 (a–c), subclone RCJ 3.2.4 (d–f), and second-step subclones RCJ 3.2.4.1 and RCJ 3.2.4.4 (g–l) at 3, 9, and 16 days in vitro. Note the different cell morphologies at day 3 (a,d,g, and j) and the tissue-like appearance of RCJ 3.2 and 3.2.4.4 at 16 days (c and l). ×80.

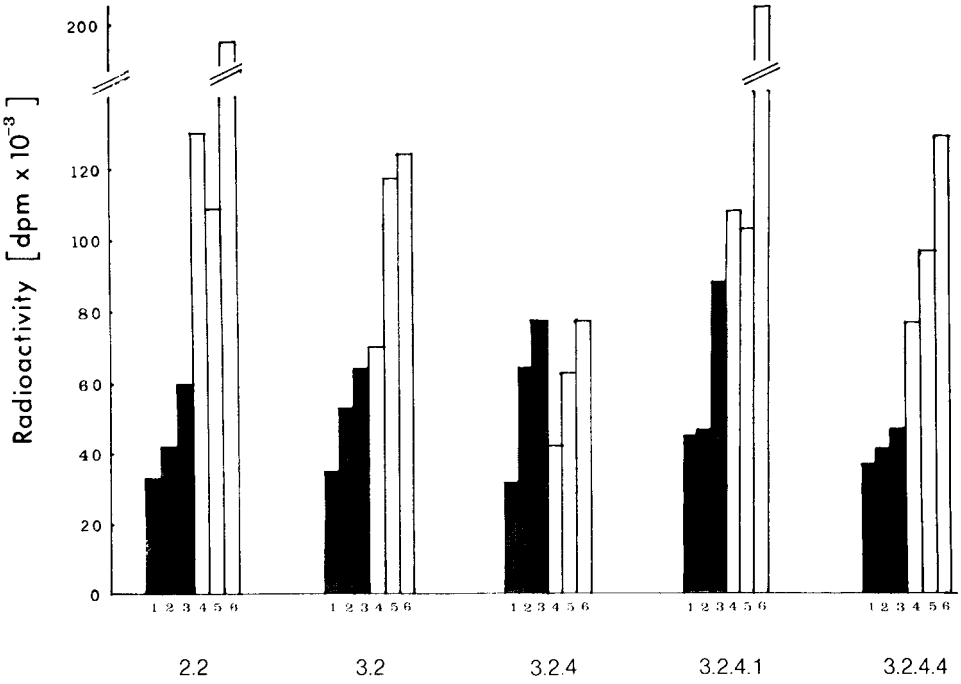
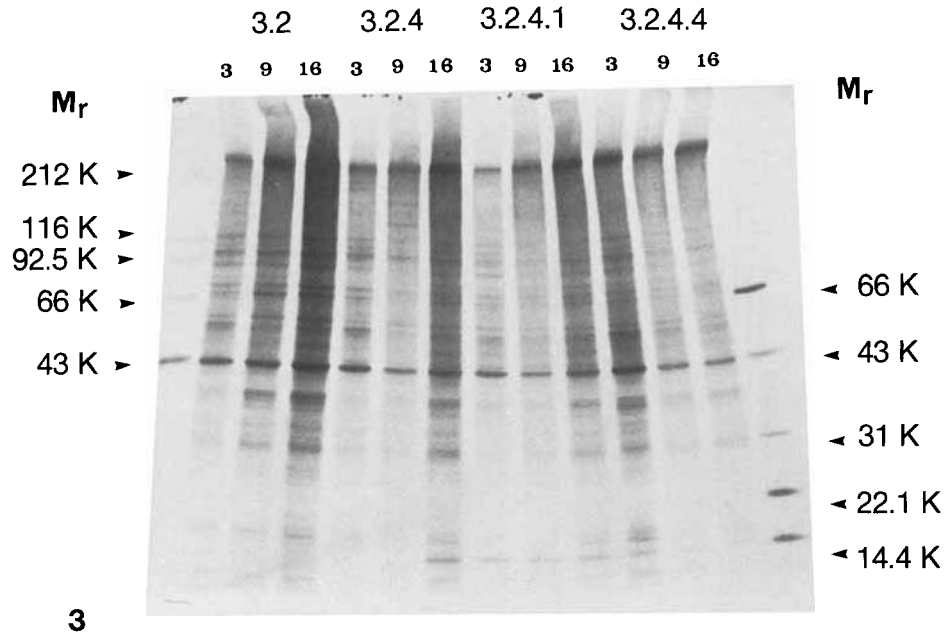


Fig. 2. Comparison of cellular and extracellular protein synthesis by the various bone cell populations at different cell densities. Cells RCJ 2.2, 3.2, 3.2.4, 3.2.4.1, and 3.2.4.4 were plated in triplicate 35-mm dishes and analyzed at three different densities. 1,4 when approaching confluency (day 3); 2,5 when multilayered (day 9); 3,6 when multilayered with cells obscured by matrix (day 16). Protein synthesis was measured from the incorporation of [^{35}S] methionine into media (shaded blocks), matrix (not shown), and cellular (open blocks) proteins with a 30-min pulse and a 4-hr chase period. Results are the average of triplicate determinations with variation less than 5% from equivalent sample volumes.

The radiolabeled proteins in the cells, in the cell matrix, and in the culture medium were analyzed by SDS-PAGE and fluorography. The patterns of radiolabeled cellular proteins from each population were almost identical, and differences at the various time points were almost entirely quantitative (Fig. 3). Radiolabeled proteins in the matrix fraction also appeared to be qualitatively similar between the populations (Fig. 4), although some differences were apparent in the region of the gel (M_r 120–200 K) where procollagens would be expected.

In contrast to the proteins extracted from the cells and matrix, the radiolabeled proteins secreted into the medium showed markedly different profiles on SDS-PAGE (Fig. 5). Many of these differences appeared to be quantitative, but qualitative differences were also apparent. Although the nature of the proteins in the majority of the bands is unknown the proteins in the M_r 120–200K were identified as collagen precursors and intermediates from the migration of corresponding standards (see below) and susceptibility to bacterial collagenase. In addition, the fibronectin band was identified by affinity purification of the radiolabeled material on gelatin- and heparin-Sepharose by the use of a modification of the method of Hayashi and Yamada [26].

Analysis of collagen synthesis by the different cell populations showed a relatively high proportion of the radioactivity in the media to be associated with collage-



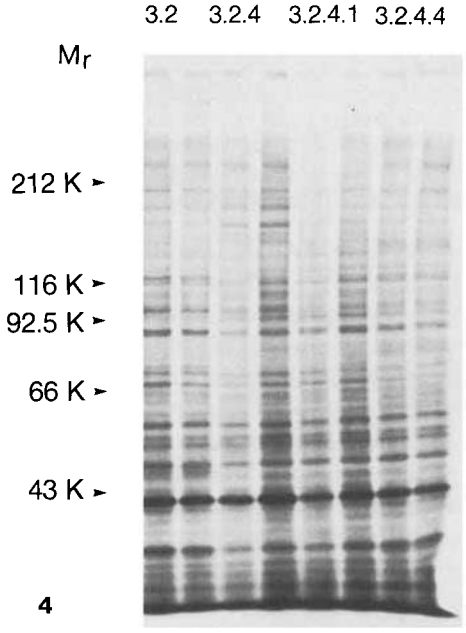


Fig. 3. Fluorograph of radiolabeled cell-layer proteins separated by SDS-PAGE. [^{35}S] methionine-labeled proteins extracted from the cell layers of the various bone cell populations with 0.5 M NH_4OH were analyzed under reduced conditions on SDS-PAGE with a 5–20% gradient gel. Samples from 3- , 9-, and 16-day cultures are shown for each RCJ population, 3.2, 3.2.4, 3.2.4.1, and 3.2.4.4, and the mobility of proteins was compared with radioactive protein M_r markers.

Fig. 4. Fluorograph of radiolabeled matrix proteins separated by SDS-PAGE. [^{35}S] methionine-labeled proteins remaining after NH_4OH extraction were analyzed on 7.5% gels for RCJ 3.2, 3.2.4, 3.2.4.1, and 3.2.4.4 cells. The fluorograph shows the patterns obtained for multilayered cells (day 9). Note the variability between the cell populations of radioactive bands in the M_r 160,000 region.

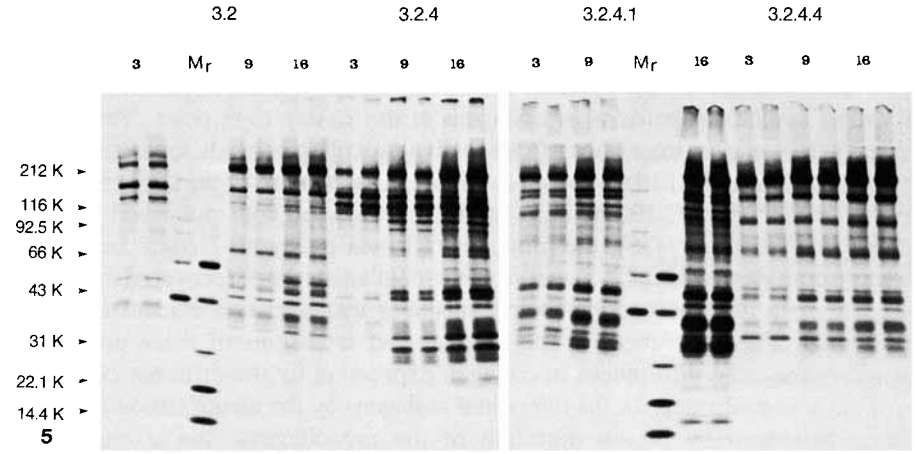


Fig. 5. Composite fluorographs of radiolabeled media proteins separated by SDS-PAGE on gradient gels. [^{35}S] methionine-labeled proteins in the culture media of pulse-chased cells were analyzed on 5–20% gradient gels for RCJ 3.2, 3.2.4; 3.2.4.1, and 3.2.4.4 cell populations. Samples from the 3-day (3), 9 day (9), and 16-day (16) labeling times are compared.

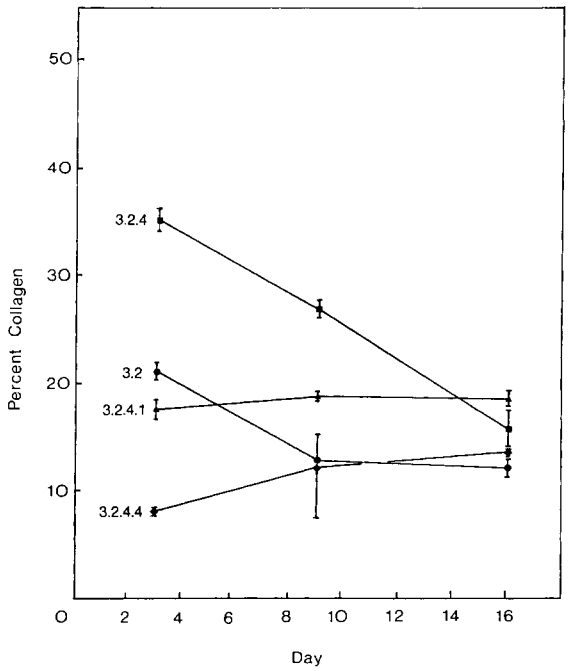


Fig. 6. Analysis of collagen synthesis by the various bone cell populations. Radiolabeled medium proteins were digested with purified bacterial collagenase and the percentage of digested (7% TCA soluble) protein was determined for RCJ 3.2 (●-●), 3.2.4 (■-■), 3.2.4.1 (▲-▲), and 3.2.4.4 (♦-♦) cells at 3, 9, and 16 days. Vertical bars represent standard errors from triplicate assays.

nous proteins (Fig. 6). Both RCJ 3.2 and the RCJ 3.2.4 cells, which had the highest amount of collagen synthesis at day 3, showed proportionately lower amounts of collagen production at the 9 day and 16 day time points, whereas RCJ 3.2.4.1 and 3.2.4.4 demonstrated proportionately higher collagen synthesis, such that all clones synthesized similar proportions of collagens at the 16-day time point. This result is reflected in the relative intensities of radioactive procollagen bands separated on 7.5% gels, a fluorograph of which is shown in Figure 7. By the use of purified radiolabeled procollagens for type I, III, and IV collagens, together with published results on procollagens for type V [26], the procollagen bands in Figure 7 could be identified with some confidence. Notably, most of the procollagens were recovered as complete molecules, with little processing to intermediates and α chains evident in the 4-hr chase period. The differences in the patterns and intensities of these procollagens indicated remarkable differences in collagen expression by the different cell populations. This was confirmed for the interstitial collagens by the identification of collagen α chains produced by pepsin digestion of the procollagens, the α chains being separated by SDS-PAGE and compared with the positions of known standards by delayed reduction (Fig. 8).

Clone RCB 2.2 synthesized primarily α1(I)-trimer collagen as has been described previously [25]. However, in this study, a small amount of α1(V) was also evident at day 3. Subsequent analyses at days 9 and 16 demonstrated the synthesis of type III collagen with lower amounts of α1(I)-trimer and the apparent loss of the α1(V) band (Table I) such that type III collagen and α1(I)-trimer were synthesized in similar quantities at day 16. Populations RCJ 3.2 and RCJ 3.2.4 synthesized predominantly type I collagen with appreciable amounts of type III collagen (approx. 5% and 22%, respectively) and small amounts of type V collagen (Fig. 8, Table I). In contrast

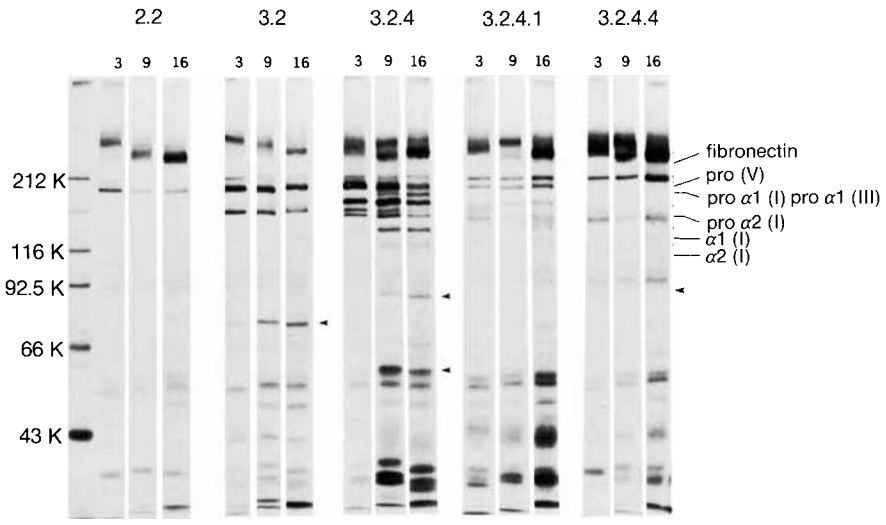


Fig. 7. Composite fluorograph of radiolabeled media proteins separated by SDS-PAGE on 7.5% cross-linked gels. Samples shown in Figure 5 were analyzed on 7.5% gels to emphasize differences in the gel patterns in the procollagen region. The positions of various procollagen standards and fibronectin used to aid in the identification of the radioactive proteins are indicated. Arrowheads point to unidentified proteins that suggest that qualitative differences exist between the cell populations.

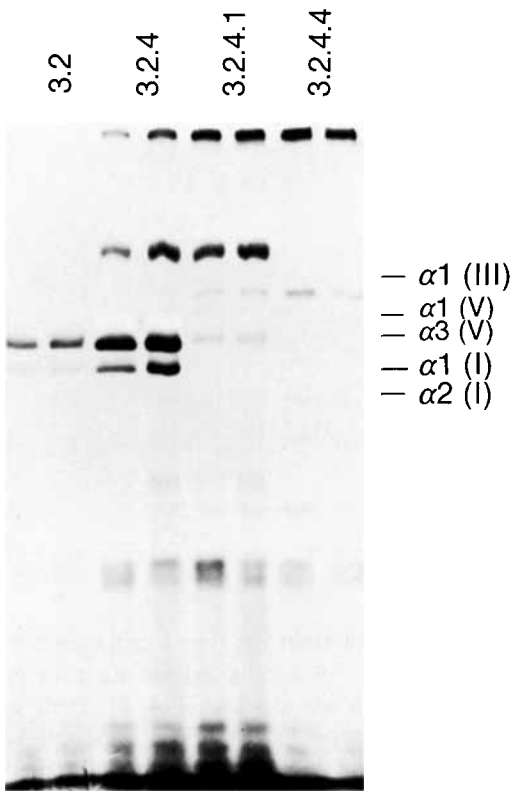


Fig. 8. Analysis of collagen types synthesized by the various bone cell populations. [^{35}S] methionine-labeled proteins from culture media of cells (9-day) were digested with pepsin and the collagen α chains separated by SDS-PAGE with 7.5% gels and delayed reduction. The positions of standard type I, III, and V collagen α chains are indicated. Note the pepsin-resistant protein bands migrating faster than the α chains.

to RCB 2.2 cells, the relative proportions of the interstitial collagens did not change significantly with time in culture. RCJ 3.2.4.1 cells synthesized primarily type III collagen with small amounts of type V collagen. The material in the α1(I) position may represent some α1(I), α2(V), or α1(III) chains in which interchain S-S bridges had not been formed. Population RCJ 3.2.4.4 did not appear to synthesize any interstitial collagens, the only identifiable collagen being type V, which in these cells appears to be a homotrimer of α1(V) chains.

Although the major bands could be identified as interstitial collagens, the fluorographs did show a number of additional pepsin-resistant bands migrating faster than the collagen α chains. These were prominent particularly in populations RCJ 3.2.4.1 and RCJ 3.2.4.4 labeled at day 16. Since pepsin digestion of type IV collagen results in extensive fragmentation, analysis for the synthesis of type IV collagen was performed by immunocytochemical and immunoprecipitation procedures.

Immunocytochemical analysis of RCJ 3.2 and RCJ 3.2.4 cells showed specific intracellular staining for type I and III collagens (Fig. 9),with weaker staining for type V collagen and faint staining with type IV collagen and laminin antibodies (not

TABLE I. Relative Proportions of Pepsin Resistant Collagen α Chains Synthesized by Clonal Bone Cells

Cell population	Culture period (days)	Percentage of collagen α chains		
		Type III	Type V	Type I
RCJ 3.2	3	5	< 0.5	95
	9	6	ND	94
	16	6	ND	94
RCJ 3.2.4	3	21.5	4.5	74
	9	26	ND	74
	16	26	1.0	73
RCJ 3.2.4.1	3	95	5	ND
	9	93	7	ND
	16	93	7	ND
RCJ 3.2.4.4	3	ND	100	ND
	9	ND	100	ND
	16	ND	100	ND
RCB 2.2	3	< 0.5	4	96 (α1)
	9	25	<4	75 (α1)
	16	45	ND	55 (α1)

ND, not detected.

shown). RCJ 3.2.4.1 cells did not stain for type I collagen but stained strongly for type III collagen, whereas RCJ 3.2.4.4 cells did not stain for type I, although some faint staining for type III collagen was evident (Fig. 9). Both second-step subclones also showed good staining for type IV collagen, although variations in staining intensities were evident in different cells (Fig. 10).In addition, these same subclones also stained with antisera raised against laminin and against basement membrane proteoglycan, the latter showing a cell surface fibrillar pattern together with an intracellular punctuate staining (Fig. 10). All cell populations examined also showed staining for fibronectin, which was observed both intracellularly and in fibrillar structures outside the cells (not shown).

The synthesis of type IV collagen and laminin was also indicated by immunoprecipitation of [^{35}S] methionine-labeled proteins from the chase media of RCJ 3.2.4.1 and RCJ 3.2.4.4. With rabbit antitype IV antibodies, two closely spaced bands of M_r ~165,000, which were susceptible to bacterial collagenase digestion, were precipitated. Laminin antibodies immunoprecipitated radiolabeled bands in the M_r ~400,000 and 200,000 region (results not shown).

DISCUSSION

When clonal populations of embryonic rat bone cells are routinely subcultured and maintained over extended time periods, distinct changes in phenotype occur. In general, we have found that following the initial isolation of bone cell clones, those populations demonstrating a response to PTH gradually lose PTH responsiveness, with some populations concomitantly become more responsive to PGE_2 (Heersche and Aubin, unpublished). Also, whereas most of the populations direct over 98% of their collagen synthesis toward the production of type I collagen, type III collagen synthesis was frequently increased on routine subculture, and some of the populations became more fibroblastic in shape. These observations reflect a tendency of bone cell

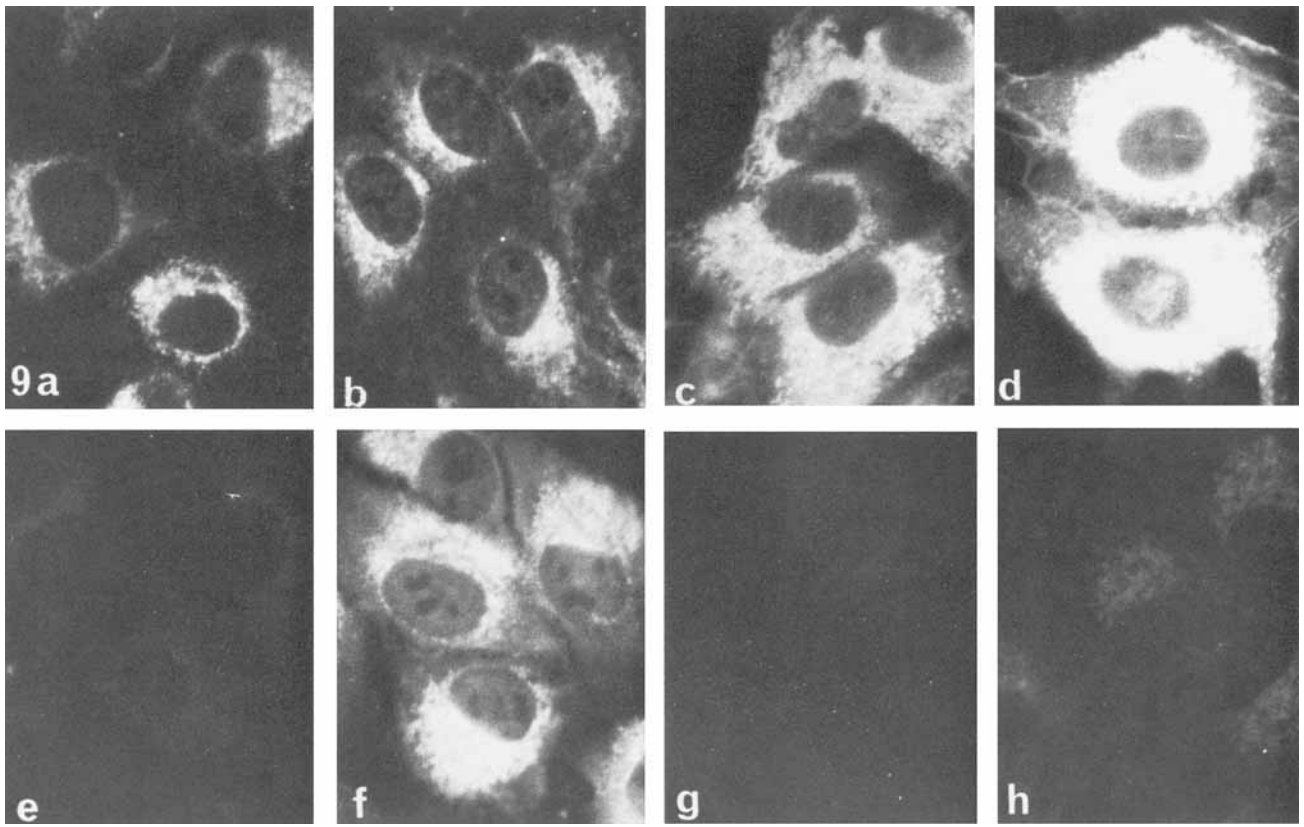


Fig. 9. Immunofluorescence photomicrographs of preconfluent cultures of bone cell clones labeled with affinity-purified antibodies to type I and III collagens. Clone RCJ 3.2 labels strongly for type I (a) and type III collagen (b); RCJ 3.2.4 also forms type I (c) and type III collagen (d). RCJ 3.2.4.1 is basically negative to type I (e) but responds strongly for type III collagen (f), while RCJ 3.2.4.4 is negative to type I collagen (g), and at this density, labeled weakly with type III antibody (h). ×315.

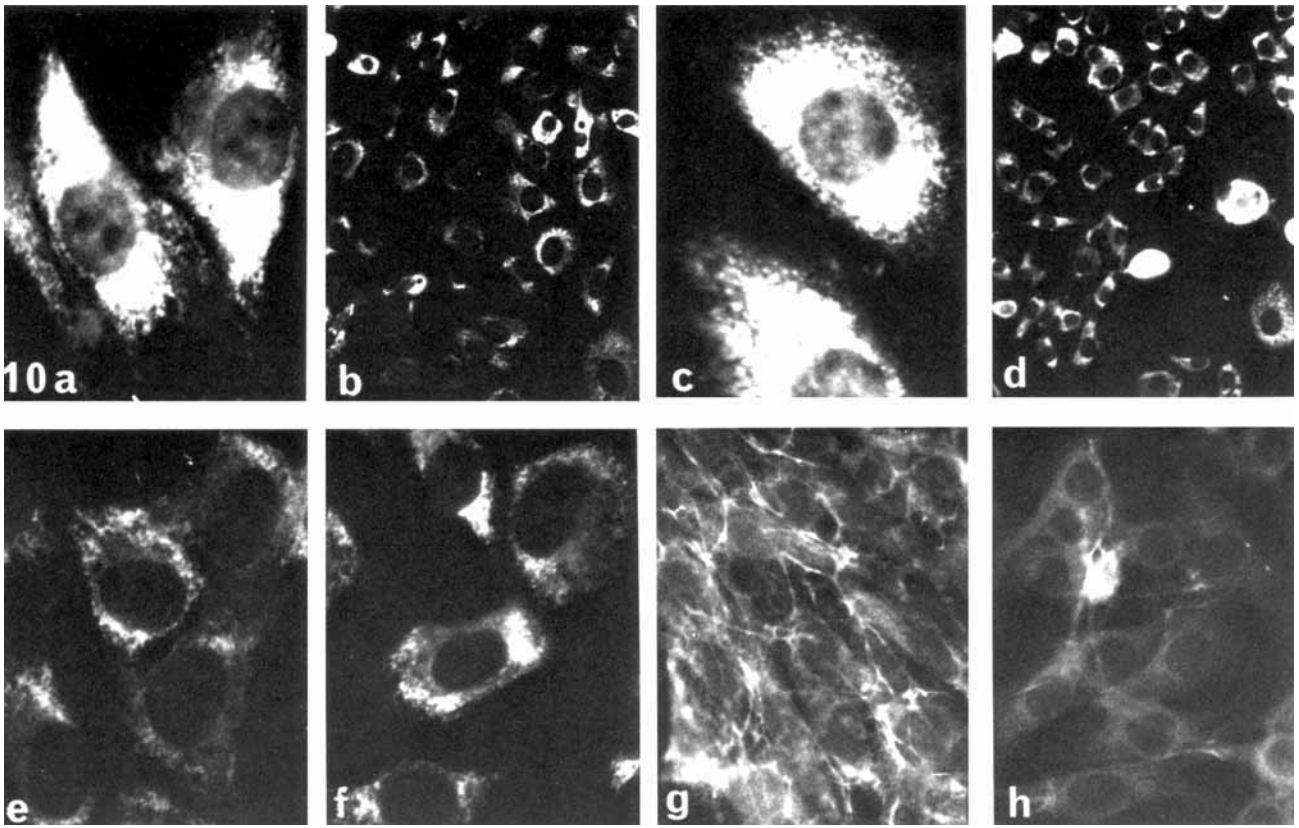


Fig. 10. Immunofluorescence of preconfluent cultures of bone cell clones labeled with antisera to type IV collagen (1:40), laminin (1:40), and basement membrane proteoglycan (1:20). Both RCJ 3.2.4.1 (a and b; ×315 and ×80) and RCJ 3.2.4.4 (c and d; ×315 and ×80) stain strongly for type IV collagen; however, the intensity is variable. Both second-step subclones stain for laminin giving a punctate appearance (e and f, × 315) and for basement membrane proteoglycan, where both extracellular and intracellular localization is evident (g and h; ×200).

populations to change toward a more fibroblastic phenotype and in this respect are similar to chondrocytes maintained in monolayer culture [3,28,29].

Density-dependent modulation of phenotype in the cell populations we have studied is evident from the qualitative and quantitative differences in proteins secreted by individual populations at different population densities. This was most readily apparent in the synthesis of collagen by RCB 2.2 cells. At confluence, these cells secreted almost entirely α1(I)-trimer with small amounts of type V collagen. However, when the cells became multilayered, the type V collagen was reduced, and type III collagen was synthesized in amounts almost equivalent to those of the α1(I)-trimer. These results, together with the less pronounced cell density-dependent changes in collagens synthesized by the RCJ series of cells, indicate that the qualitative differences in the collagens and perhaps also in the majority of noncollagenous proteins are due to culture condition modulation of the phenotype. While somatic mutations and gene loss could account for some of the qualitative differences, functional changes in other cultured cells reported to date have largely been attributed to phenotypic modulation [30,31].

This study further documents heterogeneity of phenotype among subpopulations within clonally derived populations. Heterogeneity is particularly evident in comparisons of the profiles of secreted proteins of the RCJ populations (Fig. 5). Notably, RCJ 3.2.4.1 does not appear to synthesize type I collagen and RCJ 3.2.4.4 does not synthesize any of the interstitial collagens. In addition, the differential staining intensities for type IV collagen and laminin in individual cells (Fig. 10) are indicative of intraclonal heterogeneity. Consequently, the phenotypic changes associated with long-term culture, besides being modulated by culture and density conditions, may also be affected by differential growth rates of subpopulations within a clone.

It is interesting to note that the most obvious changes in protein synthesis occurred in the secreted (media) proteins, with some changes also evident in the proteins extracted with the cell matrix. In contrast, cellular proteins extracted with ammonium hydroxide showed little change, either within or between cell populations. The cellular proteins largely comprise the constitutive or "housekeeping" proteins, whereas "tissue specific" or the "luxury" proteins might predominate in the matrix and media proteins [32]. Housekeeping proteins are thought to be essential for the survival and growth of cells such that many changes would not be tolerated [32]. In addition, the genes for these proteins are thought to be replicated at a stage of the cell cycle different from that of the tissue specific proteins [33].

The differences in collagen gene expression in the various cell populations demonstrate striking changes associated with long-term subculture. The apparent switch to the synthesis of α1(I)-trimer in RCB 2.2 cells has been discussed previously [25]; however, the cell density-dependent modulation of type III collagen in these cells was not observed at that time. Interestingly, collagen expression in the RCJ cells was not altered as dramatically by changes in cell density. The RCJ 3.2.4 subclone differed from the parent population in having a marked increase in the relative proportion of type III collagen being synthesized. However, the apparent loss of type I collagen expression in RCJ 3.2.4.1 cells and both type I and III collagens in the RCJ 3.2.4.4 cells, both of which are subclones of RCJ 3.2.4, were the most obvious differences observed. The synthesis of type V collagen as the principal collagen type for RCJ 3.2.4.4 has also been reported in a Chinese hamster lung fibroblast (CHL) clone [34], in a rhabdomyosarcoma cell line [35], and in Chinese hamster ovary

(CHO) cells [36]. Similar to the CHL cells, RCJ 3.2.4.4 cells produce type V collagen as an α1(V) homotrimer, but in contrast to the CHL cells, the bone cells secrete this collagen into the culture medium. The synthesis of type III collagen as the primary collagen by RCJ 3.2.4.1 cells appears to be unique. While it is known that cell shape changes can be related to different protein synthesis patterns [3,37], it is not known whether the observed changes in collagen or other secreted proteins are a cause or consequence of the different cell morphologies in these bone cells. Although a number of epithelial cell types have been shown to synthesize interstitial collagens [38,39] and fibronectin [40], proteins typically synthesized by connective tissue cells, the synthesis of basement membrane proteins by cells of connective tissue origin has been reported only in an embryonic human lung cell line, W1-38 [41], and cell lines derived from Ewings sarcoma [42], which have been shown to synthesize type IV collagen. Since it is recognized that certain specialized cells have the capacity to express multipotentiality and, in some cases, perhaps also totipotentiality [43], the synthesis of type IV collagen, laminin, and basement membrane proteoglycan in these cells may result from the activation of genes that would otherwise be dormant in connective tissue cells.

Preliminary evidence suggests that the RCJ 3.2, 3.2.4, 3.2.4.1, and 3.2.4.4 populations, but not the RCB 2.2 cells, had become tumorigenic on either the chorioallantoic membrane of the chick embryo or in immune-suppressed, thymectomized mice (Aubin JE, Heersche JNM, unpublished observations). It is presently not known whether acquisition of immortality and tumorigenicity preceded, followed, or were concomitant with changes in collagen gene expression. Although viral and chemical transformation of connective tissue cells is often accompanied by a decrease in collagen synthesis and perhaps also changes in the types of collagen synthesized, the level of collagen synthesis in the RCJ cells is quite high, and at high cell density is similar for each population (Fig. 6). Whether or not changes in collagen expression do correlate with transformation, it is likely that at least some of the alternations observed in the protein profiles in this study are part of the same series of chromosomal changes that result in the spontaneous transformation of these rodent cell lines. Current studies with these cells are directed toward the clarification of the mechanisms involved in the changes observed.

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