

CHARACTERIZATION OF RABBIT STROMAL FIBROBLASTS DERIVED FROM RED AND YELLOW BONE MARROW

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Hematopoiesis occurs only in an environment where a permissive stroma exists. The hematopoietic stromal microenvironments of bone marrow and spleen in mice are not identical. The ratio of granulopoiesis to erythropoiesis differs in the two organs, whereas the population of pluripotential stem cells is the same. Thus, the stroma not only is important for lodgement of the pluripotential stem cells, but also determines the type of hematopoiesis (1). Furthermore, Friedenstein et al. (2, 3) have shown that fibroblasts cultured from marrow and spleen, when retransplanted under the kidney capsule of an autologous rabbit, can regenerate a structure characteristic of the organ of origin. Marrow fibroblasts form hemopoietic marrow and spleen fibroblasts form lymphoid organs. This finding suggests that it is the stromal cells of bone marrow, mainly fibroblasts, that transfer the specific hematopoietic microenvironment.

Particularly pertinent to our study is Tavassoli and Crosby's (4) observation that red and yellow marrow differ in hemopoietic potential upon ectopic implantation. A subcutaneous implant of autologous red marrow in the rabbit generates red marrow in the ossicle that forms at the implantation site, whereas a similar implant of yellow marrow generates yellow marrow (5). Using the Friedenstein fibroblast culture method (2, 3), Patt et al. (6) demonstrated a similar sequence of events: when stromal cells subcultured from red or yellow marrow are implanted into ectopic sites, they form ossicles whose hemic cellularity mirrors the cellularity of the marrow used for culture. The lesser capacity of stromal cells from yellow marrow to establish a microenvironment conducive to persisting hematopoiesis is direct evidence for an intrinsic difference between red and yellow marrow in the phenotypic expression of stromal elements. Thus, the fibroblasts grown *in vitro* differed in their potential for supporting hematopoiesis *in vivo*. Patt et al. (6) hypothesized that the extracellular matrix of such stromal cells provides information necessary for supporting specific kinds of hematopoiesis.

Used a coordinated biochemical and morphological approach, we have characterized the red and yellow bone marrow fibroblasts before their implantation beneath the renal capsule. Here, we describe electron microscopic and enzyme

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histochemical studies of cultured rabbit stromal cells derived from red and yellow bone marrow, and compare their profiles of type-specific procollagens, the major synthetic products of fibroblasts.

Materials and Methods

Bone Marrow Samples. Marrow was obtained under sterile conditions from surgically exposed femora of three New Zealand white male rabbits, each weighing 3.2 ± 0.1 kg, or tibiae of three New Zealand white male rabbits, each weighing 4.0 ± 0.2 kg. An incision was made over the knee, and the marrow was removed by inserting polystyrene tubing (inner diam, 2 mm) into the femoral (red marrow) or tibial (yellow marrow) shaft. The tissue collected in the tubing was extracted by perfusion with HBSS. A segment of marrow from the proximal end of the femur or the distal end of the tibia was minced with scissors and then dispersed into a suspension with DMEM with glutamine, supplemented with 20% FCS, penicillin, streptomycin, and neomycin (tissue culture medium). The suspension was centrifuged at 1,000 g for 10 min. The fat layer was removed, and the pelleted cells were resuspended in the tissue culture medium in single-cell suspension. Nucleated cells were counted with a hemocytometer (6).

Culture System for Obtaining Bone Marrow Fibroblasts. Duplicate cultures of $8\text{--}16 \times 10^6$ bone marrow cells were grown in 25-cm² Corning flasks containing 10 ml of tissue culture medium. In some cases, the medium also contained 10^{-7} M hydrocortisone sodium succinate. The flasks were gassed with 5% CO₂, sealed, and incubated at 37°C; after 1 wk, half of the tissue culture medium was replaced with fresh medium. At confluence (14–16 d), the cultures were treated with trypsin-EDTA (Gibco, Grand Island, NY) for 5 min at 37°C; the dispersed cells were then washed with tissue culture medium and counted. First-passage cultures were set up in either 75-cm² Corning flasks with 1 or 4×10^6 cells, or 25-cm² Corning flasks with 0.3 or 1.2×10^6 cells. The tissue culture medium was identical with that used in the primary culture. The flasks were gassed with 5% CO₂, sealed, and incubated at 37°C. After 3 d, the heavier concentration of cells plated was post confluent and the lighter concentration was 80% confluent.

Implantation of First-passage Fibroblasts Under the Renal Capsule. Bone marrow fibroblasts ($2\text{--}4 \times 10^6$ cells) from primary cultures, grown with or without hydrocortisone, were grown in 75-cm² flasks for 4 d, and were collected after trypsin-EDTA treatment. The cells were then washed and counted with a hemocytometer. The pelleted fibroblasts from both types of cultures were implanted separately under the renal capsule of the original marrow donor. This was done, with the rabbit under anesthesia, by making a small incision in the capsule, lightly puncturing the renal cortex with a probe to facilitate angiogenesis, and inserting a sterile Plexiglas ring (3-mm height, 6-mm inner diam), which was then filled with $\sim 4\text{--}8 \times 10^6$ fibroblasts, and slid under the capsule. Implants were removed at ~ 50 d, along with a sample of marrow from the previously undisturbed proximal femur (6).

Histologic Studies. Bone marrow taken from the area adjacent to the marrows used for culture, the ossicles recovered after the renal implantation, and bone marrows obtained from the undisturbed femora when the ossicles were recovered, were processed as previously described (7).

Transmission Electron Microscopy of First-passage Cells. Cells and matrix adhering to tissue culture dishes were fixed for 1 h at 22°C in 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, containing 1% sucrose. They were then processed as previously described (8).

Enzyme Histochemical Studies of First-passage Cells. The cultured cells were immediately placed in cold (4°C) paraformaldehyde in phosphate buffer, pH 7.4. 4 h later, they were scraped from the dishes, pelleted, transferred to phosphate wash buffer, and further processed for embedding as described (9). The entire procedure, from fixation through embedding, was carried out at 4°C. The hardened blocks were sectioned at 2 μ m with a Sorvall JB4 microtome, stained with hematoxylin, eosin, and azure for morphology, and

tested for the enzymes α -naphthyl butyrate esterase (α NBE),¹ naphthol-AS-D chloroacetate esterase (CAE), and alkaline phosphatase (AP). For further details, see Beckstead et al. (9).

Culture of Skin Fibroblasts. Skin fibroblasts were cultured from skin biopsy specimens of adult male rabbits with a body wt of 3 kg. The cells were grown as described above for bone marrow fibroblasts, except that 10% FCS was used. To obtain adequate numbers of cells, we used cells from the third or fourth passage in our studies. The metabolic labeling studies were performed 4–5 d after plating.

Studies on Procollagen Synthesis. All cells were cultured with 20% FCS and penicillin, streptomycin, and neomycin.

Metabolic Labeling. Cultures were preincubated for 24 h in ascorbic acid (25 μ g/ml), and were then exposed (24 h, 37°C) to ascorbic acid and L-2,3,4,5-[³H]proline (35 μ Ci/ml; 32–36 Ci/mmol). The lathrogen β -aminopropionitrile (50 μ g/ml) was added to prevent collagen crosslinking. After 24 h, the medium containing the secreted procollagen was removed and processed as described (10, 11).

DEAE-cellulose Chromatography for Procollagens. DEAE-cellulose chromatography was performed to separate and quantitate the radiolabeled procollagens synthesized by cultured cells and secreted into the medium, as previously described (10, 11). After the cells had been labeled, the media were processed and dialyzed. Saturated ammonium sulfate was added to make the final concentration 30% (vol/vol), and 3 mg of unlabeled carrier collagen (Vitrogen; Flow Laboratories, McLean, VA) was added to the resulting suspension. The precipitate was dialyzed and applied to a column of DEAE-cellulose (1.0 \times 8 cm). A linear gradient (0–0.2 M) of NaCl (total vol, 175 ml; flow rate, 7.2 ml/h) was used. Fractions of 2.5 ml were collected; 0.1-ml samples of fractions from red marrow cultures and 0.25-ml samples of fractions from yellow marrow cultures were removed for counting of radioactivity. 3 ml of Aquasol were added, and samples were counted in a Beckman LS8000 Counter, with a counting efficiency of 17.6% for the tritiated material.

Results

The primary culture consisted of red and yellow marrow cells cultured for 14–17 d, with or without hydrocortisone. After 14–17 d, cells were trypsinized, replated at two different densities, and cultured for an additional 4–5 d (the first-passage culture). The age of the cells studied was therefore 18–22 d. The first-passage cells were analyzed by the three methods described below.

Examination of Renal Implant after 50 d. First-passage fibroblasts were examined morphologically at 50 d after implantation under the renal capsule to ascertain their ability to transfer the appropriate hematopoietic microenvironment. We have previously (6) demonstrated that the ossicles formed by first-passage fibroblasts obtained from cellular (red) and severely hypocellular (yellow) marrow differ markedly in medullary content. Herein, we again found that fibroblasts cultured from red marrow produced a stroma with numerous hemopoietic foci (Fig. 1a), whereas those cultured from obviously yellow marrow produced a stroma containing mainly fat cells (Fig. 1b).

Morphologic Characterization of Cells from First-passage Cultures. Enzyme histochemical examination revealed only one striking difference between the fibroblasts obtained from red and yellow bone marrows. Whereas only 1–20% of those obtained from the red marrow were positive for α NBE, (Fig. 1c) 50–80% of those obtained from the yellow marrow were positive (Fig. 1d). These percentages do not include the rare, heavily stained macrophages. AP stained

¹ Abbreviations used in this paper: AP, alkaline phosphatase; α NBE, α -naphthylbutyrate esterase; CAE, naphthol-AS-D chloroacetate esterase.

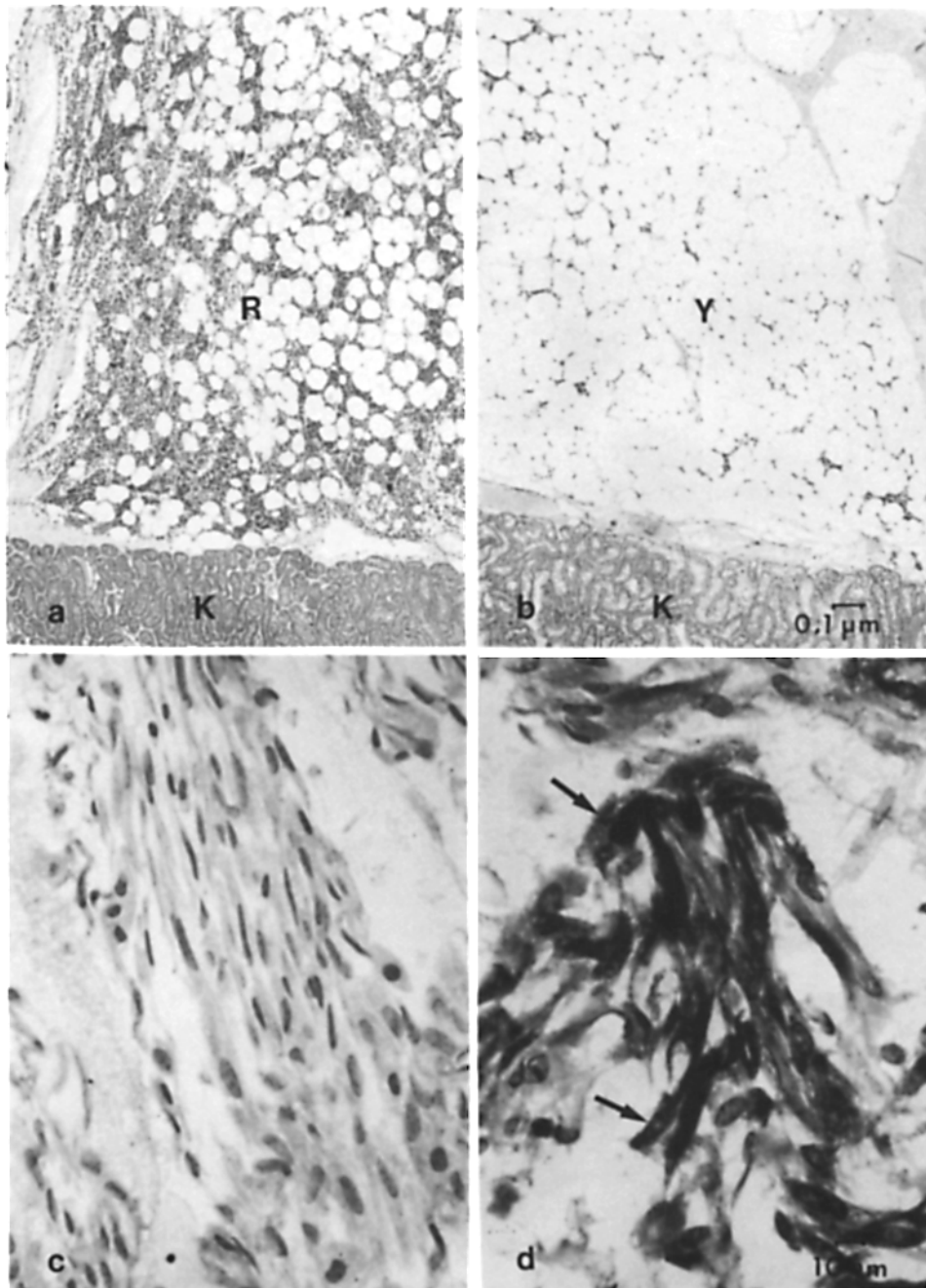


FIGURE 1. Photomicrographs of content of ossicles formed by first-passage fibroblasts after 50 d under the renal capsule. The fibroblasts from red marrow consistently produced cellular red (*R*) hemopoietic foci (*a*), whereas those from fatty marrow (*b*) produced hypocellular yellow foci (*Y*). *K*, kidney. $\times 40$. Photomicrographs of first-passage fibroblasts cultured from red (*c*) and yellow marrow (*d*) and stained for α NBE. Note the presence of dark reaction product (arrows) in the majority of the fibroblasts cultured from yellow marrow (*d*). $\times 400$.

very few (1–5%) of the cells from either source. Negativity for CAE indicated that no neutrophils or mast cells were present.

When examined by electron microscopy, the first-passage fibroblasts obtained from red (Fig. 2) and yellow (Fig. 3) bone marrow showed no consistent morphologic differences, except that the latter tended to have more dilated endoplasmic reticula. Lipid inclusions were infrequently observed, and very little extracellular matrix was seen between the cells. Occasional macrophages were present.

Ability to Secrete Various Types of Procollagen. Cells in vivo synthesize an extracellular matrix. Collagen, the major matrix protein, is secreted in a soluble precursor form, procollagen, and is then processed to the mature collagen form as it becomes incorporated into the matrix. In tissue culture, cells secrete most of their procollagen into the medium, where it accumulates and avoids undergoing the usual processing reactions.

We compared procollagen secretion by cultured stromal cells from red and yellow bone marrow (Fig. 4, *a* and *b*) and by cultured rabbit dermal fibroblasts (Fig. 4*c*). The dermal cells had the expected profile, with a prominent peak of type I procollagen and a small amount of type III procollagen. Another peak contained predominantly acidic glycoproteins and proteoglycans. The presence of hydrocortisone in the culture medium produced no change in the profile for dermal fibroblasts (data not shown). This profile is similar to chromatograms obtained previously for human dermal fibroblasts (12–14). The elution positions of the type-specific procollagens were identified previously using known standards (14).

In the profile for fibroblasts from red marrow, type III procollagen was the major species (Fig. 4*a*), and its peak was more prominent than that of type I procollagen. >60% of procollagen synthesis was devoted to type III, whereas <40% was devoted to type I in dermal fibroblast cultures. As was the case for dermal fibroblasts, the presence of hydrocortisone in the culture medium had no major effects on the profile. The profile for the cells from yellow bone marrow (Fig. 4*b*) also had a prominent peak of type III procollagen. Because they did not grow well without hydrocortisone, their profile under this condition of culture is not shown. For both red and yellow bone marrow cultures, the peak of proteoglycans and acidic glycoproteins was proportionately greater than that for dermal fibroblast cultures.

These studies were performed several times to determine the range and variability of procollagen production. In experiments with dermal fibroblasts from three rabbits (Table I), the ratios of procollagens I/III averaged 62:38. In the red bone marrow cultures, the ratio of procollagens I/III ranged from 30:70 to 40:60, the converse of the ratio for the dermal fibroblasts. Variations in the ratio of procollagens I/III have been reported for preconfluent and postconfluent cultures (15), and for cells cultured in the presence and absence of corticosteroids (16, 17). Neither of these variables had major effects on the procollagens I/III ratio in red bone marrow cultures (Table II). In yellow bone marrow cultures from three animals, the procollagens I/III ratios were similar to those for red marrow cultures, with a predominance of type III (Table III). Again, the ratios in preconfluent and confluent cell cultures differed little.

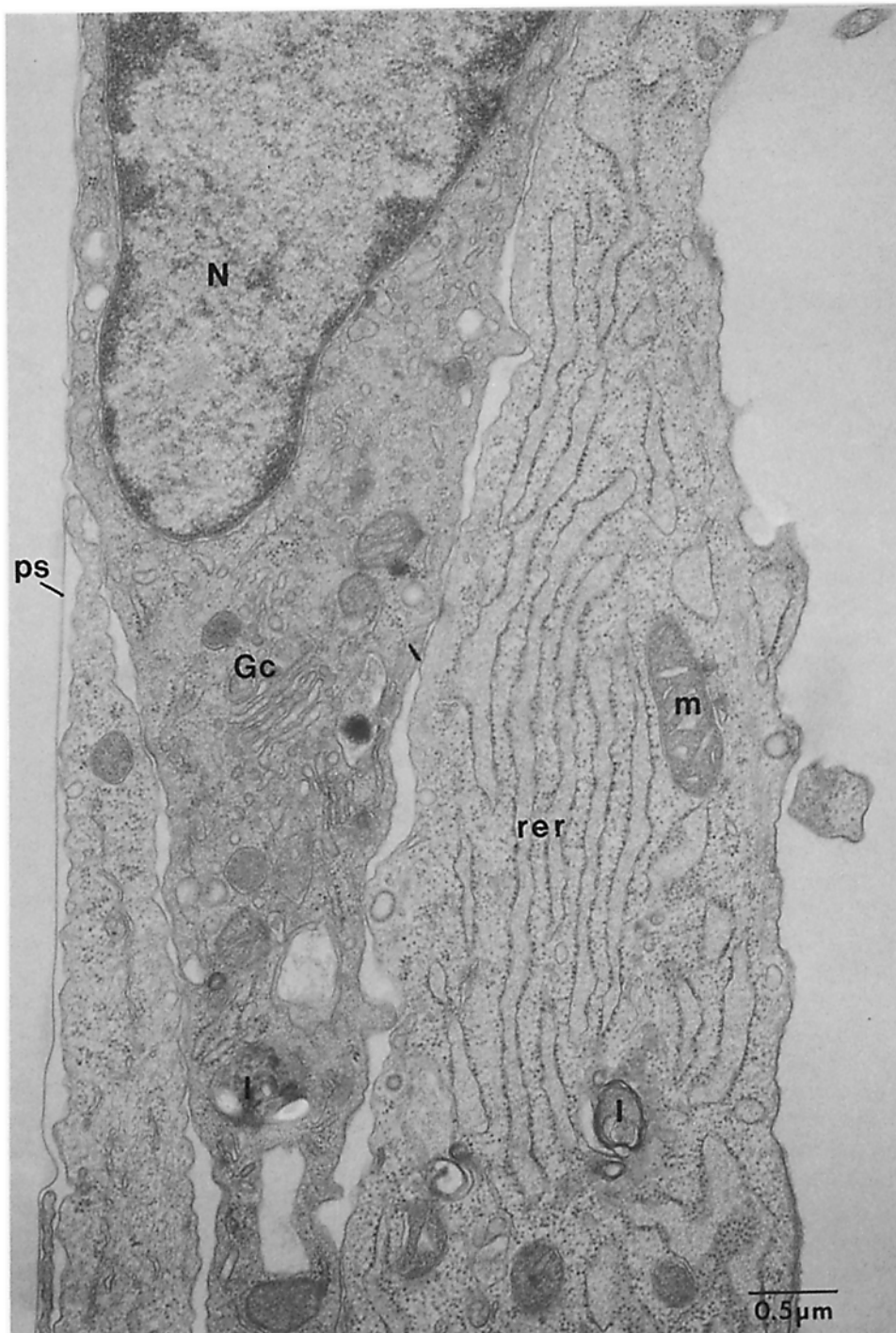


FIGURE 2. Transmission electron micrograph of the first-passage cultured cells from red bone marrow. Most of the cells are long and slender fibroblast-like cells with moderate amounts of rough endoplasmic reticulum (*rer*). The perinuclear Golgi complex (*Gc*) consists of five or six cisternae. Moderate numbers of secondary lysosomes (*l*) are present. *m*, mitochondria; *ps*, plastic surface; *N*, nucleus. $\times 24,000$.



FIGURE 3. Transmission electron micrograph of the first-passage cultured cells from yellow bone marrow. The cells have a fibroblast-like appearance very similar to that of the cells in Fig. 2, but the rough endoplasmic reticulum (*rer*) is more dilated than in Fig. 2. *N*, nucleus; *m*, mitochondria; *Gc*, Golgi complex; *ps*, plastic surface; *l*, lysosome. $\times 21,500$.

Discussion

To characterize the sparse stromal cells of bone marrow, we have used the technique of Friedenstein and coworkers (2, 3) to obtain an enriched culture of fibroblasts. We have shown that when first-passage fibroblasts derived from red and yellow bone marrow are transplanted under the renal capsule, they allow red and yellow marrow to reform, respectively. When we examined the cultured cells from red and yellow marrow by electron microscopy, we found that both types had developed into spindle-shaped fibroblasts with little intracellular lipid. They differed strikingly, however, in their contents of esterase, which was present in most of the cells derived from yellow marrow, but in only a few of those derived from red marrow. The enzymatic findings are of interest because it is now recognized that preadipocytes contain this esterase at certain stages of maturation. For example, Hausman and Thomas (18) have observed esterase in differentiated, but not undifferentiated adipose cells in white adipose tissue *in vivo*. In addition, in 3T3-L1, a fibroblast line that synthesizes collagen before its adipocyte conversion, Novikoff et al. (19) found little or no esterase activity in undifferentiated cells, but documented a dramatic increase in staining during maturation. Although the physiologic role of this esterase activity is unknown, it has been suggested (19) that esterolytic activity is a property of α -glycerolphosphate dehydrogenase. Allen et al. (20), using the mAb F4/80, which stains mouse macrophages, have shown that the fat cells of bone marrow do not originate from the monocyte/macrophage line. They have suggested, rather, a parent-progeny relationship between fibroblast-like reticulum cells and adipocytes. Furthermore, Kodama et al. (21) have cultured an AP⁺ preadipocyte cell line derived from mouse bone that induces the proliferation of hemopoietic stem cells over a 3-wk period. In summary, the accumulated data suggest that bone marrow stromal fibroblasts may be preadipocytes, and that those derived from yellow marrow have the phenotype of more differentiated adipocytes. Unlike the bone marrow stromal cells of the rat, mouse (22), and man (9), rabbit bone marrow reticulum cells do not stain for AP except near the endosteum (D. F. Bainton, personal observation). In this regard, it is of note that the fibroblasts that we examined in this study, both from red and yellow marrow, had little AP. Tavassoli and Friedenstein (23) reported similar findings in the rabbit.

Despite the biologic and enzymatic differences mentioned above, red and yellow bone marrow fibroblasts had similar profiles of procollagen synthesis. Type III procollagen was predominant in each type of culture. In a previous study (24) of collagen synthesis by cultured mouse bone marrow stromal cells, however, type I collagen was predominant, and the ratio of type I to type III was 5:1. These cells were cultured in a continuous Dexter culture system for several weeks, and collagen was analyzed by SDS-polyacrylamide gels using labeled proline. The difference in species, or the major differences in technique, including a prolonged period of culture and the great heterogeneity of the cells present in the studies on the mouse, may account for the difference between these findings and ours.

Type III collagen is the predominant collagen in cultured smooth muscle cells (11, 25-28), as well as cultured endothelial cells, both vascular (29-31) and corneal (14). Thus, these types of cells are clearly distinguished from dermal

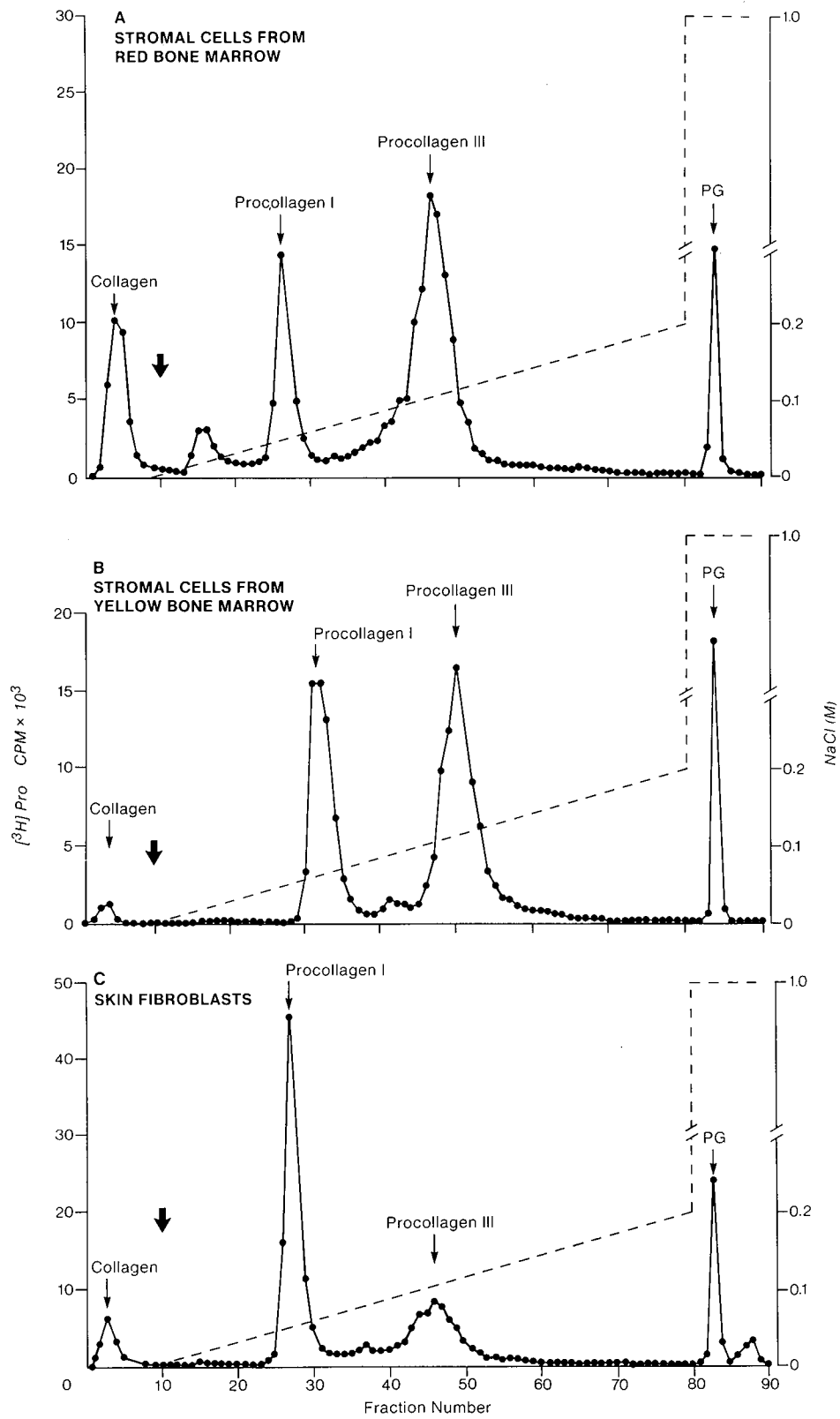


TABLE I
Ratio of Procollagen Types in Cultures of Rabbit Dermal Fibroblasts

Animal	Procollagen type (%)	
	I	III
1	68	32
2	64	36
3	56	44
Average	62	38

Cells were cultured and labeled in the continuous presence of 10^{-7} M hydrocortisone sodium succinate.

TABLE II
Ratio of Procollagen Types in Cultures of Rabbit Red Bone Marrow Stromal Cells

Culture condition	Animal	Procollagen type (%) in cultures			
		With hydrocortisone		Without hydrocortisone	
		I	III	I	III
Preconfluent	4	20	80	41	59
	5	29	71	28	72
	6	42	58	32	68
	Average	30	70	34	66
Confluent	4	28	72	41	59
	5	52	48	27	73
	6	Culture lost		42	58
	Average	40	60	37	63

fibroblasts, which synthesize predominantly type I collagen. More specifically, as we observed in rabbit dermal fibroblasts, human dermal fibroblasts synthesize types I and III collagen at a ratio of ~3:1 under the usual tissue culture conditions (12–14). Immunofluorescence studies have shown that a single fibroblast can synthesize the two types of collagen simultaneously (12). Thus, it is not necessary to invoke two separate classes of fibroblasts to account for the synthesis of the two types of collagen. Type III collagen, also known as reticular collagen, is prominent in fetal tissues, in rapidly proliferating tissue like that found in early wound healing (32–35), and in tissue subject to pathologic processes such as early cirrhosis (36, 37). Thus, it can be surmised that type III collagen is prominent in the fetal fibroblast and fibroblasts stimulated to divide rapidly. Furthermore, the ratio of types I/III in bone marrow stromal cells was similar

FIGURE 4. DEAE-cellulose chromatograms of [3 H]proline-labeled proteins secreted into culture medium by rabbit stromal cells from red (A) and yellow (B) marrow, and by rabbit skin fibroblasts (C). Samples were processed and chromatographed as described in Materials and Methods. The large arrows indicate the start of the NaCl gradient. The first peak in each run represents unabsorbed fractions containing predominantly collagens. The two peaks corresponding to types I and III procollagen are indicated. In A, the small peak of radiolabeled material that was eluted between fraction numbers 15 and 17 corresponds to p_c collagen, partially processed procollagen from which the amino terminal peptide has been cleaved, but to which the carboxy-terminal peptide is still attached. PG, proteoglycan.

TABLE III
Ratio of Procollagen Types in Cultures of Rabbit Yellow Bone Marrow Stromal Cells

Culture conditions	Animal	Procollagen type (%)	
		I	III
Preconfluent	7	43	57
	8	28	72
	9	47	53
	Average	39	61
Confluent	7	52	48
	8	30	70
	9	44	56
	Average	42	58

Cells were grown and labeled in the continuous presence of 10^{-7} M hydrocortisone sodium succinate. Cells did not grow well without the steroid supplement.

to the ratios of collagen found in fetal skin (38), and of procollagen found in endothelial and smooth muscle cell cultures.

From these studies, we cannot conclude with certainty whether these cultured bone marrow stromal cells are fibroblasts or are more closely related to endothelial cells. That endothelial cells are associated with bone marrow is well appreciated (39). From the morphologic point of view, the bone marrow stromal cells do not resemble endothelial cells. They do not show ultrastructural features of endothelial cells, such as Weibel-Palade bodies. Furthermore, type IV procollagen is eluted from DEAE-cellulose columns by the initial wash buffer (40, 41), and we observed no major peak of type IV procollagen in the DEAE-cellulose chromatograms of the culture media for red and yellow bone marrow fibroblasts. In this regard, the cells are unlike endothelial cells. Their profile, with peaks of types I and III procollagen, resembled the profile of the dermal fibroblasts, cells known not to make a basement membrane.

We favor the hypothesis that the stromal cells of bone marrow represent an immature or fetal fibroblast. It is the function of normal bone marrow to maintain a fetal situation in that hemopoietic stem cells must differentiate throughout the life of the animal. In addition, bone marrow reconstitution after mechanical depletion has been shown (42) to resemble ontogenic development; that is, after marrow is washed out of a femur, bone is formed and resorbed, and a matrix is reformed which provides for normal hematopoiesis. Furthermore, our studies with chemical inducers of differentiation have shown that rabbit red bone marrow stromal cells can be stimulated to mature in culture. After maturation, their chromatographic profile of procollagen synthesis resembles that of mature dermal fibroblasts (R. Stern and D. Bainton, unpublished experiments). This change in phenotype may be the sequence of events in pathologic conditions such as myelofibrosis.

Finally, there is a growing consensus that collagen itself is probably not responsible for specific stimulatory effects on hemopoiesis. It is more likely that

collagen forms a structural framework for the deposition of other organ-specific extracellular matrix components (43, 44). The identification of these specific components of the matrix in various organs that modulate hemopoiesis, possibly tissue specific proteoglycans (45), awaits further investigation.

Summary

Rabbit stromal fibroblasts subcultured from red and yellow bone marrow and implanted beneath the renal capsule form ossicles the hemic cellularity of which mirrors the cellularity of the marrow used for culture. Although the cultured red and yellow marrow cells are similar in fine-structural appearance, they differ strikingly in enzymatic content of α -naphthylbutyrate esterase, which is abundant only in the cells derived from yellow marrow. Other observers (20, 21) have proposed that stromal fibroblasts are preadipocytes, and this data suggests that those derived from yellow marrow have the phenotype of more differentiated adipocytes. On the other hand, fibroblasts derived from red and yellow bone marrow show no differences in their profiles of procollagen synthesis. Both types of fibroblasts secrete type III procollagen as the major species, with a I/III ratio of 1:3; in contrast, rabbit dermal fibroblasts have a prominent peak of type I procollagen. The similarity of stromal cells derived from red and yellow bone marrow in procollagen synthesis suggests that the collagen part of the extracellular matrix is not the only basis for their intrinsic difference in capacity for hematopoiesis.

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