BONE FORMATION INDUCED IN MOUSE THIGH BY CULTURED HUMAN CELLS

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ABSTRACT

Cultured FL human amnion cells injected intramuscularly into cortisone-conditioned mice proliferate to form discrete nodules which become surrounded by fibroblasts. Within 12 days, fibroblastic zones differentiate into cartilage which calcifies to form bone. Experiments were conducted to test the hypothesis that FL cells behave as an inductor of bone formation. In the electron microscope, FL cells were readily distinguished from surrounding fibroblasts. Transitional forms between the two cell types were not recognized. Stains for acid mucopolysaccharides emphasized the sharp boundary between metachromatic fibroblastic and cartilaginous zones and nonmetachromatic FL cells. ^{35}S was taken up preferentially by fibroblasts and chondrocytes and then deposited extracellularly in a manner suggesting active secretion of sulfated mucopolysaccharides. FL cells showed negligible ^{35}S utilization and secretion. FL cells, labeled in vitro with thymidine-^{3}H, were injected and followed radioautographically, during bone formation. Nuclear label of injected FL cells did not appear in adjacent fibroblasts in quantities sufficient to indicate origin of the latter from FL cells. The minimal fibroblast nuclear labeling seen may represent reutilization of label from necrotic FL cells. It is suggested that FL cells injected into the mouse thigh induced cartilage and bone formation by host fibroblasts.

INTRODUCTION

When cultured cells derived from human amnion (FL line) (12) were injected intramuscularly (i.m.) into cortisone-treated ICR/Ha mice, tumors developed which contained cartilage and bone (2). In earlier light microscopy studies (2), it was shown that transplanted FL cells multiplied and remained morphologically distinct as tumor nodules throughout a 12 day period of growth and osteogenesis. Fibroblastic cells appeared at the periphery of the FL cell colonies within a day or two after iniection of the FL cells, and as the tumor nodules of the FL cells grew, the fibroblasts increased rapidly in number. After 1 wk of tumor growth, many peripheral fibroblasts rounded up and became enveloped in a cartilaginous intercellular matrix, which calcified. No bone, cartilage, nor intercellular matrix was seen between FL cells (2).

On the basis of our light microscopic studies, we suggested that the FL cells probably induced mouse fibroblasts to secrete a matrix which subsequently became cartilaginous and then osseous (2). The experiments described below were undertaken in an effort to support or reject this suggestion.

MATERIALS AND METHODS

FL tumors were produced in cortisone-injected 15-g female ICR/Ha mice by i.m. injection of $1.2\text{-}5 \times 10^6$ FL amniotic tissue culture cells, as described elsewhere (2). In one experiment, 20×10^6 FL cells which had been heated to 56°C for 10 min were injected into each of six cortisone-injected mice.

Electron Microscopy

Tumors from 10 animals were fixed in Veronal-acetate-buffered osmium tetroxide (21), embedded in Epon, sectioned with diamond and glass knives using LKB and Porter-Blum microtomes, stained with lead hydroxide (18, 25), and examined in the RCA EMU-3F electron microscope.

Identification of Metachromatic Acid Mucopolysaccharides

Sections of FL tumors from 26 animals were cut at 4 μ, and stained for the presence of acid mucopolysaccharides with 0.01% toluidine blue (20), and with the Mowry modification of the colloidal iron stain (19). Five tumors were stained with acridine orange after sections were treated with ribonuclease (24).

The uptake and distribution of $^{35}SO_4$ was studied in FL tumors as an indication of the synthesis and localization of sulfated mucopolysaccharides. Sixteen animals with 7- to 10-day-old intramuscular tumors were injected intraperitoneally (i.p.) with 1–5 μc of ^{35}S-labeled sodium sulfate (New England Nuclear Corp., Boston, 200 μc/mmole) in H_2O per gram of body weight. They were then sacrificed at 10, 30, and 60 min, and at 3, 8, and 24 hr after isotope injection had been made, two to four animals per interval. The tumors obtained from animals injected with $^{35}SO_4$ were subdivided. One-half of each was fixed for 24 hr in 10% aqueous solution of formaldehyde (USP) buffered with 0.1 M phosphate (pH 7.0), and the other half was fixed in a 10% aqueous solution of formaldehyde (USP) which was saturated with barium hydroxide (pH 12.7). The latter fixative dissolves out much of the sulfated mucopolysaccharides of cartilage and precipitates the inorganic sulfates as insoluble barium sulfate (8). Since the buffered formalin solution does not appear to have this property (8), a comparison of the same tumor after fixation in the two solutions permits one to estimate roughly the amount of labeled sulfate present in the form of acid mucopolysaccharides.

Preparation of Tritium-Labeled FL Cells

24 hr prior to injection of FL cells into mice, the medium in the culture flasks containing the FL cells was replaced by a similar medium containing 0.05 μc/ml of tritiated thymidine (Schwartz Bioresearch, Inc., Orangeburg, New York, 360 mc/mmole). After 24 hr of exposure to the medium containing thymidine-^3H, the cells were washed twice with tritium-free medium to remove unincorporated isotope. Radioautographs of cultures of labeled FL cells revealed that 95% of the nuclei were "labeled" (defined as the presence of five or more supranuclear grains of reduced silver).

Following in vitro exposure to thymidine-^3H, the cells were scraped from the flasks by rubber "policemen," sedimented at 1000 rpm for 10 min, and resuspended in tritium-free medium for injection. After i.m. injection of labeled FL cells took place, animals were sacrificed at 24-hr intervals throughout the 12-day period of tumor growth.

Abbreviations

ER, endoplasmic reticulum
GZ, Golgi zone
Li, lipid droplet
PM, plasma membrane
M, mitochondrion
R, ribosomal rosette

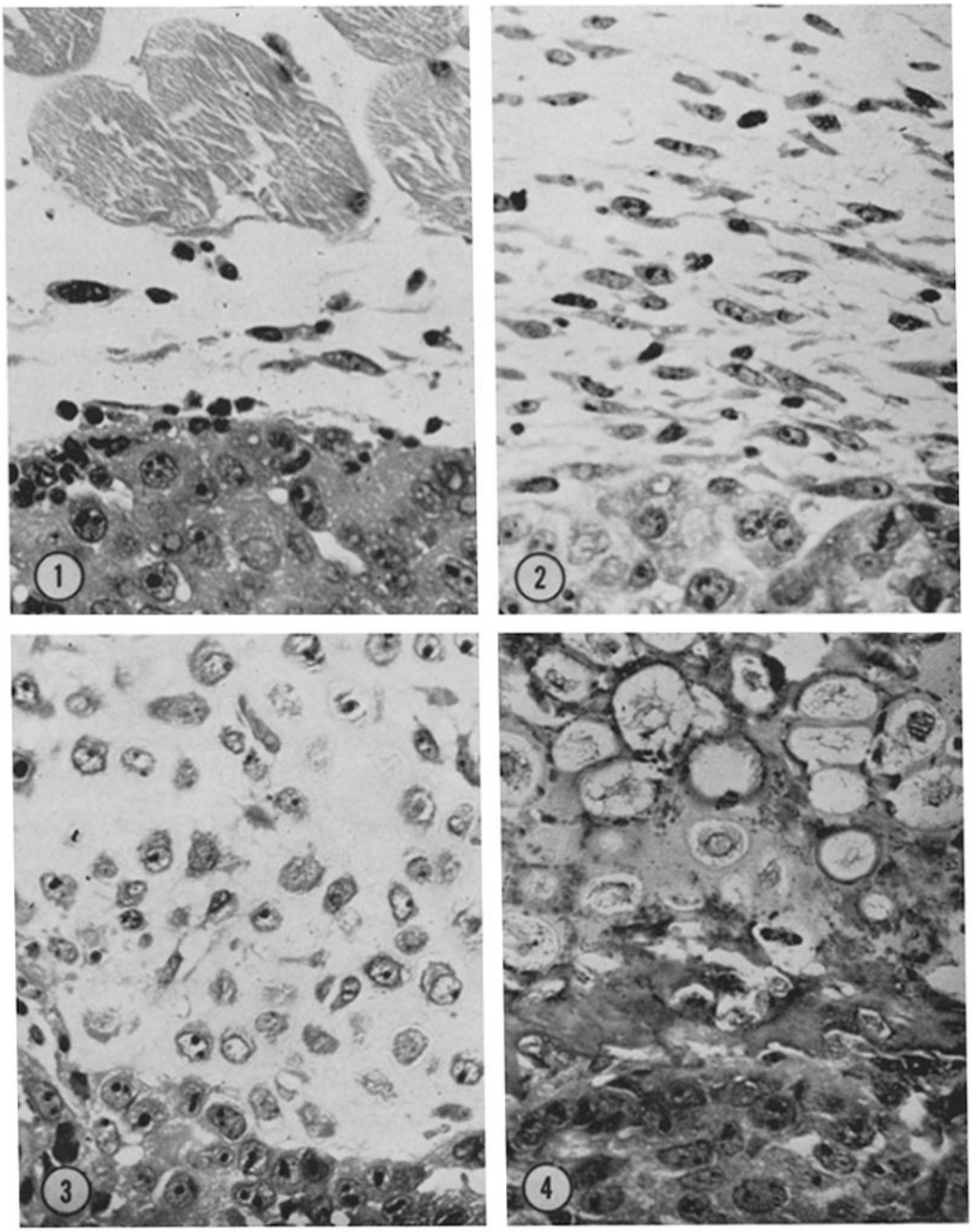
FIGURES 1–4 Histologic sections from FL mouse thigh tumors showing the sequence of cartilage and bone formation. Lower portion of each figure contains FL cells.

FIGURE 1 2 days after FL cell injection a few fibroblasts are present between FL cells and skeletal muscle (upper portion). × 500.

FIGURE 2 5 days after FL cell injection. The fibroblastic zone (upper) is markedly increased. × 500.

FIGURE 3 8 days after FL cell injection. An intercellular cartilaginous matrix is deposited between fibroblasts which round up, assuming the appearance of chondrocytes (upper). × 500.

FIGURE 4 12 days after FL cell injection. Endochondral calcification is present. The chondrocytes appear hypertrophic, and some are degenerating (upper). The appearance of FL cells (bottom) is unchanged. × 500.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Preparation of Radioautographs

When the tumor tissue had been dehydrated and embedded in paraffin, histologic sections were cut at 2 μ from tumors labeled with thymidine-^{3}H or with $^{35}SO_4$ and from nonlabeled control tumors. The sections were mounted on gelatin "subbed" slides, deparaffinized, and then coated with warm (48°C) Kodak NTB-2 nuclear track liquid emulsion. After a 2 wk exposure period at 4°C, the slides were developed with Kodak DK-50 for 5 min at 68°F. They were stained with hematoxylin and eosin.

Nuclear Counting Procedures

In each tumor studied, the numbers of reduced silver grains in the radioautographic emulsion overlying 100 FL cell nuclei and overlying the nuclei of 100 adjacent fibroblasts were counted. Tumors from at least two, and as many as five animals were counted for each day of tumor development.

FL cells used for counting were located at the edges of FL cell nodules.

Fibroblasts used for nuclear counting were located immediately adjacent to FL cells which had been counted. If transformation from FL cell to fibroblast were occurring, then fibroblasts immediately adjacent to FL cells would presumably be derived from the latter. Therefore we selected these fibroblasts for counting, in order to test the transformation hypothesis. Fibroblasts were identified by their elongate shape, oval nucleus, and general lack of adherence.

To determine whether or not fibroblasts adjacent to FL cells were representative of the fibroblast band as a whole, we made similar counts over fibroblast nuclei at increasing distance (0–10 μ, 10–20 μ, etc.), from the FL-fibroblast junction in 3- and 4-day-old tumors. An eyepiece micrometer was used to estimate distance from the FL cells.

Background radioactivity was estimated by counting nonnuclear grains in microscopic areas containing labeled FL cells and adjacent fibroblasts. Nonnuclear background was counted by using an eyepiece micrometer in a total area of 10^5 μ^2 in each microscopic section, and average counts ranged from 0.3 to 1.8 grains per 100 μ^2. Only sections averaging fewer than two nonnuclear grains per 100 μ^2 were used for nuclear counting.

RESULTS

Injection of FL cells resulted in the formation of proliferating FL cell nodules, observed microscopically, which became progressively invested with fibroblasts The latter underwent chondro-osseous differentiation (Figs. 1–4) within 12 days. Injection of the control heated FL cells failed to produce tumors or evidence of cartilage or bone formation in any of the six animals injected.

Electron Microscopy

Transplanted FL cells, observed in the electron microscope, appeared round and closely opposed to each other. The nuclei were large with irregular borders and contained one or more prominent, irregular nucleoli. The cytoplasm (Fig. 5) contained numerous free ribosomes arranged in rosettes, and sparse rough-surfaced endoplasmic reticulum. The Golgi zones were smaller and more inconspicuous than those of fibroblasts.

In sharp contrast was the fine structure of fibroblasts and chondrocytes adjacent to FL cells (Fig. 6). These could be easily distinguished by their abundant rough-surfaced endoplasmic reticulum and large Golgi zones. Unlike the rounded, closely opposed FL cells, the fibroblasts were often elongated and usually were separated from one another by a collagenous matrix. Fibroblast and chondrocyte nuclei were usually ovoid when sectioned obliquely. They were generally smaller than FL nuclei and contained moderate-sized nucleoli. Transitional or intermediate cell types between fibroblasts and chondrocytes and the FL cells were not identified.

Metachromatic Staining

A well-defined boundary could be distinguished between FL cells and fibroblasts after 7- through 12-day-old FL tumors were stained with toluidine blue or with the Mowry modified colloidal iron stain. Fibroblastic and cartilaginous elements stained strongly for acid mucopolysaccharides with all three techniques utilized, while FL colonies showed little or no staining (Fig. 7).

Sulfur-35 Utilization

^{35}S was taken up almost exclusively by fibroblasts and chondrocytes adjacent to FL colonies, and only a small amount of labeled sulfate was taken up by FL cells (Fig. 8). Most of the radioactivity associated with fibroblasts and chondrocytes was removed by fixation in the barium hydroxide–saturated formalin (Figs. 9 *a* and *b*), but this procedure had very little effect on the small radioactivity associated with the FL cells (Figs. 10 *a* and *b*).

During the 1st hr following intraperitoneal injection of $^{35}SO_4$, radioactivity was greater within the fibroblasts and chondrocytes than in the surrounding matrix (Fig. 11). 3 hr after injection, radioactivity was approximately equal in fibroblasts and in matrix, and after 24 hr the radioac-

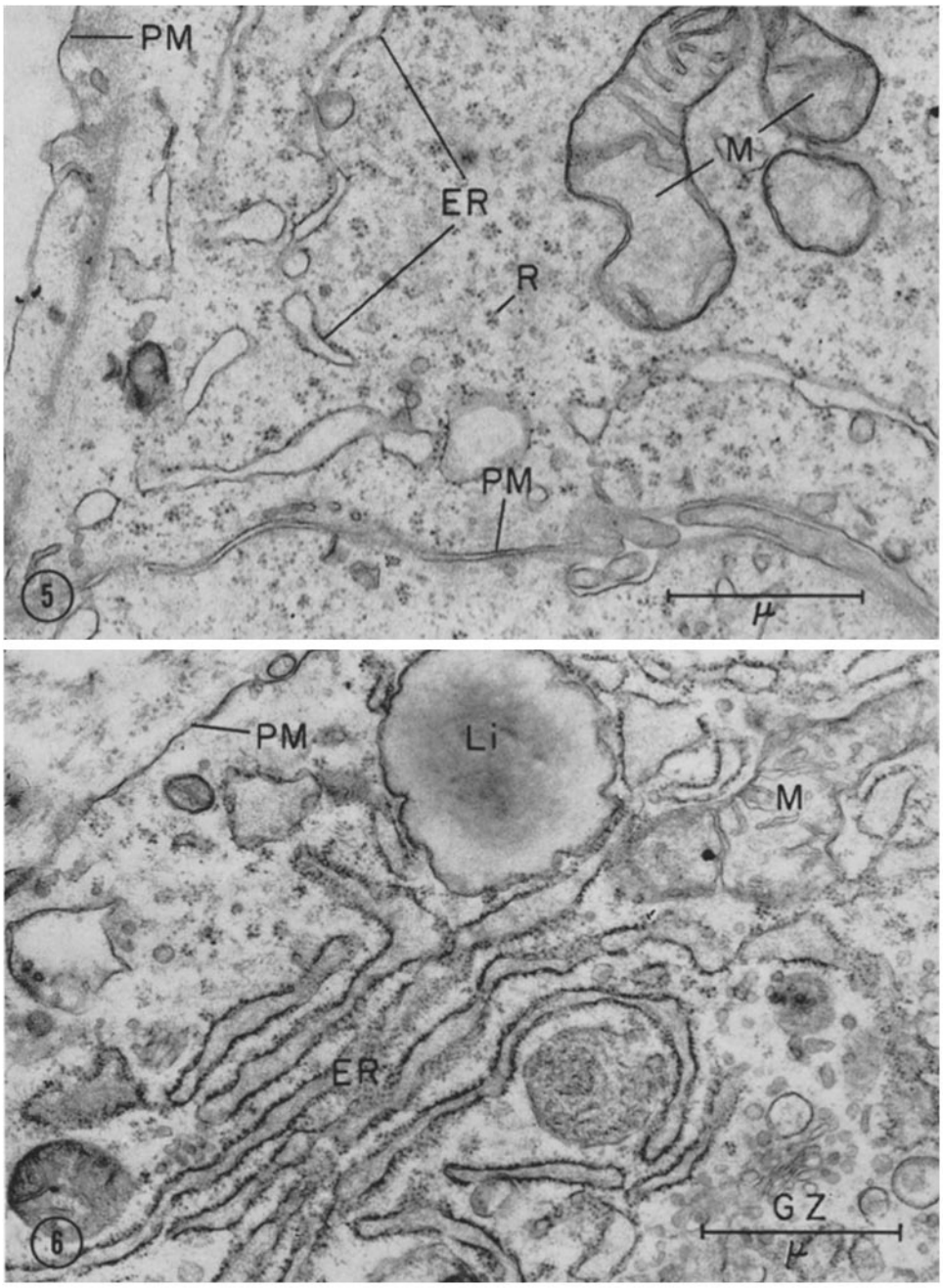

FIGURE 5 Electron micrograph (mouse thigh tumor) showing the fine structure of FL cell cytoplasm with characteristic free ribosomes forming rosettes (*R*), and sparse rough endoplasmic reticulum (*ER*). × 27,000.

FIGURE 6 Electron micrograph (mouse thigh tumor) demonstrating the cytoplasm of a fibroblast which, in contrast to FL cell cytoplasm of Fig. 5, contains abundant rough endoplasmic reticulum (*ER*) and prominent Golgi zone (*GZ*). × 27,000.

tivity was predominantly in the matrix (Figs. 8 and 12). The small amount of activity present within the FL cells showed little change during the same 24 hr interval.

Fate of Tritium-Labeled FL Cells after Intramuscular Injection into Mice

At the time of injection, following 24 hr exposure to thymidine-^{3}H, 95% of FL cells were labeled, and many showed marked nuclear activity (Fig. 13). Because of such heavy labeling, nuclear counts were not done on FL cells before injection.

1 day following injection, FL cell nuclear labeling was still quite high (77% of nuclei labeled) (Figs. 14 and 15). However, the proportion of FL cell nuclei labeled decreased daily, declining to 2.5% by the 8th day following FL cell inoculation, and thus reached control background levels seen in nuclei of injected FL cells not exposed to thymidine-^{3}H (average for 10 days = 1.7% nuclei labeled).

Nuclear activity in fibroblasts adjacent to thymidine-^{3}H–labeled FL cells (Fig. 15) was very low on day 1 (1.5% nuclei labeled), and remained low throughout the 12 day period of osteogenesis. Slight elevations were seen in fibroblast nuclear labeling on days 3–6, reaching a peak on day 3 (10% of nuclei labeled). Thereafter, the per cent of fibroblast nuclei labeled descended to the background level seen in fibroblasts adjacent to control FL cells injected without exposure to thymidine-^{3}H (average for 10 days = 0.0% fibroblast nuclei labeled).

The highest per cent of labeled nuclei present in fibroblasts (10% of nuclei labeled) which was seen on day 3 did not approach that seen in FL cells on the same day (65.5% of nuclei labeled). It should be noted that nuclear labeling in fibroblasts never exceeded levels of nuclear labeling seen in FL cells even after 12 days when the radioactivity of the latter was negligible (Fig. 15).

Daily changes in mean grain count over nuclei of injected FL cells and over nuclei of adjacent fibroblasts (Fig. 16) paralleled the changes, described above, in per cent of nuclei labeled (Fig. 15).

Nuclear labeling in fibroblasts at increasing distances up to 100 μ from the FL cell margin was similar to that seen in fibroblasts adjacent to FL cells.

Tritium labeling did not appear to inhibit or increase the ability of FL cells to induce bone formation since seven out of seven tumors produced by injection of labeled FL cells and allowed to grow for at least 10 days contained what appeared to be the usual amount of bone and/or cartilage.

DISCUSSION

Delineation of FL Cells from Bone-Forming Fibroblasts

The histologic, electron microscopic, histochemical, and radioautographic data presented above emphasize the marked morphological and functional differences between transplanted FL cells and surrounding fibroblasts.

The light and electron microscopic preparations clearly delineated FL cells from the adjacent fibroblastic zone (1–3). The electron microscopic appearance of the FL cells within tumors (3) was similar to that of FL cells in culture (11). Their cytoplasm was relatively undifferentiated with many free ribosomes, sparse ergastoplasm, and small Golgi zones. The cytoplasm of fibroblasts, on the other hand, contained prominent ergastoplasm and Golgi zones, thus presenting the picture seen in cells undergoing protein synthesis (22).

Metachromatic stains demonstrated the association of a chondroid matrix, rich in acid mucopolysaccharides, with fibroblasts and chondrocytes but not with FL cells. These stains also emphasized the sharp boundary between the two cell types. Studies with $^{35}SO_4$ showed a pronounced concentration in fibroblasts and chondrocytes as opposed to the low incorporation into FL cells. The sequential appearance of ^{35}S first within fibroblasts and then in the intercellular matrix suggested that these cells were active in the synthesis and secretion of sulfated material, presumably sulfated mucopolysaccharides.

The disappearance of most ^{35}S activity in the chondrogenic zones after fixation in the barium hydroxide–saturated formalin indicated the association of ^{35}S with acid mucopolysaccharides.

The small amount of ^{35}S taken up by the FL cells which appeared to remain intracellularly and was not dissolved out by barium hydroxide-saturated formalin solution suggests that these cells were relatively inactive in the production of the chondroid matrix.

Thus, it would appear the FL osteogenetic tumors are composed of two distinct cell populations: transplanted FL cells which do not secrete a chondroid matrix, and fibroblasts which become

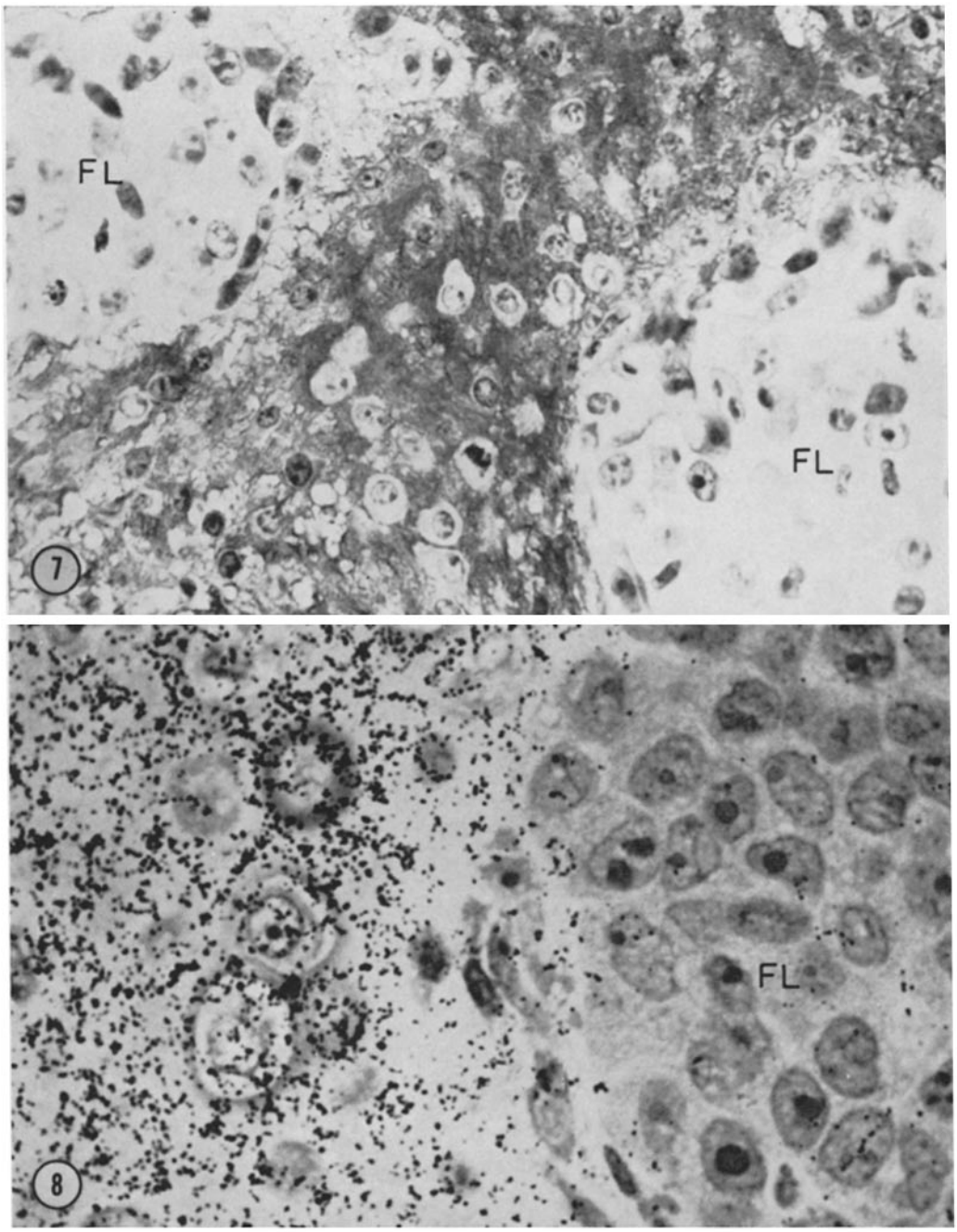

FIGURE 7 Histologic section of mouse thigh tumor. Dark-staining acid mucopolysaccharides (blue green in original preparations) are almost exclusively present in the central fibrocartilaginous zone. FL colonies (*FL*) remain essentially unstained. Hale colloidal iron procedure. × 500.

FIGURE 8 Radioautograph of mouse thigh tumor. ^{35}S is taken up selectively into the fibrocartilaginous zone. Radioautograph made from a tumor obtained 24 hr after intraperitoneal injection of $^{35}SO_4$. Note concentration of radioactivity in the matrix between cartilage cells, and the sparse activity seen in FL cells (right). × 770.

chondrocytes and which apparently have the ability to synthesize a metachromatic cartilaginous matrix. Our failure to find transitional forms between FL cells and fibroblasts is evidence against transformation of the former into the latter.

Bone Induction by Labeled FL Cells

FL cells injected i.m. after labeling with thymidine-^{3}H and followed radioautographically through osteogenesis did not appear to become incorporated into osteogenic zones.

Assuming transformation of FL cells into fibroblasts, one would expect radioactivity in fibroblasts to approach or exceed that seen in the progenitor neighboring FL cells, depending upon the generation time of the two cell types in question. This was definitely not the case in FL osteogenetic tumors (Fig. 15). Although a very low level of radioactivity was observed in fibroblasts during days 3-6, the nuclear activity seen at this time did not approach levels seen in FL cells, nor exceed FL cell activity in a fashion which would suggest a precursor relationship. If transformation from FL cell to fibroblast were occurring, one might expect the fibroblasts nearest to the FL cell tumor margin to have a higher nuclear activity than fibroblasts located farther away from the tumor margin. Such did not appear to be the case in preliminary counts of nuclear activity in fibroblasts at increasing distances from the FL-fibroblast junction. Therefore, transformation is considered unlikely.

An interesting aspect of these experiments was the slightly increased average nuclear label in fibroblasts adjacent to thymidine-^{3}H-containing FL cells in days 3–6 tumors. Although this radioactivity did not approach that seen in adjacent FL cells, microscopic examination revealed some fibroblasts which appeared to demonstrate more than background activity. If this difference is real, it could represent reutilization of labeled FL cell DNA, or FL cell DNA breakdown product. The phenomenon of DNA reutilization has been suggested in a number of other systems (4–7, 9, 10, 16, 23), in which tritium-labeled DNA, available as purified DNA (4, 16, 23) or available in nuclei of infused leukocytes (5, 7) or in degenerating liver cells or lymphocytes (6, 23), appeared in the nuclei of rapidly dividing transplanted tumor cells, or in dividing cells of the gut, testis, etc. An obvious parallel to these observations exists in our own experiments in which degenerating FL cells are typically present in the center of FL colonies (2), and could serve as a source of diffusible label to multiplying fibroblasts.

Possible Mechanisms of Bone Induction

Similarities exist between FL cell-induced osteogenesis in mice and the induction of bone formation in the rectus sheath of dogs by transplanted mucosa of the urinary bladder or gall bladder (14, 15). In all three systems, proliferating epithelial cells serve as a stimulus, skeletal muscle is a susceptible site for implantation, and ossification occurs in young or adult animals. The mechanism(s) of induction has not been worked out for urinary bladder and gall bladder mucosae, and an

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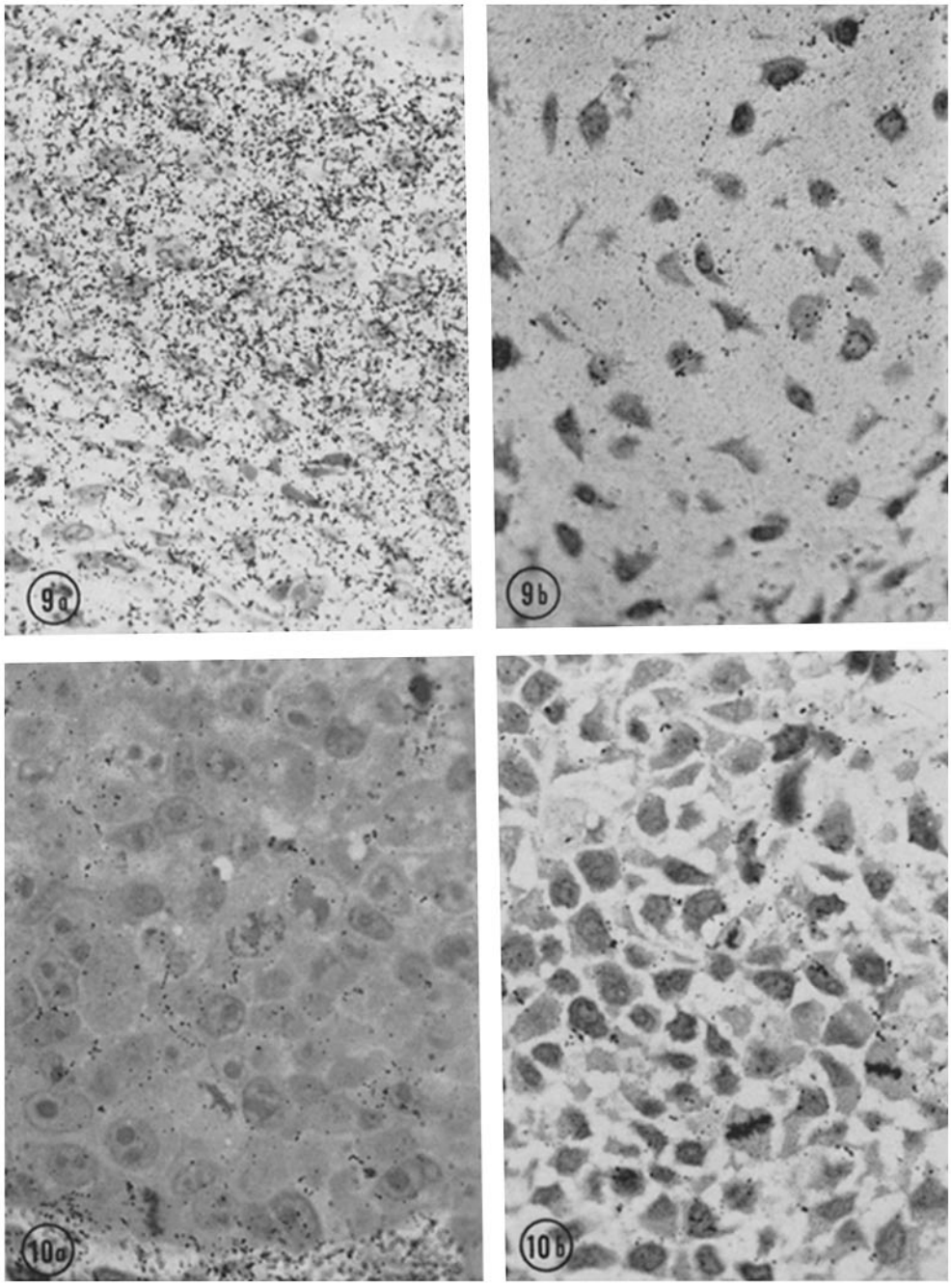
FIGURE 9 *a* Radioautograph of fibroblasts and chondrocytes surrounding an FL tumor, 8 days after injection of FL cells and 24 hr after i.p. injection of $^{35}SO_4$. Fixation in buffered formalin. Compare with Fig. 9 *b*. × 650.

FIGURE 9 *b* Radioautograph of portion of the same tumor as in Fig. 9 *a*, but fixed in barium hydroxide–saturated formalin. Relatively little radioactivity is present in the fibrocartilaginous zone, suggesting association of ^{35}S with acid mucopolysaccharides, known to be soluble in this fixative. × 650.

FIGURE 10 *a* Radioautograph of FL cells in portion of the same tumor as in Figs. 9 *a* and *b*. Fixation in buffered formalin. Relatively few grains are present in FL cells. More are present at junction of FL cells and cartilage area (bottom). × 650.

FIGURE 10 *b* Radioautograph of portion of same tumor as in Figs. 9 *a* and *b*, and 10 *a*. Fixation in barium hydroxide–saturated formalin. Small amount of radioactivity associated with FL cells approximates amount seen in Fig. 10 *a*. Findings suggest that FL cell ^{35}S was present as inorganic sulfate (insoluble in barium hydroxide–saturated formalin) and not bound to acid mucopolysaccharides. × 650.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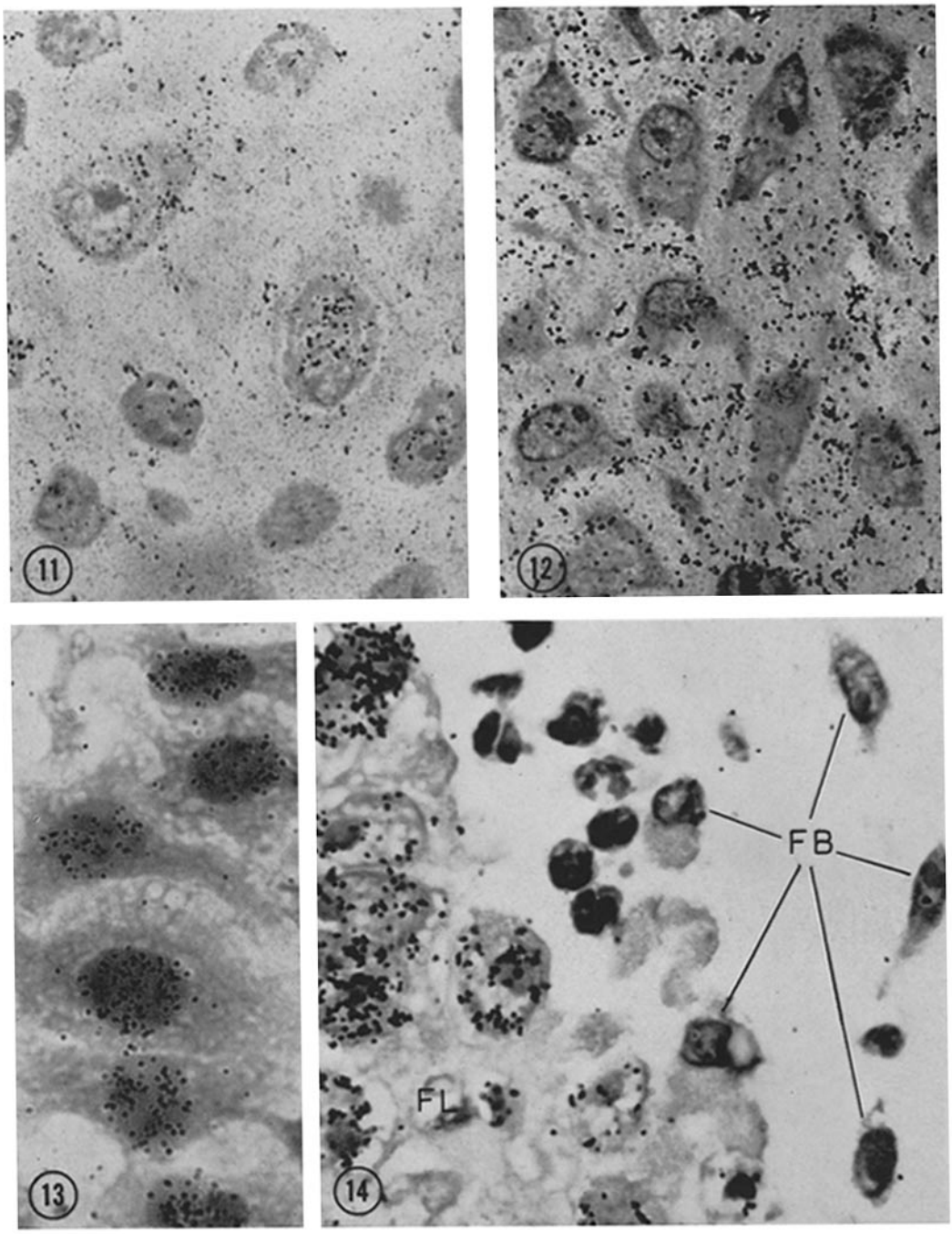

FIGURE 11 Radioautograph, mouse thigh tumor. Intracellular localization of ^{35}S in chondrocytes 10 min after i.p. injection of $^{35}SO_4$. × 1000.

FIGURE 12 Radioautograph, mouse thigh tumor. Predominantly extracellular localization of ^{35}S, in cartilaginous matrix, 24 hr after i.p. injection of $^{35}SO_4$. × 1000.

FIGURE 13 Radioautograph. Nuclear radioactivity in FL cells in vitro following a 24-hr exposure to culture medium containing thymidine-^3H. × 1200.

FIGURE 14 Radioautograph, mouse thigh tumor. 1 day following i.m. injection of thymidine-^3H labeled FL cells (*FL*). Prominent FL cell nuclear radioactivity (left) vs. insignificant fibroblast (*FB*) nuclear activity. × 1200.

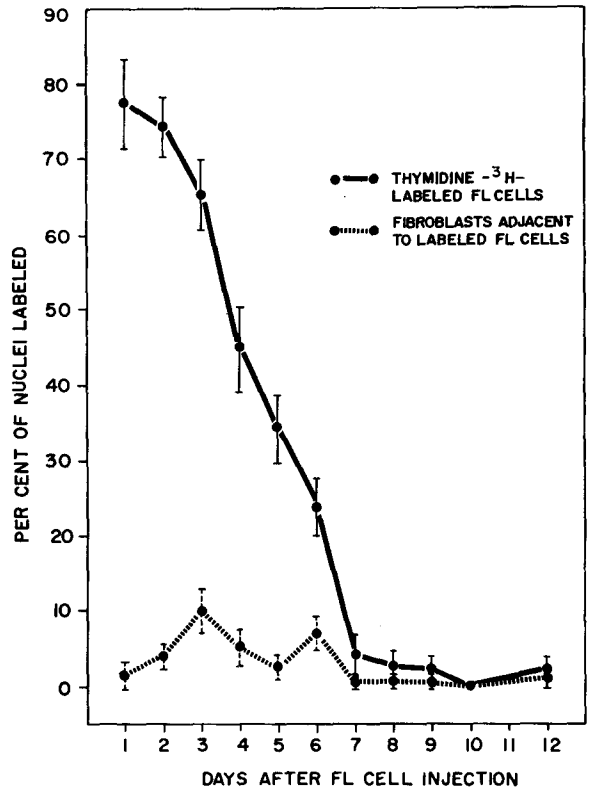

FIGURE 15 Percentage of nuclei labeled in FL cells and in adjacent fibroblasts at daily intervals following injection of thymidine-^{3}H-labeled FL cells. Vertical bars represent two times standard error.

The difference in per cent of nuclei labeled in the two populations and the lack of cross-over in values suggest that fibroblasts were not derived from FL cells. The elevation in activity seen in fibroblasts on days 3–6 may reflect reutilization of labeled DNA breakdown product from necrotic FL cells.

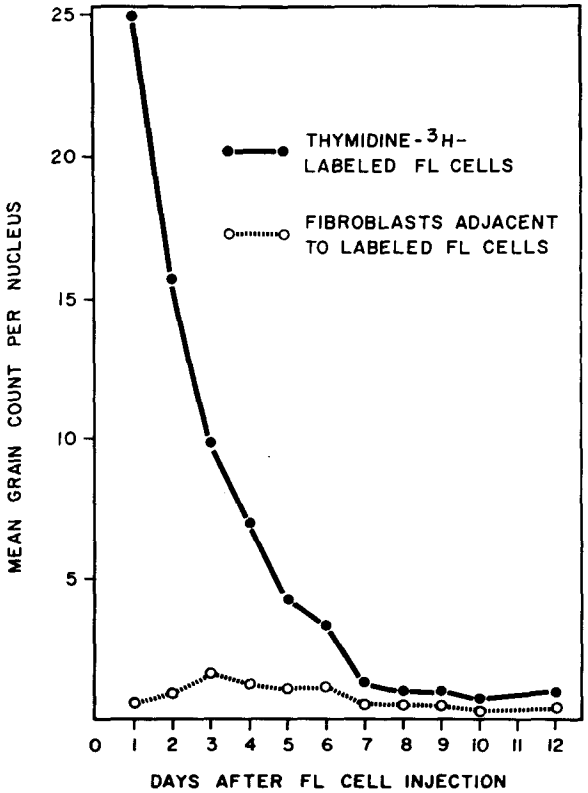

FIGURE 16 Mean grain count per nucleus of thymidine-^{3}H-labeled FL cells and of adjacent fibroblasts at daily intervals following FL cell injection.

Daily changes in mean grain count parallel changes in per cent of nuclei labeled (Fig. 15), further suggesting that fibroblasts are not derived from FL cells.

understanding of FL cell induction could possibly shed light on all of these systems.

Bone induction has not been shown to be a property unique to FL cells, and other tissue culture cells from various species should be tested for growth and bone-inducing capability. It is possible that FL cells contribute no inducing substance, but merely cause sufficient mechanical damage at the cellular level to bring in large numbers of fibroblasts, some of which possess the property of cartilaginous differentiation.

It is also possible that FL cell induction requires direct cell-to-cell contact between FL cells and susceptible fibroblasts. This appears to be the case in the induction of embryonic lens development by the optic vesicle (26).

The possibility should be considered that a chemical substance, similar to an embryonic inductor, may pass from FL cells to sensitive fibroblasts, causing the latter to differentiate into chondrocytes. Such a diffusible substance with the ability to induce cartilage development in embryos has been shown to be elaborated by the ventral spinal cord and notochord. In this system, the inductor, which is able to function in vitro across a millipore filter barrier, has been isolated in nucleotide fractions and has been shown to be of low molecular weight (17).

If a diffusible bone-inducing substance is elaborated by FL cells, it could act by entering host fibroblasts and directing metabolic pathways toward cartilage matrix production, or it could "condition" the chemical environment of the fibroblast, perhaps by interaction with a substance or substances already present, producing reaction products which lead to bone formation (13).

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