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# The Establishment and Characterization of an Immortal Cell Line With a Stable Chondrocytic Phenotype

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**Abstract** A cell line was developed from the transplantable Swarm rat chondrosarcoma (RCS) and has been maintained in continuous monolayer tissue culture for a number of years. This long term-cultured (LTC) cell line exhibits the morphological and biochemical characteristics of chondrocytes and resembles the RCS tumor by electron and light microscopy. The cell line differs from the original tumor cells in that about 90% of the sulfated macromolecules are retained in the LTC extracellular matrix as compared to 30% by primary cultures of cells from the RCS tumor. An interesting and useful feature of this cell line is that it contains clonal populations of cells which differ in the quality and quantity of matrix produced. Two such clones serve to illustrate the diversity of cell types within the LTC cell line. One termed Rex accumulates an intensely staining matrix around it, while the other, Ng, accumulates a matrix, that remains virtually unstained. The chondrocytic nature and ease of cloning make these cells ideal for biochemical analysis of the chondrocyte and its extracellular matrix. J. Cell. Biochem. 89: 992–1004, 2003. © 2003 Wiley-Liss, Inc.

**Key words:** chondrosarcoma; chondrocytes; extracellular matrix; metabolism; proteoglycan

The study of constituents of hyaline cartilage has been greatly facilitated by the model system of the Swarm rat chondrosarcoma (RCS). These chondrocytes are maintained as a transplantable tumor in rats [Choi et al., 1971]. Characteristic of these chondrosarcoma chondrocytes is the production of many macromolecules common to the extracellular matrix (ECM) of hyaline cartilage including aggrecan, the large aggregating chondroitin sulfate proteoglycan [Kimura et al., 1979; Kimura et al., 1981], type II collagen [Kucharska et al., 1990], type IX collagen [Arai et al., 1992], link protein [Oegema et al., 1977], hyaluronan (HA) [Mason et al., 1982], COMP [Mörgelin et al., 1992], and fibronectin [Kimata et al., 1982]. These RCS tumor cells have been used for the isolation and study of other cartilage molecules such as matrix metalloproteinases [Moses and Shing, 1994] and CD-RAP [Bosserhoff et al., 1997]. Typically, molecules are isolated directly from the tumor or after short-term culture of dispersed cells [Calabro and Hascall, 1994]. Additional topics of study making use of this tumor include the biosynthesis of ECM molecules [Thonar et al., 1983; Fellini et al., 1984; Kimura et al., 1984; Calabro and Hascall, 1994], the interactions between ECM molecules [Faltz et al., 1979; Kimura et al., 1980; Hascall and Kimura, 1981; Lohmander et al., 1983], the organization of molecules within the matrix [Hascall and Kimura, 1981], and the effects of hormones and growth factors on ECM metabolism [Stevens et al., 1981; Foley et al., 1982; Seong et al., 1994; Matsumura et al., 2000]. It is an excellent source of protein for antibody

Abbreviations used: RCS, Swarm rat chondrosarcoma; LTC, long term–cultured chondrosarcoma; ECM, extracellular matrix; HA, hyaluronan; COMP, cartilage oligomeric matrix protein; CD-RAP, cartilage-derived retinoic acid-sensitive protein; RHT, ruthenium hexamine trichloride; DMG, Dulbecco's Modified Eagle media with 4.5 g glucose/L; FBS, fetal bovine serum; CSS, calf supplemented serum; PBS, phosphate buffered saline; EDTA, ethylene diamine tetraacetic acid; CS, chondroitin sulfate; ΔDi-0S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose; ΔDi-HA, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-glucose; ΔDi-4S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose; and ΔDi-6S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose.

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preparation [Caterson et al., 1985; Kittelberger-Ewert et al., 1988; Calabro et al., 1992], or a source of messenger RNA for cDNA sequencing, cell-free translation, and promoter analysis of cartilage genes [Doege et al., 1986; Doege et al., 1987; Hering and Sandell, 1988; Rhodes et al., 1991]. It is useful in the study of oncology [Di Cesare et al., 1998; Fang et al., 2000; Fang et al., 2001].

An obstacle in the use of this system has been contamination of fibroblast-like cells when this line is cultured in monolayer over 1 week [Kucharska et al., 1990]. Suspension culturing of RCS chondrocytes in an uncharged matrix of agarose has shown retention of chondrocytic phenotype over a 20 day period [Sun et al., 1986; Kucharska et al., 1990]. While this method also provides flexibility in terms of culturing time and physical manipulation of cultures, there remain constraints in daily culture maintenance as well as the interference of the agarose with biochemical examination of the cell-produced matrix.

To answer this we have developed a new long term-cultured (LTC) cell line from the rat tumor, which can be maintained entirely in monolayer. These cells produce a ruthenium-stainable matrix, as well as biochemically identifiable macromolecules similar to those of RCS and of hyaline cartilage. Western blot analysis of LTC cell culture extracts has demonstrated the presence of type II procollagen [Fernandes et al., 2001]. Analysis of tritium-labeled cell cultures demonstrates production of only alpha 1 bands, but no alpha 2 bands [Fernandes et al., 1997]. Further demonstration of chondrocyte-like gene expression is the expression of COL2A1 and COL9A2 mRNA, but no expression of COL10A1 nor COL1A1 [Mukhopadhyay et al., 1995]. In addition, this line grows quickly, doubling in about 1 day during log phase. This line is useful for biochemical and molecular biological analysis of the ECM [Mukhopadhyay et al., 1995; Bosserhoff et al., 1997; Fernandes et al., 1997; King et al., 1997; Yamauchi et al., 1999; Fernandes et al., 2001].

Clonal cell lines developed from this long term culture have shown that LTC contains a number of minor populations of cells with characteristics unlike the parent line. One specific difference is in the different levels of synthesis and sulfation of proteoglycans. We present the biochemical charaterization of the parent line (LTC) and two of the cloned cell lines (Rex, Ng) and illustrate their usefulness as model systems for the chondrocyte.

## MATERIALS AND METHODS

### Materials

Collagenase was purchased from Worthington Biochemical Corporation (Lakewood, NJ); tissue culture supplies were from Life Technologies (Life Technologies, Inc., Rockville, MD), Invitrogen Corp. (Rockville, MD) with the exception of the following: all sera were obtained from Hyclone Laboratories, Inc. (Logan, UT); dispase from Calbiochem-Novabiochem Corp. (La Jolla, CA); and all tissue culture dishes were from Falcon Division of BD Biosciences (Bedford, MA). Radioisotope was obtained from Amersham Pharmacia Biotech (Piscataway, NJ); PD-10 and Superose 6 columns were also from Amersham Pharmacia Biotech; Permanox chamber slides from Nalge Nunc, International (Rochester, NY); RHT from Alfa Aesar (Ward Hill, MA); Ecoscint A scintillation fluid from National Diagnostics (Atlanta, GA); Chondroitinase ABC from ICN Pharmacueticals, Inc. (Costa Mesa, CA). Unless otherwise indicated, all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO), and were of the highest quality available.

### Maintenance of Tumor and Preparation of Primary Cultures

Tumors of the RCS were propagated by serial subcutaneous implantation [Kimura et al., 1979]. For in vitro culturing, homogenized tumor tissue was dispersed by sequential trypsin (0.25%) and collagenase (0.5%) digestion. The media used in all procedures unless otherwise specified was Dulbecco's Modified Eagle's Medium (DMG) and 3.7 g/L sodium bicarbonate, and 50 μg/ml gentamycin sulfate, pH 7.2.

### Development of Long Term Culture (LTC)

Cells were obtained from suspension cultures of rat tumor cells prepared as described previously [Saito et al., 1988]. Cells were liberated by trypsin/collagenase digestion and plated at $10^7$ cells/100 mm culture dish. Only detached cells were harvested and subcultured until free of the fibroblast-like cells, which were still present in the nodular cultures. Monolayer cultures were initiated from a trypsin/collagenase

dispersion of these fibroblast-free floating cells. This chondrocyte culture was maintained in monolayer culture for several years with no sign of fibroblasts. Typical culturing conditions included ~1.5 ml media/$10^6$ cells/day with 10% (v/v) FBS or CSS and incubation at 37°C, 5% $CO_2$, and 95% humidity. Monolayers were split once a week.

### Cloning Procedures

Dispersed and filtered (20 μm sterile nylon mesh) chondrocytes were serially diluted to ~1 cell/400 μl DMG with 20% FBS, and 200 μl were placed in each well of two 96-well tissue culture dishes. After overnight incubation, each well was examined for attached cells. Wells containing only one cell were followed for the next week. The number of cells present in each well was recorded periodically.

### Preparation of Cultures for Light Microscopy

One million dispersed cells in 1.5 ml media with 20% FBS were allowed to adhere to Permanox chamber slides ($43 \times 20$ mm$^2$) using the culturing conditions above or were suspended in agarose with 10% FBS. After 4–5 days these cultures were fixed in 1% glutaraldehyde with 0.4% RHT (30 min for monolayer, 2–4 h for agarose samples) followed by PBS washes [Hunziker et al., 1983; Hunziker and Graber, 1986]. Cultures were stained with 0.01% toluidine blue containing 0.1% RHT (2 h for monolayer, overnight for agarose) then mounted with glycerol. Light microscopic photographs were obtained with Hoffmann optics.

### Preparation of Cultures for Electron Microscopy

LTC chondrocytes were plated at 2.5 to $3 \times 10^6$ cells per 35 mm dish. Every day the spent media was centrifuged, and fresh media was added returning pelleted cells to the culture. After 9 or 10 days the culture medium was centrifuged, and the cell pellet and the monolayer culture were fixed and stained separately in 1.5% glutaraldehyde, 1% paraformaldehyde, and 0.4% RHT in 0.1 M cacodylate buffer for 2 h, washed, then post-fixed with 1% osmium tetroxide and 0.4% RHT for 2 h, then dehydrated [Hunziker et al., 1983]. The monolayer tissue was scored in small squares (about 5 mm$^2$) with a sharp blade prior to addition of propylene oxide [Larramendi, 1988]. Individual thin sections were placed on carbonized grids and stained with uranyl acetate. Stained sections were examined and photographed with a JEM-100CX electron microscope.

### Preparation of Cultures for DNA Assay

Three million cells were plated in 60 mm tissue culture dishes in 10 ml DMG with 20% FBS. Culture medium was not changed for the duration of the growth period.

Each day, three dishes were selected at random for DNA assay. Media was removed and centrifuged to collect non-adherent, "floater" cells. Fresh papain solution (500 μg/ml 100 mM $NaH_2PO_4$, 10 mM EDTA, 10 mM cysteine HCl, pH 7.0, sterile filtered) was added to the floaters and dish and incubated overnight at 60°C. The extracts were collected by scraping the dish surface with a rubber policeman.

DNA was quantified in a dye binding assay [Kim et al., 1988]; 30 μl of sample extract was briefly mixed with 3 ml of the working dye solution (Hoechst 33258 at 0.2 μg/ml in 20 mM Tris base, 1 mM EDTA, 100 mM NaCl, pH 7.4) and examined for fluorescence in a Turner fluorimeter with excitation and emission filtration near 365 nm and 458 nm, respectively.

### Preparation of Agarose Cultures

Dispersed LTC chondrocytes were mixed in agarose to a final concentration of $1 \times 10^5$ cells/ml 2% agarose (w/v DMG without serum). This agarose/cell mixture was poured between glass plates (Bio-Rad Mini-Protean II Slab gel) to form an $80 \times 80 \times 1$ mm$^3$ culture and was quickly solidified at 4°C then divided into three equal pieces. One piece was placed in 10 ml DMG with 10% FBS, one in 10 ml DMG with insulin (10 μg/ml), and the third in 10 ml DMG without additives.

For each culture, an area (approximately $12 \times 10^{-3}$ cm$^2$) near the center of the agarose gel was examined under 10× magnification. The total number of cell groups (single cells + colocolonies) within this area was determined as well as the number of cells within each group.

### Monolayer Culturing With Insulin or Serum and Proteoglycan Assay

LTC chondrocytes were grown at $2.5 \times 10^5$ cells per 2 cm$^2$ well of a multi-well tissue culture dish. These cultures were fed daily with DMG containing one of the following: FBS (10% v/v), CSS (10%), insulin (10 μg/ml with bovine serum albumin 10 μg/ml as carrier) or DMG alone.

Each day, one dish containing each media group in triplicate was extracted with 125 μl 2 N NaOH then neutralized with 100 μl 4 N acetic acid. Extracts were clarified by centrifugation, diluted and examine by glycosaminoglycan assay [Farndale et al., 1982]. Briefly, unknown solutions were mixed with 1,9-dimethymethylene blue (in a formate buffer). Absorptions of unknown solutions were immediately measured and compared to dilutions of cartilage chondroitin sulfate standard.

### Isotopic Labeling of Monolayer Cultures

Isotopic labeling was used in a number of metabolism experiments described below. RCS chondrocytes, LTC chondrocytes, or chondrocytes from the clonal lines were dispersed to single cells as described previously and plated at 2.5 to $3 \times 10^6$ cells per 35 mm dish and allowed at least 2 h to adhere. The exact number of cells varied between experiments, but was held constant within experiments. Less than 3% of original plating number was lost when plating media (with 20% FBS) was replaced with radiolabeled media containing 5 or 50 μCi/ml $^{35}S$-$SO_4$ or 100 μCi/ml 6-[$^3H$]-glucosamine with 10% FBS. Cultures were labeled with sulfate for 30 min to 24 h or with tritium for 16 h. Spent media was removed from cultures immediately after the labeling period.

### Measurement of Radiosufate Incorporation

Media samples were mixed 1:1 with 2× extraction buffer (1× = 4 M GuHCl, 50 mM $Na_2SO_4$, 50 mM Tris-HCl, pH 7.5). The pelleted floaters were suspended in 2 ml 1× extraction buffer and returned to the culture dish containing the cell matrix layer for 1 h at 4°C. The cell extract was then removed, and the dish was rinsed twice with 0.5 ml buffer and scraped with a rubber policeman.

Macromolecular radioactivity was quantified after a sample containing 0.25 ml of either labeled media or labeled cell extract was added to a PD-10 column (Sephadex G-25 M) and washed and eluted with the extraction buffer [Stevens et al., 1981].

### Precipitation of Glycosaminoglycans

Cultures labeled with radioactive sulfate or tritium for 16 h were extracted overnight in 4 M GuHCl, pH 11.5, then neutralized with 6 N HCl. Unincorporated isotope was removed from cell extracts by molecular sieve chromatography. Chondroitin sulfate carrier (50 μg CS chains/sample) was added prior to ethanol precipitation of glycosaminoglycans [Mason et al., 1982]. Precipitates were suspended in water.

### Thin Layer Chromatography

Portions of tritiated precipitates described above were pre-digested with papain (25 μg/ml 0.1 M $NaH_2PO_4$, 5 mM EDTA, 5 mM cysteine HCl) for 3 h at 37°C prior to polysaccharide digestion with chondroitinase ABC (0.4 units per 100 μl reaction in 0.01 M Tris HCl, pH 8.0, 0.01 M NaF) for 1.5 h, 37°C. These disaccharides ($2 \times 10^5$ cpm/sample) were dried and resuspended with the addition of 50 μg unlabeled bovine trachea disaccharides in the same buffer. Samples were applied to a sheet of plastic backed cellulose [E. Merck; distributed by Alltech Associates, Inc. (Deerfield, IL)] and chromatographed by first desalting overnight in 52% n-butanol, 32% ethanol then eluting several hours in n-butanol: glacial acetic acid: 2 N NaOH (2:3:1). Standard disaccharides [Δdi-0-S, Δdi-4-S, and Δdi-6-S from Seikagaku Fine Biochemicals (Tokyo, Japan), and HA prepared with chondroitinase ABC] were run together adjacent to tritiated samples. The solvent front migrated 145 mm above the sample origin. The width of each migration path was cut every 5 mm from 10 mm below the origin to the solvent front, solubilized in HCl and analyzed by liquid scintillation. These methods for measuring unsaturated disaccharides were developed and validated by Mason et al. [1982].

### Column Chromatography

Tritiated precipitates were pre-digested with papain for 3.5 h at 60°C. Digests were chromatographed directly or after 5 h of digestion at 37°C with Streptomyces hyalurolyticus hyaluronidase (0.03 U/μl with sodium acetate added to 0.1 M). The FPLC Superose 6 column was eluted with 0.5 M sodium acetate, pH 7.0 (0.4 ml/min). Ten drop fractions (~0.2 ml) were collected. The $^3H$-HA content was calculated from the difference between fractions prepared with or without hyaluronidase.

## RESULTS

### Characteristics of the Long Term Culture

The LTC parent line was developed from the RCS tumor using specific culturing and

selection conditions. Cells from a suspension culture maintained for 18 months were used to isolate a cell line [Saito et al., 1988]. Cells were isolated by sequential trypsin/collagenase digestion and plated on plastic tissue culture dishes. Detached cells were harvested and passaged separately until no fibroblast-like cells remained. This LTC cell line was maintained for several years in tissue culture with passages occurring about once a week.

Observed characteristics included a rounded shape, which flattened as cells adhered to the tissue culture plastic (similar to the original tumor cells). These cells increased in number rapidly, and their progeny also appeared as either slightly flattened cells adhering to the substratum or as rounded "floaters." Many groups of floating cells were viewed a few days after passage. When the monolayer was dispersed for passage, the cells in solution returned to their rounded morphology. Batches of cells were slowly frozen in media containing dimethlysulfoxide and then stored in liquid nitrogen vapor. Re-suspension of frozen cells in warm medium containing 20% FBS typically recovered over 80% viable cells.

Toluidine blue staining was present over the culture dish but was not uniformly distributed. Many cells appeared unstained with foci of heavily stained cells interspersed (Fig. 1A). Higher magnification showed that the distribution of stain was not uniform even within groups of cells and suggested that even in the case of cells positive for proteoglycan production, individual cells exhibited different levels of accumulation (Fig. 1B,C).

Images taken using electron microscopy were obtained from 9- and 10-day monolayer cultures of the LTC cell line (Fig. 2). Monolayers and the cell floaters collected from their centrifuged media were prepared with a fixative containing 0.4% RHT, a cationic dye known to prevent the loss of proteoglycans typical of common fixation procedures [Hunziker et al., 1983]. Fixed, embedded samples of monolayer or pelleted floaters were sectioned and stained. Sections through the 9-day monolayer culture showed rounded cells with a prominent ruthenium stain about the cell surface, extensive ECM, and thick areas of RHT-precipitated proteoglycans (Fig. 2A). Cellular features included a large nucleus, large mitochondria, numerous Golgi, very extensive endoplasmic reticulum, several vesicles, glycogen deposits, and lipid droplets.

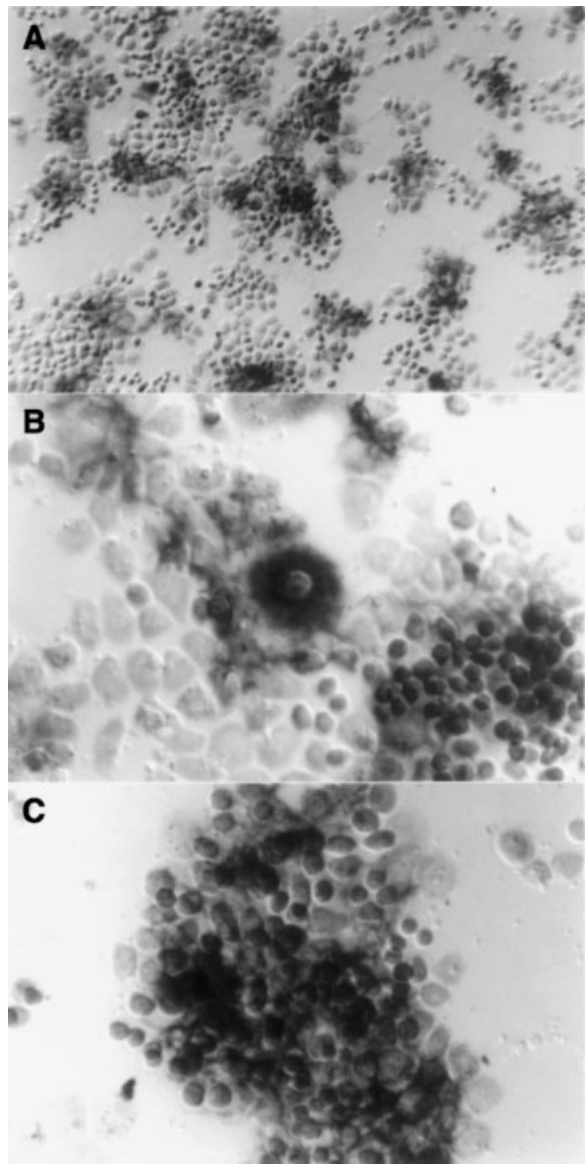


**Fig. 1.** Toluidine blue stained LTC chondrocytes in monolayer. Low density monolayers were fixed and stained after 4 days of growth on plastic slides in DMG with 20% FBS. These cells exhibited different levels of matrix accumulation and retention of stain. **A**: 10× magnification; (**B**) 30×; (**C**) 30×. All images were taken using Hoffmann optics.

The features were quite similar to those observed of RCS cells [Hascall, 1980]. Sections of the pelleted LTC floaters showed similar morphology (Fig. 2B).

The average rate of growth by the LTC chondrocytes was determined by estimating the rate of increase in DNA per culture (Fig. 3). This experiment demonstrated a very short lag phase in growth following plating procedures. Cultures were found to double in about 1 day

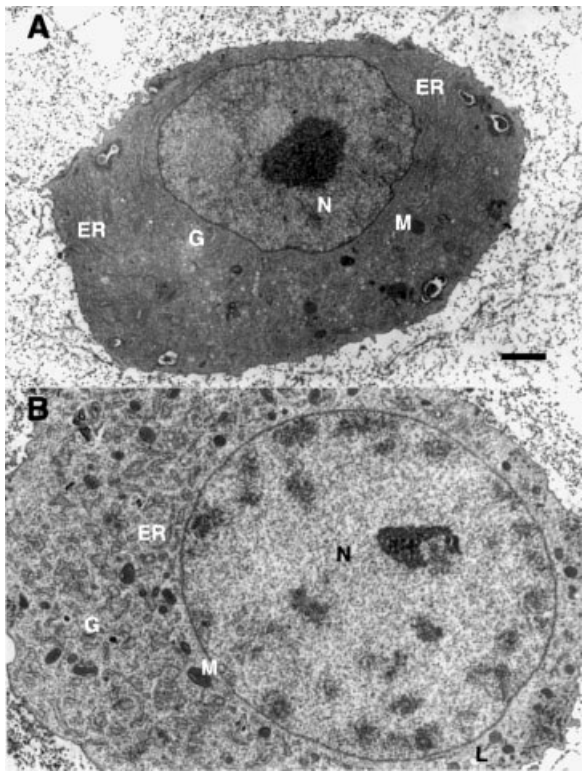


**Fig. 2.** Electron micrographs of LTC chondrocytes. **A**: A cell from 9 day monolayer culture; (**B**) a cell from pelleted floaters of 10 day monolayer culture media. Note the large nucleus (N), mitochondria (M), Golgi (G), endoplasmic reticulum (ER), and lipid (L). Bar equals 10 μm.

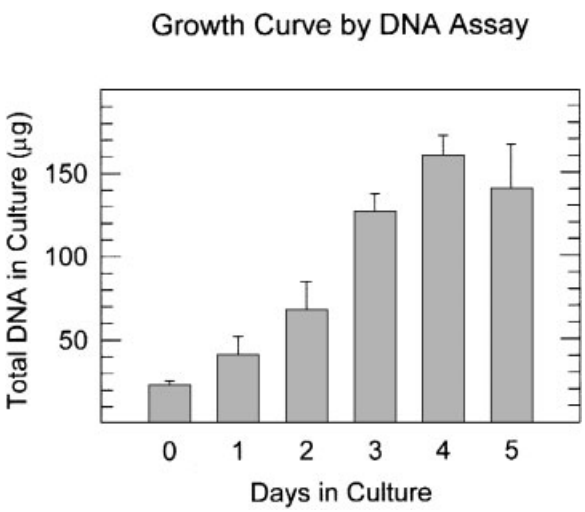


**Fig. 3.** Relative DNA content of monolayer LTC cultures. Six dishes per day (three dishes on day 5), $3 \times 10^6$ cells per 35 mm dish were extracted with papain after growth in 10 ml DMG (with 20% FBS). Total DNA content per dish was measured against calf thymus DNA standard using the Hoechst 33258 DNA assay. Error bars represent standard deviation.

from day 1 to day 4 (log phase). At day 5, the growth rate slowed, perhaps due to insufficient nutrients as the media was not changed in order to retain floating cells.

### Effect of Insulin and Serum

In a separate growth study, LTC chondrocytes were suspended in a 2% agarose matrix ($1 \times 10^5$ cells/ml) in which media was changed every other day without loss of cells. Low initial cell densities were chosen to facilitate distinction of individual cells and colonies. Also studied was the effect of insulin at 10 μg/ml without serum in contrast to growth in media with 10% fetal bovine serum or media alone (Fig. 4). The cultures grown in FBS showed an ability to produce colonies, and average colony size appeared to increase in number steadily, even out of range of precise determination after 8 days. In the cultures supplemented with insulin, up to 38% colonization was seen (number of colonies divided by the sum of colonies and single cells). However, there were rarely more than two cells per colony; at 15 days the average colony size was 1.43 cells. In the agarose culture which received unsupplemented media doublets were rarely present although a few cells did produce a

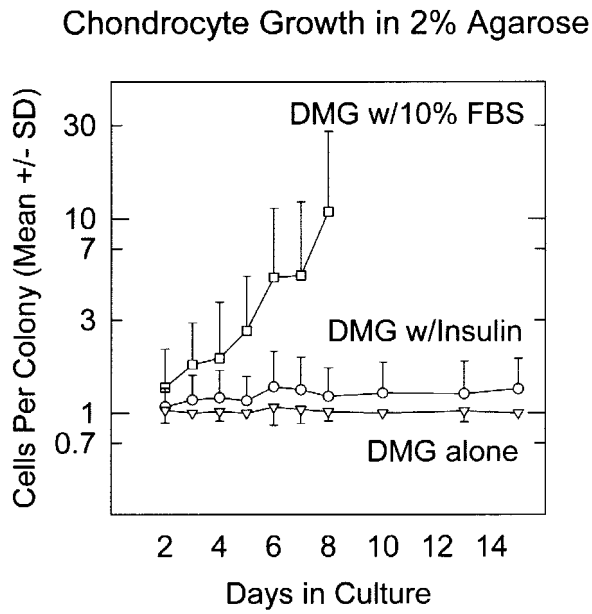


**Fig. 4.** Chondrocyte growth in 2% agarose. Agarose cultures of LTC chondrocytes ($1 \times 10^5$ cells/ml 2% agarose) were incubated for 2 weeks in DMG with either 10% fetal bovine serum, insulin 10 μg/ml, or neither. Average number of cells per colony was determined by directly counting a $12 \times 10^{-3}$ $cm^3$ area under 10× magnification. LTC cells survived in insulin supplemented, serum-free medium and produce a matrix but did not proliferate.

matrix visible under phase-contrast microscopy. Many of the insulin treated cells produced a clearly visible matrix. All of the colonies observed in the serum supplemented agarose culture produced matrix about the colony.

### Cloning

The ability of these LTC chondrocytes to produce colonies in the presence of serum was examined further. Isolated cells were cultured in separate tissue culture wells for 1 week without change of media. Typically 60–80% of single cells were grown to colonies, typically doubling in number every day without coculture (Fig. 5). Cultures which contained one cell 1–2 h after plating and greater than two cells at day 4 were considered colonies and were included in Figure 5, a compilation of data from six experiments.

### Proteoglycan Synthesis

Proteoglycan biosynthesis was examined by measuring the incorporation of radiosulfate in macromolecules. After removal of unincorporated isotope by molecular size exclusion, distribution of isotope incorporated between the media fraction and the cell extract was compared in 24 h cultures of RCS cells and LTC cells (Fig. 6). Much of the macromolecular radiosulfate accumulated in the media fraction of the RCS cultures, while in the LTC cultures the cell layer retained the majority of incorporated counts. RCS distributed 69% to the media and 30% to the cell extract. Conversely, only 14% of the sulfate incorporated by the LTC was found in the media with the remaining 86% in the cell extract.

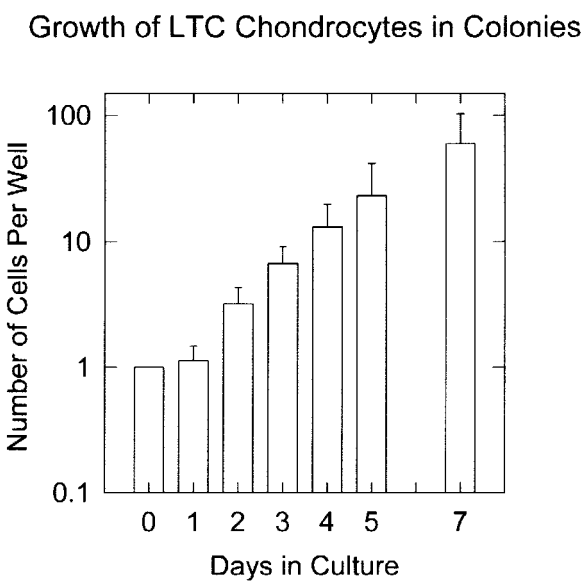


**Fig. 5.** Growth of LTC chondrocytes in colonies. Isolated cells were cultured in separate wells of 96-well tissue culture dishes for 1 week without change of media (200 µl DMG w/20% FBS). Cultures which contained one cell 1–2 h after plating and greater than two cells at day 4 were considered colonies and were included in this figure which represents a compilation of data from six experiments. While most colonies that formed survived beyond 1 week, some were out of range due to high density proliferation and were not included as day 7 data points. Error bars indicate standard deviation of data.

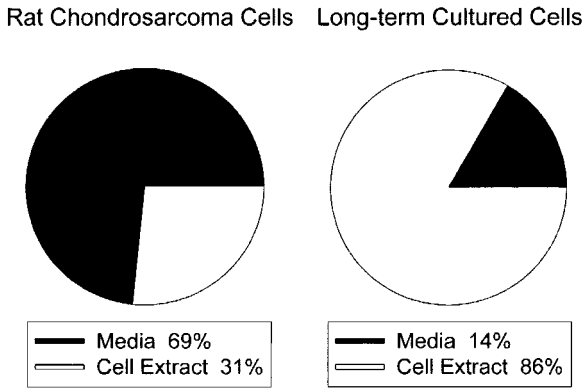


**Fig. 6.** Distribution of radiosulfate incorporation by the tumor chondrosarcoma in culture and the LTC chondrocytes. Duplicate cultures were plated at $3 \times 10^6$ cells/35 mm dish overnight and labeled the next for 9 h. **A**: RCS chondrocytes in short term monolayer culture. **B**: LTC chondrocytes in short term monolayer culture. The RCS was nearly opposite the LTC in matrix distribution.

The rate and distribution of radiosulfate incorporation over time following plating were examined in the LTC (Fig. 7). During the first 8 h of label (10–18 h after plating) the cells incorporated sulfate at a steady rate of about $2.6 \times 10^5$ cpm/h/$10^6$ cells. Between 8 and 24 h of label the rate of incorporation increased about 47%, possibly due to conditioning of the medium or cell division. At 30 min only about 3% was found in the media, whereas after 24 h about 12% of sulfate was found incorporated in molecules released to the media. As demonstrated in the previous experiment, the LTC retains the majority of sulfated macromolecules in its matrix.

Proteoglycan biosynthesis in response to media supplements was examined with the use of a glycosaminoglycan assay. Cultures of the LTC line were grown in DMG containing either insulin, CSS, or FBS (Fig. 8). All three supplements individually enhanced biosynthesis over that of cultures grown in media alone.

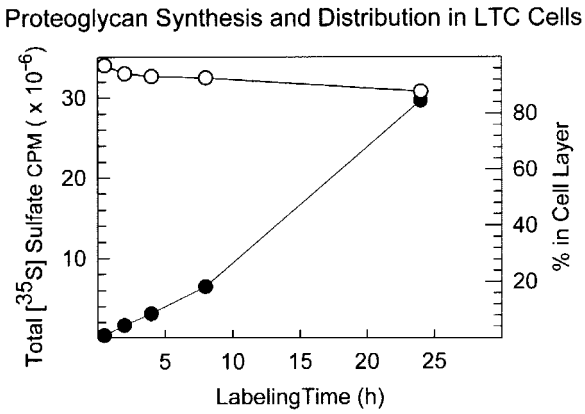


**Fig. 7.** Proteoglycan synthesis and distribution in LTC cells. Three million cells were labeled with $^{35}$S-sulfate 10 h after plating for 0.5, 2, 4, 8, and 24 h. Samples were in duplicate. Filled circles: total incorporation over time. Data reported are the sum of incorporation in the cell and matrix extract and media extract. Open circles: percent of counts found in the cell and matrix extract. Error bars represent standard deviation of data.

There was very little difference in biosynthesis between the two types of serum. Insulin at 10 μg/ml was shown to significantly increase biosynthesis over baseline to nearly the rate of serum supplemented cultures.

### Heterogeneity of the LTC Parent Line

The heterogeneity of the LTC chondrocyte line was seen in toluidine stained monolayer cultures viewed at high magnification. This effect was more dramatic in the stained cultures of chondrocytes suspended in agarose (Fig. 9A). Here cells isolated by agarose divided, producing isolated colonies. Within colonies the matrix staining was relatively even. However, some colonies stained dark while a few stained only lightly regardless of colony size. Clonal lines developed from isolated cells in monolayer were suspended in agarose and stained after 5–6 days of growth. When stained, most clonal cultures showed homogeneous staining throughout. Many clonal lines retained the cationic stain (Fig. 9B). In a few clonal lines the ECM stained only slightly or not at all with

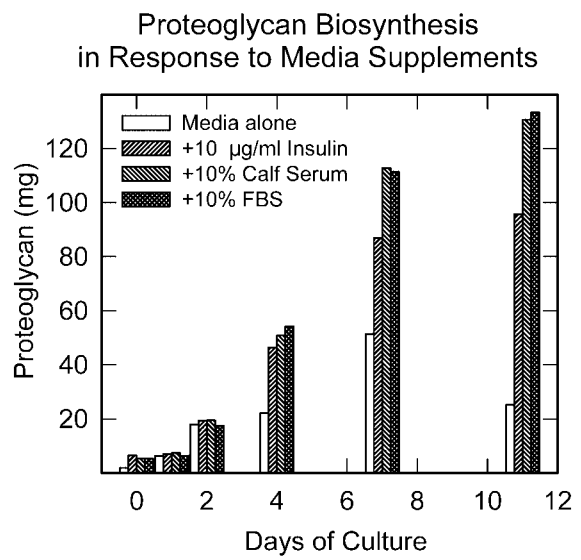


**Fig. 8.** Proteoglycan biosynthesis by the LTC in response to media supplements. Small cultures were daily fed media alone or media containing 10 μg/ml insulin, 10% CSS, or 10% FBS. Cell extracts were analyzed for proteoglycan using the dimethylmethylene blue glycosaminoglycan assay. The data represent the average of cultures in triplicate.

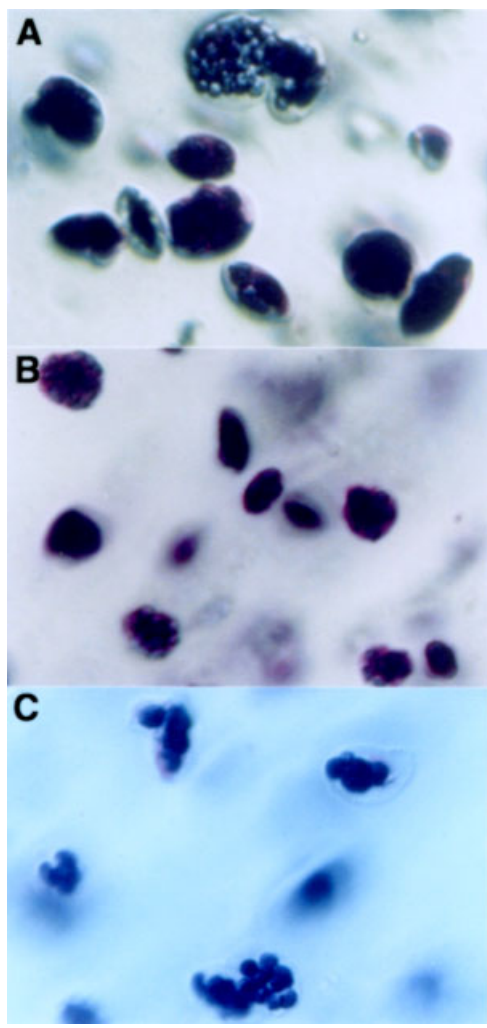


**Fig. 9.** Toluidine blue stained clonal lines. Agarose cultures of isolated clones were grown 5–6 days then were fixed and stained overnight. (10× magnification). All images were taken using Hoffmann optics. **A**: LTC parent line. **B**, **C**: Two individual clonal lines. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

toluidine blue, yet it could be observed with Hoffmann optics (Fig. 9C).

### Radiosulfate Incorporation by Clonal Chondrocyte Lines

Two clonal lines obtained by the monolayer cloning procedure were chosen for either intense toluidine blue staining of the ECM (Rex) or for the lack of matrix staining (Ng). Sulfate labeling of monolayer cultures showed much higher proteoglycan synthesis in clone Rex than the parent LTC and slightly lower synthesis in clone Ng (Fig. 10). In all three cultures at least 82% of incorporated macromolecules were found in the cell layer extracts.

### Characterization of Glycosaminoglycans

Analysis of disaccharides obtained from chondroitinase ABC digested glycosaminoglycan chains showed additional differences between the clonal and LTC lines and were in agreement with the differences demonstrated by toluidine blue staining and sulfate incorporation studies. Thin layer chromatography of disaccharides showed that Rex, the high staining clone, produced a larger ratio of Δdi-4-S compared to the parent LTC (Table I). A dramatically different profile was shown for clone Ng which produced a notable amount of chondroitin disaccharides (Δdi-0-S) not present in clone Rex and much higher than reported for the tumor RCS [Mason et al., 1982]. The Rex clone had the lowest ratios of Δdi-6-S:CS and Δdi-HA:CS of the three lines. The production levels of hyaluronate calculated as a ratio of $^3$H-[Δdi-HA]: $^3$H-[Δdi-0-S + Δdi-4-S + Δdi-6-S] for LTC, Rex, and Ng were each much lower (~70% less) than that reported for the cell layer extract of RCS in large scale chondrocyte cultures using the same technique of analysis [Mason et al., 1982].

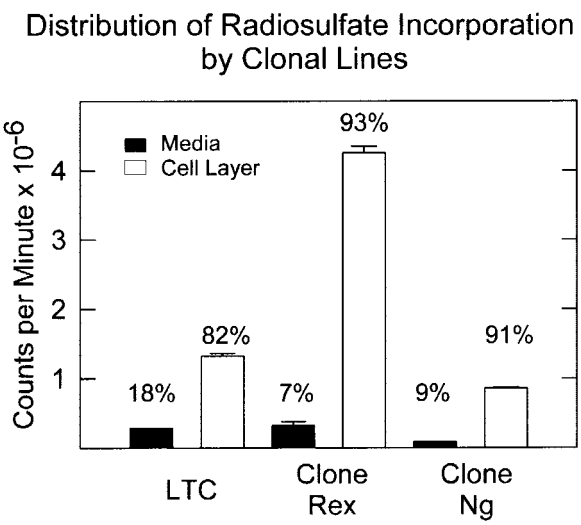


**Fig. 10.** Distribution of radiosulfate incorporation by parent LTC and Rex and Ng clonal lines. A total of $3 \times 10^6$ cells/35 mm dish were labeled 16.5 h with 5 μCi/ml $^{35}$S-sulfate in sulfate-free media. Error bars represent standard deviation. The Rex clonal cells incorporated the most sulfate, the Ng clonal cells incorporated the least.

Levels of HA produced by the cell lines were further investigated with column chromatography of isolated tritiated glycosaminoglycan chains that were either undigested or digested with the HA-specific lyase Streptomyces hyaluronidase (Fig. 11). Cultures of Rex, Ng, or parent LTC were labeled overnight with $^3$H-glucosamine. Cell layers were removed and incubated with papain to digest protein. Portions of each papain digest were chromatographed on Suparose 6 columns (closed circles). HA from these cells was sufficiently large to appear in fractions at or around the void volume ($V_0$). The long chondroitin sulfate chains appeared around fraction 50, oligosaccharides peaked around fraction 66, and the total column volume ($V_t$) appeared at fraction 73. Portions of each papain digest were further cleaved with Streptomyces hyaluronidase. These were also chromatographed on Suparose 6 columns (open circles).

For each cell line the HA peak disappeared from $V_0$ after hyaluronidase treatment, while a peak just preceding $V_t$ expanded proportionally. The profile of the undigested samples from the parent LTC suggested that HA contributed 7% of $^3$H-cpm. The void volume of the undigested material from clone Rex was much less (4%). In clone Ng a slightly higher percentage (8%) of tritium was found in the HA position, and total tritium incorporation by Ng was nearly three times higher than the other two.

In addition, the elution of clone Ng chondroitin sulfate chains was three fractions after LTC and eight fractions after clone Rex. The basis for these differences was not investigated further, although it is likely that at least a portion of the differences was due to the smaller hydrodynamic size of unsulfated glycosaminoglycan on gel chromatography. It is likely that the earlier elution of chondroitin sulfate from Rex compated to LTC was due to larger chain sizes as the ratio of sulfated to unsulfated disaccharide in the two cell lines was nearly identical.

**TABLE I. Ratios of Disaccharides Within Clonal Lines Rex and Ng Compared to Parent Line, LTC**

| | Chondroitin sulfate | | | Hyaluronate |
|---|---|---|---|---|
| Chondrocytes | Δdi-6-S:CS | Δdi-4-S:CS | Δdi-0-S:CS | Δdi-HA:CS |
| Parent LTC | 0.062:1 | 0.828:1 | 0.110:1 | 0.088:1 |
| Clone Rex | 0.039:1 | 0.924:1 | 0.038:1 | 0.063:1 |
| Clone Ng | 0.048:1 | 0.607:1 | 0.345:1 | 0.095:1 |

Chondrocytes were cultured with $^3$H-glucosamine for 16 h. Cell layer extracts were treated with papain then chondroitinase ABC prior to thin layer chromatography alongside disaccharide standards. The values represent the proportion of radioactivity recovered that was in each disaccharide spot. Note the high proportion of Δdi-0-S produced by the Ng clone.

## DISCUSSION

The LTC chondrocyte line that we present here is similar to the cells of the transplantable RCS tumor. They both exhibit the rounded morphology of chondrocytes. They have similar cellular organelles consistent with their high production of ECM molecules of hyaline cartilage (Fig. 2). Cellular detail of this subculture includes features found in cells which frequently secrete macromolecules including extensive endoplasmic reticulum and Golgi apparatus for protein post-translational modification as well as many secretory vesicles. Ruthenium staining of the cultured cells produces extensive precipitation of the ECM components of proteoglycan and fibrillar collagen. These same characteristics have made the RCS a useful and popular model for the study of chondrocyte metabolism.

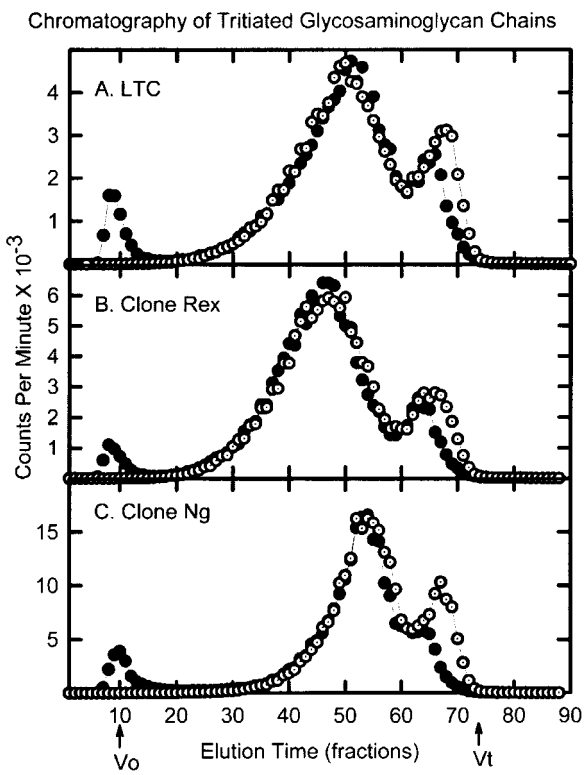


**Fig. 11.** Suparose 6 chromatography of glycosaminoglycan chains undigested (closed circles) or digested with Streptomyces hyaluronidase (open circles). **A**: Parent LTC cell extract. **B**: Clone Rex cell extract. **C**: Clone Ng cell extract. $V_o$, fraction 9. $V_t$, fraction 73. The buffer was 0.5 M sodium acetate, pH 7.0, flowing at 0.4 ml/min.

LTC is a sub-line begun from a suspension of the RCS cells and is an additional alternative to the time consuming dissection of articular joints for chondrocyte harvest. Furthermore, LTC is an improvement over the RCS as a cell model for chondrocytes. First, the LTC is much easier to handle. No animals are required to maintain the line. Standard tissue culture enzymes of trypsin and collagenase are employed. Cultures dispensed at subconfluent densities form a monolayer on tissue culture plastic in which cells appear rounded then slightly flattened and polygonal as cells adhere to the dish surface. Within 1 week the culture is overgrown with rounded and ovoid cells 2–3 layers thick. This is consistent with our studies showing that these cells begin to multiply in logarithmic phase within 3 days with a doubling rate of about 1 day (Fig. 3). These cultures are dispersed weekly with trypsin and collagenase and split 1:2 or 1:3. The second improvement is a more stable chondrocytic phenotype. The LTC can be cultured in either a 2-(monolayer) or 3-(agarose) dimensional culture system without loss of phenotype or fibroblast overgrowth. RCS cultures, however, have the drawback of contamination by fibroblasts which can only be avoided by culturing the RCS in a three-dimensional scaffold [Stevens et al., 1981]. Cells of the LTC line are viable and chondrocytic after liquid nitrogen storage. The LTC produces molecules

similar to those of the RCS and of hyaline cartilage. However, a large improvement over the RCS is that the LTC cells retain these molecules in the cell layer (Fig. 6). Having closer-to-nature matrix accumulation may be important due to the influence of the matrix upon cellular metabolism.

Additional characteristics presented in this study demonstrate that the LTC line is an excellent cell line. As is the case with RCS cells [Stevens et al., 1981], LTC cells remain viable and produce ECM molecules under serum-free conditions (Figs. 4, 8). Insulin added to serum-free media at 10 μg/ml also stimulates LTC cells to produce proteoglycan at rates greater than media alone and nearly as great as cultures supplemented with either type of serum. The ability to grow competent LTC chondrocytes permits experimentation under defined culture conditions.

A serendipitous feature is the ability to produce clonal lines of the LTC parent line (Fig. 5). We show that when the LTC line is dispersed to single cells and grown in regular medium plus serum without the use of co-culturing, conditioned media, or additional growth factors, over 60% of single cells grow into colonies of greater than four cells within 4 days with an average of 68 cells per colony at 1 week of culture without change in media. These data imply the possibility of establishing clonal lines as well as facilitating screening for mutants or cells with otherwise unique characteristics. Clearly this cell line creates more flexibility in biochemical and genetic manipulations.

We have created several clonal lines and found that the LTC is a heterogeneous mixture of chondrocytes producing distinctive extracellular matrices. We demonstrate this with the characterization of two clonal lines that differ markedly in their ability to produce a toluidine blue-stainable matrix (Fig. 9). The continued presence of these multiple cell types within a population of cells suggests that these clonal cell lines must be able to modulate their growth behavior in the presence of other clonal types or that their rate of cell division is virtually identical and prevents one cell type from dominating the culture and displacing the other cell types.

Furthermore, the LTC and its clonal lines are valuable model systems for the study of cellular glycosylation events. In addition to being very easy to culture under defined conditions, the cells produce glycosylated proteins at a high rate. For the parent LTC we show that synthesis of total proteoglycan increases over 25 h of label to $3 \times 10^5$ cpm/h/$10^6$ cells, and little of this is lost to the medium. At 30 min only 3% of incorporated isotope is found in the medium fraction rising at 24 h to 12%.

Analysis of clonal lines Rex and Ng demonstrates that these cells exhibit distinct morphological differences in terms of matrix synthesis and proteoglycan production (Figs. 9, 10). It is interesting to note that neither synthesize glycosaminoglycans which can be considered identical to that produced by the LTC (Table I and Fig. 11).

This variation of sulfation levels between the clonal lines can be exploited for glycosylation studies. The clonal line, Ng, is used for the study of cellular sulfation of macromolecules by taking advantage of its unusually high chondroitin (Δdi-0-S) production [Kimura et al., 1995; Morcuende et al., 1996; Zhang et al., 1999]. On the other hand, clonal line Rex appears closer to "normal" chondrocytes with its lower HA:CS ratio and higher Δdi-6-S production compared to Ng (Table I).

The two methods used here to estimate HA synthesis are in close agreement, and the results demonstrate that the parent line and the two clonal lines measured have much lower ratios of HA to total glycosaminoglycan synthesis: Rex $(\sim 4\%) <$ LTC $(\sim 7\%) <$ Ng $(\sim 8\%) \ll$ RCS (12–20%). This difference in HA production may explain why the RCS cells are unable to retain their ECM in culture. The imbalance may result in aggrecan being assembled with monomers spaced further apart on the unbranched HA chains leading to smaller aggregates that diffuse to the media. A similar explanation was postulated by Mason et al. [1982]. Widely spaced monomers would decrease the molecular connections possible between monomers and other ECM molecules [Olin et al., 2001]. Excess HA, by steric hindrance, might limit the molecular connections between other ECM molecules within the pericellular collagen matrix as well.

As our knowledge of the interdependency between ECM molecules in cartilage function and metabolism becomes larger and more complicated, the need for a suitable cell model becomes greater. The parent LTC cell and the clone Rex are more useful than the tumor RCS

in biochemical and molecular studies of cartilage because LTC and Rex have sulfation ratios, HA content, and ECM retention closer to cultured chondrocytes. Most of those applications for which the RCS tumor cell was employed can be simplified using the LTC line or its clonal lines; applications including, but not exclusive to: isolation of mRNA for genetic analysis, preparation of ECM proteins as antigens for antibody production, discovery of unknown molecules, particularly those that exist in small amounts in the native cartilage. It should be noted, however, that these cells are from a transplantable tumor and are therefore abnormal and should be used as a model system for chondrocytes as appropriate.

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