Conditioning of Native Substrates by Chondroitin Sulfate Proteoglycans during Cardiac Mesenchymal Cell Migration

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Abstract. It is generally proposed that embryonic mesenchymal cells use sulfated macromolecules during in situ migration. Attempts to resolve the molecular mechanisms for this hypothesis using planar substrates have been met with limited success. In the present study, we provide evidence that the functional significance of certain sulfated macromolecules during mesenchyme migration required the presence of the endogenous migratory template; i.e., native collagen fibrils. Using three-dimensional collagen gel lattices and whole embryo culture procedures to produce metabolically labeled sulfated macromolecules in embryonic chick cardiac tissue, we show that these molecules were primarily proteoglycan (PG) in nature and that their distribution was class specific; i.e., heparan sulfate PG, the minor labeled component (15%), remained pericellular while chondroitin sulfate (CS) PG, the predominately labeled PG (85%), was associated with collagen fibrils as "trails" of 50–60-nm particles when viewed by scanning electron microscopy. Progressive "conditioning" of collagen with CS-PG inhibited the capacity of the template to support subsequent cell migration. Lastly, metabolically labeled, PG-derived CS chains were compared with respect to degree of sulfation in either the C-6 or C-4 position by chromatographic separation of chondroitinase AC digestion products. Results from temporal and regional comparisons of in situ–labeled PGs indicated a positive correlation between the presence of mesenchyme and an enrichment of disaccharide-4S relative to that from regions lacking mesenchyme (i.e., principally myocardial tissue). The suggestion of a mesenchyme-specific CS-PG was substantiated by similarly examining the PGs synthesized solely by cardiac mesenchymal cells migrating within hydrated collagen lattice in culture. These data were incorporated into a model of "substratum conditioning" which provides a molecular mechanism by which secretion of mesenchyme-specific CS-PGs not only provides for directed and sustained cell movement, but ultimately inhibits migration of the cell population as a whole.

MESENCHYMAL cell migration constitutes one of the fundamental mechanisms of morphogenesis. The in situ migration of mesenchyme occurs within a three-dimensional extracellular matrix (ECM),[1] of which the collagen fibrils serve as a scaffold or substratum for many migrating cell populations. Cell translocation within this lattice has been envisioned as the cumulative result of repeating, synchronous cycles of positive and negative cell–substratum interactions (i.e., attachment and detachment, respectively) (8, 30, 41). Thus, molecular events that modify the interaction(s) between the cell surface and the collagenous substratum may constitute key regulatory mechanisms during mesenchymal cell migration.

The adhesive glycoprotein fibronectin (Fn) is thought to function in situ as an intermediate, bridging molecule linking cell to substratum (e.g., native collagen) (17, 46). Accordingly Fn-coated plastic has been used as an experimentally defined substrate in culture systems yielding much information concerning the interaction(s) between the cell surface and Fn (6, 10, 36). For example, in cultures of fibroblast cell lines there is an enrichment of certain macromolecules (sulfated proteoglycans [PGs], hyaluronate, glycoproteins) in artificially induced substrate-attached material (SAM) vs. that of the cell fraction (36). Furthermore, the proportional distribution of PG classes and hyaluronate within SAM varies with the length of time allowed for attachment (35). While short-term cultures yield material representing re-

1. *Abbreviations used in this paper*: AV, atrioventricular canal; CM, cardiac mesenchyme; CS, chondroitin sulfate; CHase, chondroitinase; CHase-R and CHase-S, CHase-resistant and -sensitive, respectively; ΔDi-6S, 2 acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-6-O-sulfo-D-galactose; ΔDi-4S, 2 acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulfo-D-galactose; ΔDi-0S, 2 acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose; ECM, extracellular matrix; Fn, fibronectin; GAG, glycosaminoglycan; HS, heparan sulfate; OT, outflow tract; PG, proteoglycan; SAM, substrate-attached material; S-GAG, sulfated GAG; V, ventricle.

cently formed attachment sites, long-term cultures contain a mixed population of attachment and former-attachment (i.e., "detachment") sites. The greatest proportion of heparan sulfate PG (HS-PG) is associated with recently formed SAM, while chondroitin sulfate PGs (CS-PGs) and hyaluronate are proportionally greater in long-term SAM (35).

These observations, in part, led Rollins and Culp to suggest that HS-PG is involved during the initial phase of cell attachment to an Fn-coated substratum, while CS-PG and hyaluronate function during detachment (35). Although evidence supports a role for HS-PG during cell attachment to Fn (20), a satisfactory mechanism through which CS-PG and/or hyaluronate induce cell detachment has not yet been suggested (1, 2, 18). This may reflect the fact that the array of ECM constitutive elements present during positive and negative cell-substratum interactions in situ are lacking from in culture model systems that use experimentally defined, planar substrates. It is feasible, even likely, that the functional significance of cellular macromolecules in migration is dependent upon the substratum provided; e.g., a collagen-binding molecule may be rendered functionally inert in a collagen-free system. Thus, despite the challenges of dealing with in situ migratory systems, ultimately, attempts to mechanistically probe migratory phenomena must ensure that the "natural setting" of the interface between cell and endogenous substratum are more optimally retained. In this study, we have explored such considerations in an attempt to determine the functional significance of why virtually all migrating mesenchymal cells (be they derived from ectoderm or mesoderm) synthesize, in situ and in culture, sulfated macromolecules during active translocation (for review see reference 40). It has been generally proposed that, in an undetermined manner, mesenchymal cells use sulfated macromolecules to arrest their own movement to promote subsequent cytodifferentiation (40).

To examine this question of sulfated glycosaminoglycan (S-GAG) synthesis by migrating mesenchyme and still retain an in situ component, we have used the early embryonic chick heart as a model system. The advantage of this system is that the in situ pattern of mesenchyme formation and migration can be closely approximated in three-dimensional cultures using collagen gel lattices. The pertinent specifics of the system are these: the early heart tube is initially entirely epithelial; a continuous endothelium, at specific timepoints and in specific regions, "seeds" mesenchyme into a basement membrane matrix produced by a peripheral epithelium, the myocardium (9, 15, 29, 31). The myocardial basement membrane (historically termed "cardiac jelly") which provides the in situ substratum contains fibrillar collagen, glycosaminoglycans (GAGs), Fn and other uncharacterized glycoproteins (14, 24, 25, 31). As shown in Fig. 1, the endothelium of the atrioventricular canal (AV) (but not that of the ventricle [V]) "seeds" mesenchymal cells which progressively "condition" the basement membrane matrix with S-GAG (28, 29) during migration. Thus, by comparing "premigratory matrix" with "postmigratory matrix" (i.e., stages 17 vs. 20) as well as mesenchyme-free (V) vs. mesenchyme-containing regions (i.e., AV and outflow tract [OT]), we have obtained biochemical and morphological evidence that migrating cardiac mesenchyme (CM) cells secrete S-GAGs as PGs which are either associated with cell surfaces (HS-PG) or the surrounding basement membrane matrix (CS-PG). Regional and temporal variation in the positional sulfation pattern of CS occurred in situ and correlated with the appearance of CM, suggesting that the mesenchyme secretes a CS-PG different from that made by other cardiac tissues (myocardium). Similar examination of CS from isolated CM migrating within collagen gel cultures further substantiated this suggestion while morphological evidence indicated that this CS-PG is directly positioned upon collagen fibrils as "trails" of 50–60-nm particles. From these data, it is proposed that a CS-PG/collagen interaction constitutes a biological mechanism whereby cell attachment is prevented via "substratum masking," a hypothesis supported by the restricted capacity of "conditioned" collagen to support continued cell translocation.

Materials and Methods

All reagents were analytical grade. Materials were purchased as follows: $Na_2[^{35}S]O_4$, [^{3}H]thymidine, and NEN-963 aqueous fluor from New England Nuclear (Boston, MA); collagenase from Calbiochem-Behring Corp.

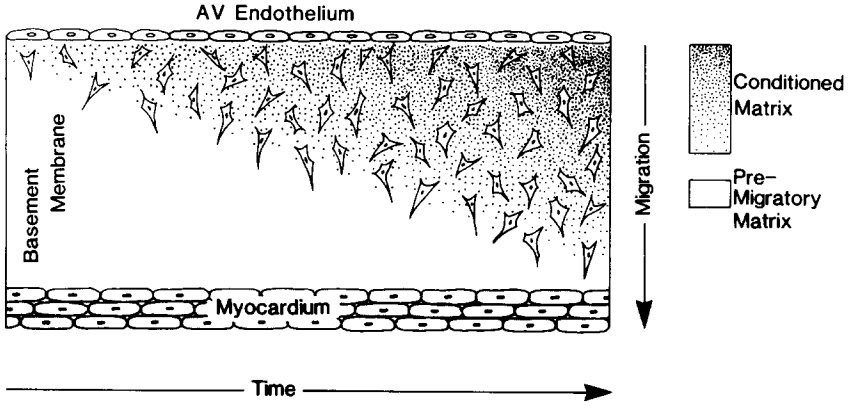


Figure 1. Model of directional migration. It is proposed that substrate "conditioning" provides a mechanism imparting directional guidance at the histological level to the migrating mesenchymal cell population. The number of accessible substratum sites for cell attachment in any given area of ECM is dependent upon the degree of "substratum masking," which in turn reflects the number of cells having traversed that area. Hence, at any given point in time during migration, subendothelial collagen fibrils (i.e., point of origin) will be "conditioned" or "masked" to a greater degree than fibrils nearest the myocardium (i.e., point of destination). The resultant gradient of conditioned ECM increases the probability of a cell establishing an attachment site within a less-conditioned area of matrix, i.e., distal to the endothelium, and thereby serves as a directional guide during cell translocation.

(La Jolla, CA); chondroitinase (CHase) AC, ABC, heparitinase, chondroitin sulfates A and C, and disaccharide standards (ΔDi-6S, ΔDi-4S, ΔDi-0S) from Miles Laboratories Inc. (Naperville, IL); medium 199, penicillin–streptomycin–fungizone, and chick serum from Gibco (Grand Island, NY); insulin–transferrin–selenium from KC Biological Inc. (Lenexa, KS); plastic tissue culture dishes from Falcon Labware (Oxnard, CA); PD-10 columns and Sepharose CL-6B from Pharmacia Fine Chemicals (Piscataway, NJ); and, cellulose thin layer chromatographic plates from Eastman Kodak Co. (Rochester, NY).

Culture Conditions

Whole Embryo Culture, Labeling, and Collection. Embryos were maintained in whole embryo culture in order to increase accuracy of staging and ensure rapid uptake of radioactive isotope. Culture chambers were made by adhering the cover of a 35-mm^2 tissue culture dish to the inside bottom of a 60-mm^2 petri dish. The center well (i.e., 35-mm^2 lid) was filled with 6.0 ml of medium 199 containing Earle's salts, 1% chick serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2.5 μg/ml fungizone. The chamber (covered with the petri dish lid) was incubated in a humidified atmosphere of 95% air/5% CO_2 at 37°C, thus allowing the medium to equilibrate before embryonic transfer.

Fertilized White Leghorn chicken eggs were incubated in a forced air incubator with a humidified atmosphere at 38°C. An embryo of the appropriate stage (i.e., either stage 17$^+$, 18$^-$ or stage 20$^-$; Hamburger and Hamilton [11]) was transferred with blastodisc intact using a sterile filter paper ring (inside diameter 32 mm; outside diameter 50 mm; Whatman #1; Whatman Inc., Clifton, NJ) to an equilibrated embryo culture chamber, returned to the incubator for 30 min to remove adherent yolk granules before transferring to a fresh culture chamber.

Radioactive inorganic sulfate (50 μCi in 25 μl sterile H_2O) was administered to the dorsal (upper) surface of each blastodisc and incubation continued for 5 h. Development of the embryos proceeded normally during the stages examined as determined by the acquisition of stage-specific structures and characteristics as described for the chick embryo by Hamburger and Hamilton (11). Whole hearts were removed in warm (37°C) Tyrode's balanced salt solution and prepared for autoradiography or further dissected into AV, OT, and V regions. These regions were then analyzed by either gel filtration or thin layer chromatography.

Collagen Gel Cultures. Collagen gels were formed by the polymerization of rat tail collagen (types I and III; final concentration 1.86 mg/ml) in 35-mm^2 tissue culture dishes according to the procedure described previously (3). The gels were washed repeatedly with medium 199 supplemented with 1% chick serum and insulin (5 μg/ml)–transferrin (5 μg/ml)–selenium (5 ng/ml) followed by equilibration under incubation conditions as described above. The myocardium was dissected from stage 21 CM-forming regions (i.e., AV, OT). The endothelium, mesenchyme, and associated ECM was freed of myocardium (confirmed by histological and direct observations of living cultures) explanted to collagen gels and allowed to attach for 24 h at which time non-adherent material was removed. Incubation continued for an additional 48 h resulting in an enriched population of mesenchymal cells within the collagenous lattice. The gels were rinsed with fresh media before the addition of 100 μCi $Na_2[^{35}S]O_4$. Incubation was continued for an additional 48 h.

Collagen gels were either detergent extracted or digested with highly purified collagenase (final concentration, corrected for medium trapped within the gel, 25 U/1.5 ml; 37°C; 2 h). Detergent extracts were examined by gel filtration chromatography while the collagenase-digested gels were subjected to CHase digestion and the GAG products analyzed by thin layer chromatography (both methodologies subsequently detailed). Additionally, intact collagen gel cultures were prepared for either autoradiography at the light microscopic level or scanning electron microscopy as described below.

Biochemical Methodologies

Gel Filtration Chromatography. Samples of each region (n = 15–18) or collagen gel cultures were extracted for 24 h at room temperature in 0.2% SDS in sodium acetate buffer (0.15 M; pH 5.8) containing protease inhibitors (final concentrations: phenylmethylsulfonyl fluoride [PMSF], 0.1 mM; pepstatin A, 0.01 mM; N-ethylmaleimide, 10 mM; and, EDTA, 10 mM). Unincorporated label was removed from in situ samples using a Sephadex G-25 (Pharmacia PD-10) column and by dialysis for the collagen gel cultures. The high molecular weight material was divided into aliquots which either (*a*) remained as an untreated control, (*b*) was reduced under alkaline borohydride conditions (0.05 M NaOH, 1 M $NaBH_4$; 20 h; 45°C) (4), or (*c*) were subjected to hydrolysis with nitrous acid (80 min; room temperature) (22). Treated and untreated aliquots were subsequently chromatographed on Sepharose CL-6B columns (115 cm × 1.4 cm) equilibrated in sodium acetate buffer (0.15 M) containing 1 mM each of magnesium and calcium chloride, and 0.2% SDS (final pH 5.8). 2-ml fractions were collected, 15 ml of aqueous fluor added (NEN-963), and radioactivity was determined by liquid scintillation counting (Beckman LS7000; Beckmann Instruments, Inc., Fullerton, CA).

GAG Isolation. Proteoglycans were precipitated with TCA (10% wt/vol; overnight; 5°C) after brief sonication (10–15 s; 5°C) of in situ cardiac regions or collagenase-digested collagen gel cultures. The release of GAG chains from their respective core proteins using alkaline borohydride reduction (as described above) was confirmed by their subsequent recovery in the acid-soluble fraction (using the acid precipitation conditions described above). Carrier GAG (100 μg each of chondroitin sulfates A and C) was added to each analysis before precipitation with alcoholic potassium acetate (3 vol of 5% potassium acetate [wt/vol] in absolute ethanol; overnight; 5°C). The pellet (dried under a stream of N_2 gas) was resuspended in 0.02 M NaCl (1.0 ml), and the GAGs selectively precipitated by bringing the sample to a final concentration of 1% (wt/vol) with cetylpyridinium chloride which was later removed by dissociating the insoluble salt complex with 2 M NaCl/ethanol (100:15). The resultant pellet, subsequently washed with absolute ethanol and dried as above, represented purified GAG.

Enzyme Digestions and Thin Layer Chromatography. Each isolated GAG sample was exhaustively digested (i.e., two 12-h digestions) with CHase AC (0.2 U in 100 μl Tris-enriched buffer, pH 7.3 at 37°C) (38) after removing an aliquot which without the addition of enzyme was identically treated and served as a buffer control. Enzyme-sensitive and -resistant material was isolated following the addition of carrier GAG (100 μg each chondroitin sulfates A and C) by precipitation with absolute ethanol (3 vol) into ethanol-soluble and -insoluble fractions, respectively. The latter was dried and digested as above except with CHase ABC (pH 8.0) (38). Enzyme-sensitive and -resistant fractions were isolated as above.

The digestion products (i.e., ethanol-soluble fractions) were examined by thin layer chromatographic procedures modified from Wasserman et al. (45) and Vogel and Kendall (44). Subsequent to ethanol evaporation, samples were resuspended in distilled H_2O, spotted on thin layer chromatography plates (cellulose, 0.25 mm thickness), desalted in n-butanol/ethanol/H_2O (52:32:16), and developed in n-butanol/acetic acid/1 M NaOH (2:3:1). Buffer controls remained at the origin and disaccharides were identified according to co-migration with known standards. Each sample lane was cut into 40 0.5-cm segments, each segment was eluted in 0.5 M HCl at 60°C for 12 h, dissolved in scintillation fluor (Ready-Solv; Beckman Instruments, Inc.), and radioactivity was determined using a Beckman LS6800 scintillation counter. Samples were quench corrected and proportional distributions between 6- and 4-sulfation positions were determined as the ratio of unsaturated disaccharide-6S to that of -4S (ΔDi-6S/ΔDi-4S). The standard deviation of four experimental runs/region/stage was determined, and the statistical significance between CM-forming and mural regions of this ratio was examined during early and late stages of CM migration using the Student's t test.

Morphological Procedures

Autoradiography. Hearts labeled by whole embryo culture were excised, rinsed in Tyrode's balanced salt solution, and fixed for 2 h in 3% glutaraldehyde in 0.065 M cacodylate buffer containing 0.25% cetylpyridinium chloride. Tissues were subsequently rinsed, dehydrated in graded alcohols, embedded in paraplast, and sectioned at 4 μm. Before dipping in Kodak NTB-2 emulsion, representative sections of each stage were digested with CHase ABC (2.5 U/ml, Tris-HCl, pH 8.0, 4 h) or sequentially with CHase ABC followed by heparitinase (5 U/100 μl 0.1 M sodium acetate, pH 7.0 containing 0.01 M calcium acetate for 16 h at 37°C) (23). The above enzymes were applied directly to a deparaffinized tissue section in a 10-μl drop of acetate or Tris buffer alone. Sections were stored at 4°C for 10 d and developed in Dektol D-19 using previously published procedures (12).

For labeling in collagen gel cultures, stage 17 AV explants (endothelium and myocardium only) were established on collagen gels using procedures described above and as further detailed in Bernanke and Markwald (3). At this stage, endothelial cells grow across the surface of the gel and begin to "seed" mesenchymal cells into the gel lattice after 12 h in culture. The myocardium was removed after 6 h and radiolabeled $Na_2[^{35}S]O_4$ (50 μCi/ml medium, 804 mCi/mMol; New England Nuclear) subsequently added to the medium at the time mesenchymal seeding began. Incubation was continued for variable periods of time until cells had reached the bottom of the gel lattice (usually 72 h for a 2-mm gel). Accordingly, collagen gel lattices were selected for processing in which cell migration had proceeded to differ-

ent depths. For each period, cultures were rinsed five times in medium without serum, fixed for 1 h in the same fixative used above for whole hearts, rinsed overnight in several changes of buffer and embedded in Epon/Spurr mixture using established procedures (26). Selected cultures were treated with CHase ABC (2.5 U/ml; Miles Laboratories, Inc.) for 4 h before embedding in plastic. Blank gels (i.e., cell-free) which were incubated for corresponding time periods in complete, labeled medium were rinsed and either digested with CHase ABC (identical conditions as above) or treated with the digestion buffer minus enzyme (i.e., buffer control), and subsequently processed as described above. Semi-thin, 1-μm sections were dipped in emulsion and developed as described for in situ heart tissues.

In a separate group of experiments, a second stage 17 explant was positioned upon the surface of a collagen gel previously "conditioned" by a first explant for 4 d. To facilitate the removal of surface endothelial cells (the source of mesenchyme), the first explant attached and formed an endothelial monolayer on a nylon raft (180-μm^2 meshwork). With explant intact and dorsally positioned, the raft was placed on a collagen gel (see Fig. 6 *a*). Mesenchymal cells readily seeded the gel through the mesh (Fig. 6 *b*) and after 4 d the surface-associated endothelium was removed. Cultures were rinsed (five times) in medium 199 (no serum) and subsequently incubated for 12 h in medium 199, pH 7.2, with or without either CHase ABC (2.5 U/ml) or heparitinase (50 U/ml). Cultures were thoroughly rinsed (~3 ml × 3) with complete media before receiving the second explant. The latter, obtained from an embryo labeled 7 h with [^{3}H]thymidine (100 μCi) in whole embryo culture (sufficient time for most endothelial nuclei to incorporate label, Fig. 6 *c*), was allowed to "seed" a conditioned gel for 24 h (sufficient time for some cells to normally reach the base of the gel). Cultures were fixed, sectioned, and prepared for autoradiography as described above.

Scanning Electron Microscopy. Companion collagen gel cultures established, collected and fixed exactly as described for autoradiographic processing were prepared for scanning electron microscopy. Gels (either with or without cells) were dehydrated, critically point dried in CO_2 using a Polaron device, layered with a 2-nm film of gold/palladium in a cold stage Polaron 5000 sputter-coater fitted with film thickness monitor, and examined in an Hitachi HS-500 scanning electron microscope at 25 kV. Some gels were mechanically disrupted with tungsten needles (3) to aid in viewing the interior of the collagen gels. Controls included blank gels incubated in complete medium (including serum) or gels treated with chondroitinase ABC as above before dehydration.

Results

Detergent extracts of cardiac mesenchyme-forming (i.e., AV, OT) and mural (i.e., V) regions from both early and late stages of migration gave virtually identical profiles when chromatographed on Sepharose CL-6B (Fig. 2, top). In all cases the majority of radiolabeled material (86–88%) eluted at the void volume, and upon reduction with alkaline borohydride the elution position shifted to a K_{av} of 0.20, indicating its PG nature (19). Similar results were observed with sulfated macromolecules isolated from collagen gel cultures of CM-derived cells (Fig. 2, bottom). The percentage of excluded radioactivity was reduced compared with that from in situ–labeled regions, yet was also entirely susceptible to alkaline borohydride treatment. The slightly included peak, thought to represent non-protein-associated GAG chains, probably reflects metabolic breakdown of PGs. The difference in labeling times between collagen gel and whole embryo cultures (i.e., 48 h and 5 h, respectively) may have accounted for the relative increase of this peak in material isolated from the collagen gel cultures.

Nitrous acid hydrolysis, which specifically cleaves glycosidic linkages unique to heparin/HS sequences (22), was used to determine the proportional distribution between S-GAG classes (Fig. 2) (i.e., CS and HS as nitrous acid–resistant and -sensitive material, respectively). Differences in the ratio of CS to HS were not observed regionally or temporally, with HS representing the minor S-GAG compo-

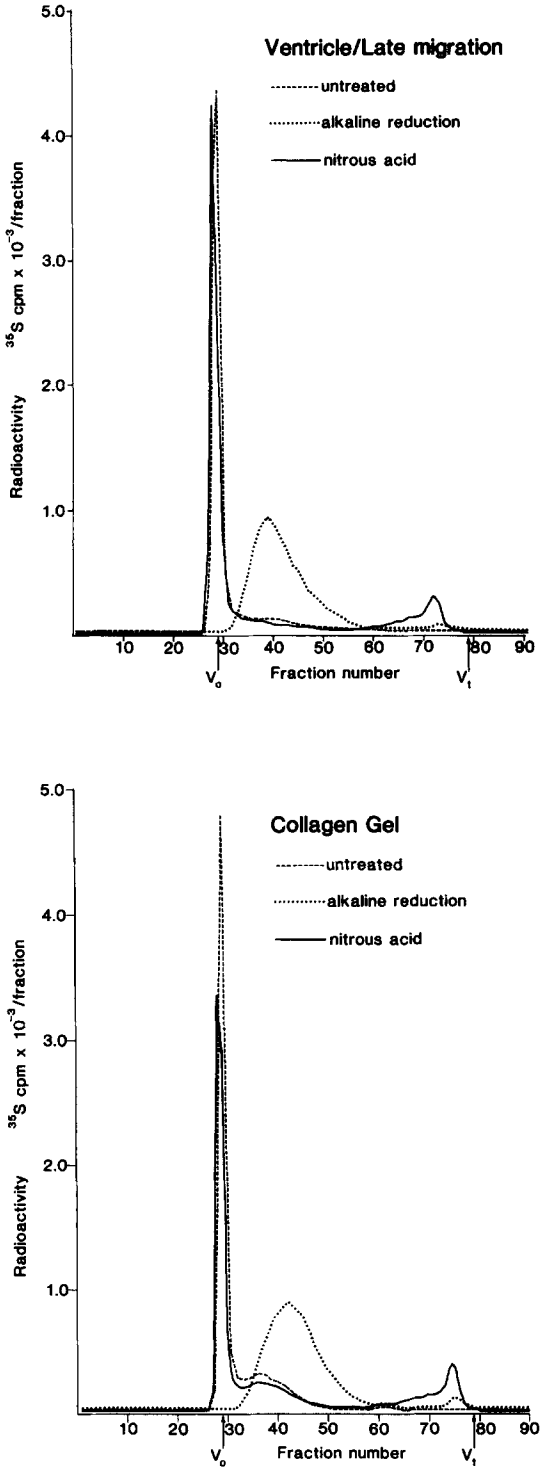


Figure 2. Molecules metabolically labeled with $Na_2[^{35}S]O_4$ were detergent extracted from (*top*) cardiac regions or (*bottom*) CM migrating within a collagenous lattice. Aliquots from each were chromatographed on a column of Sepharose CL-6B (115 cm × 1.4 cm) equilibrated with an acetate buffer containing 0.2% SDS (pH 5.8) either as an untreated control or subsequent to alkaline borohydride reduction or nitrous acid hydrolysis. In both cases (*a* and *b*), the majority of untreated labeled material eluted at the void volume and was PG as indicated by the shift in elution position upon reduction. Nitrous acid hydrolysis indicated that <20% (15 and 17%, respectively) of label was incorporated into heparin/HS sequences. Similar results were obtained with AV and OT regions (data not shown).

nent (11–12% and 15% for CM-forming and mural regions, respectively). Thus, radioactive inorganic sulfate was predominately incorporated into CS in all regions examined during early and late stages of CM migration (Fig. 2, top). Mesenchyme migrating within the collagen gel culture system (Fig. 2, bottom) reflected a similar distribution between S-GAG classes with 17% of the radiolabeled material being nitrous acid sensitive (i.e., HS).

To ensure that the nitrous acid–resistant radiolabeled material was in fact CS, isolated PG-derived GAG chains were digested sequentially and exhaustively with CHases AC and ABC. The combined digestions released 80–85% of the radiolabeled material (data not shown) in agreement with the above nitrous acid sensitivity data. Additionally, <5% of the total radioactivity in the GAG fraction was susceptible to CHase ABC subsequent to exhaustive digestion with CHase AC, indicating the presence of little or no dermatan sulfate (data not shown).

The ΔDi-6S/ΔDi-4S ratio was determined for each region and stage on the basis of thin layer chromatographic separations of CHase AC digestion products. The results (Fig. 3) indicate that CS from the non–CM-forming, ventricular region (an enriched myocardial source) was sulfated to a greater extent in the C-6 position and this pattern of sulfation was constant between the early and late stages of CM migration. In contrast, CS from the CM-forming regions (i.e., AV, OT) exhibited a reduction in the ΔDi-6S/ΔDi-4S ratio from

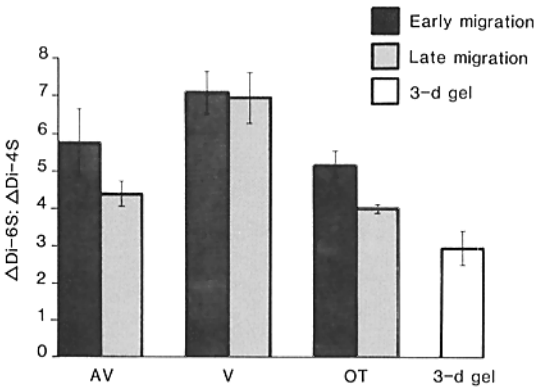


Figure 3. $Na_2[^{35}S]O_4$ metabolically labeled, PG-derived GAG chains isolated from CM-forming (i.e., AV, OT), non–CM-forming (i.e., V), and collagen gel cultures of CM were exhaustively digested with CHase AC. The digestion products were separated by thin layer chromatography and identified by co-migration with known standards. The ΔDi-6S/ΔDi-4S ratio from in situ–labeled samples indicated regional and temporal variability in the positional sulfation pattern of CS-GAG chains. The ratio from the V region was temporally constant and higher than its corresponding value from CM-forming regions, and the difference was statistically significant during the stage of late migration (LM) (using the Student's *t* test; AV/LM vs. V/LM, $P \geq 0.001$; OT/LM vs. V/LM, $P \geq 0.01$). In contrast, the ratio from the AV and OT regions exhibited an intraregional statistically significant stage-dependent reduction which coincided with the appearance and migration of CM (AV/EM [early migration] vs. AV/LM, $P \geq 0.05$; OT/EM vs. OT/LM, $P \geq 0.01$). The suggestion of a mesenchymal cell-specific CS was further substantiated by examining the ΔDi-6S/ΔDi-4S ratio from collagen gel cultures of CM-derived mesenchymal cells (i.e., myocardial free). Each value represents the mean of four experimental runs/region/stage and its corresponding standard deviation.

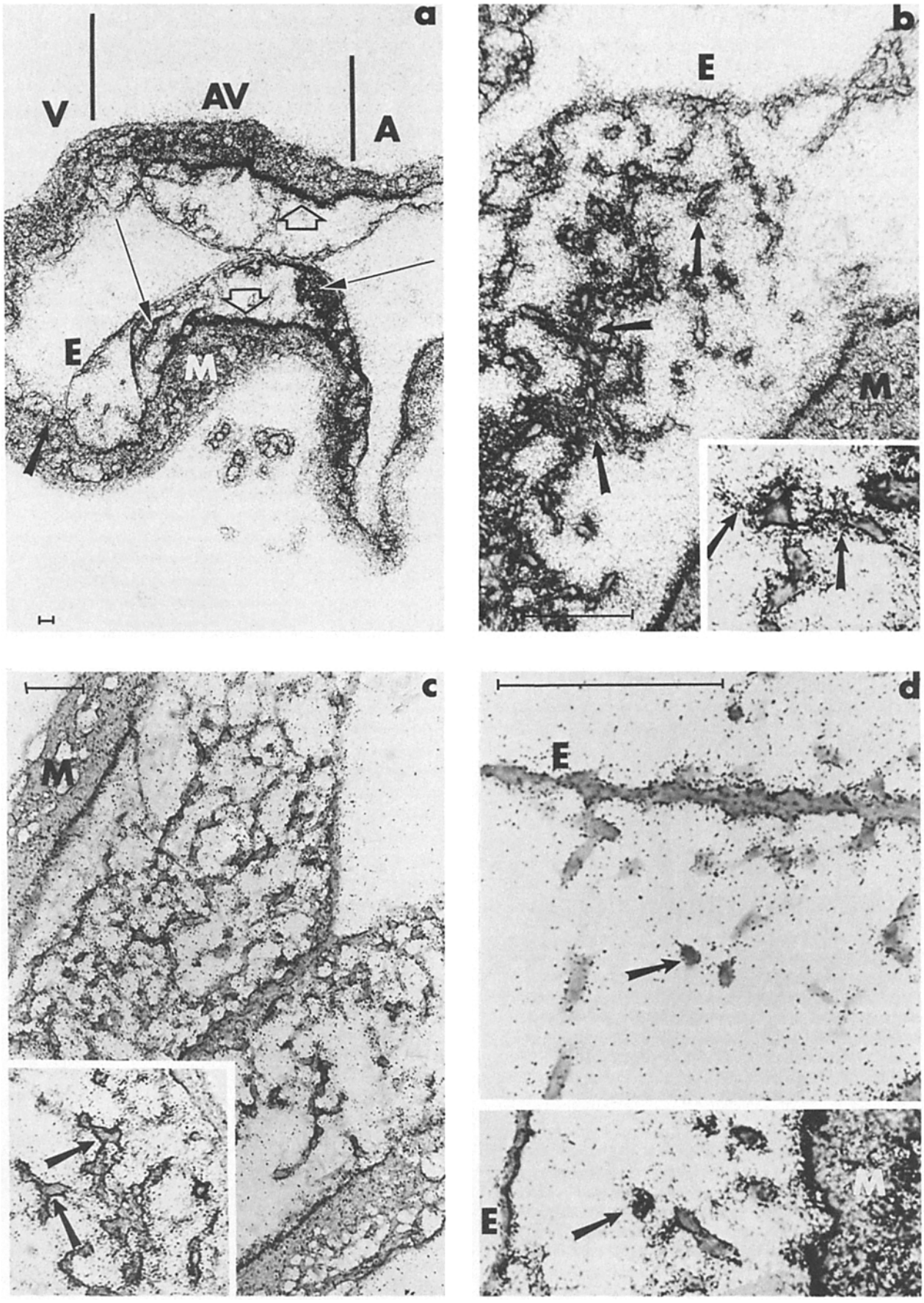
that observed for the V, as well as an intraregional time-dependent reduction coinciding with the appearance and migration of CM.

These data indicated a structural heterogeneity of PG-derived CS chains which was regionally and temporally specific, not only suggesting the possibility of more than one CS-PG, but that such variety represents cell specificity; namely, a CS enriched in ΔDi-6S being a myocardial derivative while a CS with a relatively greater proportion of ΔDi-4S being of endothelial and/or mesenchymal origin. Analysis of in situ cell products precluded resolution of this suggestion due to the presence of mixed cell populations. However, a three-dimensional culture system void of myocardial cells was used to examine the ΔDi-6S/ΔDi-4S ratio from CM-derived mesenchymal cells actively migrating within a hydrated collagen lattice. Further reduction of this ratio relative to that from in situ cardiac regions (Fig. 3) substantiated the suggestion of cell-specific CS-PGs.

The synthetic source(s) and histological localization of PGs metabolically labeled with $Na_2[^{35}S]O_4$ was examined at the light microscopic level in autoradiographically prepared longitudinal tissue sections. Digestion of serial sections with specific glycosidases permitted identification of certain grain distributions as either CHase-sensitive (CHase-S) or -resistant (CHase-R). In general, silver grains were distributed throughout the length of the heart tube in association with all cell types during both stages examined (Fig. 4), indicating more than one cell population as a synthetic source of S-PGs. Both CHase-S– and CHase-R–labeled material were associated with the myocardial basal laminae and CM cells. In the latter case the localization pattern was specific relative to the CM cell. CHase-R material intensely localized around the circumference of the cell and was diminished or removed by digestion with heparitinase, indicating an HS-PG cell surface association. In contrast, CHase-S–labeled material extended peripherally from the cell into the ECM creating a "halo" of grains, a pattern consistent with that of a secretory cell product and with prior in situ studies that CM cells modify the ECM during migration via CS-PG secretion (28, 29).

Confirmation of the latter was sought by examining areas of the collagen gel for "conditioning" with $^{35}SO_4$-labeled matrix deposited by migrating cells that were continuously supplied with the precursor during active migration (Fig. 5). No indication of any organized or focalized, extracellular patterns of silver grains was observed in autoradiographs of cultures in which migratory activity had not occurred (i.e., a low-level, homogenous grain distribution representing a background level of trapped, unincorporated label was observed in cell-free gels and in gels established with non–mesenchyme-forming explants). In cultures of actively migrating cells that had been labeled for 4 h with $Na_2[^{35}S]O_4$, most cells, in particular their elongated cell processes, were heavily labeled pericellularly. Extended periods of continuous labeling (24–72 h) resulted in the deposition of CHase-S, clustered grain foci in cell-free areas of the gel that had clearly been traversed by migrating cells. Often the pattern of extracellular grain clusters took the form of elongated, sinuous profiles resembling "tracks" that were not in association with any resolvable cell processes and were most conspicuous in areas where cell migration had been extensive.

To determine what effect, if any, the "tracks or trails" representing conditioned collagen had upon the migratory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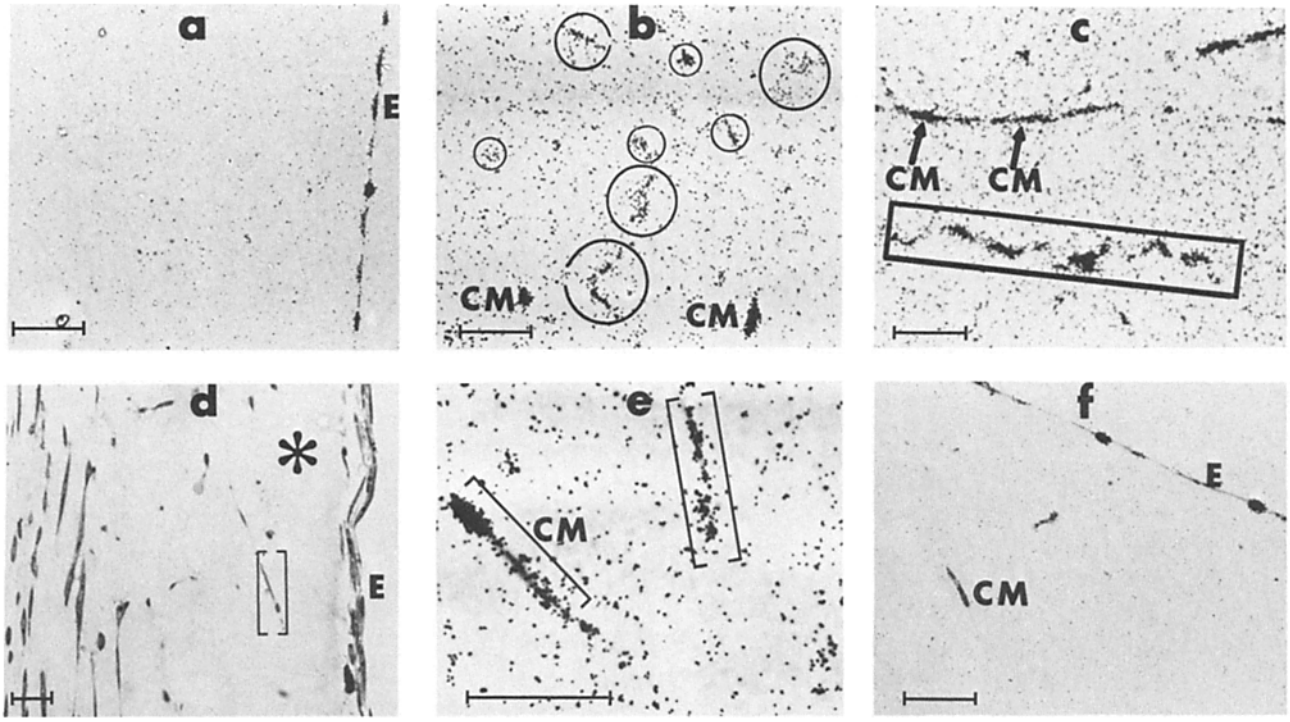
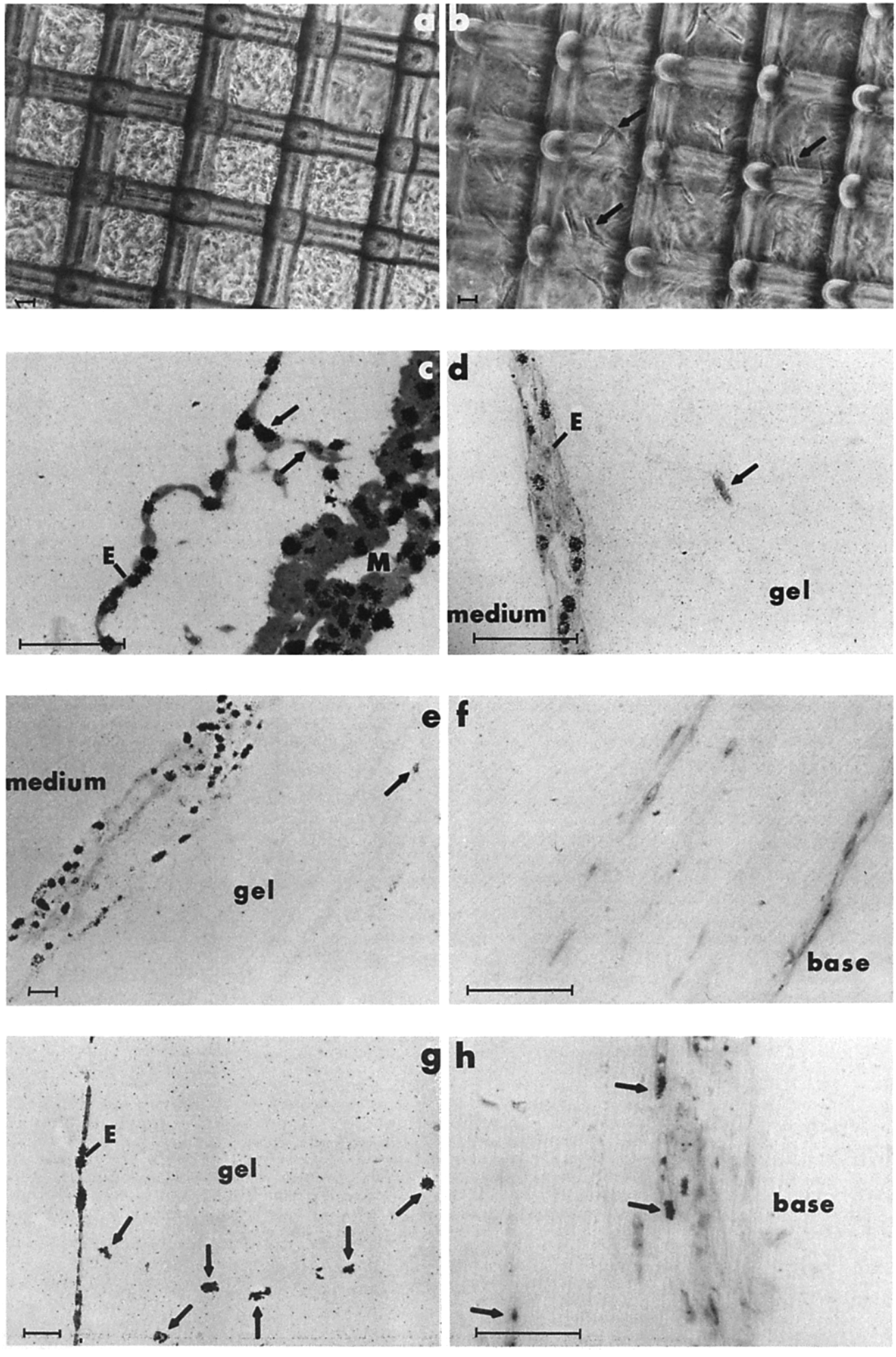


Figure 5. Autoradiographs of collagen gel cultures continuously labeled with $Na_2[^{35}S]O_4$. (*a*) 72-h V explant in which the endothelium (*E*) does not seed mesenchyme into the gel lattice; only a uniform pattern of background labeling occurs within the gel in the absence of cell migration. (*b*) 48-h, AV explant in which a "wave" of seeded cardiac mesenchyme cells (*CM*) have actively migrated through the gel in a direction starting from the top of the micrograph to their present position; the randomized, homogeneous pattern of grains is replaced by the formation of focalized clusters of linear profiles (*circles*). All areas enclosed by circles were examined by Normaski optics and determined to be independent of any resolvable cellular association. (*c*) 72-h, AV explant; CM cells are subendothelial in a region similar to that denoted by an asterisk in an adjacent toluidine blue–stained, serial section shown in *d*; the rectangle encloses an undulating series of clustered grains resembling a "trail" located in a cell-free region of the gel through which an earlier population of CM cells had passed. (*d*) Orientation micrograph of a 72-h AV explant in collagen gel culture; note the first population of seeded CM cells has reached the base of the gel and "stratified" leaving a broad, rather cell-free region into which a second population of cells formed by the endothelial (*E*) monolayer will pass. The area enclosed by brackets is enlarged in *e*. (*e*) Open bracket denotes the typical labeling pattern of CM cells in which elongated cell processes are characterized by association with extensive pericellular grains; as suggested in the model diagram (see Fig. 8), the detachment of such appendages would leave behind cell-free linear deposits similar to the one shown within the closed brackets. (*f*) A 48-h AV culture similar to *b*, except treated with CHase AC; note the absence of clustered grain foci. Bars, 0.5 μm.

activity of a second explant (having the potential to form a new migratory cell population), cultures were established as described in Materials and Methods whereby the surface monolayer of the original explant could be easily and completely removed (confirmed by histological analysis) (Figs. 6, *a* and *b*). The original explant was cultured for 4 d producing cultures in which most of the mesenchyme had migrated to the lower half of the gel and organized into several, tendon-like strata (see Fig. 5 *d*). Typically, few cells were present under the surface of the monolayer after the first 48 h after explantation, indicating that "seeding" activity had ceased. However, if transferred to a fresh collagen gel culture (using the raft as a carrier) seeding of new mesenchyme occurred (data not shown).

Positioning a second explant (labeled with [^{3}H]thymidine) directly upon the site of the original explant (monolayer removed) resulted in modified "seeding" behavior in 11 of 13 such cultures. Surface endothelium grew out of the explant onto the gel surface but, thereafter, most often failed to seed mesenchyme in the normal fashion. After 24 h, either the monolayer had "stratified" upon itself (never seen heretofore in control cultures) (Fig. 6 *d*) or a group of cells formed an aggregate of closely associated cells within the upper region of the gel. All cultures were serially sectioned through the extent of the labeled endothelial outgrowth to confirm altered seeding. In all situations where seeding was modified, no labeled nuclei were found near the base of the gel (Fig. 6, *e* and *f*). However, pretreatment of the "conditioned" gel with

Figure 4. Autoradiographs of longitudinally sectioned hearts from chick embryos labeled with $Na_2[^{35}S]O_4$ for 5 h in whole embryo culture; no counterstain used. (*a*) Stage 19 heart denoting atrial (*A*), atrioventricular (*AV*), and ventricular (*V*) regions; hollow arrow denotes the heavily labeled myocardial basal lamina of the AV region, whereas the solid arrow denotes a group of newly seeded and heavily labeled CM cells. *E*, endothelium; *M*, myocardium. (*b*) Higher magnifications of migrating CM cells from a late stage 19 AV region; note that each mesenchymal cell is outlined by a sharp border of silver grains from which a "halo" of additional grains extend peripherially. (*c*) CHase ABC digestion; note the pericellular borders of all cells remain sharply delineated by CHase-R label, but that the "halos" are CHase-S. (*d*) Sequential digestion with CHase and heparitinase; (*inset*) CHase only. Heparitinase removes or diminishes the pericellular CHase-R grains, particularly noticeable on cells cut tangential to their surface (*arrows*). Bars, 1.0 μm.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chondroitinase ABC for 12 h enabled the endothelium to seed mesenchyme in all cultures. Yet, in only 9 of 17 were labeled nuclei observed at the base of the gels (Fig. 6, *g* and *h*).

Direct visual confirmation by scanning electron microscopy of matrix modification via CS-PG was obtained directly through use of the collagen gel culture system. As previously described (3), CM cells move from a surface position toward the base of the gel where movement ceases followed by the formation of tendon-like strata (e.g., see Fig. 5 *d*). Movement of CM cells through native collagen was accompanied by the deposition of granular material upon collagen fibrils (Fig. 7). The granular material measured 50–100 nm in diameter (after sputter-coating) and, in established cultures (i.e., those in which several "waves" of cells had passed) virtually all collagen fibers (and cell surfaces) were "conditioned" with granular components (Fig. 7 *c*). Treatment with CHase ABC released many of the granules from association with fibrillar collagen and resulted in the formation of amorphous foci of extracellular material ostensibly "bound" to coalesced collagen microfibrils (Fig. 7 *d*). This suggested that one component in the "trails" of granular material was CS-containing material.

Discussion

Cell migration is translocation from point of origin to destination (i.e., versus positional relocation due to differential growth). This phenomenon is believed to result from a coordinated series of positive and negative cell–substratum interactions (8, 30, 41), each complex and incompletely understood. For mesenchymal cells, translocation is accompanied by synthesis of S-GAGs. Using whole embryo culture to produce in situ labeling of S-GAGs, we found the latter to be incorporated into two PG species: HS-PG and CS-PG.

Concerning HS-PG, it together with Fn has been implicated in culture as one bridging molecule linking cell membrane to substratum. Specifically, HS-PG is thought to function during the formation of "close-contacts" (6, 21), the purported initial stage of cell–Fn attachment (via the respective and oppositionally located heparin/HS and collagen-binding domains of Fn). However, several independent laboratories have recently shown that at least one type of cell surface receptor influences cell adhesion via interaction with the more interiorly located cell-binding domain(s) of Fn (6, 7, 10, 33).

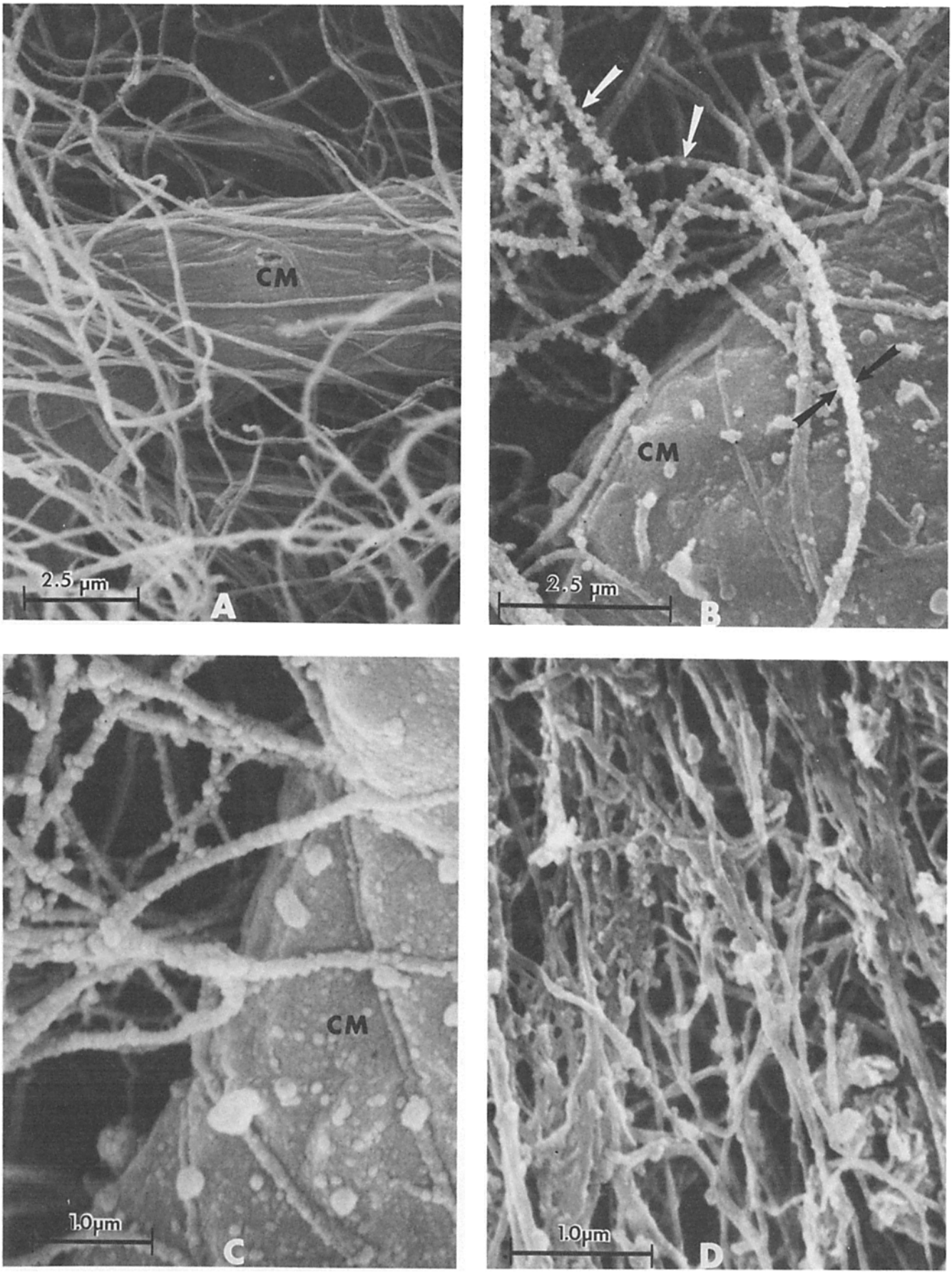
Consistent with these observations and the proposed role of HS-PG in cell–Fn attachment is the current study which demonstrated that, during active migration (i.e., cell translocation), CM cells synthesized cell surface–associated HS-PGs both in situ as well as in collagen gel culture. It has previously been shown that collagen (14) and Fn (13) are constituents of the myocardial basement membrane (historically termed cardiac jelly). Hence, the proposed requisite molecules for the initial phase of cell attachment have been identified in an in situ system characterized by the continual, repeating cell–substratum interactions of its mesenchymal cell population. Thus, we have shown in our model of in situ cell migration (Fig. 8) HS-PG and Fn as one mechanism for promoting attachment to collagen.

In addition to HS-PG, it was observed that under the same conditions CM synthesized CS-PGs whose sulfation pattern differed from that of the non-CM-forming ventricular region, suggesting that the observed heterogeneity was cell specific (i.e., myocardial-derived CS-PG vs. CM-derived CS-PG), a concept supported by the work of Carrino and Caplan (5). They have demonstrated structural similarity between the CS-PG isolated from skeletal muscle cells in culture and one of several CS-PGs isolated from in situ skeletal muscle tissue. The skeletal muscle–specific CS-PG is predominantly sulfated (~90%) in the C-6 position and, therefore in that regard, similar to a CS-PG isolated from non–mesenchyme-forming cardiac regions. Heterogeneity of CS-PG profiles isolated from tissue samples is believed to be due to the presence of more than one cell type. GAG chains of a putative nonmuscle CS-PG are sulfated in the C-6 position to a lesser degree (~60%) than that of its muscle CS-PG counterpart (5). In accordance with these observations, the present data indicated that myocardial CS-PG was primarily sulfated in the C-6 position relative to that derived from CM.

The structural heterogeneity of CS-PGs may be related not only to synthetic source, but to functional diversity as well. Observations from both in situ and in culture systems suggest involvement of either CS-PG and/or hyaluronate during the process of negative cell–substratum interactions (1, 2, 36, 40), yet models elucidating mechanisms at the molecular level have not been forthcoming. This may be related to the fact that current concepts of cell–substratum interactions are largely based upon data gathered from "collagen" -free culture systems. While seen in culture to accumulate in cellular "footprint" material (i.e., remnants of cells representing former-attachment sites) on Fn-coated plastic (36), CS-PGs may be functionally different or inert in the absence of collagen fibrils.

It is accepted that collagen fibrils are a scaffold upon and through which many mesenchymal cell populations migrate in situ. We propose that the negative cell–substratum interactions, upon which cell translocation depends, be considered two (though not necessarily unrelated) events: (*a*) detach-

Figure 6. Explant "switching" experiment. (*a*) A monolayer is shown that has formed after 24 h in culture across the surface of a nylon raft which is resting on the top of a collagen gel, the myocardium having been removed. (*b*) Same as *a*, except the objective was focused beneath the raft to show that mesenchymal cells (*arrows*) had been seeded through the raft. (*c*) AV region of a stage 17 heart that had been labeled in whole embryo culture with [^{3}H]thymidine for 7 h. Note that most endothelial (*E*) and a few newly formed mesenchymal cells (*arrows*) are labeled. *M*, myocardium. (*d*) Same as *c*, except placed for 24 h upon a collagen gel previously conditioned 4 d with a previous, unlabeled explant of which the endothelium associated with a raft had been removed. Note the "stratified" layer of labeled endothelium (*E*). The arrow denotes an unlabeled cell that probably formed from the first explant. (*e*) Same as *d*, except the labeled endothelium appears to have been able to seed mesenchyme that have remained close to the surface, except for the cell indicated by the arrow. No counterstain was used. (*f*) The base of the gel shown in *e*, except stained with toluidine blue, demonstrates that nuclei were all unlabeled. (*g*) A collagen gel, initially conditioned with a previous explant, was treated with CHase ABC before positioning of the second, labeled explant. Note that after enzyme treatment, the endothelium (*E*) forms a monolayer typical of routine cultures and that several mesenchymal cells (*arrows*) have invaded the gel. (*h*) The base of the gel shown in *g*, except stained with toluidine blue, indicates that cells with labeled nuclei (*arrows*) are distributed among the unlabeled cells from the first explant. Bars, 0.5 μm.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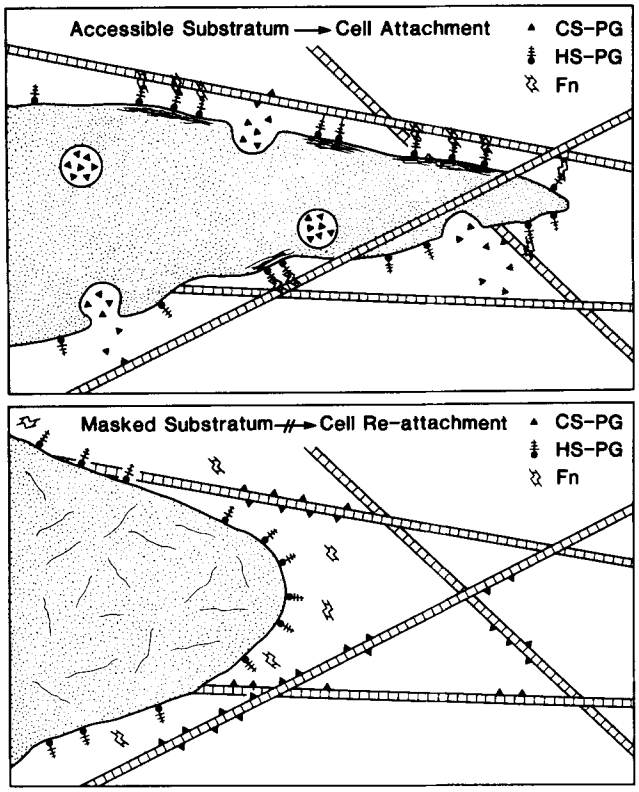

Figure 8. CS-PG substrate masking model. The postulated requisite molecules for one type of positive cell–substratum interaction in situ are as follows: (*a*) cell surface–associated HS-PG; (*b*) fibronectin; and (*c*) collagen microfibrils, each of which has been identified in situ during mesenchymal cell migration. Cell translocation is believed to be the cumulative result of a synchronous series of attachment–detachment events, but can only be assured if a mechanism exists whereby static cycles of cell–substratum interaction are prevented (i.e., attachment–detachment–re-attachment at the same site). We propose that a mesenchyme-specific CS-PG functions during migration to prevent re-attachment by "conditioning" the immediate extracellular environment via a CS-PG–collagen interaction, hence "masking" the substratum.

ment (i.e., labilization of a cell–collagen attachment site); and (*b*) substratum masking for the purpose of preventing re-attachment. Any net movement of a cell (i.e., distance traveled) is logically ensured only if a mechanism exists whereby a cell is prevented from undergoing static cycles of attachment/detachment/re-attachment to the same collagen fibril site. As proposed in our model (Fig. 8), one mechanism by which such considerations might be ensured is through the secretion and subsequent interaction of mesenchyme-derived CS-PG with collagenous substrata.

The present data support this hypothesis by indicating that a mesenchyme-specific CS-PG, enriched in ΔDi-4S residues, associates with collagen during cell migration. As a result, collagen becomes progressively "conditioned" by CS-PG deposited as "trails" (not "footprints" as in planar substrates) during cell passage. Whether the increased ratio of ΔDi-4S to ΔD-6S is important in promoting this association is not clear. However, the affinity of cartilage CS-PG for native collagen under physiological conditions has been recently documented (39, 43). The capacity of CS-PG to associate with collagen may, however, vary with cell type as well as with the composition of other endogenous extracellular matrices.

"Conditioning" collagen as proposed in the model with CS-PG is potentially significant at both the cellular level as well as the tissue level. Not only would the conditioned matrix increase the probability that a cell will form an attachment at an exposed collagen site, but when expanded to the histological level the number of exposed sites would be inversely proportional to the number of cells having migrated through any given area of matrix, and therefore to the degree of substratum masking, as confirmed by the autoradiographs in Fig. 5. Several implications can be drawn, all of which may directly relate to the unknown significance as to why most, if not all, migrating mesenchyme synthesize and secrete S-GAGs. First, deposition of the CS-PG could serve to labilize attachment sites mediated by Fn (34). In this regard, CS-PG would function directly in mediating detachment as implicated by Rich et al. (34). Second, the gradient of conditioned matrix could provide directionality to a migratory cell group in a manner independent of chemotactic stimuli (Fig. 1). Consistent with this concept is the recent, preliminary observation of Newgreen et al. (32) that migrating neural crest cells which normally avoid the perichordal, CS-rich ECM will invade the area upon treatment with CHase ABC. Thirdly, progressive conditioning would be expected to ultimately inhibit sustained migration of a cell group as the available sites for attachment decreased below threshold requirements for sustained movement. In situ and in collagen gel culture, cessation of movement correlates with the formation of tendon-like strata. Thus, substratum conditioning may constitute one mechanism of imprinting positional information ("patterning") characteristic of tissue type (especially fibrous connective tissues).

A critical test of this hypothesis would be to examine the capacity of a collagen gel to support cell movement after directly conditioning the gel with varied amounts of native, endogenous CS-PGs. Presently this has not been technically feasible. To substitute with CS-PG of another tissue source may constitute only an experiment in evolution, proving only that cells can adapt or react to previously unseen molecules (18, 42). Instead, we sought to examine our hypothesis by letting one cardiac mesenchyme population "naturally" condition a gel and then examine for effects upon the formation and migration of a second cell population. The inhibitory responses (Fig. 6) and the partial reversal by CHase treatment of the conditioned gel are consistent with, but do not prove the hypothetical model.

In a related approach, Kinsella and Fitzharris (16) "layered" a putative endothelial monolayer (previously cultured

Figure 7. Scanning electron micrographs of CM-derived cells at various depths of migration into a collagen gel lattice. (*a*) Area of a gel in which CM cells have just initiated migratory activity; collagen microfibrils are relatively free of associated material. (*b*) Granular deposits (*arrows*) are associated with gel microfibrils contacting CM cells; note the collagen-associated granular material which persists in areas of the gel through which this cell has passed in its migration from surface to gel base. (*c*) Region of a gel from a long-term culture in which migration has stabilized with the formation of tendinous strata (see Fig. 5 *d* for orientation); note that granular material "coats" most collagen microfibrils and CM cell surfaces. (*d*) Same as *c* except treated with CHase.

on plastic) upon the endothelial lining of an older AV explant whose matrix had presumably been naturally conditioned by in situ mesenchymal cell migration. They observed that variable numbers of donor cells penetrated the endothelial lining to invade the cellularized explant. They concluded that such limited invasiveness would argue against the concept of matrix conditioning affecting mesenchymal cell migration. However, their data is actually consistent with our hypothesis in that donor cells predictably ceased migration at variable depths within the host pads. The physiological relevance of these data is questionable since normally non-invasive ventricular endothelium was observed to invade the AV explants. This suggests that either culture conditions modified donor cells or, more likely given their experimental design and our own experience (37), their monolayers contained the highly invasive epicardial cell population.

To summarize, our data provide one explanation as to why most mesenchymal cells synthesize S-GAGs during active translocation. That purpose being to condition collagen with a CS-PG that could function initially to sustain movement (by inhibiting static cycles of cell–collagen interaction) but with time, ultimately to restrict movement of a whole cell population (by masking available sites for cell–collagen interaction).

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