Proteoglycans and Glycosaminoglycans Induce Gap Junction Synthesis and Function in Primary Liver Cultures

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Abstract. Intercellular communication via gap junctions, as measured by dye and electrical coupling, disappears within 12 h in primary rat hepatocytes cultured in serum-supplemented media or within 24 h in cells in a serum-free, hormonally defined medium (HDM) designed for hepatocytes. Glucagon and linoleic acid/BSA were the primary factors in the HDM responsible for the extended life span of the electrical coupling. After 24 h of culture, no hormone or growth factor tested could restore the expression of gap junctions. After 4–5 d of culture, the incidence of coupling was undetectable in a serum-supplemented medium and was only 4–5% in HDM alone. However, treatment with glycosaminoglycans or proteoglycans of 24-h cultures, having no detectable gap junction protein, resulted in synthesis of gap junction protein and of reexpression of electrical and dye coupling within 48 h. Most glycosaminoglycans were inactive (heparan sulfates, chondroitin-6 sulfates) or only weakly active (dermatan sulfates, chondroitin 4-sulfates, hyaluronates), the weakly active group increasing the incidence of coupling to 10–30% with the addition of 50–100 µg/ml of the factor. Treatment of the cells with 50–100 µg/ml of heparins derived from lung or intestine resulted in cells with intermediate levels of coupling (30–50%). By contrast, 10–20 µg/ml of chondroitin sulfate proteoglycan, dermatan sulfate proteoglycan, or liver-derived heparin resulted in dye coupling in 80–100% of the cells, with numerous cells showing dye spread from a single injected cell. Sulfated polysaccharides of glucose (dextran sulfates) or of galactose (carrageenans) were inactive or only weakly active except for lambda-carrageenan, which induced up to 70% coupling (albeit no multiple coupling in the cultures). The abundance of mRNA (Northern blots) encoding gap junction protein and the amounts of the 27-kD gap junction polypeptide (Western blots) correlated with the degree of electrical and dye coupling indicating that the active glycosaminoglycans and proteoglycans are inducing synthesis and expression of gap junctions. Thus, proteoglycans and glycosaminoglycans, especially those found in abundance in the extracellular matrix of liver cells, are important in the regulation of expression of gap junctions and, thereby, in the regulation of intercellular communication in the liver. The relative potencies of heparins from different tissue sources at inducing gap junction expression are suggestive of functional tissue specificity for these glycosaminoglycans.

Gap junctions are specialized regions of intercellular contact containing membrane channels through which cells exchange signalling molecules and metabolites (for reviews see references 3, 46). Recent progress in the field of gap junction research includes chemical and immunological characterization of the channel-forming protein, identification of the roles that these channels may play in normal and abnormal tissue function, and elucidation of controls acting on expression and operation of gap junctions (3, 48).

There is abundant evidence for a substantial degree of plasticity in the function and presence of gap junctions between cells. Gap junction channels can be opened or closed relatively quickly by physiological and pharmacological treatments (for review see reference 46) and, over a longer time course in in vivo studies, formation and loss of gap junctions can be stimulated by various factors including certain hormones (for review see 48). Both types of regulation of gap junctions may be to some degree attributable to posttranslational processing of the gap junction protein by modifications such as phosphorylation (45). However, in some cases an earlier step in expression of gap junction protein is presumably responsible because certain hormonal effects are inhibited by protein and mRNA synthesis inhibitors (17, 25).

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Gap junctions between liver cells also display a remarkable degree of plasticity in terms of turnover of the gap junction protein. The half-life of the gap junction protein is 5–10 h in normal liver (13) which is very rapid for membrane proteins. Moreover, liver undergoes a dramatic decrease in anatomically defined gap junctions, in electrotonic coupling, and in gap junction protein soon after partial hepatectomy. The liver cells then recover their gap junction protein both anatomically and functionally within a few days of liver regeneration (50, 52, 53).

Culturing of normal cells under classical cell culture conditions results in the rapid loss, within a few hours, of gap junctions (47).[1] The development of new culture conditions (1, 12, 20, 23, 35–37) that permit the retention of differentiated functions in primary liver cultures has resulted in the discovery of factors, certain glycosaminoglycans (GAGs)[2] and proteoglycans (PGs), that affect the synthesis and expression of gap junctions. In the studies reported here, we demonstrate that dissociated pairs of hepatocytes undergo processes kinetically similar to those that follow partial hepatectomy. Spread of intracellularly injected current and dye (Lucifer Yellow CH) disappear soon after dissociation, and the rate of disappearance depends on the conditions in which the cells are cultured. Thereafter, dye and electrotonic coupling and levels of gap junction protein in primary liver cultures maintained on tissue culture plastic return very slowly and to a limited extent. However, reexpression is dramatically accelerated and augmented by treatment with specific GAGs and PGs.

Materials and Methods

Sources of Glycosaminoglycans

Reference standards for GAGs (dermatan sulfate, chondroitin sulfate, heparan sulfate, heparin, and hyaluronate) were provided by Dr. Martin Mathews and Dr. J. A. Cifonelli of the University of Chicago (Contract NO1-AM-5-2205) from the National Institutes of Health.

Some GAGs and anionic polysaccharides were obtained from Sigma Chemical Co. (St. Louis, MO) including chondroitin 4-sulfate from whale cartilage; chondroitin 6-sulfate from shark cartilage; dermatan sulfate from porcine skin; heparins from porcine intestinal mucosa or from bovine lung; and hyaluronic acid from human umbilical cord. The anionic polysaccharides lambda-carrageenan from *aciculaire* and *pistillata* of the genus, *Gigartina*, iota-carrageenan from *Eucheuma spinosa*, kappa-carrageenan from *Eucheuma cottonii*, and dextran sulfate (8 and 500 kD) were also from Sigma Chemical Co.

Purification and Chemical Characterization of Liver-derived Heparin

A heparin-containing fraction was also isolated from mature bovine liver. Fresh bovine liver was extracted in 4 M guanidine hydrochloride (GdmCl), 0.05 M EDTA, 0.15 M sodium acetate, pH 7, containing protease inhibitors at 0°C for 3 h, a protocol previously described (41, 42). The mixture was centrifuged at 8,000 rpm for 1 h. The supernatant was filtered, then subjected to equilibrium density gradient centrifugation in 2.5 M cesium chloride and 3 M GdmCl at 40,000 rpm for 68 h at 5°C. The gradient was cut into six equal fractions called D1 through D6 (42). The fractions of highest buoyant density (D1 and D2), which contained most of the uronate, were pooled and subjected to gel chromatography on Sepharose CL-4B. The fractions were monitored for uronate by absorbance at 280 nm, and by SDS-PAGE on 5–20% gradient slab gels which were stained with toluidine blue. A uronate-containing fraction was isolated which contained polydisperse, low molecular weight GAG chains (M_r 10,000–20,000), which appeared as a broad, heavily stained band on toluidine blue–stained gradient slab gels. The GAG in this fraction bound to antithrombin III affinity columns. The heparin-containing fraction was further purified by digestion with ribonuclease, followed by chromatography on DEAE-Sepharose in 6 M urea, using a 0–1 M NaCl gradient. A single unimodal peak which was high in uronate and showed no absorbance at 280 or 260 nm was eluted at 0.8 M NaCl. The fraction was recovered and its susceptibility to digestion with heparinase and to heparitinase was examined.

Specifically, the heparin-containing fraction was digested with heparinase and with heparitinase, and the amount of unsaturated uronate residues formed was determined by the increase in A_{232} and by the thiobarbituric acid assay (42). No unsaturated uronate residues were formed after treatment with heparitinase, which readily degraded heparan sulfate standards. No unsaturated uronate residues were formed after treatment with chondroitinase AC. Less than 2% of the dry weight of the material was digested with chondroitinase ABC. Approximately 25% of the dry weight of the fraction consisted of heparin which was cleaved to unsaturated uronate residues with heparinase. The heparin-containing fraction was not characterized further biochemically. The remainder of the heparin-containing fraction was used for studies of its effects on cell-cell communication and gene expression.

Purification and Chemical Characterization of Proteoglycans

Dermatan sulfate proteoglycans (DS-PGs) were isolated from mature bovine articular cartilage as previously described (42). The preparation used in the present studies consisted of a mixture of DS-PGI and DS-PGII isolated after gel chromatography on Sepharose CL-4B in 4 M guanidine hydrochloride. This preparation contains DS-PGI and DS-PGII in approximately equal amounts and is free of cartilage-specific proteoglycan monomer based on ELISA using an antiserum to cartilage-specific proteoglycan monomer from mature bovine articular cartilage. Chondroitin sulfate proteoglycans, sometimes called cartilage-specific proteoglycans, were isolated as the A1A1D1D1 fractions from mature bovine articular cartilage (43), calf nasal cartilage (49), and bovine fetal epiphyseal cartilage (9) as previously described.

Animals

Sprague Dawley rats (200–250 g) were purchased from Marland Farms (New York), maintained under standard conditions, and used for preparation of hepatocytes. Littermates to the rats used for preparation of liver cultures were used for in vivo controls.

Preparation of Cell Suspensions

Rat hepatocytes were prepared by the standard liver perfusion procedure of Berry and Friend (6) using the buffer and perfusion mixture of Leffert et al. (28).

Culture Conditions

Substrata. Cells were plated directly onto 35-, 60-, or 100-mm tissue culture plastic dishes (Falcon Labwares, Oxnard, CA).

Media. Hepatocytes were cultured in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. This medium was supplemented with 10% FBS (Gibco) to produce serum-supplemented medium (SSM), or with a defined mixture of trace elements, hormones, and growth factors (see below) to produce a serum-free, hormonally defined medium (HDM). The development of this HDM has been previously described (12, 20, 35, 37). The medium into which the cells were plated and allowed to attach for 4 h consisted of SSM supplemented with the hormones used in the HDM (SSM/HDM).

Hormones and Trace Elements Used in the Hormonally Defined Medium

The hormones, growth factors, and trace elements, their commercial sources and concentrations used in the hormonally defined medium are as follows: epidermal growth factor (Collaborative Research), 50 ng/ml; insulin (Sigma Chemical Co.), 10 μg/ml; glucagon (Sigma Chemical Co.), 20

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[1] Saez, J. C., W. A. Gregory, E. L. Hertzberg, and D. C. Spray, manuscript submitted for publication.

[2] *Abbreviations used in this paper*: DS-PG, dermatan sulfate proteoglycan; GAG, glycosaminoglycan; g_j, junctional conductance; HDM, hormonally defined medium; PG, proteoglycan; SSM, serum-supplemented medium.

µg/ml; linoleic acid/BSA (Sigma/Pentax), 5 µg/ml; prolactin (Sigma Chemical Co.), 20 mU/ml; growth hormone (Sigma Chemical Co.), 10 µU/ml; zinc (Johnson Matthey), 0.1 nM; copper (Johnson Matthey), 0.1 µM; and selenium (Johnson Matthey), 0.3 nM.

Linoleic acid was prepared in stock solutions of 1 mg/ml in 1% fatty acid-free BSA (Miles Laboratories, Naperville, IL); if added without the BSA, the linoleic acid is quite toxic to the cells.

Morphological Studies

Cultures of hepatocytes plated under various conditions were maintained for 4-5 d and then studied using phase-contrast optics on a Nikon inverted phase Diaphot microscope to characterize the cell culture populations morphologically. The cultures were evaluated by several of the investigators independently. Representative cultures were selected and photographed using phase microscopy.

Electrophysiological Techniques

Cell pairs (which comprised a small significant fraction of the cultured cells) were impaled with microelectrodes (20-30 MΩ, filled with 3 M KCl or potassium citrate) connected to WPI (New Haven, CT) or homemade high impedance electrometers with active bridge circuits. Electrode resistance was balanced before cell entry and was adjusted as necessary after penetration by neutralization of the fast initial resistive phase in the voltage trace in response to a 30-ms current pulse. Current pulses were applied alternately to the two cells and conductances of junctional and nonjunctional membranes were calculated by applying the pi-tee transform to measured input and transfer resistances (2). These techniques were used to qualitatively evaluate coupling in confluent cell cultures. Lucifer Yellow CH (5% wt/vol, in 150 mM LiCl), a fluorochrome with size near the permeability limit of the channel (1.2 × 1.4 nm) and high quantum yield, was iontophoretically injected into one cell (100 ms, 0.1-1 nA pulses). Spread to adjacent cells was evaluated and photographed after 1 min. At least 20 cells were assayed per condition per experiment. Each experiment was repeated at least three times.

Western Blots

Rat hepatocytes were cultured in HDM or SSM with and without supplementation with various GAGs, PGs, or anionic polysaccharides: chondroitin sulfate proteoglycan (10-20 µg/ml), DS-PG (10-20 µg/ml), heparins (10-100 µg/ml, depending on source of heparin; see tables), and lambda-carrageenan (10-20 µg/ml). After 5 d in culture, cells were harvested by scraping, pelleted, solubilized (50 mM Na_2CO_3, 2% SDS, 50 mM DTT) and samples containing 5×10^5 cells were subjected to SDS-PAGE as described by Hertzberg and Skibbens (22). Subsequent to electrophoresis, the resolved proteins were electrophoretically transferred to nitocellulose and processed for Western blot analysis. The primary antibody was affinity-purified sheep anti-rat liver gap junction protein described previously (21). Antibody binding to the 27-kD gap junction polypeptide was determined by autoradiography of the nitrocellulose sheet after incubation with rabbit anti-sheep IgG and ^{125}I-protein A.

Molecular Hybridization Assays

Hybridization assays (Northern Blots) were used to determine the abundance of mRNA encoding the gap junction protein compared to a common gene, β-actin. The normal hepatocytes were plated onto 100-mm culture dishes under the conditions specified and maintained for 5 d with daily medium changes. In each experiment, cells were pooled from 3-4 dishes per culture condition. The cells were washed twice with 40 ml of cold PBS, and the cells were removed from culture dishes with a rubber policeman, pelleted, and total RNA isolated using the guanidinium/hot phenol method of Feramisco et al. (16). RNA samples were resolved by electrophoresis through 1% agarose, submerged slab, denaturing gels in MOPS buffer. RNA was transferred to Gene Screen (New England Nuclear, Boston, MA), and the RNA-containing filters were prehybridized and then hybridized with appropriate probes. The cDNA clones complementary to specific mRNAs (listed below) were radioactively labeled by primer extension, as described by Feinberg and Vogelstein (15). ^{32}P dCTP (specific activity of 3,200 Ci/mmol) was included to obtain a specific activity of $8\text{-}12 \times 10^8$ cpm/µg of DNA. DNAs used in hybridization included DNA complementary to mRNA encoding gap junction protein (obtained from David Paul, Harvard University, MA) (32) and rat β-actin and PBR322 (obtained from J. Darnell, The Rockefeller University) (10).

Protocol of Studies

The freshly isolated cells were plated at 10^4/35-mm dish, at 5×10^5/60-mm dish, or at 5×10^6 per 100-mm dish in SSM/HDM. After 18 h, the cultures were rinsed and maintained thereafter in HDM or SSM as dictated by the experimental design. The medium was changed every day. Evaluation of cultures for cell-cell communication by dye coupling, electrical coupling, or Western blots was done after 4, 12, 18, 24, 48, 72, and 96 h of culture. Evaluation of cultures for gap junction mRNA was done after 96 h of culture.

Results

Electrotonic and Dye-Coupling in Primary Liver Cultures in HDM vs. SSM

Pairs of freshly dissociated rat hepatocytes were strongly coupled with respect to the dye Lucifer Yellow CH and were also strongly coupled electrotonically. Within the first 5 h after plating, no change as compared to freshly dissociated cells was observed in incidence of dye coupling or in strength of electrotonic interactions. These results were similar to previously reported findings (47).[1]

However, dye and electrical coupling disappeared within 7.5-9 h (Fig. 1) when dissociated hepatocytes were cultured on tissue culture plastic and in medium supplemented with serum (SSM). After 10 h, dye coupling was undetectable in cells cultured in SSM at any density. In HDM, dye coupling was still strong at 10-12 h (Fig. 1) but disappeared by 20 h. Measurement of junctional conductance (g_j) between pairs of cells demonstrated that the disappearance was gradual in both SSM and HDM, but the time course of disappearance was markedly different between the two groups (Fig. 1). At 7.5-9.5 h, g_j values for cells in HDM were not different from g_j's between freshly dissociated cells (0.63 ± .08 µS, SD, $n = 26$ vs. 0.65 ± 0.09 µs, SD, $n = 21$). Cells in HDM progressively lost junctional conductance, so that g_j was reduced by 60% at 14.5-16.5 h and by 90% at 19-20 h. By contrast, g_j for cells in SSM at 7.5-9.5 h was much lower (0.08 ± 0.01 µS, $n = 14$) and by 14.5-16.5 h was barely detectable (<0.5% of controls).

Components in HDM That Maintain Cell-Cell Communication

To determine which components in HDM were responsible for the prolonged lifetime of dye and electrical coupling between pairs of hepatocytes during the first 24 h of culture, each of the components in HDM was added to SSM or was subtracted from HDM. In addition, the effects of each component alone was tested in the presence and absence of serum. Only two of the factors in HDM, glucagon and the linoleic acid/BSA complex, were effective in extending the lifetime of gap junctions from 7 to 20 h. Addition of glucagon and linoleic acid/BSA to SSM elevated g_j to nearly normal levels, and the deletion of glucagon and linoleic acid/BSA from HDM eliminated the dye coupling in primary liver cultures tested at 7-10 h after seeding (Fig. 1). These results indicate that glucagon and linoleic acid are two of the factors responsible for maintenance of coupling in HDM compared to SSM. However, it is clear from the histogram in Fig. 1 that other factors are also involved: g_j at 7.5-9.5 h is stronger in HDM in which glucagon and linoleic acid/BSA is deleted than in SSM, and g_j measured at 14.5-16.5 h and 19-20 h is stronger in HDM than in SSM + glucagon +

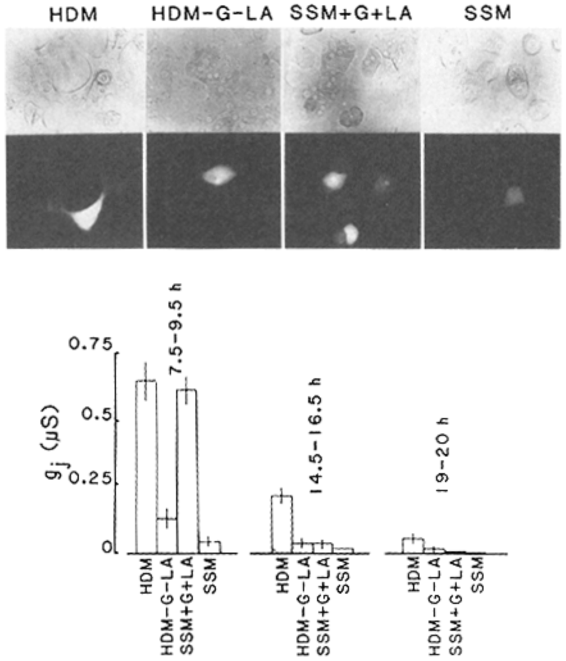

Figure 1. Electrical and dye coupling between pairs of hepatocytes disappeared within 7–20 h in cells cultured in SSM or HDM. Coupling was prolonged in SSM supplemented with glucagon (*G*) and linoleic acid–BSA complex (*LA*) and diminished in HDM in which these factors are deleted. Thus, the rate of disappearance of the gap junctions was most affected by these components in the HDM. (*Top*) Dye coupling in the various media and measured at 14–16 h. Each pair of photographs includes an upper photo with Hoffman modulated contrast optics and a lower photo of the same field with epifluorescence. In HDM and SSM + G + LA, dye injected into one cell spread to adjacent cells; in SSM and HDM-G-LA, there was no detectable spread after 5 min. (*Bottom*) Histogram showing calculated junctional conductance (g_j) in an experiment in which sister cultures were examined at ~5-h intervals. Lines at the top of bars represent standard deviations, *n* being greater than 20 in each case. Treatments are indicated below the bars and include hormonally defined medium (*HDM*) from which glucagon and linoleic acid were subtracted (*HDM-G-LA*) and serum-supplemented medium (*SSM*) to which glucagon and linoleic acid were added (*SSM + G + LA*).

linoleic acid/BSA. This suggests that factor(s) present in serum may inhibit the stimulatory effects of glucagon + linoleic acid/BSA on maintenance of gap junctions after liver dissociation.

Cell-Cell Communication in Primary Liver Cultures Treated with Glycosaminoglycans or Proteoglycans

The effects of GAGs or PGs on expression of gap junctions in cultured cells were evaluated using dye coupling as the initial assay. Cells were cultured for 3–7 d on tissue culture plastic and in HDM supplemented with 10–100 µg/ml of various PGs or GAGs listed in Table I. Induction of some dye coupling occurred in the presence of most of the GAGs and PGs tested. However, with the majority of GAGs, no response or only a weak response (5–10% coupling) was observed (see Table I). A dramatic inductive response was observed only with heparins, especially liver-derived heparin (see below), and with proteoglycans such as DS-PG or chondroitin sulfate proteoglycan in the presence of which 70–100% of the cells tested showed dye coupling (Table I, Fig. 2). Furthermore, the most active of these factors (the PGs and the liver-derived heparin) induced multiple coupling among the cells; i.e., each cell was coupled to more than one cell. This is noteworthy since the GAGs or PGs were added at a time point (24 h) at which there was no detectable dye or electrical coupling in cells cultured in either HDM or SSM (Fig. 2). Thus, these factors induced expression of functional gap junctions at concentrations of 10–20 µg/ml. At those or higher concentrations (up to 100 µg/ml), commercial heparins or highly purified heparin (Matthew's standard) and hyaluronic acids were active at inducing dye coupling in 25–60% of the cells but did not induce multiple coupling. Weak activity was demonstrated with 100 µg/ml of dermatan sulfate (20% of the cells), chondroitin 4-sulfate (20% of the cells), and chondroitin 6-sulfate (5% of the cells).

Control Studies: Screens of Sulfated Polysaccharides

Two classes of sulfated polysaccharides were used as controls: dextran sulfates (polymers of sulfated glucose) and carrageenans (polymers of sulfated galactose derived from seaweed). All of these sulfated polysaccharides caused dramatic contractions of the cell layers, even at concentrations of 1–5 µg/ml. Yet all of the sulfated polysaccharides tested but one were inactive or only weakly active at inducing expression of gap junctions, even when added at high concentrations (up to 100 µg/ml). For example, 8-kD dextran sulfate was completely inactive, and all but one of the rest of the polyanions showed only a weak inductive effect: 500-kD dextran sulfate (10–15%), kappa-carrageenan (14%), and iota-carrageenan (30%). The only anionic polysaccharide that showed a significant effect was lambda-carrageenan, which at concentrations of 10 µg/ml, induced ~70% of the cells to be coupled. No form of sulfated polysaccharide was able to induce multiple coupling or coupling of a cell to more than one neighbor.

Functional Tissue Specificity of the Glycosaminoglycans: Studies with Heparins

To evaluate potential tissue-specificity of GAGs or PGs, heparins from various tissues were retested on replicate cultures in the same experiment. Heparins from three different tissues were tested: bovine liver, bovine lung, and porcine intestine. As shown in Table II, the liver-derived heparin-induced coupling in 96% of the cells tested, and many of these were multiply coupled; that is, each cell was coupled to 3–4 cells or more. Lung-derived heparin was intermediate in activity, inducing coupling in 50–60% of the cells and infrequently inducing a multiply coupled cell. Intestine-derived heparin was the weakest of the heparins tested, inducing coupling in 22–25% of the cells and never inducing multiply coupled cells.

Kinetics of Induction of Gap Junctions by Proteoglycans

To evaluate the time course of induction of gap junctions by the GAGs and proteoglycans, matched dishes of cells cultured in SSM, HDM, or HDM plus DS-PG were assayed for dye coupling at 8–12-h intervals for 96 h (Fig. 3). The cells cultured in SSM and in HDM demonstrated the usual time course of loss of dye and electrical coupling (reduced by 90–100% during the first 20 h). In cells maintained in HDM and at high densities (7–10 × 10^6/100-mm dish), dye and electrotonic coupling disappeared in the first 20 h and then gradually increased, reaching a maximum of 40% of in vivo

Table I. Glycosaminoglycans and Proteoglycans Tested for Biological Activity on Hepatocytes

Proteoglycan or glycosaminoglycan	Source/(dose)	Morphology	Cell–cell communication*	Gap junction protein level (Western blots)
None				
SSM	–	Flattened	0	None detected
HDM	–	Flattened	4%	None detected
Dermatan sulfate	Porcine skin (Sigma Chemical Co.)	Slightly contracted	20% (10 μg/ml) 30% (100 μg/ml)	+
Dermatan sulfate	Porcine intestine (Sigma Chemical Co.)	Slightly contracted	30% (10 μg/ml) 50% (100 μg/ml)	N.T.
Dermatan sulfate	NIH Ref. STD (Matthews) (100 μg/ml)	Slightly contracted	50%	N.T.
Dermatan sulfate proteoglycan	Bovine articular cartilage (adult) (10 μg/ml)	Increased packing density	90–100%	+++
Chondroitin 4-sulfate	Whale cartilage (Sigma Chemical Co.) (1 mg/ml)	Flattened	15–45%	N.T.
Chondroitin 6-sulfate	Shark cartilage (Sigma Chemical Co.) (1 mg/ml)	Flattened	5%	N.T.
Chondroitin sulfate proteoglycan	Bovine nasal cartilage (adult) (10 μg/ml)	Increased packing density	60–80%	+++
Heparan sulfate	Bovine lung, Ref. Std. (Matthew's) (5 mg/ml)	Flattened	0%	None detected
Heparan sulfate	Bovine intestine (Linker's; 9% sulfate) (10 μg/ml)	Flattened	0%	N.T.
Heparan sulfate	Bovine intestine (Linker's; 12% sulfate) (10 μg/ml)	Flattened	0%	N.T.
Heparan sulfate	Bovine intestine (Linker's; 15% sulfate) (10 μg/ml)	Flattened	0%	N.T.
Heparin	Porcine intestinal mucosa (Sigma Chemical Co.)	Moderate–strong contraction	30–45% (20 μg/ml) 40–70% (100 μg/ml)	N.T. ++
Heparin	Bovine lung (Sigma Chemical Co.) (20 μg/ml)	Moderate–strong contraction	45–55%	N.T.
Heparin	Bovine lung, Reference Std. (Linker's) (20 μg/ml)	Moderate–strong contraction	50–60%	N.T.
Hyaluronic acid	Human umbilical cord (Sigma Chemical Co.) (100 μg/ml)	Flattened	55%	N.T.
Hyaluronic acid	Reference Std. (Matthews) (100 μg/ml)	Flattened	30–40%	N.T.
Carrageenans (polymers of sulfated galactose)				
Lambda-carrageenan	Seaweed (Sigma Chemical Co.) (10 μg/ml)	Strong contraction	70%	+++
Kappa-carrageenan	Seaweed (Sigma Chemical Co.) (50 μg/ml)	Flattened	14%	N.T.
Iota-carrageenan	Seaweed (Sigma Chemical Co.) (50 μg/ml)	Strong contraction	30%	N.T.
Dextran sulfates (polymers of sulfated glucose)				
Dextran sulfate	8 kD (Sigma Chemical Co.) (10 μg/ml)	Strong contraction	0%	N.T.
Dextran sulfate	500 kD (Sigma Chemical Co.) (2 μg/ml)	Strongest contractions	10–15%	N.T.

* Cell–cell communication was evaluated by spread of Lucifer Yellow CH molecules injected into cells. The numbers indicate the percentage of cells capable of spreading dye to their neighbors. In each experiment and in each condition, at least 20 cells were injected. The experiments were run at least three times. N.T., not tested.

levels at 120 h (none of these fills involved multiple cells). In cultures in SSM, dye coupling disappeared within 10 h after dissociation and by 120 h was still undetectable or barely detectable.

Liver cultures were treated with 10 μg/ml DS-PG starting at 24 h after plating the cells, at which time, little or no dye coupling could be observed in either HDM or SSM. By 48 h (24 h after the addition of DS-PG), an increase in dye and electrotonic coupling was observed. By 72 h (48 h of treatment with DS-PG), 80% of the cells showed coupling and in most cases, the dye transfer was to multiple cells. At 120 h (96 h of treatment), all cells treated with DS-PG were multiply coupled to their neighbors.

Expression of Gap Junction Protein and mRNA Encoding the Liver Gap Junction

To ascertain whether the coupling observed in these experiments was due to reassembly of preexisting gap junction pro-

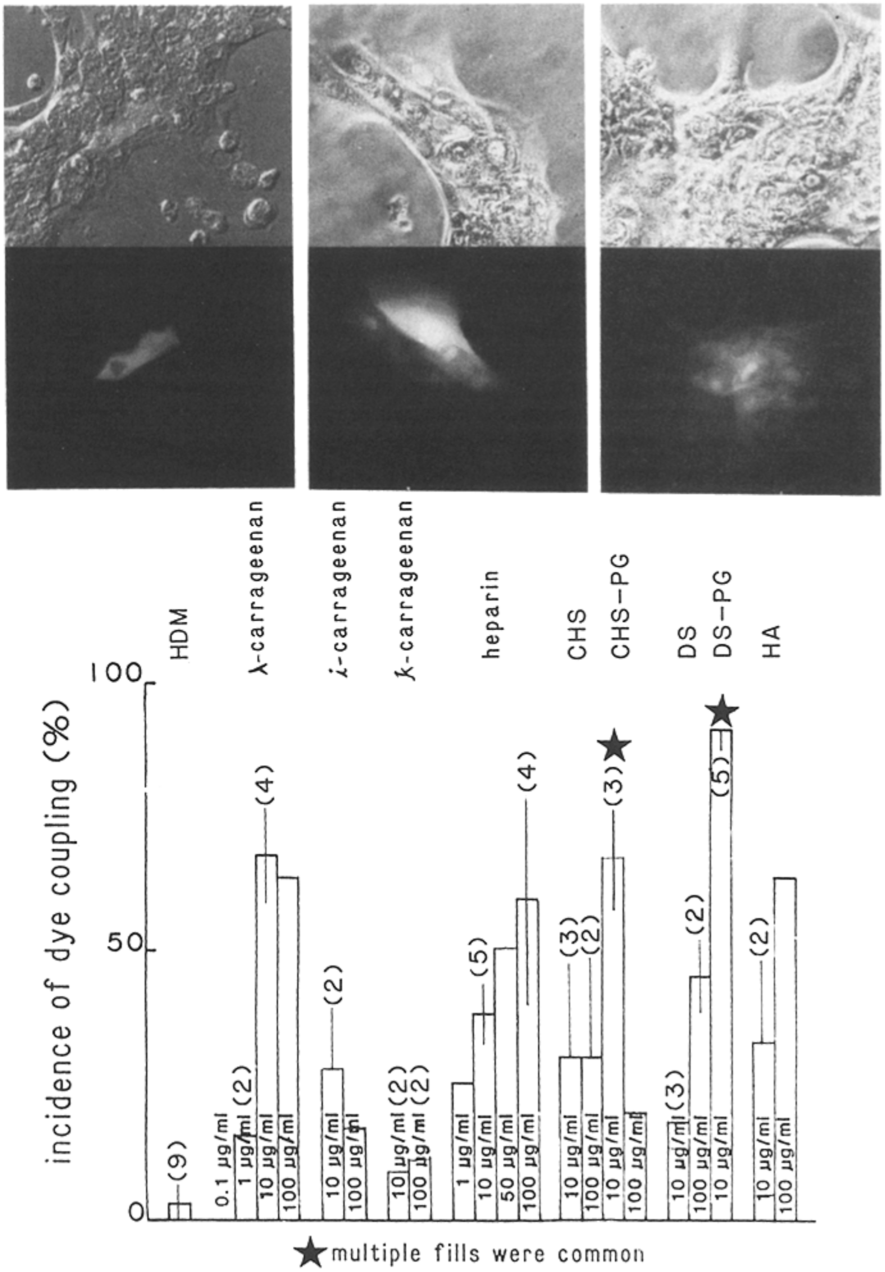

Figure 2. Influence of various GAGs and PGs on the incidence of dye coupling measured at 96 h (72 h after the addition of matrix components) in primary liver cultures. Lucifer Yellow injections into single hepatocytes cultured on tissue culture plastic and in HDM supplemented with various GAGs or PGs. (*Top*) Dye coupling was absent or very rare in cells treated with HDM (*left*), was occasional in cells treated with 10 μg/ml dermatan sulfate (*center*), and was common in cells treated with 10 μg/ml DS-PG (*right*). At the top are phase-contrast micrographs of fields of representative cultures and on the bottom are the same fields epiilluminated with a xenon arc lamp and viewed with FITC filter combinations. Lucifer Yellow injection reveals that the hepatocytes are pleimorphic under all treatments but are smaller (representing increased packing density) in the presence of proteoglycan. (*Bottom*) Incidence of dye coupling in cultures treated with GAGs and PGs. Lines atop bars represent standard deviations; *n* represents the number of pairs of sister cultures in which dye coupling of 10–12 injected cells was examined (in cases where *n* = 2, individual values from pairs of cultures were used to calculate SD).

tein or to accumulation of new protein, extracts of cultures were prepared as previously described (22). Using an antibody to gap junction protein (21), the 27-kD gap junction protein was detected in Western blots of cultures maintained in SSM, HDM, and in HDM plus various GAGs and PGs. Fig. 4 shows a representative Western blot from primary cultures maintained in SSM, HDM, and HDM supplemented with lambda-carrageenan. In Table I are given the results from Western blots on cultures treated with other GAGs and PGs. In all the studies, the amount of gap junction protein, as scored by density of the blot, correlated with the amount of dye and electrotonic coupling.

To conclusively prove that the increase in gap junction protein level was due to protein synthesis and not to increased stability of gap junction protein, the cytoplasmic abundance of mRNA encoding gap junction protein was measured using a full-length cDNA probe for gap junction isolated by D. Paul (32). As shown in Fig. 5, the in vivo cytoplasmic levels of mRNA encoding gap junction protein are high, whereas in primary liver cultures maintained on tissue culture plastic and in HDM, the gap junction mRNA is almost undetectable. Exposure of the cells to 50 μg/ml of lung-derived heparin in the HDM resulted in an increase in the abundance of gap junction mRNA to ~40% of the levels observed in vivo. As a representative of common genes, β-actin mRNA levels were assessed for comparison to the expression by gap junction protein. β-Actin levels were found to be almost undetectable in vivo in quiescent rat liver and increased dramatically in hepatocytes cultured in HDM, results similar to those found previously (12, 23). In response to the addition of 20 μg/ml of lung-derived heparin to the HDM used in the primary liver cultures, the β-actin levels were reduced.

Table II. Tissue Specificity in the Effects of Heparins on Gap Junction Expression

Tissue source of the heparin	Dose	Morphology	Average* % cells coupled (range)	Average % cells multiply coupled
Control: HDM; no heparin	–	At confluence, slight contraction	12% (10–15%)	None
Bovine liver‡	20 μg/ml	Strong contraction	96.3% (89–100%)	18–25%
Bovine lung (NIH Ref. Std.: Linker's)	20 μg/ml	Strong contraction	57.5% (50–60%)	<5%
Bovine lung (Sigma Chemical Co.)	20 μg/ml	Strong contraction	50% (40–55%)	None
Porcine intestine (NIH Ref. Std. Matthews)	20 μg/ml	Strong contraction	35% (29–45%)	None
Porcine intestine (Sigma Chemical Co.)	20 μg/ml	Strong contraction	23.3% (22–30%)	None

Primary liver cultures were seeded in HDM/SSM at a seeding density of 10^6 cells/60-mm dish. After 4 h, the cultures were rinsed with PBS and then given HDM with or without heparin (20 μg/ml). The medium was changed every day for 4 d at which time the cultures were assessed for dye and electrical coupling as specified in Materials and Methods.

* Cell-cell communication was evaluated by spread of Lucifer Yellow CH molecules injected into cells. The numbers indicate the average percentage of cells capable of spreading dye to their neighbors. This average derives from data from a minimum of three experiments, in each of which, under each condition, at least 20 cells were injected. Thus, each average is the result of studies on at least 60 cells. When cells were on tissue culture plastic and in SSM, there was no detectable dye coupling.

‡ A description of the purification and chemical characterization of the liver-derived heparin is given in Materials and Methods.

Morphological Effects of PGs and GAGs

In morphological studies, some of the GAGs and PGs and all of the anionic polysaccharides caused the cells to contract (see Fig. 6 and Table I), and since the cell–cell contacts remained intact, the entire cell layer contracted as a sheet. If the contraction was sufficiently strong, the cell sheet detached from the dish. Contraction of the cultures occurred by 24–72 h (depending on the PG/GAG/polysaccharide to which cells were exposed) with treatment with lung- or intestine-derived heparin (50–100 μg/ml), hyaluronic acid (100 μg/ml), lambda-carrageenan (20–50 μg/ml), or dextran sulfate (2–5 μg/ml of either 8- or 500-kD forms).

The proteoglycans (chondroitin sulfate proteoglycan and dermatan sulfate proteoglycan) did not cause such dramatic cell layer contraction but did cause an increase in the packing density of the cells. When the cells were packed more tightly, the cellular membranes were not discernible, and individual cells were recognizable primarily by their pale nuclei. Under these conditions the cell layer appeared less refractile.

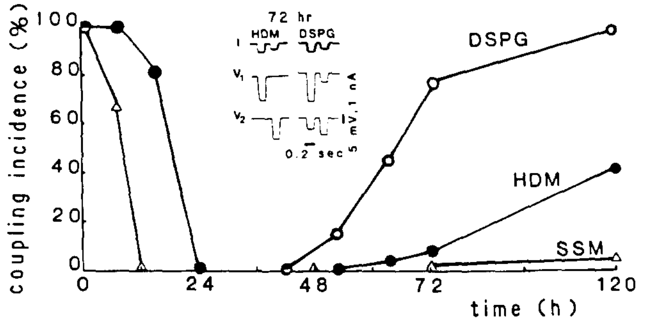

Figure 3. Time course of reappearance of electrotonic and dye coupling in primary liver cultures. In cells treated with HDM (*solid circles*) or SSM (*open triangles*), the incidence of dye coupling decreased so that very few cells were coupled under either condition by 24 h. Addition of DS-PGs at 24 h to the cultures resulted in reexpression of dye and electrical coupling beginning at ~48 h after dissociation. Thereafter, the incidence of dye coupling progressively increased in cultures exposed to DS-PG (*open circles*). The dye and electrical coupling approaches that of control cells (freshly dissociated cells) by 120 h after the addition of the DS-PG. By comparison, the incidence of coupling in cultures in HDM at the same time point was ~35–40% and in SSM was undetectable. (*Inset*) Strength of coupling was evaluated at every time point; here is shown for representative cell pairs at 72 h in culture. Traces for cells treated with HDM and DSPG correspond to current (I) injected alternately into cells 1 and 2 and resulting voltages (V_1, V_2) in the two cells.

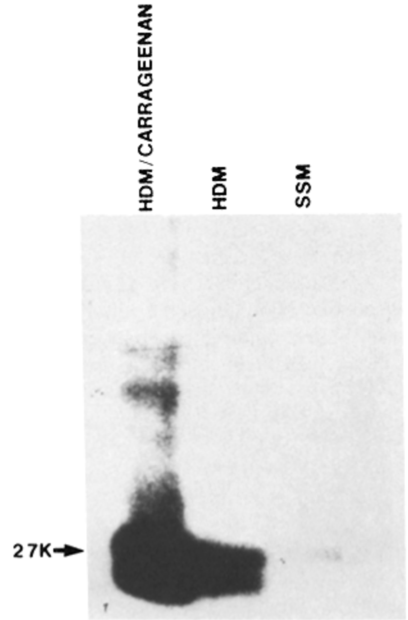

Figure 4. Western blot analysis of hepatocyte cultures in hormonally defined medium versus serum-supplemented medium. Rat hepatocytes were cultured in SSM or HDM with and without supplementation with 10 μg/ml lambda-carrageenan. After 5 d in culture, cells were harvested by scraping, pelleted, solubilized (50 mM Na_2CO_3, 2% SDS, 50 mM DTT) and subjected to SDS-PAGE. Subsequent to electrophoresis, the resolved proteins were electrophoretically transferred to nitrocellulose and processed for Western blot analysis. The primary antibody was affinity-purified sheep anti–rat liver gap junction protein. Antibody binding to the 27-kD gap junction polypeptide was determined by autoradiography of the nitrocellulose sheet after incubation with rabbit anti–sheep IgG and ^{125}I protein A.

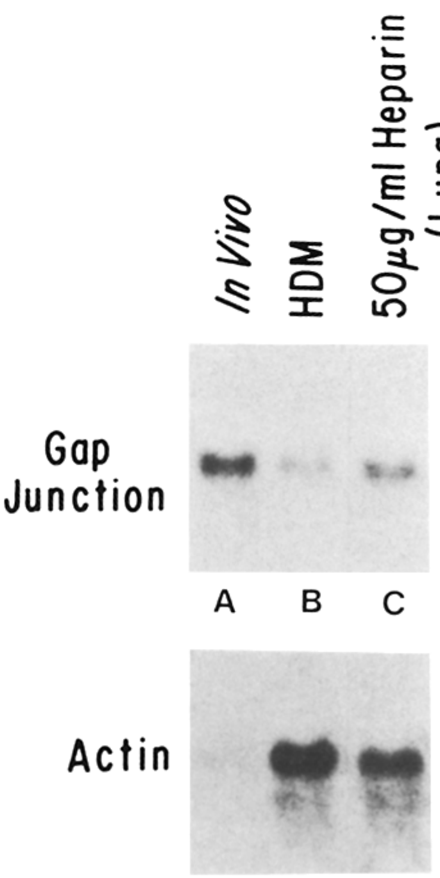

Figure 5. Abundance of cytoplasmic mRNA encoding gap junction protein and β-actin in cultured hepatocytes with or without heparin. Rat hepatocytes were cultured in HDM with or without supplementation with 50 μg/ml of bovine lung heparin (Sigma Chemical Co.). After 4 d in culture, cells were harvested by scraping and pelleted. Then RNA was isolated and purified as described in Materials and Methods. The purified RNA was subjected to electrophoresis (10 μg/lane), transferred onto Gene Screen, and hybridized with the cDNA probes radiolabeled with ^{32}P-dCTP by oligolabeling. The protocols are described in Materials and Methods. (Lane *A*) In vivo: rat liver of littermate used for preparation of the cultured liver cells (control). (Lane *B*) Primary liver culture maintained in HDM for 4 d. (Lane *C*) Primary liver culture maintained for 4 d in HDM supplemented with 50 μg/ml of bovine lung heparin (Sigma Chemical Co.).

Discussion

Influence of Regeneration on Levels of Gap Junctions In Vivo

Gap junctions, examined in thin section and freeze fracture of intact liver (52) and of freshly isolated liver cell suspensions (47), have been found between cells, where they occur as large plaques near the intercellular margins and near bile caniculi where they are interspersed among tight junctional lattices. Previous studies have shown that partial hepatectomy initiates the disappearance and reexpression of gap junctions and electrical coupling in the residual regenerating liver (31, 52, 53). It is most probable that the newly expressed gap junctions are newly synthesized (51).

Effects of Culturing Hepatocytes on Expression of Gap Junction Protein

We show here that cultured hepatocytes show a pattern of expression of gap junction protein similar to that observed in regenerating liver in vivo. As in regenerating liver, dissociated hepatocytes undergo a loss in expression of gap junctions, occurring within hours of initiation of the cultures. Certain medium and hormonal conditions affected the time course in the disappearance of gap junctions in the cultured cells but were not able to re-induce gap junctions once they had disappeared. The time course of the disappearance of gap junctions from the cell membrane after inhibition of protein synthesis (13) was strikingly similar to that measured for disappearance of junctional conductance after dissociation[1] and is consistent with that reported here for cells cultured in SSM. The disappearance of the gap junctions after liver perfusion and dissociation has been shown due to loss of synthesis of gap junction protein and due to internalization and degradation of preexisting gap junctions.[1]

In HDM or SSM to which glucagon and linoleic acid/BSA were added, the loss of the gap junctions can be slowed so that the decay of g_j after dissociation is approximately doubled. This prolongation is similar to that seen with application of membrane-permeant cAMP analogues[1] and is presumably attributable to glucagon-induced elevation of this second messenger. In primary cultures (25) and cell lines (e.g., reference 17) increased gap junction incidence and coupling strength have been shown to result from treatment with membrane permeant cAMP derivatives. Liver gap junction protein is phosphorylated by cAMP-dependent protein kinase in vitro and in cultured cells, and the phosphorylation is associated with rapid increase in junctional conductance (45).[1] How cAMP induction might slow the disappearance of gap junctions (i.e., alter the rate of internalization and degradation of gap junction protein) is unknown, although it may relate to cAMP affects on cytoskeletal components.[1] It is known that drugs, such as nocodazole, that cause disruption of cytoskeleton, also result in slowing of the degradation of gap junctions presumably by blocking the internalization process.[1]

The rapid disappearance of gap junctions in liver cultures maintained in SSM was shown due both to the absence of glucagon and linoleic acid/BSA and to other unidentified factor(s). Thus, cultures maintained in SSM containing glucagon and linoleic acid/BSA still showed a more rapid loss of gap junctions than cultures in HDM. The identity of the serum factor(s) inhibitory to cell–cell communication in primary liver cultures remains obscure. A similar inhibitory effect of SSM on cytoplasmic cAMP levels has been shown in cultured sympathetic neurons (25) and on synthesis and stability of tissue-specific mRNAs in primary liver cultures (12, 23).

Effects of Proteoglycans and Glycosaminoglycans

Whereas the medium and particular hormonal conditions could slow the disappearance of gap junctions in primary liver cultures, only certain GAGs and PGs were found capable of re-inducing expression of gap junctions in cultures devoid of them. The evidence for induction in expression of gap junctions, at a protein level, derives from dye and electrotonic studies as well as evaluation of gap junction protein lev-

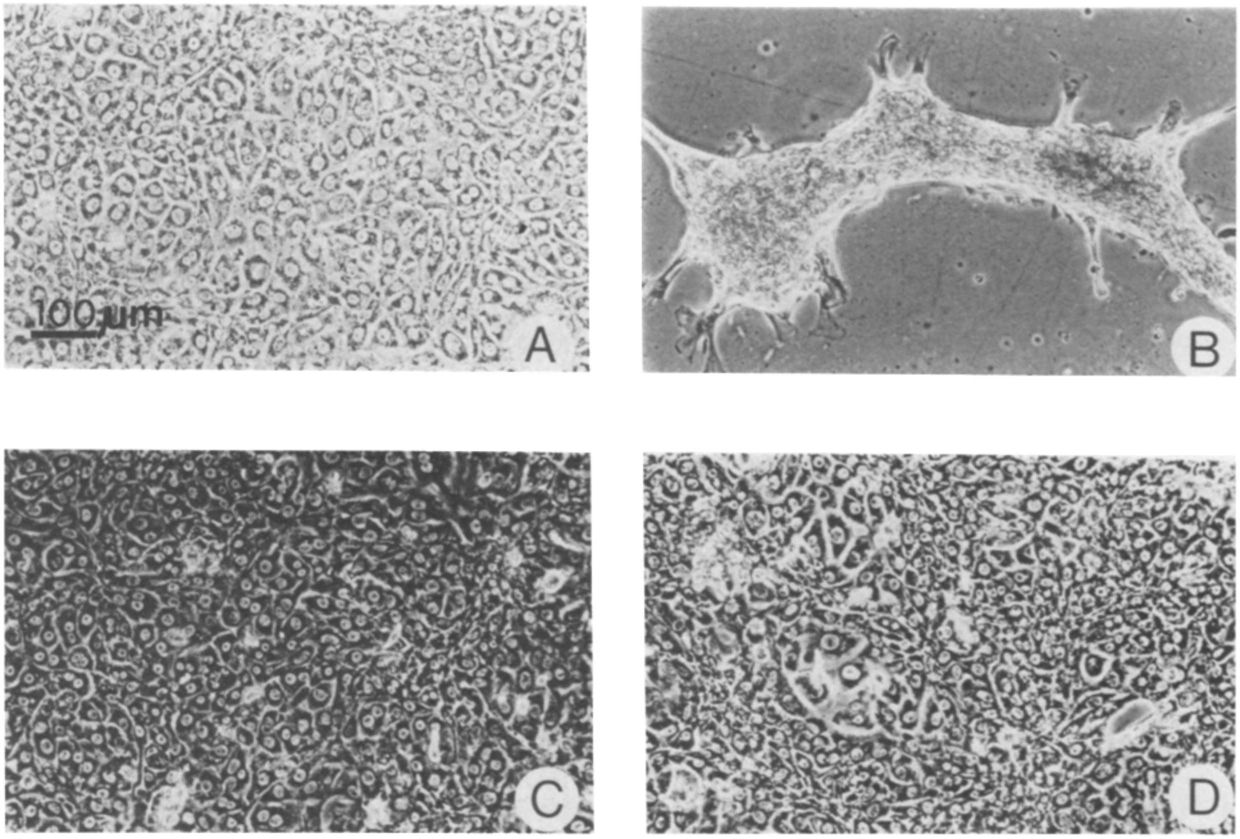

Figure 6. Influence of GAGs and PGs on cell morphology. When primary liver cultures were plated onto tissue culture plastic in HDM, cells remained squamous and flattened (*A*). When the medium was supplemented with certain GAGs or anionic polysaccharides such as lambda carageenan, the cells showed contraction of the cell sheet (*B*). An increase in packing density was observed in cultures exposed to liver-derived heparin (*C*) or to DS-PG (*D*). The response of the cells to other proteoglycans and GAGs is given in Table I.

els by Western blot analysis. The increase in gap junction protein and reexpression of gap junction functions have been shown due to effects on the synthesis of the protein, since the increased protein levels correlate with an increase in the abundance of mRNA probe using a full-length cDNA encoding gap junction protein (32). In this regard, gap junction mRNA is typical of other tissue-specific mRNAs that are not sustained in primary cultures even in a serum-free, hormonally defined medium (23, 37). In past studies this has been shown due both to loss of synthesis and to reduced half-life of the tissue-specific mRNAs in the cultured cells (Fujita, M., H. Choi, L. C. Rosenberg, and L. M. Reid, manuscript submitted for publication; 23). By contrast, the mRNAs encoding common genes, such as β-actin, are found in very low abundance in quiescent liver in vivo (23). Under culture conditions, the rates of synthesis of the mRNAs for such common genes are transiently elevated and then return to in vivo levels, but their mRNAs under most culture conditions remain highly stable. One of the few conditions found to reduce the stability of actin and other common gene mRNAs is the addition of GAGs or PGs to cultured cells (Fujita, M., H. Choi, L. C. Rosenberg, and L. M. Reid, manuscript submitted for publication). Thus, the induction of gap junction expression by GAGs and PGs is due to protein synthesis and is regulated at the level of mRNA metabolism, although it is unknown at this time if the induction is due to transcriptional or posttranscriptional regulation.

The influence of GAGs and PGs on expression of gap junctions between cultured liver cells is the first demonstration of the inductive influence of extracellular matrix components on gap junction channels in biological membranes. These data may explain the variability in coupling that are often seen as a consequence of cell density (17), since the expression of particular GAGs and PGs is strictly density dependent (14, 18, 34).

The quantitative specificity in terms of the relative potencies of particular GAGs and PGs at inducing gap junction expression and the known proteoglycan chemistry of the extracellular matrix of the liver are supportive of the interpretation of the data as indicating a physiological effect (40). The extracellular matrix in contact with hepatocytes in vivo contains mostly heparan sulfate proteoglycan and smaller amounts of DS-PG and chondroitin sulfate proteoglycan along with their corresponding GAGs (14, 24, 26, 33, 39, 40, 41). Furthermore, the most abundant species adjacent to the hepatocytes are heparin and heparin proteoglycan (14). As indicated in Table I and Fig. 3 most of the GAGs and anionic polysaccharides showed only a slight or weak biological activity inducing only ~5–20% cell–cell coupling. However, heparins, even heparins derived from tissues other than liver, were able to induce coupling in 50–60% of the cells, and liver-derived heparin induced 80–100% coupling and was the only GAG capable of inducing multiple cell–cell coupling. The two PGs tested proved much more active than their corresponding GAGs, inducing at 10 μg/ml coupling in 80–100% of the cells and inducing a high incidence of multiply coupled cells. Preliminary studies with a newly purified liver-derived heparan sulfate proteoglycan indicate that it is also highly active and much more active than its isolated GAG chains (Rosenberg, L., H. Choi, M. Fujita, D. Spray, and L. Reid, unpublished data). The increased activity of the proteoglycans over their GAG chains suggests either marked activity of the protein core by itself and/or facilitated interaction of the GAG chains with the relevant cellular target by their presentation to the cell when complexed to their protein cores. Ongoing structure–function analyses should help in distinguishing between these possible interpretations.

Studies comparing a newly purified, liver-derived heparin with heparins from the lung and intestine indicate that there is tissue-specificity in the effects of these GAGs in terms of their potency and the length of time necessary to see a response. The liver-derived heparin was the most potent, inducing coupling in virtually all of the cells and resulting in a high degree of multiple coupling even at low concentrations (10–20 μg/ml). At the same concentrations, lung-derived heparin gave an intermediate response (50% of the cell coupled) and intestinal-derived heparin gave a weak response (20–23% of the cells coupled). It will be useful to compare the relative effectiveness in the biological assays of these tissue-specific heparins with their distinctions in chemical structures to deduce the critical aspects of the structures responsible for the biological effects.

In summary, the most abundant form of GAG found in the liver, heparin, has proven the most biologically active of all of the GAGs tested at induction of gap junction expression. PGs are more active than their corresponding GAG chains. The increased potency of the PGs over their corresponding GAG chains may be due to their larger size, to their protein cores, to increased binding of the molecules to the cell surface via the protein cores or to other undefined variables.

The significance of the biological activity by lambda-carrageenan is not clear, although certainly studies of it will be useful for structure–functional analyses. At the least it suggests that the scaffolding of aminosugar–uronic acid polymer can be replaced by a polysaccharide (polygalactose). Generalizations and interpretations about structure–function relationships or about tissue specificity of the GAGs or PGs are limited due to the extremely limited availability of GAGs and PGs from multiple tissues.

The GAGs, PGs, and anionic polysaccharides showing ability to induce gap junction synthesis also cause dramatic morphological changes in the cultures. Conversely, those with no or weak activity in the gap junction assay also showed no ability to cause contraction or cell density changes in the cultures. However, the two phenomena were dissociable: there were factors (e.g., the dextran sulfates and the carrageenans) that caused marked cytoplasmic shape changes but did not cause significant increases in expression of gap junction protein. Thus, although there is currently much speculation about the effects of cytoplasmic shape on differentiation of cells (4; for review see references 35–37), these observations indicate that shape changes do not correlate strictly with gap junctional conductance changes.

Changes in the composition of GAGs and PGs in the extracellular matrix have long been shown to be associated with changes in growth and differentiation of a variety of cells (5, 7, 8, 11, 14, 18, 24, 27, 29, 30, 40). More recently, studies on cell lines and primary cultures have indicated that GAGs and PGs can have dramatic effects on attachment and morphology of cells (26, 27, 38). Furthermore, Bornstein and associates (7, 29) and Rosenberg and Karnovsky and associates (8, 11, 18, 30) have shown that addition of GAGs can cause a marked inhibition of growth in cultures of fibroblasts and of smooth muscle cells, respectively. Kawakami and Terayama (24) have shown a similar effect of a heparan sulfate proteoglycan on hepatoma cell lines, findings that complement the study by Fedarko and Conrad (14) who have demonstrated a distinct change in the chemistry of heparin produced by the cells and correlated with the state of growth of the cells. However, the data presented here are the first indicating that these matrix components are also important in regulating cell–cell communication by inducing gap junction protein synthesis and function.

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