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Heparin Inhibits the Attachment and Growth of Balb/c-3T3 Fibroblasts on Collagen Substrata

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In investigating the role of cell-extracellular matrix interactions in cell adhesion and growth control, the effects of heparin on cell-collagen interactions were examined. Exponentially growing Balb/c-3T3 fibroblasts were radiolabelled with ³H-thymidine and detached from tissue culture surfaces using EDTA, and cell attachment to various types of collagen substrata was assayed in the presence or absence of heparin or other glycosaminoglycans (GAGs) or dextran sulfate (40 K). Cells attached readily (70–90%) to films of types I and V, but not to type III collagen. The number of cells bound to types I and V collagen films was inhibited by 10–50% when heparin was present from 0.1–100 µg/ml. Cell-collagen attachment was also inhibited by dextran sulfate, and to a lesser extent by dermatan sulfate, but chondroitin sulfates A and C and hyaluronic acid showed no effect. Heparin was active even at early time points in the adhesion assay, suggesting it may disrupt cell-collagen attachment. To study the effects of heparin in modulating cell growth on collagen, growth arrested cells cultured on type I collagen films were serum stimulated in the presence of heparin or other GAGs for 3 days. Growth was inhibited (>40%) only by heparin and dextran sulfate. Interaction of heparin fragments ($M_r \leq 6$ KD) with type I collagen was analyzed by affinity co-electrophoresis (Lee and Lander, 1991) and showed higher affinity heparin binding to native as compared with denatured collagen. These data suggest that sites within native collagen may mediate Balb cell-collagen and heparin-collagen interactions, and such interactions may be relevant towards understanding heparin's antiproliferative activity in vivo and in vitro.

Heparin is a sulfated glycosaminoglycan (GAG) that possesses significant antiproliferative activities towards vascular smooth muscle cells in vivo (Clowes and Karnovsky, 1977) and in vitro (Castellot et al., 1981), and to other cell types in vitro (Lippman, 1968; Wright et al., 1985; Kardami et al., 1988). Although the exact mechanism of the action of heparin on growth remains unknown, it is possible that heparin may act intracellularly or extracellularly to affect growth related processes. For example, heparin is taken up by cells (Castellot et al., 1985), and suppresses the expression of the protooncogenes *c-fos* and *c-myc* by inhibiting a protein kinase C-dependent pathway for mitosis in fibroblasts (Wright et al., 1989) and smooth muscle cells (Pukac et al., 1990). On the other hand, heparin could also act extracellularly on growth, by interacting with various components of the extracellular matrix (ECM) known to contain heparin binding sites, including thrombospondin (Lawler and Slayter, 1981), fibronectin (Yamada et al., 1980), laminin (Sakashita et al., 1980), and certain of the collagens (Keller et al.,

1986; Tsilibary et al., 1990). Thus, heparin may affect cell growth by directly binding ECM components, or displacing endogenous heparin-like molecules from the ECM, and thereby disrupting cell-substratum attachments which are believed to be required for mitosis (Campisi and Medrano, 1983; O'Neill et al., 1986). Indeed, abundant evidence suggests a role for cell surface proteoglycans in cell adhesion (e.g., Lark and Culp, 1984; Woods et al., 1986; Gill et al., 1986). Consistent with this hypothesis, it has been reported that heparin inhibits cell-collagen attachment of mammary epithelial (Koda et al., 1985) and vascular smooth muscle cells (LeBaron et al., 1989), possibly by displacing cell surface heparan sulfate from binding sites on collagen. Alternatively, heparin may inhibit mitosis in a more indirect fashion, through effects on ECM metabolism or deposition, which may in turn affect cell

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movement and growth. In this regard, heparin has recently been shown to exert significant effects on collagen transcription (Liau and Chan, 1989), extracellular deposition (El Nabout et al., 1989), and covalent crosslinking mediated by lysyl oxidase (Gavriel and Kagan, 1988).

In the present study, we have begun to characterize the effects of heparin and other GAGs on the attachment and growth of Balb/c-3T3 fibroblasts on collagen substrata. We have also initiated a study of heparin's interactions with type I collagen. Ultimately, our goal is to determine if the binding of heparin to collagen(s) may play a role in the regulation of cell-ECM interactions of consequence to cell growth.

MATERIALS AND METHODS

Cell culture

Balb/c-3T3 fibroblasts (ATCC, Rockville, MD) were maintained in tissue culture flasks (75 mm; Falcon) at subconfluent cell densities in DMEM supplemented with 10% calf serum (Gibco), penicillin (50 U/ml), streptomycin (50 µg/ml), and glutamine (2 mM). Cultures were incubated at 37°C in a humidified tissue culture incubator in a 9% CO₂/91% air environment, and were used for experiments up to their tenth passage.

Adhesion assay

The assay used here is adapted from that of Laterra et al. (1983) and uses cells released from tissue culture substrata by EDTA exposure, not trypsin treatment, and without the presence of cycloheximide during the subsequent attachment assay. These conditions were chosen to obtain cells with relatively undisturbed cell surfaces and cell surface-associated factors which may normally play critical and complex roles during cell attachment and growth. For these experiments, exponentially growing, subconfluent cultures were fed media containing ³H-thymidine (New England Nuclear; 10 uCi/dish) for 24–48 hr. The media was removed, and the cultures were rinsed twice with CMFH, fed with fresh media, and placed into the tissue culture incubator for an additional 3 hr. The media was removed, and the cultures were rinsed twice with phosphate buffered saline without calcium and magnesium (PBS(-)), after which 10 ml PBS(-)/0.5 mM EDTA was added to each dish. The dishes were incubated at 37°C for 15 min, with constant agitation. Cells were released from the culture surface by vigorous pipetting. Cell suspensions were mixed with an equal volume of DMEM containing 2 mg/ml BSA (fraction V powder #A-7906; Sigma), and were pelleted by centrifugation at 1,000 RPM/10 min. Cell pellets were gently resuspended in 10 ml of DMEM/BSA solution, and were pelleted again, as above. The resultant pellets were resuspended and combined, cell numbers were determined using a hemacytometer, and volumes were adjusted to achieve a cell density of 0.1–0.5 × 10⁶ cells/ml. Adhesion onto collagen substrata was measured by adding 0.1 ml of the cell suspension to collagen-coated tissue culture dishes which contained 1.0 ml DMEM/BSA, plus various supplements (see below). These dishes were placed into a tissue culture incubator for various periods of time. The dishes were removed from the incubator, and

the media containing unattached cells was aspirated off and saved. Each dish was gently rinsed twice with warm phosphate buffered saline with calcium and magnesium (PBS(+)), and these rinses were pooled with the media. Cells of the media and rinse pools were pelleted by centrifugation at 1,000 RPM for 5 min in a refrigerated centrifuge, the supernatant aspirated off, and then 0.5 ml of 1% SDS/0.1 N NaOH was added to the cell pellet, as well as to the cells remaining on the culture dishes. Both fractions were incubated overnight at 37°C, followed by radioactivity determination by liquid scintillation counting. Percent adhesion onto each dish was calculated as radioactivity of the attached cell fraction divided by the total radioactivity present in the attached plus the unattached cell fractions.

Collagen substrata

Preparation of collagen films was modified from that of Kleinman (1982). Collagens were solubilized at 1.0 mg/ml in 0.5 N acetic acid, with very gentle stirring overnight at 4°C. Plastic bacteriological dishes (35 mm; Falcon) received 1.0 ml of DMEM, to which was added 0.1 ml of the above collagen solution, with mixing. The acetic acid was neutralized by the addition of 0.5 N NaOH solution. The dishes remained at room temperature for no longer than 20 min, and the liquid was aspirated off, followed by two rinses with PBS(+). When adhesion onto denatured collagen was studied, collagen in 0.5 N acetic acid was incubated at 50°C for 1 hr, before coating of the dishes, as above. In some experiments adhesion assays were performed on bacteriological plastic, 96-well flat bottom microtiter dishes (#9017; Costar, Cambridge, MA), with 0.05 ml collagen solution used to coat each well. The adhesion assays were begun immediately, before drying of the collagen films.

Efficiency of collagen coating

To measure the amount of collagen coated onto 35 mm bacteriological dishes, radioiodinated type I collagen was first prepared. A 21 µl aliquot of collagen (dissolved at 2.4 mg/ml in 0.2 N acetic acid) was added to a glass tube pre-coated with 18 µg iodogen, and containing 100 µl of 0.25 M phosphate buffer (pH 7.5). Then 1.5 mCi of Na¹²⁵I (carrier free; NEN) was added to the sample and allowed to incubate for 5 min at room temperature with intermittent agitation. The collagen was separated from unincorporated isotope by gel filtration over a G-25 column, equilibrated, and eluted with 0.5 N acetic acid. The specific activity of incorporation was determined to be 20 µCi/µg, or about two atoms of ¹²⁵I per native collagen molecule. This radio-labelled collagen was diluted 1:100 with native unlabelled collagen in 0.5 N acetic acid, and following the coating and rinsing of 35 mm dishes with the collagen (see above), the collagen remaining on the dishes was removed with 0.5 N NaOH/1% SDS, and CPMs were determined by liquid scintillation counting.

Isolation of type I collagen

Ten Long Evans male rats were sacrificed at day 35, and their tails were removed and stored at -70°C until use. After thawing, the tails were rinsed in 70%

ethanol, and the skin was dissected away. Tail tendons were removed, rinsed three times in 50 ml of distilled water, and extracted in 300 ml of 0.5 N acetic acid for 2 days at 4°C with stirring. All of the following procedures were conducted at 4°C. The material was clarified by centrifugation at 30,000g/1hr, and the collagen was precipitated from the supernatant by the addition of 7.5% NaCl, with stirring overnight. The collagen was pelleted by centrifugation at 30,000g/1 hr, and redissolved in 0.5 N acetic acid overnight, as before. The collagen was precipitated with NaCl and redissolved in acetic acid twice more, as described above. The acid soluble collagen was centrifuged at 50,000g/1 hr, and the clear supernatant was dialyzed against 12 L distilled water overnight, and lyophilized, with the final yield of collagen being 0.32 g.

Cell growth assays

In some experiments, the effects of GAGs on cell growth on collagen substrata were examined. For this purpose, bacteriological culture dishes were collagen-coated using sterile solutions, and the dishes were placed under ultraviolet light for 15 min, before their use. Balb/c-3T3 cells were trypsinized from subconfluent cultures and were plated at subconfluent densities onto the collagen-coated dishes in 1.5 ml media. After tissue culture incubation overnight, cultures were growth arrested by the addition of 1.5 ml DMEM containing 0.3% calf serum and were placed back into the incubator for 3 days. The media was then removed, and cells were fed DMEM containing 10% calf serum, with or without various GAG supplements, and were placed back into the incubator. Three-and-a-half days later, cell numbers were determined as follows. Culture dishes were rinsed twice with Ca^{2+} - Mg^{2+} free Hanks solution, and 1.0 ml of collagenase (1 mg/ml; Worthington) in Hanks Hepes was added to each dish, followed by a 1 hr incubation at 37°C. At this time, a single cell suspension could be generated by vigorous pipetting of the solution in the culture dish. Cell numbers were determined using a Coulter counter.

GAGs

Heparin (porcine intestinal mucosa; $M_r = 16$ K) was from Choay Institute, Paris; chondroitin sulfates B (dermatan sulfate) and C (from porcine skin and shark cartilage, respectively), hyaluronic acid (type III; human placenta), and dextran sulfate ($M_r = 40$ K) were from Sigma (St. Louis, MO). Polysaccharides were suspended in distilled water at 100× concentrations and were used directly in the adhesion studies. In the cell growth studies, the supplements were added to the culture media, and these solutions were filter sterilized before use.

Collagens

Collagen types I (rat tail tendon), III, and V (human placenta) were purchased from Sigma, and type I collagen (rat tail tendon) was isolated in our laboratory (see above). Collagen purity was assessed by SDS-PAGE (4% separating/6% stacking) and Coomassie Blue staining.

Affinity co-electrophoresis

The interaction between low molecular weight heparin fragments and type I collagen was analyzed by the affinity co-electrophoresis (ACE) technique, recently developed by Lee and Lander (1991). Fluorescein derivatization and iodination of heparin (porcine intestinal mucosa; Sigma), isolation of heparin chains of $\leq 6,000 M_r$, and the electrophoresis of the heparin fragments through agarose wells each containing one in a series of concentrations of protein was performed as described (Lee and Lander, 1991). For these experiments the collagen (rat tail tendon collagen isolated in our laboratory) was dissolved in 0.5 N acetic acid at 2.4 mg/ml, followed by serial dilution of the samples into 0.5 N acetic acid. Native collagen and those samples denatured by subsequent incubation at 50°C/1hr were neutralized by the addition of an equal volume of 0.5 N NaOH immediately before mixing of the samples with warm 2× concentrated electrophoresis running buffer and agarose, and subsequent pouring of the agarose wells. After electrophoresis the gels were dried and the heparin migration front was visualized by autoradiography.

RESULTS

Adhesion to collagen substrata

To test the possibility that heparin inhibits growth by interfering with cell-substratum interactions, we decided first to examine the effects of this GAG on the adhesion of Balb/c-3T3 fibroblasts to collagen, because of the importance of collagen in cell adhesion and growth (see Kleinman et al., 1981, for review), and because several of the collagens are known to contain specific heparin or heparan sulfate proteoglycan binding sites (Stamatoglou and Keller, 1982; Keller et al., 1986; Koda et al., 1985). Thus, exponentially growing fibroblasts were detached from the culture surface, then added back to culture dishes coated with type I collagen, in the presence or absence of heparin (100 $\mu\text{g/ml}$), and adhesion was examined as a function of time. It was found that Balb cells adhered to the collagen in a time-dependent fashion, and that in the presence of heparin, cell attachment was significantly inhibited at all time points (Fig. 1). Because a similar effect was detected at all time points in the subsequent experiments cell adhesion to purified collagens was studied at 60–90 min.

Collagen specificity

Balb/c-3T3 fibroblasts adhered well to substrata of collagen types I or V, with at least 60–70% of the total cells attached within 60–90 min (Fig. 2). Heparin inhibited the attachment of fibroblasts to type I collagen by about 40–50%, and to type V collagen by about 90% (Fig. 3). Substrata of denatured type I collagen or native collagen type III did not promote cell adhesion, with only 35% and 23% of total added cells adherent at 60 min, and no effect of heparin was observed on cell attachment to either of these substrata (Figs. 2, 3). In some experiments (see below), the effects of heparin on cell adhesion to type I collagen were variable, but heparin when present at 100 $\mu\text{g/ml}$ consistently showed an inhibition of adhesion to this substrata by $\geq 40\%$.

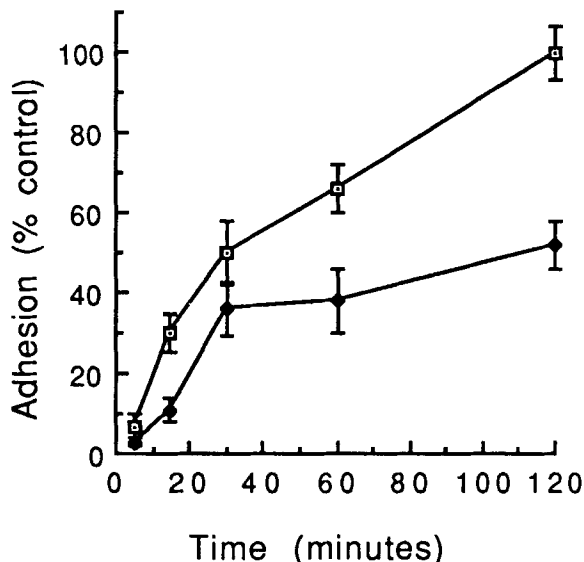


Fig. 1. Effect of heparin on fibroblast adhesion to type I collagen. Cells were detached from the tissue culture substrata, and their adhesion to type I collagen films was measured in media in the absence (open squares) or presence (closed diamonds) of heparin at 100 µg/ml. Here as in other figure legends, percent adhesion denotes total radioactivity of the attached cell fraction divided by the total radioactivity present in the attached plus unattached cell fractions. At all time points, heparin showed a significant inhibition of adhesion ($P < .05$).

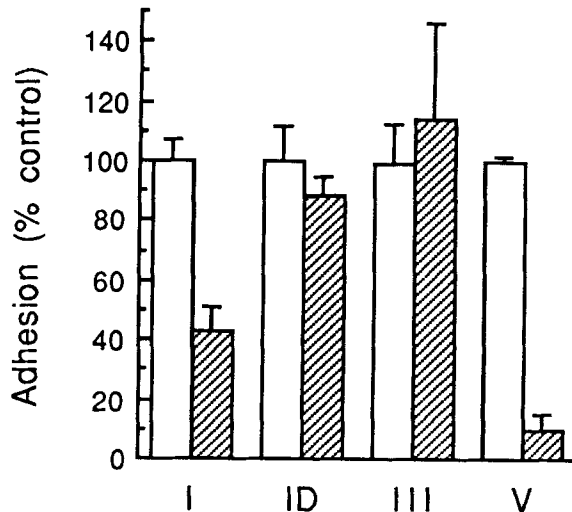


Fig. 3. Effect of heparin on fibroblast adhesion to native (I) or denatured (ID) collagen type I, III (III), and V (V). Suspended fibroblasts were added to collagen films in media in the absence or presence of heparin (100 µg/ml), and adhesion was measured after 60 min. The percent of total adhesion in media without heparin (open bars) and in heparin containing media (striped bars) was determined, using the values for control adhesion on the various substrata as 100% in each case (see Fig. 2.) Heparin significantly inhibited cell attachment to native collagen types I and V ($P < .01$).

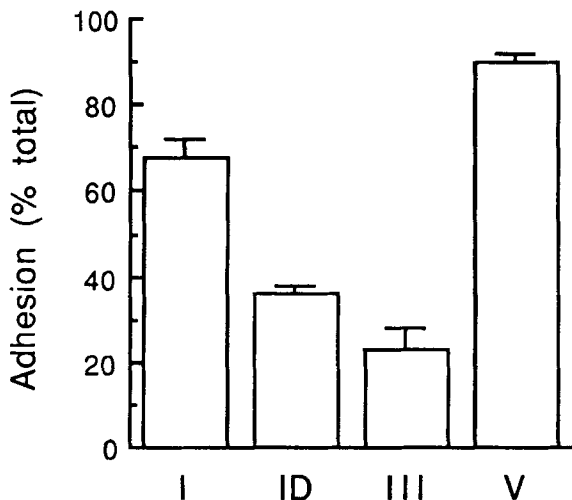


Fig. 2. Fibroblast adhesion to collagen films. Cells were detached from the tissue culture substrata, and their adhesion to films of native (I) or denatured (ID) type I, or native type III (III) or V (V) collagen was measured at 60 min, cell adhesion is expressed here as percentage of total cells adherent.

Collagen purity

The purity of the collagen samples used in this study was assessed by SDS-PAGE electrophoresis. All of the samples displayed their characteristic collagen chain compositions, and insignificant amounts of contaminating proteins (Fig. 4). The rat tail tendon collagen

isolated in our lab showed significantly less contamination as compared with the sample of type I collagen obtained from a commercial source, although both samples proved to be equally effective in promoting fibroblast attachment.

Efficiency of collagen coating

The amount of type I collagen coated onto the bacteriological plastic dishes was measured using radioiodinated collagen as a tracer. It was found that of the 100 µg of collagen added to the dishes during coating, about 400 ng/cm² remained on the dish at the beginning of the adhesion assay. For the denatured collagen, significantly less remained on the dish (100 ng/cm²), and consistent with this fact, these dishes did not appear to have the same wettable surface as observed on dishes coated with native collagen or collagens type III or V. Thus, it is possible that the poor cell attachment observed on denatured collagen substrata (see above) could be due to insufficient amounts of protein coated onto the substrata. It was also found that a 1 hr exposure of the native collagen film to heparin (100 µg/ml) did not displace collagen from the substratum.

Heparin dose response

To determine the concentration of heparin required to exert the maximal inhibition of Balb adhesion, we measured adhesion as a function of heparin dose (Fig. 5). Even at a dose of 1.0 µg/ml, heparin showed a significant effect on cell adhesion onto type I collagen, with the maximal inhibition of cell adhesion observed at heparin doses of >50 µg/ml.

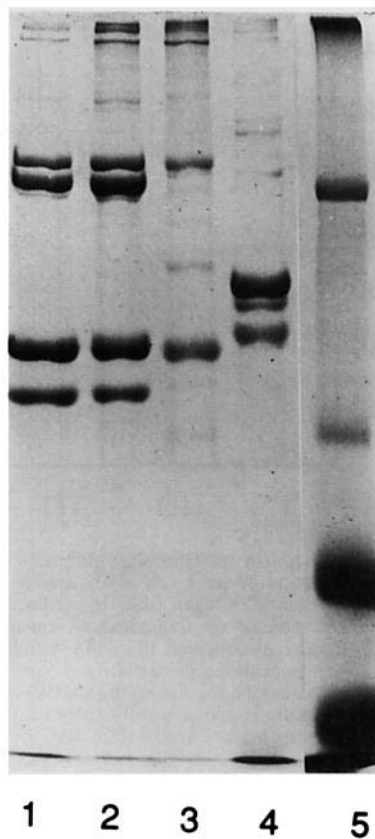


Fig. 4. The collagens used in the present study were subjected to SDS-PAGE (6% separating gel) and visualized by Coomassie blue staining. **Lane 1:** Rat tail type I collagen isolated in our lab; other collagens were from Sigma. **Lane 2:** Rat tail type I. **Lane 3:** human placenta type III. **Lane 4:** human placenta type V. **Lane 5:** M_r markers of 205, 117, 77, and 47 KD.

GAG specificity

The specificity of the heparin effect on adhesion was studied by assaying the effects of various other GAGs, and the highly sulfated synthetic polysaccharide, dextran sulfate (40 K), on the adhesion of fibroblasts to type I collagen (Fig. 6A). Only dextran sulfate inhibited adhesion to a similar extent as heparin (52 and 49% inhibition, respectively). Chondroitin sulfate type B (dermatan sulfate) showed a significant but lesser degree of inhibition (30%). The other GAGs tested had no effects.

Growth on collagen substrata

Since we were interested in the relationship between the effects of heparin on cell-collagen interactions and growth, we examined the activities of GAGs on the growth of cells cultured on type I collagen. In these experiments cells were plated onto type I collagen films, serum starved for 3 days, and then given the various GAG supplements in normal growth media containing 10% calf serum. Cell numbers were assayed 3.5 days later. It was found that heparin inhibited growth on collagen films by nearly 50%, and that none

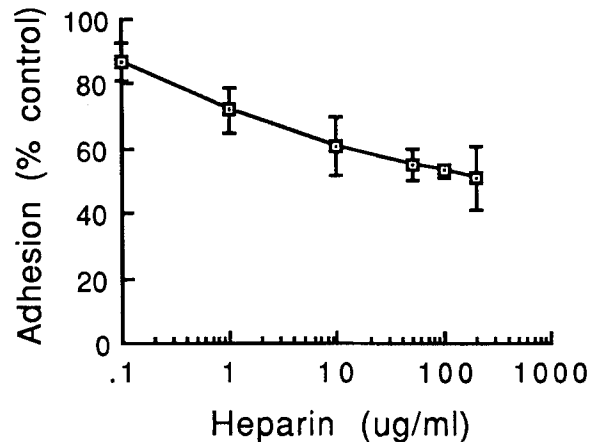


Fig. 5. Dose dependence of the heparin effect on fibroblast adhesion to collagen type I. Suspended fibroblasts were added to collagen films in media without heparin, or media containing heparin (0.1–200 $\mu\text{g/ml}$), and adhesion was measured after 60 min. At heparin doses of $\geq 1 \mu\text{g/ml}$, cell adhesion was inhibited as compared with the controls ($P < .05$).

of the other GAGs exerted significant effects (Fig. 6B). Dextran sulfate also inhibited the growth of fibroblasts on collagen, to a similar extent as did heparin.

Heparin-collagen interactions

The finding that native type I collagen supported significant cell-collagen attachment, and that this attachment was inhibited by heparin (see above), suggested the involvement of native collagen in mediating cell adhesion, and at least one aspect of collagen-heparin binding. Thus, as a first step towards understanding the nature of the heparin-collagen interactions of possible relevance to cell attachment and growth on collagen, we have begun to study heparin-collagen binding by the technique of affinity co-electrophoresis (ACE), recently developed by Lee and Lander (1991). Radioiodinated heparin fragments (≤ 6 KD) were subjected to electrophoresis through agarose wells each containing one in a series of collagen concentrations. After electrophoresis, the degree of retardation of heparin at the various protein concentrations was used to estimate the K_d of heparin-collagen binding. In such electrophoretograms, the binding K_d can be estimated by the protein concentration at which the electrophoresed molecule (i.e., heparin) is half maximally shifted from being totally retarded at high protein concentrations, to fully mobile at low protein concentrations. To analyze the gel of the native collagen, for example (Fig. 7), note that in wells containing collagen at a concentration of 100–1,000 nM, all of the heparin within the wells is retarded (for comparison, note that the labeled material passing between the wells marks the mobility of free heparin). At collagen concentrations of 5–25 nM, a shift in heparin mobility occurs, with the K_d falling somewhere in this range. At the collagen concentration of 1 nM, practically all of the heparin is fully mobile. By such analysis it was found that heparin has a higher affinity to native

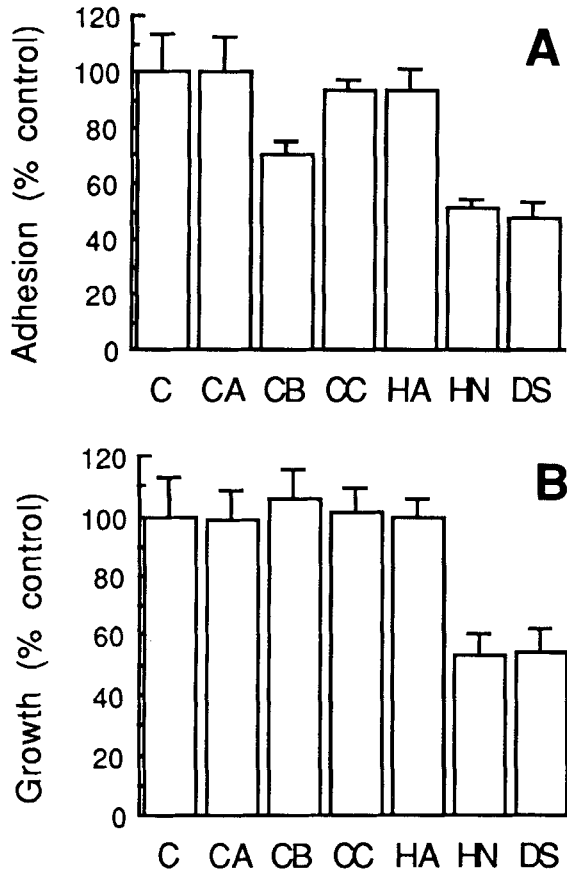


Fig. 6. **A:** Effects of GAGs and dextran sulfate on fibroblast adhesion to type I collagen. Suspended fibroblasts were added to collagen films in the presence of media without supplements, or media containing the various polysaccharides (100 μ g/ml), and adhesion was measured after 60 min. Adhesion was inhibited by heparin, dextran sulfate, and chondroitin sulfate type B ($P < .05$). **B:** Effects of glycosaminoglycans and dextran sulfate on the growth of fibroblasts cultured on type I collagen films. Cells were cultured on type I collagen films, growth arrested by serum starvation, then were released from growth arrest and fed media with or without the various polysaccharides. Final cell numbers were determined after 3.5 days. Chondroitin sulfate types A, B, and C, CA, CB, and CC; control, C; dextran sulfate, DS; heparin, HN; hyaluronic acid, HA. Growth was inhibited by heparin and dextran sulfate ($P < .05$).

(5 nM $< K_d < 25$ nM) as compared with denatured (50 nM $< K_d < 250$ nM) type I collagen (Fig. 7). In both gels the heparin front shifts not as a discrete band, but rather exhibits a broad range of mobilities at collagen concentrations near the value of K_d . A similar effect has been seen in the analysis of heparin-fibronectin binding by ACE and results from the fact that heparin molecules are not homogenous but exhibit a wide range (about twentyfold) of affinities for fibronectin (Lee and Lander, 1991). We have recently confirmed that the electrophoretic behavior in Figure 7 results from heterogeneity in heparin's affinity for type I collagen (unpublished observations).

DISCUSSION

In the present study, we have begun to characterize cell-collagen interactions with respect to cell-substra-

tum attachment and growth control, and the effects of the GAG heparin on these processes. It was found that Balb/c-3T3 fibroblasts attach readily to substrata of native type I and V collagens, but not to native type III collagen. These data indicate that there is a collagen-type specificity involved in the binding of fibroblasts to collagen, and that cell-collagen interactions (as mediated by a plasma membrane receptor or cell surface-associated ECM molecule such as fibronectin or proteoglycans) may depend upon the presence of determinant(s) in the native collagen triple helix. Indeed, it has been shown that the attachment of a variety of cell types to collagens shows a clearly defined collagen-type specificity (Kleinman et al., 1981). Furthermore, other workers have demonstrated that the native collagen triple helix is required for mammary epithelial (Koda et al., 1985) and smooth muscle (LeBaron et al., 1989) attachment to type I and V collagens, respectively, and for integrin receptor-mediated binding to type I collagen (Dedhar et al., 1987).

Here it was demonstrated that heparin significantly inhibits the attachment of Balb/c-3T3 fibroblasts to type I and V native collagens, but did not affect the limited adhesion that was observed on type III collagen. With respect to the types I and V collagen data, these results indicate that the activity of heparin on cell-collagen attachment, like the process of cell-collagen attachment itself, may require the presence of determinant(s) in specific native collagen molecules. Interestingly, most of the fibril forming interstitial collagens including types I, II, III, and V have been shown to bind heparin and heparin-like molecules (Stamatoglou and Keller, 1982; Koda et al., 1985; Keller et al., 1986; LeBaron et al., 1989), although the specific structural identity of the GAG binding sites, and the physiological function of such binding, remains unknown. Of great relevance is the previous demonstration that heparin binds to a native type I collagen affinity column, but did not appreciably bind to denatured collagen (Keller et al., 1986). These authors also showed that the c-terminal fragment of type I collagen, as generated by vertebrate collagenase digestion, contains the most significant heparin binding activity. It has been shown that the ectodomain of a heparan sulfate proteoglycan binds to native type I collagen with an apparent K_d of 1 nM, and was displaceable by heparin, but that the ectodomain did not bind significantly to denatured collagen (Koda et al., 1985). In the present study we have measured the affinity of heparin-collagen binding by affinity co-electrophoresis, and report that native type I collagen binds heparin with a higher average affinity ($K_d = 5-25$ nM) as compared with denatured collagen ($K_d = 50-250$ nM). It is interesting to compare the measured heparin-collagen affinity to the concentration-dependence of heparin inhibition of cell attachment to collagen substrata. Assuming a single binding site on low molecular weight heparin for collagen, the measured affinity of 5-25 nM implies that small amounts of collagen will be half-saturated with heparin at heparin concentrations of 0.3-0.15 μ g/ml. Half-maximal inhibition of cell attachment, in contrast, required about 2 μ g/ml of heparin. The difference between these two values could be explained in several ways. First, the amount of

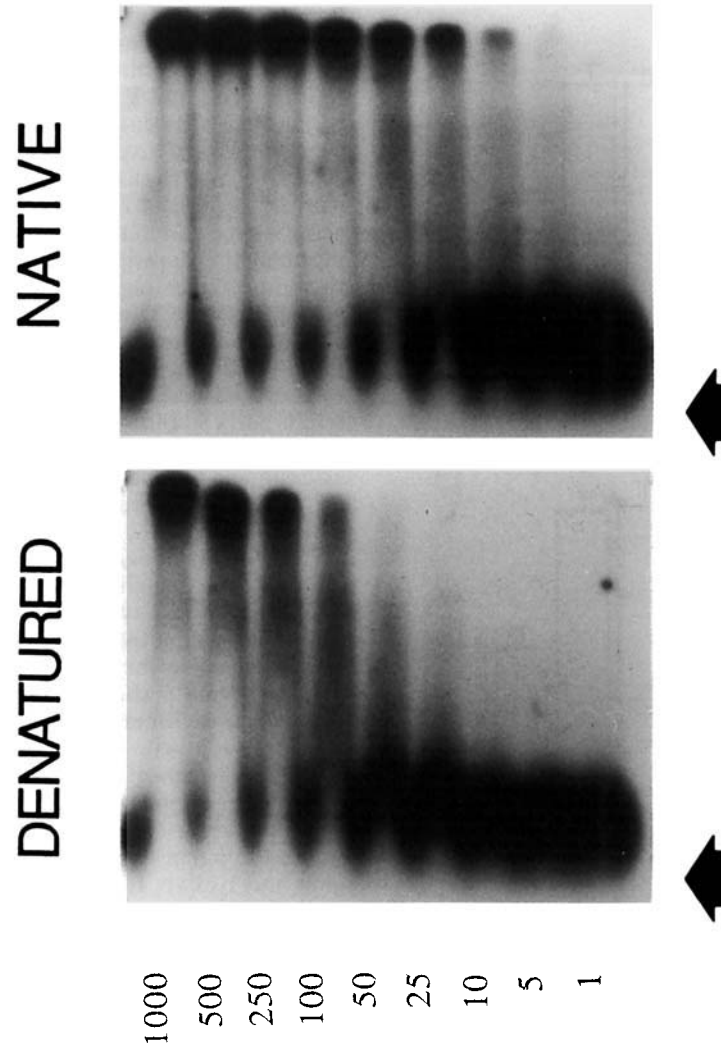


Fig. 7. Affinity co-electrophoretic analysis of the interactions between low molecular weight heparin fragments and type I collagen. Radioiodinated heparin fragments ($M_r \leq 6$ KD) were subjected to electrophoresis through agarose wells, each containing one in a series of concentrations of native or denatured collagen. Concentrations of collagen are in nM; note that for these calculations the M_r of fully processed type I collagen was taken to be 300 KD, which is an

approximation of the actual M_r (see Bornstein and Sage, 1980, and Butkowski et al., 1982, for a discussion of type I collagen M_r determinations). Heparin showed higher affinity binding to native collagen ($5 \text{ nM} < K_d < 25 \text{ nM}$) as compared with denatured collagen ($50 \text{ nM} < K_d < 250 \text{ nM}$). The upper margin on each photograph represents the origin, and the forward boundary of the free heparin front is indicated (arrowheads).

collagen adsorbed onto the substratum in this assay may be far in excess of the amount required to permit cell adhesion; if so, blocking adhesion might require achieving near full saturation of collagen with heparin. Consistent with this possibility it has been reported that as little as $0.1 \mu\text{g}$ of collagen (about forty times less than used here) supported nearly maximal attachment of Chinese hamster ovary cells (Kleinman et al., 1979). Alternatively, it may be the case that substratum-bound collagen binds heparin more weakly than collagen in solution. A third possibility is that there are subpopulations of heparin with significant collagen binding activities which are not relevant to cell attachment. Finally, if heparin acts on attachment by displacing a cell surface heparan sulfate proteoglycan from a binding site on collagen, and if this cell surface-collagen interaction involves cooperative binding

events of much greater efficiency, and with higher affinities than that of heparin-collagen binding, then very large amounts of exogenous heparin may be required to inhibit cell-collagen attachment.

In addition to the importance of collagen type specificity in mediating Balb-collagen attachment, the heparin effect on this attachment was also shown to be specific in that only two other of the tested polysaccharides were active. The synthetic polysaccharide dextran sulfate was equal to heparin in its inhibitory activity, and dermatan sulfate also inhibited adhesion, but to a lesser extent. Possibly the GAG-collagen interaction responsible for a disruption of adhesion requires a significant conformational flexibility of the polysaccharide, which is thought only to be conferred upon the GAGs containing sulfated iduronic acid such as heparin and dermatan sulfate (Casu et al., 1988). Dextran

sulfate is a polymer of D-glucose residues, containing up to three sulfate groups per glucose. This polysaccharide also mimicks the activity of heparin in several other biological systems (LeBaron et al., 1989; San Antonio et al., 1987; Bernfield et al., 1984) possibly because its randomly placed sulfate moieties and flexibility may form sequences within the polymer with GAG-like properties, at least in terms of their protein binding and functional activities. The effects of heparin and other polysaccharides on the growth of Balb cells cultured on type I collagen was studied, with the finding that the two agents with the greatest effect on attachment, heparin and dextran sulfate, were also potent inhibitors of growth. However, based on the fact that dermatan sulfate was not antiproliferative, but was inhibitory to cell-collagen adhesion, no conclusions at present can be made concerning the possible relationship between the activities of GAGs on cell attachment and growth on collagen substrata. Finally, the inhibitory activity of heparin on the growth of Balb fibroblasts on collagen was not remarkably different from that observed when these cells were grown on a tissue culture plastic substrata (unpublished observation). This latter observation could argue for the possibility that the effects of heparin on growth are independent of the culture substrata used. On the other hand, even with cells grown on plastic, heparin may act through an extracellular matrix which is cell surface-associated or deposited on the substrata by the fibroblast.

Heparin was found to inhibit cell adhesion even at early times during the adhesion assay, when rounded cells were settling onto the collagen film, and before they had formed extensions and spread onto the substrata. This finding suggested that heparin inhibits cell-substratum attachment. The fact that cell-collagen attachment was inhibited by no more than 40–50% even at high doses of heparin indicates that the cell population used in the present study may be heterogeneous with respect to the expression of specific cell-substratum adhesion molecules. Additionally, it is possible that individual cells may express an attachment mechanism which contains heparin sensitive and insensitive components. The former could be mediated by a cell surface proteoglycan, as has been shown for cell-collagen adhesion of mammary epithelial cells (Koda et al., 1985); possibly exogenous heparin interferes with the binding of the cell surface proteoglycan to GAG binding site(s) on type I collagen. Similarly, heparin could interfere with known binding interactions between cell surface-associated fibronectin and collagen (Engvall and Ruoslahti, 1977; Kleinman and McGoodwin, 1976; Klebe, 1974). The heparin insensitive attachment could be mediated by members of the integrin family of receptors, which are thought to be responsible for cell binding to types I and IV collagens (Dedhar et al., 1987; Kramer and Marks, 1989). Indeed, the binding of mammary epithelial cells to fibronectin can be mediated by a cell surface proteoglycan, or by a cell surface receptor with integrin-like characteristics (Saunders and Bernfield, 1988). Clearly, however, there may be some overlap in the activity of heparin on the mechanisms involved in fibroblast-collagen attachment. For example, heparin could specifically inhibit

cell surface proteoglycan binding to collagen by interacting with GAG binding site(s) on collagen, but also could disrupt integrin-collagen binding in adjacent sites by steric hindrance, and electrostatic repulsion by virtue of its size and high charge density. Such a model was previously suggested to explain the inhibition of tumor cell attachment to fibronectin by a chondroitin sulfate proteoglycan (Brennan et al., 1983). Further work must be done to better define the role of heparin and heparan sulfates in fibroblast-collagen adhesion, and to determine how such a GAG-dependent adhesion mechanism may relate to growth.

NOTE ADDED IN PROOF

We (J.S.A., M.J.K., and A.D.L.) have recently observed that labeling heparin by an alternative procedure, involving radioiodination of a tyramine group coupled to the polysaccharide's reducing end, results in heparin that binds type I collagen with a somewhat lower affinity than that observed in Figure 7. A detailed analysis of this effect will be presented in a study in preparation for submission.

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