Heparin Modulates the Organization of Hydrated Collagen Gels and Inhibits Gel Contraction by Fibroblasts

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Abstract. We studied the effects of extracellular matrix components on fibroblast contraction of hydrated collagen gels. After 4-h incubations, heparin-containing collagen gels contracted only 10% compared with 50% contraction of control gels. Contraction was not affected by hyaluronic acid, dermatan sulfate, or fibronectin, implying that the activity of heparin was specific. The possibility that heparin inhibited attachment of the cells to the gels was ruled out. Also, addition of heparin to the incubation medium had no effect on contraction. Microscopic examination showed that control collagen gels were composed of a uniform network of interlocking fibrils of similar sizes. Heparin-containing gels, on the other hand, were highly variable with some collagen bundles containing 5–6 collagen fibrils and other regions containing amorphous material. Unlike the control gels, the fibrils of heparin-containing gels were not continuously interconnected. Based on the results, we propose that fibroblasts attach normally to the collagen fibrils of heparin-containing gels and attempt to contract the gels, but the mechanical forces exerted by fibroblasts on individual collagen fibrils cannot be propagated throughout the gels.

FIBROBLASTS cultured in hydrated collagen gels were shown to acquire a bipolar morphology and cytoskeletal organization typical of fibroblasts in vivo, but quite distinct from fibroblasts cultured on glass or plastic surfaces (8, 25). During culture, the cells contracted the collagen gels (1, 23) and reorganized the collagen fibrils into a dermal-like tissue (2) or into structures resembling ligaments and tendons (3, 24). Artificial dermis produced by this method has been combined with epidermal cells into a skin equivalent and used for skin grafting (16).

Because fibroblast reorganization of hydrated collagen gels is an in vitro process that leads to the development of in vivo–like tissue, there has been considerable interest in this system as a model in which to study fibrosis, connective tissue organization, and wound repair. Consequently, we started to investigate the mechanism by which the collagen gels are contracted by fibroblasts. We found that gel contraction did not involve collagen synthesis, degradation, or covalent cross-linking (14). At first, collagen fibrils were bound individually and in clusters at the cell surface and were surrounded by cellular microvilli and blebs (12). Subsequently, rearrangement of the fibrils occurred in two steps. There was an actin cytoskeleton-dependent, mechanical rearrangement of the fibrils, and the rearranged fibrils were stabilized in place by the cells. Later, changes in the intermolecular bonding between collagen fibrils led to fibril stabilization by noncovalent collagen–collagen interactions, and the cells no longer were necessary for maintenance of this state (14, 15).

One conclusion of our previous studies was that components secreted by cells, e.g., fibronectin, proteoglycans, were unnecessary for contraction of the collagen gels (15). It was possible, however, that these noncollagenous connective tissue components might modulate gel contraction. Such modulation could play an important role during dermal remodeling when there are major changes in the matrix components present. For instance, during cutaneous wound repair, high levels of hyaluronate can be found in the matrix initially, but later on proteoglycans containing sulfated glycosaminoglycans, such as dermatan sulfate, become predominant (4). High levels of fibronectin can be found in the wound region associated at first with the plasma clot and later on with type III collagen in granulation tissue (10). Heparin, on the other hand, has been reported to be elevated in fibrotic and scar tissues (5). Consequently, it was of interest to determine if these noncollagenous matrix components affected collagen gel contraction by fibroblasts. To make this determination, gels were prepared by polymerizing collagen in the presence of hyaluronic acid, dermatan sulfate, heparin, or fibronectin, and the ability of fibroblasts to contract the gels was measured. The results of these studies are reported herein.

Materials and Methods

Cells and Other Materials

Human skin fibroblast cultures were established from foreskins obtained at circumcisions. Cells were cultured in 75 cm² tissue culture flasks (Falcon Labware, Oxnard, CA) using growth medium composed of DME (GIBCO, Grand Island, NY), 20 mM Hepes buffer, 1% penicillin/streptocyin/Fungi-

sone (GIBCO), 50 µg/ml gentamicin (GIBCO), and 20% FBS (GIBCO). The cultures were incubated in a humidified incubator containing an atmosphere of 5% CO_2 and 95% air. Fibroblast cultures were harvested using 0.025% trypsin/0.02% EDTA solution (GIBCO). Cultures were discarded before the 15th passage.

Human plasma fibronectin was obtained from the New York Blood Center. ^{3}H-Fibronectin was prepared by reductive alkylation as described previously (11). The specific radioactivity was 11,000 cpm/µg. Heparin (porcine, grade I, 170 U/ml), dermatan-sulfate (porcine, chondroitin-sulfate B), and hyaluronic acid (human, grade 1) were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of Hydrated Collagen Gels

Hydrated collagen gels were prepared as before (12, 14). Briefly, Vitrogen 100 collagen (Collagen Corp., Palo Alto, CA) was adjusted to physiological ionic strength and pH with 10× PBS (1.5 M NaCl, 0.1 M sodium phosphate, pH 7.2) and 0.1 M NaOH while maintained at 4°C. The final collagen concentration, unless indicated otherwise, was 1.5 mg/ml. Noncollagenous extracellular matrix components dissolved in 1× PBS were added at the concentrations indicated. Aliquots (0.2 ml) of the neutralized collagen and collagen/matrix component solutions were placed in 35-mm tissue culture dishes (Falcon Plastics, Cockeysville, MD) and occupied an area outlined by a 12-mm diam. circular score. Gels were polymerized by raising the temperature to 37°C and incubating the samples for 60 min.

Measurement of Matrix Component Incorporation into Collagen Gels

Polymerized collagen gels containing extracellular matrix components were washed by incubating the gels with 3 ml of PBS for 24 h. The amounts of heparin, hyaluronic acid, and dermatan sulfate that remained associated with the washed gels were measured using the carbazole procedure (6). Briefly, the gels were dissolved in 1 ml concentrated sulfuric acid containing 0.025 M Na tetraborate. The samples were boiled 10 min and cooled. Then 0.2 ml of ethanol containing 0.125% carbazole were added and the samples were boiled an additional 15 min and cooled again. Adsorbancy of the samples was determined at 530 nm and compared with standard curves obtained using known amounts of the same matrix components. Blank samples contained collagen gels but no added matrix components.

The amount of fibronectin that remained associated with washed gels was measured directly using radiolabeled fibronectin. Samples were dissolved in 1% SDS, mixed with 10 ml Budget Solve (Research Products International Corp., Elk Grove Village, IL), and counted in a Nuclear Chicago Mark II scintillation spectrophotometer.

Measurement of Gel Contraction

Cells (5 × 10^{4}) in 0.1 ml growth medium supplemented with 10% FBS were placed on top of the polymerized gels and incubated for 30 min at 37°C. During this time the cells attached to the gels. Then, an additional 3 ml of medium was added, which completely covered the gels. Since the gels were attached to the underlying culture dishes, their contraction by fibroblasts resulted in a decrease in gel thickness with no change in gel diameter (12). Gel thickness was measured on a Zeiss Invertoscope D equipped with a Mitutoyo dial test indicator (0.01–100 mm) as described previously (14). The plane of focus was adjusted from the bottom to the top of the gels, and the distance of stage movement recorded from the dial test indicator. This method was found to be reproducible to 0.02 mm. A 50% decrease in gel thickness measured microscopically corresponds to a 50% decrease in gel volume measured by retention of [3]H$_2$O. Numerical data presented are the averages of duplicate experiments, and the variation between duplicates was <4%.

Microscopy

Transmission and scanning electron microscopy were performed as before (12). Briefly, samples for transmission electron microscopy were fixed for 2 h at 22°C with 2% glutaraldehyde, 1% paraformaldehyde, and 1% tannic acid in 0.1 M Na cacodylate (pH 7.4), postfixed for 30 min at 22°C with 1% aqueous OsO$_4$, and stained en bloc with 1% aqueous uranyl acetate for 30 min at 4°C. After dehydration, specimens were embedded in Epon 812. Thick sections were stained with 1% toluidine blue and examined and photographed using a Zeiss Photomicroscope III. Thin sections were observed and photographed with a Philips 300 electron microscope. Samples for

Table I. Retention of Extracellular Matrix Components Copolymerized with Collagen in Hydrated Gels*

Extracellular matrix component	Amount added	Amount retained
	mg/ml	mg/ml
Hyaluronic acid	1.0	0.52
	0.1	0.065
Dermatan sulfate	1.0	0.19
	0.1	0.022
Heparin	0.1	0.055
	0.05	0.034
Fibronectin	0.1	0.057
	0.05	0.026

* Collagen solutions at 4°C were brought to physiological ionic strength and pH and mixed with noncollagenous matrix components dissolved in PBS. The mixtures, which contained 1.5 mg/ml collagen and matrix components as indicated, were polymerized by raising the temperature to 37°C and incubating the samples for 60 min. Subsequently, the gels were washed with a 15-fold excess of PBS for 24 h after which the amount of noncollagenous matrix components remaining in the gels was measured. Other details are described in Materials and Methods.

scanning electron microscopy were fixed and postfixed as above except tannic acid was omitted. Dehydration was accomplished with ethanol and critical point drying was performed with liquid CO_2. Specimens were coated with ~20 nm of gold/palladium using a sputter coater and examined and photographed with a JEOL JSM-840 I scanning electron microscope.

Results

Effects of Extracellular Matrix Components on Collagen Gel Contraction

Preliminary experiments were performed to determine the concentration range of extracellular matrix components that could be used to prepare modified gels. Collagen solutions (1.5 mg/ml) at physiological ionic strength and pH and at 37°C polymerized rapidly in the presence of 1 mg/ml hyaluronic acid or dermatan sulfate. Similar concentrations of heparin or fibronectin, however, inhibited gel polymerization, an observation that is consistent with previous studies (19, 22, 27). In the presence of 0.1 mg/ml heparin or fibronectin, gels were able to form. Therefore, experiments with heparin and fibronectin were carried out with lower concentrations of matrix components than experiments with hyaluronic acid and dermatan sulfate.

Quantitative measurements showed that at the concentrations tested, the amounts of matrix components incorporated into the gels were proportional to the amounts added (Table I). Since the 0.2-ml gels were washed with 3 ml of medium, we expected that only 6% of the added components would have been found in the gels if the added components were free to diffuse into the medium. Significantly, however, more than half of the added hyaluronic acid, heparin, and fibronectin, and ~20% of the added dermatan sulfate remained with the gels after washing.

Control collagen gels and collagen gels copolymerized with matrix components were incubated with fibroblasts. After 4 h, the control gels contracted by 50% (Fig. 1). Gels prepared in the presence of dermatan sulfate, hyaluronic acid, or fibronectin contracted at the same rate as control gels. There was, however, a marked inhibition of contraction with heparin-containing gels. Gels polymerized in the presence of
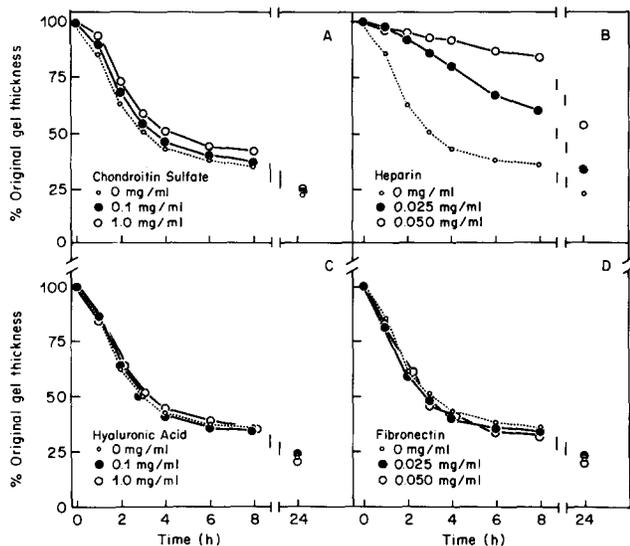
Figure 1. Contraction of gels containing collagen copolymerized with other extracellular matrix components. Gels containing collagen and extracellular matrix components as indicated were incubated with fibroblasts. The gel thickness was measured at the times specified. Other details are in Materials and Methods.

0.025 mg/ml heparin contracted ∿20% in 4 h, and gels polymerized in the presence of 0.05 mg/ml heparin contracted only ∿10% in 4 h.

One possible explanation for the inhibition of gel contraction by heparin was decreased cell attachment to the heparin-containing collagen gels, but control experiments with radiolabeled cells showed that by 30 min, 95% of the cells had attached to the gels with or without heparin (data not shown). Also, cells attached to the heparin-containing gels were able to spread (see below). Moreover, we found that the effect of heparin required that it be polymerized in the gels. For instance, there was no inhibition of gel contraction by fibroblasts when 0.1 mg/ml heparin was added to the incubation medium instead of polymerized in the gels (Fig. 2). In fact, addition of heparin to the incubation medium at 0.01 or 0.05 mg/ml promoted gel contraction slightly.

Morphological Features of Control and Heparin-containing Collagen Gels Contracted by Fibroblasts

During the above experiments we observed marked differences between the arrangement of cells on the control and heparin-containing gels. Control gels that had been con-
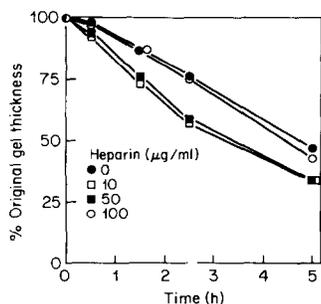
Figure 2. Contraction of collagen gels with heparin added to the incubation medium. Collagen gels prepared in the absence of heparin were incubated with fibroblasts. Heparin was added to the incubation medium at the concentrations indicated. The gel thickness was measured at the times specified. Other details are in Materials and Methods.
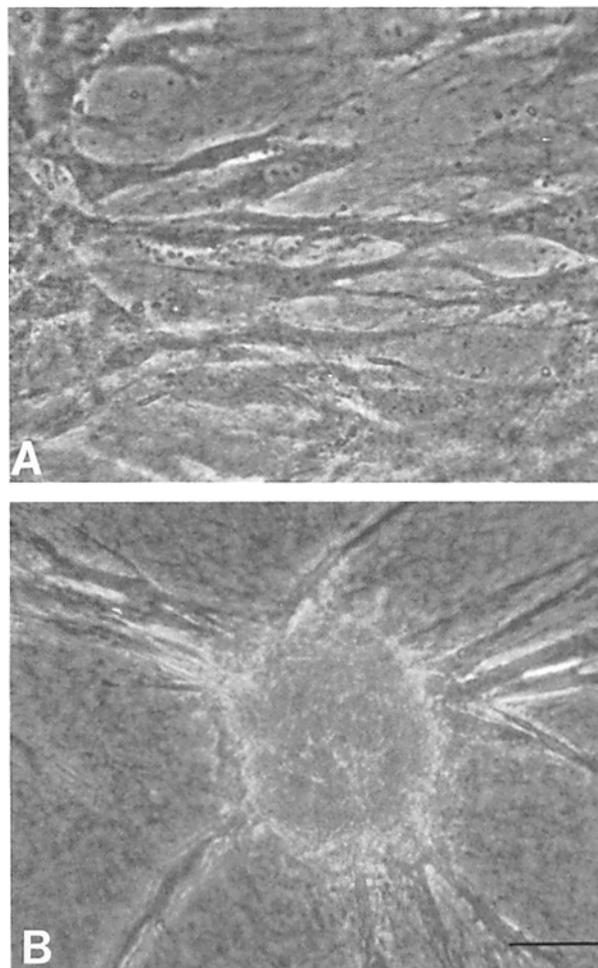
Figure 3. Distribution of cells on the surfaces of control and heparin-containing collagen gels. Phase contrast photomicrographs were taken of samples from the experiment described in Fig. 1. (A) Cells incubated for 24 h on control collagen gels were uniformly distributed on the surfaces of the gels. (B) Cells incubated for 24 h on gels of collagen copolymerized with heparin (0.05 mg/ml) were in clusters on the surfaces of the gels. Spreading of individual cells was similar on both substrata. Other details are in Materials and Methods. Bar, 50 μm.

tracted for 24 h showed a uniform distribution of cells on the gel surface (Fig. 3 A). With heparin-containing gels, however, the cells were mostly in large clusters (Fig. 3 B). Usually, when cells form clusters rather than remain as individuals on a substratum, it is because the cell–cell interactions are stronger than the cell–substratum interactions (9). But the morphological appearance of individual cells on control and heparin-containing gels was similar, i.e., well-spread and often bipolar (Fig. 3), which indicated that cell adhesion was strong to both types of substrata.

Another possible explanation for the cell clusters was that the heparin-containing gels were unstable and were pulled apart by the cells during contraction. Sections from control gels that were incubated with cells for 24 h showed evenly distributed collagen fibrils throughout the gel matrix (Fig. 4 A). The collagen fibrils were densely packed and aligned parallel to the gel surface in marked contrast to the loose, random arrangement of fibrils in control gels that were in-
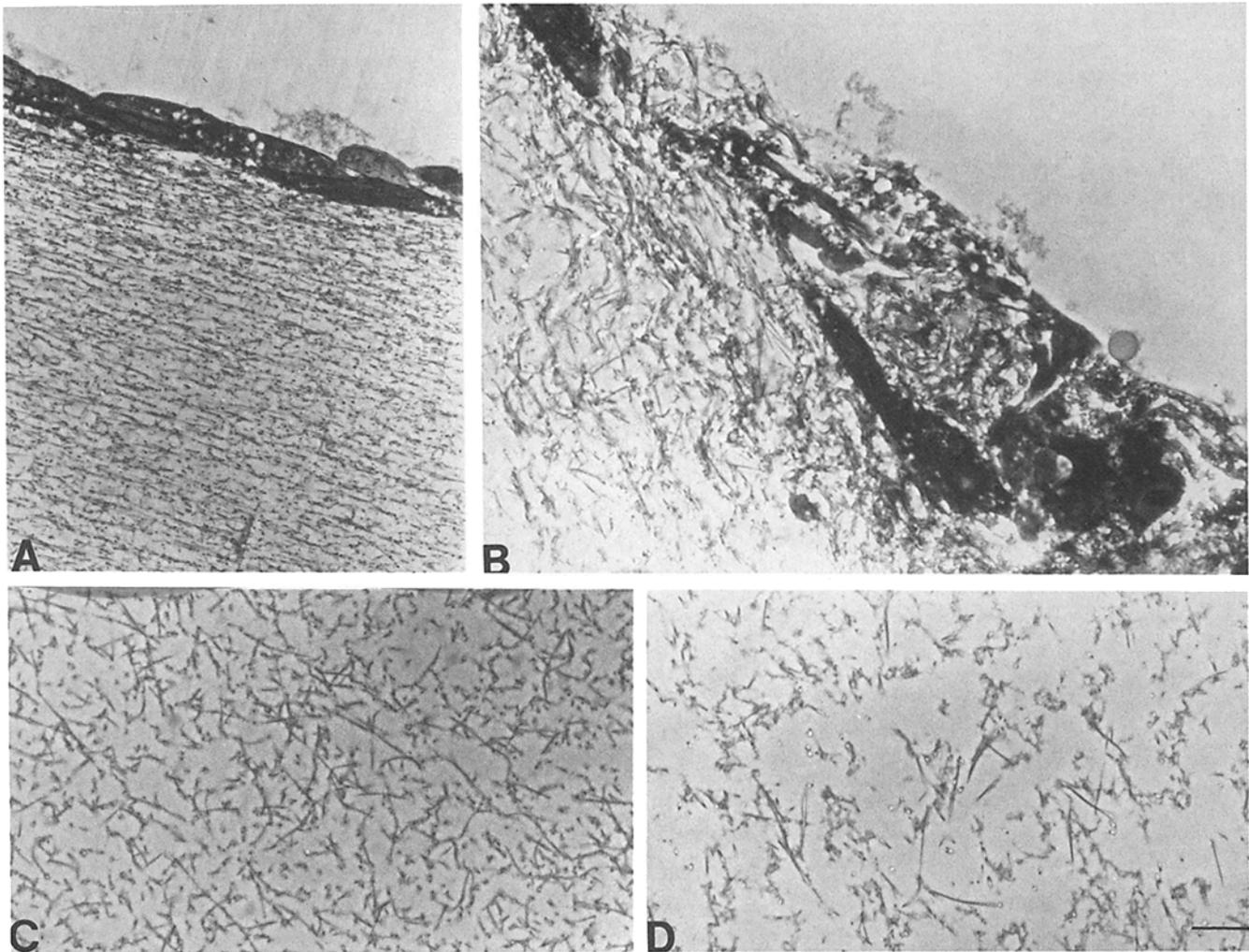
Figure 4. Distribution of collagen fibrils in control and heparin-containing collagen gels incubated in the presence and absence of cells. Control collagen gels (*A* and *C*) and 0.05 mg/ml heparin-containing collagen gels (*B* and *D*) were incubated for 24 h with (*A* and *B*) or without (*C* and *D*) cells. Fibroblasts covered the surfaces of the control gels, and the collagen fibrils were densely packed and well organized compared with gels incubated without cells (*A* vs. *C*). Fibroblasts were in clusters on the surfaces of the heparin-containing gels, and the collagen fibrils were densely packed but only in the immediate vicinity of the cells (*B*). There appeared to be larger spaces between fibrils in the heparin-containing gels than in the control gels (*D* vs. *C*). Other details are in Materials and Methods. Bar, 10 μm.

cubated for 24 h in the absence of cells (Fig. 4 *C*). In heparin-containing gels incubated with cells for 24 h, the collagen fibrils were densely packed only in the immediate vicinity of the cells, but the fibrils were not aligned (Fig. 4 *B*). Distal to the cell clusters, the arrangement of collagen fibrils appeared similar to that observed when heparin-containing gels were incubated for 24 h in the absence of cells (Fig. 4 *D*). It seemed likely, therefore, that fibroblasts were able to reorganize proximally located collagen fibrils of heparin-containing gels, but reorganization was not propagated to the collagen fibrils throughout the remainder of the gel.

Another difference between the control and heparin-containing gels was that the latter appeared to contain larger spaces between fibrils (Fig. 4 *D* compared with 4 *C*). The difference in packing of collagen fibrils in control gels and heparin-containing gels was confirmed by scanning electron microscopy. In control gels, the collagen fibrils tended to be

uniformly distributed (Fig. 5 *A*) and were individual or intertwined in pairs (Fig. 5 *C*). All the fibrils were interconnected with each other and there were no loose ends. In marked contrast, the heparin-containing gels showed a wide range of structures, including amorphous material (Fig. 5 *B*). Instead of intertwining pairs, the fibrils were combined in variable arrangements, sometimes with five or six fibrils bundled together (Fig. 5 *D*) and these bundles appeared to end in the amorphous material (Fig. 5 *B*, *arrow*). Moreover, the fibrils were not uniformly interconnected with each other since many loose ends were evident (Fig. 5 *D*, *arrows*).

Finally, observations of the fibrils in control and heparin-containing gels were made by transmission electron microscopy. The periodicity of banding was the same for fibrils in control gels (Fig. 6 *A*) as heparin-containing gels (Fig. 6 *B*). It is noteworthy that the intensity of fibril banding was more prominent in the heparin-containing gels, which may be a consequence of heparin binding to the fibrils. Consistent
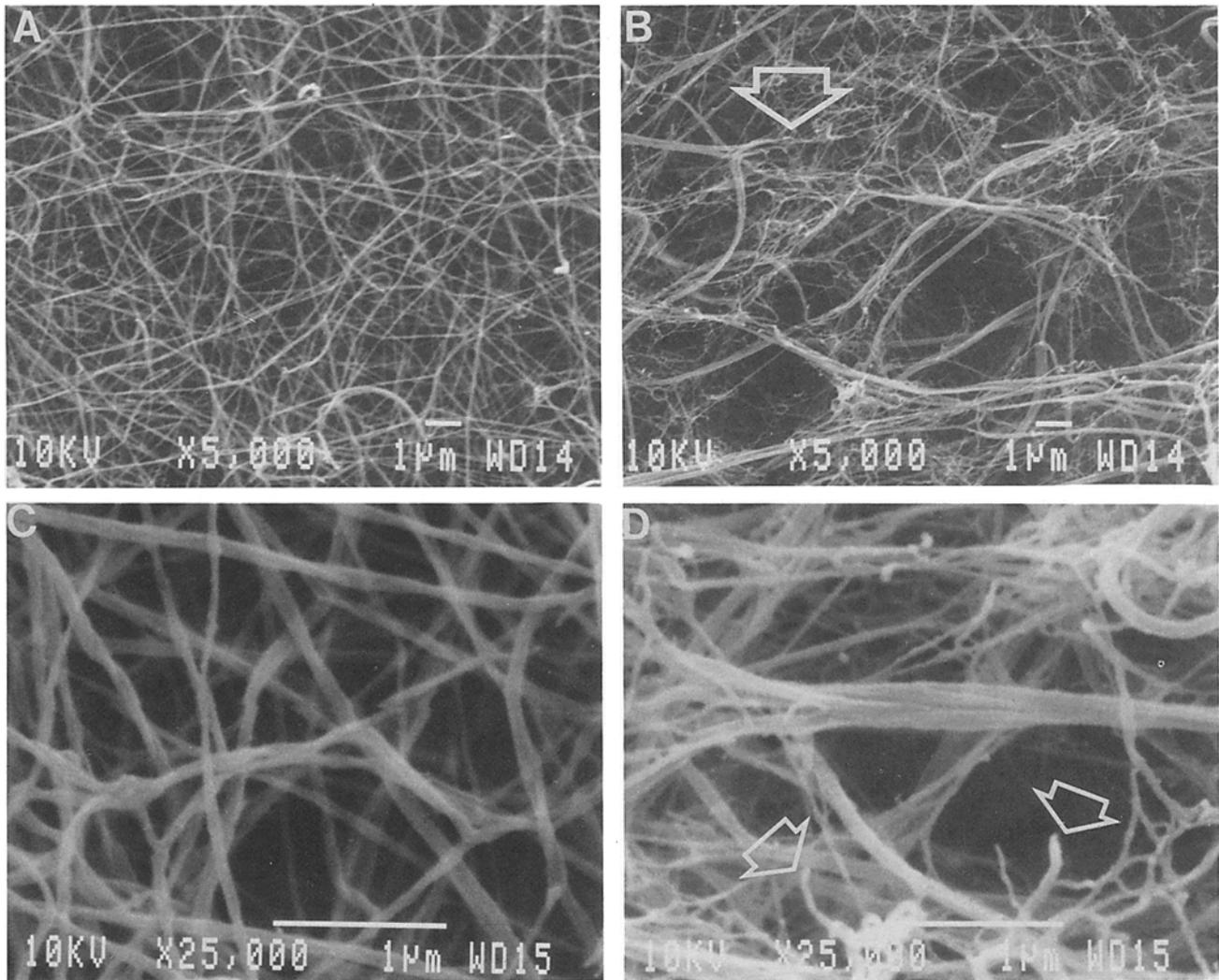
Figure 5. Collagen fibril packing in control and heparin-containing collagen gels examined by scanning electron microscopy. (*A*) Control gels were composed of a regular network of uniformly packed collagen fibrils. (*B*) Gels containing collagen copolymerized with 0.05 mg/ml heparin were composed of an irregular network of fibrils that terminated in amorphous material (*arrow*). (*C*) Higher magnification of control gels showed that most collagen fibrils were present as individuals or intertwined in pairs and interconnected with each other so that no free ends of the fibrils could be seen. (*D*) Higher magnification of heparin-containing gels showed diverse packing with some bundles containing five or six collagen fibrils, and there were many fibrils with free ends not interconnected to other fibrils (*arrows*). Other details are in Materials and Methods.

with the scanning electron microscopic observations, increased lateral associations between collagen fibrils were observed in the heparin-containing gels (Fig. 6 *B*).

Discussion

We studied the effects of hyaluronic acid, dermatan sulfate, fibronectin, and heparin on collagen gel contraction by fibroblasts. These matrix components, which may be important in dermal synthesis and remodeling during wound repair, were added with collagen during polymerization. Significant amounts of each of the components were incorporated into the gels and could not be removed by washing in excess buffer for 24 h. Since heparin and fibronectin markedly decreased the rate of polymerization, as has been reported by others (19, 22, 27), a lower concentration of these matrix

components was used than of hyaluronic acid and dermatan sulfate. A marked inhibition of contraction occurred with collagen gels that were formed in the presence of 0.05 mg/ml heparin. In 4 h, fibroblasts contracted these gels only 10% compared with 50% contraction of control gels during the same time. Similar inhibition was not obtained with 10-fold higher concentrations of hyaluronic acid or dermatan sulfate, implying that the effect of heparin is very specific.

Some investigators have found that soluble heparin inhibits fibroblast adhesion to fibronectin-coated substrata (18, 21, 26). In addition, it has been reported that cell surface heparan sulfate is required for formation of focal cell adhesions (17, 20, 28). Nevertheless, such results may not be applicable to fibroblast adhesion to collagen gels since this adhesion process is fibronectin independent (13). Indeed, we found that cells attached to heparin-containing gels as well as to
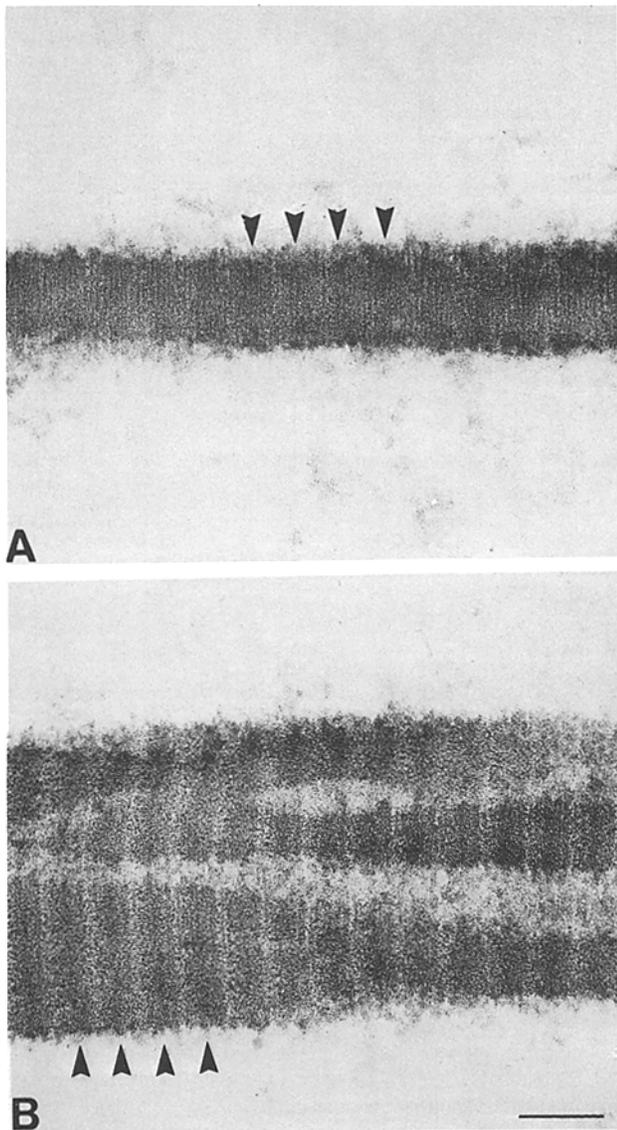
Figure 6. Periodicity of collagen fibrils in collagen and heparin-containing collagen gels examined by transmission electron microscopy. (*A*) Fibrils in control collagen gels and (*B*) fibrils in gels of collagen copolymerized with 0.05 mg/ml heparin showed the same periodicity of banding (*arrows*), although the banding of fibrils in the heparin-containing gels was more pronounced. Also, the fibrils in the heparin-containing gels showed extensive lateral associations. Other details are in Materials and Methods. Bar, 0.1 μm.

control gels. Moreover, heparin did not inhibit contraction unless it was copolymerized with collagen. That is, addition of heparin to the incubation medium had no effect on contraction. Even 20-fold higher concentrations of heparin (100 U/ml, ∼0.7 mg/ml) added to the incubation medium only partially inhibited the ability of cells to contract collagen gels (93% gel contraction with control incubation medium compared with 67% gel contraction with heparin added to the incubation medium) (7). It can be concluded, therefore, that inhibition of collagen gel contraction by heparin is not a consequence of inhibition of fibroblast adhesion to collagen fibrils. Rather, as will be discussed below, it seems likely that the fibroblasts attach normally to the collagen fibrils and attempt to contract the gels.

In trying to understand the mechanism by which heparin inhibits collagen gel contraction, it is useful to consider that gels can be contracted not only by fibroblasts that are uniformly distributed throughout the gel matrix (1, 23), but also by fibroblasts that are located asymmetrically on the upper or lower gel surface (12). With fibroblasts on the upper surfaces of the gels, which was the arrangement used for the studies described in this paper, contraction begins proximally to the cells and spreads distally throughout the gel (12). That is, the mechanical effect of cells pulling on proximal collagen fibrils could be propagated to fibrils located distal to the cells.

Based on the above considerations, we believe that the decreased contraction of heparin-containing gels can be explained by the changes in collagen fibril organization that occurred in the presence of heparin. That is, control collagen gels were composed of a uniform network of interlocking collagen fibrils, but this network was disrupted in heparin-containing gels. The latter showed variations in collagen packing from bundles that contained five to six fibrils to regions of amorphous material, and there were many fibrils that terminated freely and were not interconnected to other fibrils.

Considering the structural differences between control and heparin-containing gels, one can imagine much different results when cells apply mechanical forces to the gels. In control gels, the forces would be transmitted throughout the gels by the interlocking collagen network. The cells would maintain their spacing on the gel surfaces and there would be an increase in collagen density throughout the gels. In heparin-containing gels, the forces would be transmitted through the collagen network until reaching a weak spot, e.g., regions of amorphous material or loose ends. Then the gels would pull apart and the cells would collapse into local clusters. Collagen density would be increased only in the immediate vicinity of the cells.

It is intriguing to speculate on the possible in vivo relevance of our observations. As mentioned in the Introduction, significant amounts of heparin can be found in tissues undergoing fibrosis and in scars (5). The presence of heparin at these sites during collagen polymerization may result in local changes in the collagen matrices that affect the ability of fibroblasts to remodel the matrix. Therefore, the abnormal arrangement of collagen fibrils and decreased tensile strength of scar tissue may be, in part, a consequence of the discontinuous packing of collagen fibrils promoted by heparin.

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