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The Mechanism of Precartilage Mesenchymal Condensation: A Major Role for Interaction of the Cell Surface with the Amino-Terminal Heparin-Binding Domain of Fibronectin

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Using low magnification Hoffman Modulation Contrast microscopy to rapidly identify precartilage mesenchymal condensations in chick limb bud cultures, we have determined the effect on condensation number of treatments disruptive of the interaction of cell surface components with endogenously produced fibronectin. A monoclonal antibody directed against the amino-terminal heparin-binding domain of fibronectin reduced the number of condensations by more than 50%, as did the oligopeptide gly-arg-gly, which is a repeated motif in that fibronectin domain. In contrast, monoclonal antibodies directed against the collagen- and integrin-binding domains of fibronectin, or oligopeptides containing the fibronectin integrin-recognition sequence arg-gly-asp-ser, had no significant effect on condensation number. Addition of *Flavobacterium* heparinase to cultures also reduced condensation number by more than 50%. Alcian blue staining of sulfated proteoglycan was greatly reduced in differentiated cultures that had been exposed to treatments that reduced condensation number. Taken together with the accompanying study, which directly demonstrates an adhesive interaction between the amino-terminal domain of extracellular fibronectin and heparin-like molecules on the surfaces of latex bead probes, the data presented here strongly indicate a major role for the corresponding cell-matrix interaction in mediating precartilage condensation in limb mesenchyme.

INTRODUCTION

The formation of tight mesenchymal aggregates, or precartilage condensations (Fell and Canti, 1934; Thorogood and Hinchliffe, 1975), is the earliest morphogenetic event associated with the position-dependent differentiation of skeletal structures in the developing vertebrate limb (Newman, 1988). Such aggregates also form in cultures of limb mesenchyme (Ede *et al.*, 1977; Newman, 1977; Ahrens *et al.*, 1977) and are thought to be required for subsequent chondrogenic differentiation, although this requirement can be circumvented by certain treatments that modulate cell shape (Zanetti and Solursh, 1984).

The mechanism of condensation formation is poorly understood, but the accumulation of extracellular matrix macromolecules, such as fibronectin (Dessau *et al.*, 1980; Tomasek *et al.*, 1982; Frenz *et al.*, 1989) and tenascin (Mackie *et al.*, 1987), at sites of precartilage condensation *in situ* and *in vitro*, suggests that local adhesive interactions between the mesenchymal cell surface and the extracellular matrix could play an important role in mediating this process. By using cell-sized polystyrene latex beads coated with various molecules as probes of the adhesive environment within cultures of limb mesenchyme, we have shown that an adhesive interaction occurs between heparin-like molecules of the bead surface and the amino-terminal domain of cellular fibronectin concentrated at foci of condensation (Frenz *et al.*, 1989). The abrogation of this interaction with a domain-specific antifibronectin monoclonal antibody, or with a peptide corresponding to a repeated determinant in the fibronectin amino-terminal domain, prevents the accumulation of heparin-coated beads at condensing foci (Frenz *et al.*, 1989).

Here we show that interference with cell-fibronectin interactions by these same reagents, or removal of heparin-like components from the mesenchymal cell surface with heparinase, drastically curtails the formation of condensations in precartilage mesenchyme. Treatments that reduce condensation number also reduce the subsequent elaboration of the highly sulfated proteoglycans characteristic of cartilage. These studies provide evidence for a major role for an interaction between the amino-terminal domain of fibronectin and heparin-like cell surface molecules in the formation of condensations.

MATERIALS AND METHODS

Cell culture. Mesenchymal cells were prepared from stage 22/23 chick wing buds (Hamburger and Hamilton, 1951). Ten microliter spots (2.5×10^5 cells) were cultured in 24 well plates (Falcon 3047 or Costar 3024) in Ham's F12 medium containing 10% fetal bovine serum, as described (Frenz *et al.*, 1989). In certain experiments

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the medium was depleted of serum fibronectin (Frenz *et al.*, 1989). Living cultures were monitored by phase contrast microscopy or Hoffman Modulation contrast microscopy (Modulation Optics, Inc., Greenvale, NY) on a Zeiss IM 35 inverted microscope. Some cultures were grown in fibronectin-free medium and immunostained for endogenous fibronectin, as described (Frenz *et al.*, 1989).

Monoclonal antibodies, peptides, and heparinase. Antibodies directed against the 31-kDa amino-terminal heparin-binding domain, the 43-kDa collagen-binding domain, and the 75-kDa integrin-binding domain of fibronectin (Newman *et al.*, 1987) were added to cultures at 10 μg/ml. Peptides (gly-arg-gly (GRG)[1]; gly-arg-gly-asp (GRGD); gly-arg-gly-asp-ser (GRGDS) and gly-arg-gly-asp-ser (GRGDS) were obtained from Peninsula Laboratories, purified by reverse phase high performance liquid chromatography, and added to cultures at 15–100 μg/ml. *Flavobacterium* heparinase was a gift from J. Marcum, Massachusetts Institute of Technology. The stock solution was 1.8 units per ml (Marcum *et al.*, 1984) and was diluted to 1% or 0.1% in Ham's F-12 medium for use in cultures. Antibodies, peptides, and heparinase were present for the duration of the culture period.

Quantitative Alcian blue staining of micromass cultures. On the sixth day of culture cell layers were washed with Earle's balanced saline solution, fixed for 10 min with 3% acetic acid adjusted to pH 1.0 with HCl, and stained overnight with 0.5% Alcian blue 8GX made up in the same solution (Lev and Spicer, 1964; Hassell and Horigan, 1982). Cell layers were washed twice with 3% acetic acid adjusted to pH 1.0 to remove unbound stain, and then extracted overnight at 4°C with 0.3 ml 8M guanidinium chloride. Extracts from each well were removed to a 96-well microtiter plate and their absorbances at 600 nm determined spectrophotometrically (Hassell and Horigan, 1982), using a Biotek EIA Reader (Bio-Tek Instruments, Inc., Burlington, VT).

RESULTS

Rapid, Reliable Identification of Condensations in Living Cultures

Precartilage condensations can be recognized by phase contrast microscopic examination of high density cultures of stage 22/23 chick wing bud cells during a period of approximately 24 hr, beginning about two days after the cultures are established. During this period condensations can also be identified by their heavy intercellular accumulation of fibronectin, which can be visualized by indirect immunofluorescence (Frenz *et al.*, 1989; Fig. 1). In the phase image in Fig. 1 condensations are distinguished by characteristic features of cell packing and orientation, but these features can be difficult to discern at low magnification. In each set of experiments reported below it was necessary to count condensations in 10–15 cultures repeatedly over 6–8 hr in order to compare plateau numerical values. We therefore sought an alternative to phase contrast microscopy for identifying condensations.

A microscopic field comparable to that shown in Fig. 1, in a living 2.5-day-old culture, was photographed with Hoffman Modulation contrast optics (Fig. 2). The condensations (confirmed as such by phase microscopy) appear as dark regions against a lighter background, and can be rapidly discerned and counted using a 4X objective. The optimal slit and polarizer orientations for visualizing condensations were arrived at empirically, and differ from the orientations that optimize the "topographical" image also achievable with Hoffman optics. All condensation counts reported below were performed using this optical technique.

Effects of Antifibronectin Monoclonal Antibodies and Fibronectin-Related Peptides on Condensation Number

Monoclonal antibodies directed against the amino-terminal heparin-binding domain, collagen-binding domain, or integrin-binding domain of fibronectin (Newman *et al.*, 1987) were added at 10 μg/ml to cultures of stage 22/23 limb mesenchyme. Cultures were grown in Ham's F-12 medium containing 10% fetal bovine serum from which fibronectin had been removed (Frenz *et al.*, 1989). Condensations were counted several times between 2 and 3 days after the cultures were established. Mean plateau values for condensations are given in Table 1. Comparison of all experimental values with their matched controls indicates that only treatment with antibody 304, directed against the fibronectin amino-terminal domain, significantly reduced the condensation number. The average reduction was 58%. There was no compensatory increase in the size of remaining condensations. The effect with antibody 304 was dose-dependent; cultures containing this antibody at 5 μg/ml showed an inconsistent reduction of average condensation number relative to controls. (Data not shown.)

Similar experiments were performed using the peptides GRG and GRGD, which contain a determinant repeated in the amino-terminal domain of fibronectin, and GRGDS and RGDS which competitively inhibit interactions of fibronectin with integrin (Yamada and Kennedy, 1987; Hynes, 1987). We found that only GRG and GRGD had a significant influence on the number of

[1] Abbreviations used: GRG, gly-arg-gly; GRGD, gly-arg-gly-asp; GRGDS, gly-arg-gly-asp-ser; RGDS, arg-gly-asp-ser.

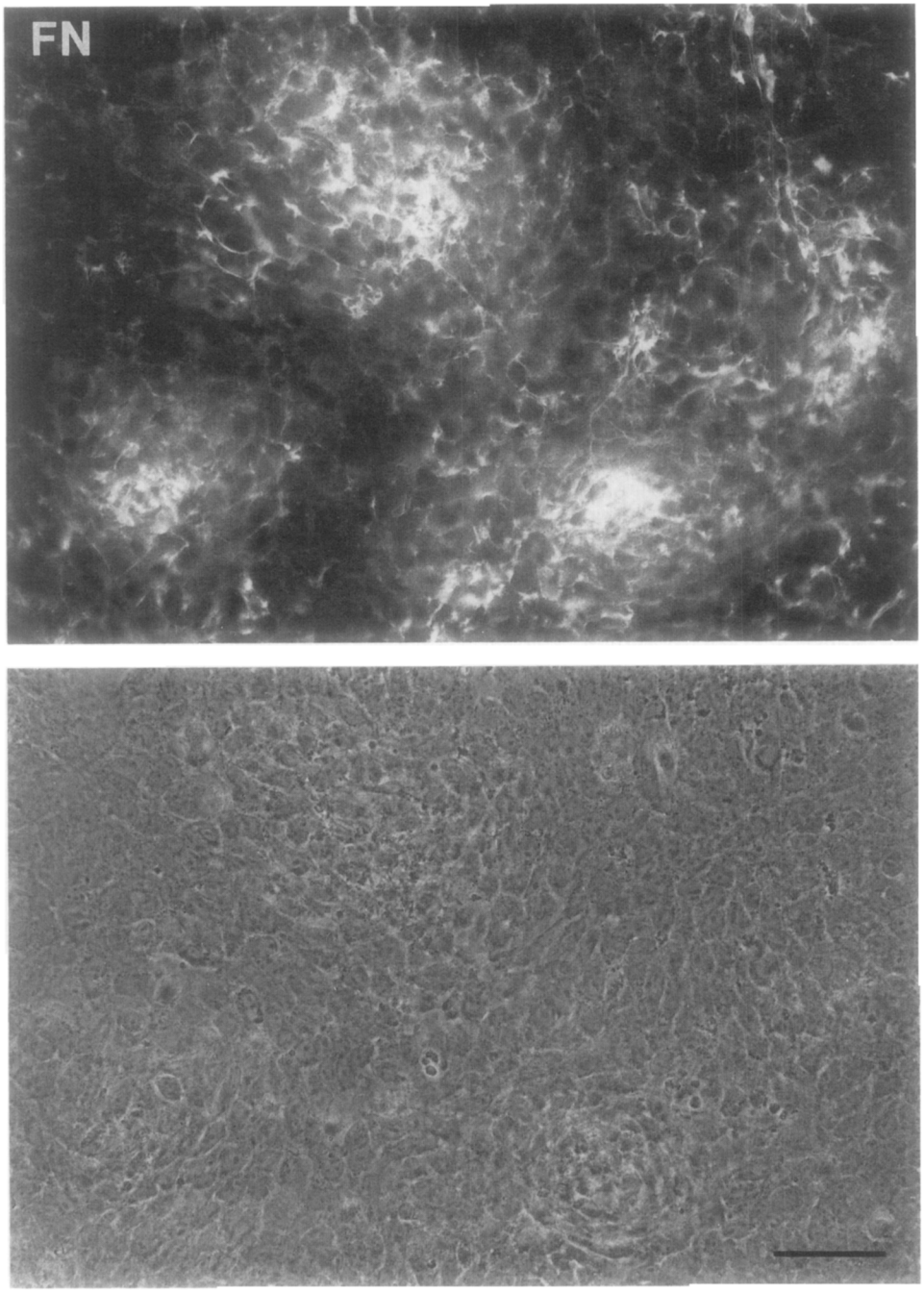

FIG. 1. Precartilage condensations in fixed stage 22/23 wing bud mesenchyme culture. Culture was fixed and immunostained for fibronectin during Day 2 after plating. (Top panel) Fibronectin localization. (Bottom panel) Phase contrast microscopy. Scale bar represents 50 μm.

condensations, reducing them by between 50 and 100% (Table 2), with no compensatory increase in the size of those that remained. However, even at GRG concentrations as high as 100–150 μg/ml it was rare for condensations to be eliminated entirely (Table 2 and unpublished experiments). Concentrations of GRGD of 10 μg/ml, and of GRG of 50 μg/ml, reduced the number of condensations only inconsistently. Representative microscopic fields in a control culture and in cultures treated with GRGD and RGDS are shown in Fig. 3.

Effect of Heparinase on Condensation Number

Precartilage mesenchymal cells were previously shown to contain heparin-like molecules on their surfaces that permitted them to interact adhesively with

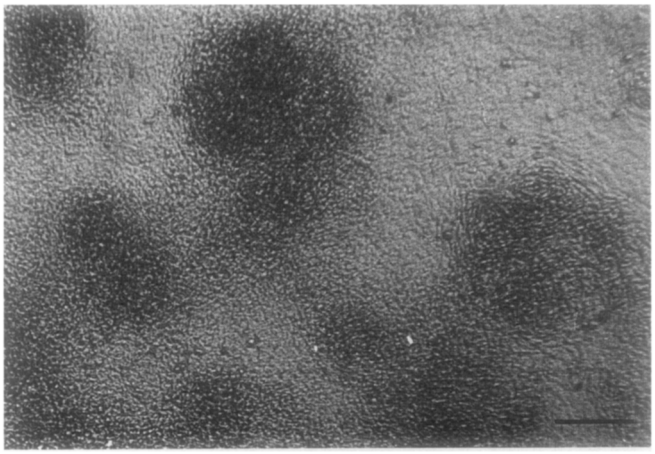

FIG. 2. Precartilage condensations in living stage 22/23 wing bud mesenchyme culture. Culture was photographed using a 4X Hoffman Modulation contrast objective during Day 2 after plating. Condensations appear as dark patches. Scale bar represents 50 μm.

TABLE 1
EFFECT OF ANTIFIBRONECTIN ANTIBODIES ON PRECARTILAGE CONDENSATIONS

Monoclonal antibody[a]	Mean condensation number[b] Experiment 1	Experiment 2	Experiment 3	% of control
304 (α31 kDa)	37	25	20	42[c]
191 (α43 kDa)	69	51	58	89[d]
333 (α75 kDa)	41	46	53	72[d]
control	77	57	60	

[a] Each antibody was present at 10 μg/ml for the duration of the culture period.
[b] Plateau condensation number recorded for 2-3 cultures per treatment group.
[c] Significantly different from control by Student's matched pair *t*-test ($P < 0.01$).
[d] Not significantly different from control.

the amino-terminal domain of fibronectin in a model extracellular matrix. These surface components could be removed by treatment with *Flavobacterium* heparinase (Marcum *et al.*, 1984; Newman *et al.*, 1987). We therefore grew mesenchyme cultures in the presence of this enzyme, and monitored condensation number between days 2 and 3. There was a consistent decline of 50–70% in the number of condensations in the presence of 0.0018 units of heparinase per ml (Table 3). Addition of up to 10 times more heparinase to the cultures did not further reduce the number of condensations (Table 3).

Effect of Peptides and Heparinase on Accumulation of Sulfated Proteoglycan

After precartilage condensations form in culture they normally develop into chondrogenic foci over the next few days (Ahrens *et al.*, 1977) producing a characteristic extracellular matrix that stains with Alcian blue at pH 1.0 by virtue of its content of highly sulfated proteoglycan (Lev and Spicer, 1964). We stained representative cultures from the peptide and heparinase experiments on the 6th day of incubation, and spectrophotometrically quantitated the amount of Alcian blue bound (Hassell and Horigan, 1982). We found that exposure of the cultures to GRG and GRGD reduced the amount of staining, and therefore, sulfated proteoglycan accumulation, by more than 50%, whereas GRGDS and RGDS had no significant effect on staining (Table 4). Heparinase, at concentration sufficient to reduce the number of condensations by half, reduced the amount of Alcian blue staining by 34% (Table 4).

DISCUSSION

Skeletal development in the vertebrate limb occurs as a sequence of cellular events, several of which appear to

TABLE 2
EFFECT OF OLIGOPEPTIDES ON PRECARTILAGE CONDENSATIONS

Peptide	Mean condensation number Experiment 1 (15 μg/ml)	Experiment 2 (50 μg/ml)	Experiment 3 (100 μg/ml)	Experiment 4 (100 μg/ml)	% of control
GRG	ND	ND	0	47	17[a]
GRGD	22	65	25	ND	31[b]
GRGDS	161	112	50	ND	87[c]
RGDS	169	134	46	ND	97[c]
Control	177	136	49	124	

Note. ND: Not determined.
[a] Significantly different from control ($P < 0.05$).
[b] Significantly different from control ($P < 0.001$).
[c] For peptide concentrations ≥50 μg/ml; not significantly different from control.

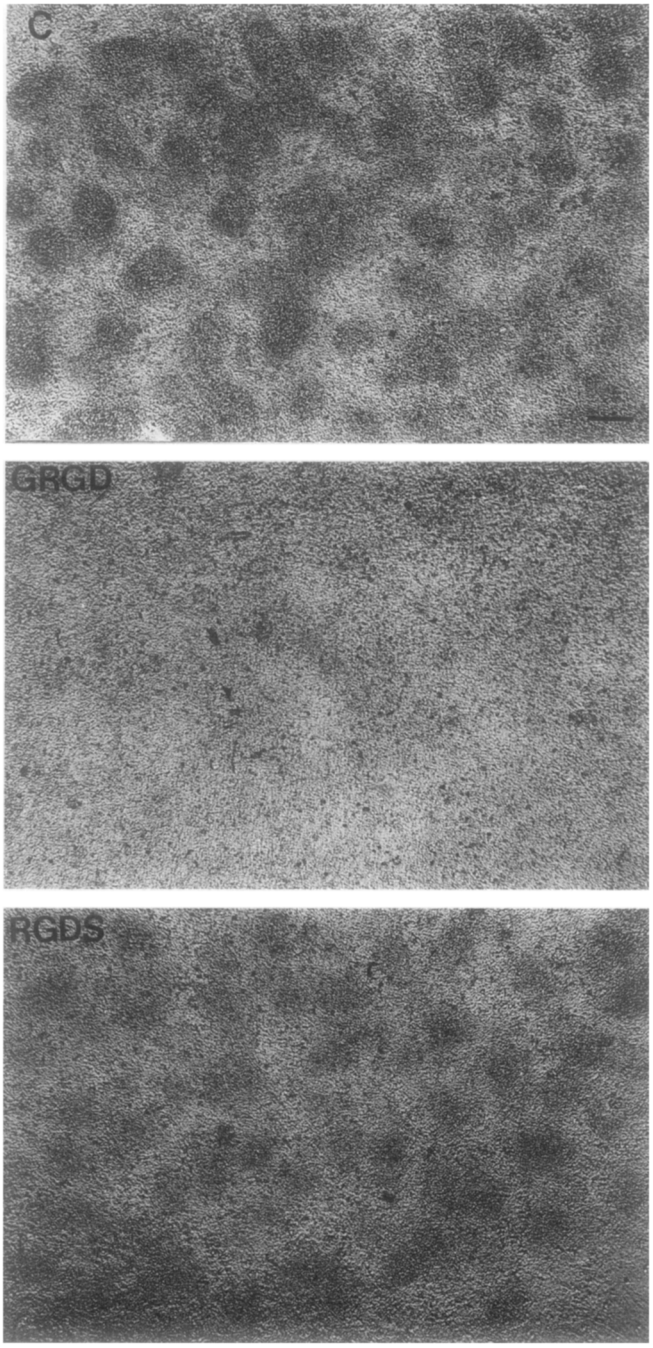

FIG. 3. Precartilage condensations in living stage 22/23 wing bud mesenchyme cultures under various conditions. Photography as in Fig. 2. (Top panel) Control culture. (Middle panel) Culture grown in presence of 50 μg/ml GRGD. (Bottom panel) Culture grown in presence of 50 μg/ml RGDS. Scale bar represents 100 μm.

constitute a causal chain (Newman, 1988). An important link in this sequence is the formation of precartilage condensations, which may stimulate the metabolic changes (such as increased cyclic AMP levels (Solursh *et al.*, 1979) that ultimately lead to cartilage-specific gene expression (Kosher *et al.*, 1986). The local synthesis and accumulation of adhesive extracellular matrix macromolecules, in particular, fibronectin, has been proposed to be a causal event in the establishment of condensations (Newman and Frisch, 1979) but this hypothesis has not previously been subjected to experimental test.

Our approach to this question was twofold. First, we have used polystyrene latex beads as probes of the intercellular adhesive environment in developing cultures of limb mesenchymal cells. We found that beads with heparin or chondroitin sulfate A or B on their surfaces can interact adhesively with components of the extracellular matrix in a manner sufficiently sensitive to local concentration differences to cause them to be passively conveyed into matrix-rich foci of mesenchymal condensation by a "sorting out" type of mechanism (Frenz *et al.*, 1989). In the case of heparin-coated beads, but not chondroitin sulfate-coated beads, the bead-matrix adhesive interaction could be abrogated by addition to the cultures of a monoclonal antibody directed against the amino-terminal heparin-binding domain of fibronectin. This interaction could also be abrogated by the oligopeptides GRG and GRGD (Frenz *et al.*, 1989), which are related to a repeated determinant in the same fibronectin domain.

These results suggested that the amino-terminal domain of fibronectin in the matrix of developing mesenchyme was potentially accessible for interaction with cells containing heparin-like molecules on their surfaces (such as the mesenchymal cells themselves (Newman *et al.*, 1987)). The second component of our approach, therefore, was to attempt to interfere directly with condensation formation by using the antibody and peptide reagents which were effective in inhibiting the heparin-coated bead-fibronectin interaction. Our results parallel the results of the bead accumulation experiments in that reagents disruptive of the interaction of heparin-like molecules on the cell surface with the amino-terminal domain of fibronectin reduced the number of condensations, and reagents directed against other sites of fibronectin did not. The greater potency of GRGD over GRG in inhibiting both bead accumulation and condensation formation may represent an effect of blocking the free carboxyl group of the second glycine of the oligopeptide, which, of course, is also blocked in the native protein.

The inhibitory effect of *Flavobacterium* heparinase on condensation formation provides evidence that heparin-like molecules which are present on the mesenchymal cell surface, and are capable of interacting with the amino-terminal fibronectin domain in a model matrix (Newman *et al.*, 1987), are also the ligands that interact with this domain of fibronectin during condensation formation.

TABLE 3
EFFECT OF HEPARINASE ON CONDENSATION NUMBER

	Mean condensation number				
	Experiment 1	Experiment 2	Experiment 3	Experiment 4	% of control
Heparinase					
0.0018 U/ml	37	25	23	ND	41[a]
0.018 U/ml	ND	ND	ND	35	41[a]
Control	74	90	45	86	

[a] Significantly different from control ($P < 0.01$); all treatment groups analyzed together with matched controls.

Although formation of condensations and bead accumulation at those condensations are superficially dissimilar processes (e.g., mesenchymal cells are *not* translocated over distances of several cell diameters into condensing foci) they may nonetheless result from similar adhesive interactions. Steinberg and his coworkers have convincingly shown that a single mechanism—differential adhesion—can cause experimental preparations of cells or tissues to undergo disparate sequences of changes (e.g., sorting out of cell mixtures; tissue fragment engulfment behavior) as they evolve toward an equilibrium state determined by the minimization of overall adhesive free energies (reviewed in Steinberg, 1978). When deformable cells respond to a spatially localized increase in adhesivity, saturation of potential binding sites can be attained by increases in cell packing, with consequent cell shape changes (Sulsky *et al.*, 1984). When equipped with similar ligands on their surfaces, polystyrene beads, which cannot alter their shapes, will simply accumulate at the most adhesive regions of the cultures that they encounter as a result of random movement of surrounding cells.

The amino-terminal domain of fibronectin has previously been shown to interact adhesively with cells or polystyrene beads containing heparin-like surface moieties in a model collagen matrix system (Newman *et al.*, 1985, 1987). This effect appears to be related to a specific conformational change in the domain upon its binding to heparin (Khan *et al.*, 1988) and is inhibited by GRG containing peptides (Jaikaria *et al.*, 1987). We suggest that this heparin-mediated function of this domain is responsible for the interaction reported here and in the accompanying paper (Frenz *et al.*, 1989). A heparin-dependent adhesive mechanism is consistent with translocation and accumulation into condensation foci of heparin-coated (but not dextran sulfate-coated) beads in the studies reported in Frenz *et al.*, 1989, as well as with the inhibitory effect of heparinase on condensation formation in the present study. Although the amino-terminal domain of fibronectin also plays a role in extracellular matrix assembly (McDonald *et al.*, 1987), this function does not appear to depend on heparin-like cell surface components (McKeown-Longo and Mosher, 1985) and therefore probably involves a different interaction from that described here.

TABLE 4
EFFECT OF VARIOUS TREATMENTS ON ACCUMULATION OF SULFATED PROTEOGLYCAN

Treatment	Alcian blue stain bound (percentage of control)
GRG (100 μg/ml)	38[a]
GRGD (15-50 μg/ml)	44[a]
GRGDS (50-100 μg/ml)	95[b]
RGDS (50-100 μg/ml)	86[b]
Heparinase (0.0018 U/ml)	66[c]

[a] Significantly different from control ($P < 0.001$).
[b] Not significantly different from control.
[c] Significantly different from control ($P < 0.05$).

Finally, it must be noted that the evidence presented in these two studies, while indicating a major role for an adhesive interaction of the amino-terminal domain of fibronectin with heparin-like cell surface moieties in the mechanism of condensation formation, and excluding any significant role for fibronectin–integrin interactions in this process, does not exclude other cell–matrix interactions in contributing to condensation formation. In the first place, chondroitin sulfate-coated beads accumulated at foci of condensation (Frenz *et al.*, 1989), indicating that matrix components (probably other than fibronectin), are available in the mesenchymal matrix for interaction with this family of glycosaminoglycans. Second, extracellular matrix components in addition to fibronectin, for example, type I collagen (Dessau *et al.*, 1980) and tenascin (Mackie *et al.*, 1987), are also present at foci of precartilage condensation and provide potential adhesive sites for a variety of cell surface components. And third, it was rare for any of the treatments that disrupted the interaction between the cell surface and the amino-terminal domain

of fibronectin to completely eliminate condensations from the cultures. It is therefore probable that, as in many other developing systems, multiple mechanisms can cooperate to bring about an important end point.

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