# Initial Stages of Cell-Matrix Adhesion Can Be Mediated and Modulated by Cell-Surface Hyaluronan

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ABSTRACT    A conceptual temporal and spatial gap exists between the first encounter of a cell with an adhesive substrate and the advanced stages of focal adhesion formation. Although ample information is available on focal adhesions structure and function, the mechanism of the first interaction events and the nature of the molecules mediating them are largely unknown. In this paper we identify cell-surface-associated hyaluronan as a mediator and modulator of the first steps of adhesion of A6 and other cells to conventional tissue culture substrates as well as to the surfaces of calcium-(R,R)-tartrate tetrahydrate crystals. Treatment of A6 cells with hyaluronidase suppresses their rapid interactions with these adhesive substrates, and incubation of either the hyaluronidase-treated cells or the substrate with hyaluronan restores cell adhesion. In contrast, excess hyaluronan on both the cells and the substrate strongly inhibits adhesion. We thus propose that cell-surface-associated hyaluronan can mediate and modulate cell-matrix adhesion at the very first encounter with the substrate. It may promote it through the establishment of exquisitely stereospecific chemical interactions or inhibit it by virtue of steric exclusion and/or electrostatic repulsion.

## INTRODUCTION

Cell adhesion is a complex multi-stage process that plays a central role in the development of all metazoan organisms (Lauffenburger and Horwitz, 1996; Hynes, 1999). Studies of the molecular architecture of cell-matrix or cell-cell adhesion sites indicate that these cellular structures consist of a large number of different proteins including transmembrane adhesion receptors (such as members of the integrin family), components of the actin cytoskeleton, and interconnecting anchor proteins, which link the cytoskeleton to the membrane (Kemler, 1993; Takeichi, 1995; Critchley, 2000; Zamir and Geiger, 2001). These complexes were recently shown to function not only in mediating the physical integration of cells into tissues and organs but also in the generation and transduction of transmembrane signals that regulate many cellular functions such as cell proliferation, motility, differentiation, and apoptosis (Clark and Brugge, 1995; Yamada, 1997; Giancotti and Rouslahti, 1999; Schoenwaelder and Burridge, 1999; Geiger and Bershadsky, 2001).

The molecular complexity of most adhesive surfaces and of the adhesive machinery of the cells introduce an intrinsic difficulty in the characterization of the molecular mechanisms of adhesive interactions. To dissect the adhesive process into temporally defined molecular events, we have, over the last several years, selected to work with crystals as adhesive surface models. Crystals are particularly apt to this purpose, because they exhibit highly uniform surfaces, known at molecular and even atomic resolution. We demonstrated that cells can attach, spread, and grow on appropriate crystal surfaces, using a variety of molecular mechanisms (Hanein et al., 1993, 1994, 1995, 1996; Zimmerman et al., 1999).

One of the most striking observations was the rapid recognition and attachment of A6 cells (*Xenopus laevis* epithelial cells) to the {011} faces of calcium-(R,R)-tartrate tetrahydrate crystals (Hanein et al., 1993). A6 cells attach to these crystal surfaces within seconds of contact, but fail to spread on them, do not form focal adhesions, and die of apoptosis within a day or two (Hanein et al., 1996). This fast adhesion is mediated by protease-resistant and cytoskeleton-independent surface molecules and is stereoselective and enantioselective, insofar as it does not occur on the {011} faces of calcium-(S,S)-tartrate tetrahydrate crystals, despite the fact that the two have the same chemical composition and are structurally identical (apart from one being the mirror image of the other) (Hanein et al., 1994). Based on these and additional results it was proposed that the interactions of A6 cells with these crystal faces represent early stages in the physiological adhesion process. We suggested that such fast and early engaging interactions effectively tether cells to the surface, providing the temporal and spatial framework for slower integrin-mediated interactions to occur (Hanein et al., 1995). Subsequent efforts were aimed at the identification of the cell surface molecule(s) that mediate the earliest stages in cell-surface adhesion.

In this paper we identify hyaluronan as a primary mediator of early adhesion of A6 cells to a variety of exogenous surfaces, including, besides the above-mentioned crystals, conventional substrates such as glass or tissue culture dishes. Based on the findings reported here, we propose that cell-surface-associated hyaluronan can mediate and modulate early cell adhesion to the extracellular matrix (ECM).

# MATERIALS AND METHODS

## Crystallization

Optimal conditions for crystallization of calcium-(R,R)-tartrate tetrahydrate and calcium-(S,S)-tartrate tetrahydrate were determined, ensuring that the crystals are well-formed, homogeneous, and reproducible with respect to morphology and size. Crystallization conditions for calcium-(R,R)-tartrate tetrahydrate crystals were as follows. A solution of 30 ml of 40 mM sodium hydrogen tartrate (Sigma Chemical Co., St. Louis, MO) was mixed with 30 ml of 43 mM $CaCl_2 \cdot 2H_2O$ (Merck-Schuchardt, Darmstadt, Germany). All solutions were slightly preheated and kept warm until poured. The solution was aliquotted into 35-mm tissue culture dishes (Falcon, Becton Dickinson Labware, Plymouth, UK) either containing or not glass coverslips (Smethwick, Warley, UK) and kept at room temperature. Typically, crystals of 200–500 $\mu$m in length form within 1 day and remain attached to the dish or glass surface. The crystallization conditions for calcium-(S,S)-tartrate terahydrate crystals were the same as for the (R,R) enantiomer except for the use of potassium hydrogen D-tartrate (Fluka, Buchs, Switzerland).

For adsorption experiments, before crystallization, the solution of sodium hydrogen tartrate was purified by three to four extractions with 200 ml of chloroform (Merck-Schuchardt) in an extraction funnel. Excess chloroform was evaporated from the sodium hydrogen tartrate solution.

To avoid crystal dissolution during experiments, all media, fixation, and washing solutions were saturated with respect to the crystal being used. The saturation was achieved by overnight incubation of excess crystals with the relevant solution and filtration through a 0.2-$\mu$m filter (Schleicher and Schuell, Dassel, Germany) before use.

## Fluorescent labeling of hyaluronan

Five milligrams of hyaluronan (molecular mass = $2.7 \times 10^6$ daltons; Bio-Technology General, Rehovot, Israel) were dissolved in 3 ml of 50 mM Hepes (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonate]), pH 8.5. Ten milligrams of dansylboronic acid (Sigma) were added in aliquots over a period of 5 h, and the reaction mixture was incubated for 16 h at room temperature. The solution was centrifuged at 14,000 rpm for 5 min, and excess of unbound dansyl was removed by dialysis against PBS. The specific intensity of labeling was measured at $\lambda_{exc}$ = 340 nm, $\lambda_{emm}$ = 520 nm using a flourimeter (Shimadzu recording spectrofluorophotometer RF-540, Shimadzu Corp., Kyoto, Japan). We have used labeled hyaluronan ranging in its dansyl content from 3 to 12 moieties per 100 sugar rings. The behavior of the different preparations was virtually indistinguishable.

## Fluorescent labeling of fibronectin

Human plasma fibronectin (400 $\mu$g; Bio-Technology General, Rehovot, Israel) was dissolved in 200 $\mu$l of 0.1 M sodium carbonate buffer, pH 9.0. Rhodamine-B-isothiocyanate (13 $\mu$g; Sigma) dissolved in dry dimethylsulfoxide was added to the protein solution in small aliquots over 2 h. The mixture was covered with aluminum foil and incubated at 4°C for 6 h. $NH_4Cl$ was added to a final concentration of 50 mM after an additional 2 h of incubation. The solution was subjected to dialysis against PBS.

## Adsorption of hyaluronan, dextran, or fibronectin to tartrate crystals

Equal amounts (20–30 mg) of calcium-(R,R) and (S,S)-tartrate tetrahydrate crystals with typical size distributions (200–500 $\mu$m) were incubated for 2 h at room temperature with fluorescently labeled hyaluronan (dansyl HA) at final concentrations of 0.1–0.8 mg/ml, or fluorescently labeled fibronectin (20 $\mu$g/ml), in 500 $\mu$l of PBS, pH 7.2, tartrate-saturated solutions. The incubation with rhodamine-labeled dextran (50 $\mu$g/ml)

(molecular mass = 9300 daltons; Sigma) was for 1 h. The crystals were rinsed three times, for 5 min each, in the buffer, rapidly washed with double-distilled water, and allowed to dry. The adsorption levels were estimated by fluorescence microscopy, using a Zeiss fluorescence microscope equipped with a dansyl filter (for hyaluronan) or rhodamine filter (for fibronectin and dextran) (Zeiss, Oberkochen, Germany) and a video camera with a MSV-700L integration attachment (Applitec, Israel).

Crystals with adsorbed hyaluronan were observed either directly in air or after immersion in a solution of 1:1 anisole:benzyl acetate (whose refractive index is similar to that of the crystals). The refractive index of the crystals slightly differs for the different surfaces. The solvent mixture was therefore selected such that its refractive index would be intermediate between that of the two surface types of the crystal. Under these conditions, the level of multiple internal reflections is minimal.

## Cell culture

A6 cells (kidney epithelial cells from *Xenopus laevis*, ATCC.CCL 102) were cultured at 27°C in a humidified atmosphere of 5% $CO_2$ in air in Dulbecco's minimum essential medium (Biological Services, The Weizmann Institute) diluted 8.5:1.5 with water and supplemented with 10% fetal calf serum (Biolab, Jerusalem, Israel).

Raji and Daudi cells (human B-cell lines) and K562 cells (human chronic myelogenous leukemia) were cultured at 37°C in a humidified atmosphere of 7.5% $CO_2$ in air in Dulbecco's minimum essential medium (Biological Services, The Weizmann Institute) supplemented with 10% fetal calf serum (Biolab).

Crystals were sterilized under UV light for 2 h prior to cell seeding.

## Cell treatment with hyaluronidase

A6 cells were suspended using trypsin versene (Biolab), centrifuged, and resuspended at a concentration of ~$10^6$ cells/ml in serum-free medium. Hyaluronidase (Hyaluronidase VI-S from bovine testes; Sigma) was added to the suspended cells to a final concentration of 100 enzyme units/ml, and the cells were further incubated for 30 min at 27°C with occasional shaking. After the treatment, the cells were centrifuged, resuspended in saturated, serum-free medium, and seeded on calcium-(R,R)-tartrate tetrahydrate crystals for 10 min. The crystals were washed thoroughly to remove the unattached cells and fixed for light or scanning electron microscopy (SEM; see below).

## Cell treatment with exogenous hyaluronan

Hyaluronidase-treated cells were centrifuged, washed, and resuspended in increasing concentrations (0.01–1 mg/ml) of hyaluronan in serum-free medium. After 1 h of incubation, the cells were centrifuged, resuspended in saturated serum-free medium, and seeded on calcium-(R,R)-tartrate tetrahydrate crystals. The samples were washed thoroughly to remove unattached cells and prepared for visualization by SEM.

## Adsorption of hyaluronan on tartrate crystals

Calcium-(R,R)-tartrate tetrahydrate crystals were incubated for 2 h with increasing concentrations of hyaluronan (0.01–1 mg/ml in PBS). The crystals were rinsed three times for 5 min each with PBS and briefly washed with double-distilled water before cell plating.

## Scanning electron microscopy

Crystal-attached cells (on glass coverslips) were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer containing 5 mM $CaCl_2$, pH 7.2, for 30

min. The cells were rinsed three times, 5 min each, with 0.1 M cacodylate buffer and post-fixed for 1 h with 1% osmium tetroxide in the same buffer. The coverslips were then rinsed, dehydrated with ethanol, and critical point dried with $CO_2$ (Pelco CPD2, Ted Pella, Redding, CA). The samples were sputter-coated with gold for 6 min at 8 mA followed by 6 min at 10 mA (S150 Edwards, Sussex, UK) and examined in the scanning electron microscope, JSM-6400 (JEOL, Tokyo, Japan) operated at 15 kV.

## Adhesion of hyaluronidase-treated cells to tissue culture dishes

Hyaluronidase-treated or untreated A6 cells were suspended in serum-free or serum-containing medium and seeded in triplicate in 24 multi-well tissue culture plates (Falcon). After 10, 30, or 60 min of incubation the plates were washed three times with PBS and fixed with absolute ethanol (10 min at room temperature), followed by three additional washings with PBS. The attached cells were stained for 25 min with 10% Giemsa solution in water (Fluka) and washed with water until all excess dye was removed. The number of attached cells per square millimeter was then determined.

## RESULTS

### Selective binding of hyaluronan to calcium tartrate crystals

The selectivity manifested in the attachment of A6 cells to the {011} faces of (R,R), but not of (S,S) calcium tartrate crystals provides a unique tool for the identification of the molecular cell surface component responsible for surface recognition. For this purpose different macromolecules, including hyaluronan, dextran, and fibronectin were incubated with the two types of crystals, and the enantioselectivity in their adsorption to the crystal surfaces was monitored. Hyaluronan is a highly abundant large glycosaminoglycan with mass of up to several million daltons, composed of the disaccharide glucuronic acid-*N*-acetyl glucosamine (Weissman and Meyer, 1954), which is produced by many cell types and can be associated either with the cell surface or with the ECM (Laurent and Fraser, 1992; Toole, 2001). Hyaluronan is involved in numerous cellular processes, including cell adhesion, locomotion, inflammatory processes, and tumor invasion (for reviews see Ruoslahti, 1988; Delpech et al., 1997; Toole, 1997; Chen and Abatangelo, 1999; Toole, 2001). Dextran is a polysaccharide composed of D-glucose units and fibronectin is an Arg-Gly-Asp-containing glycoprotein involved in integrin-mediated adhesion of cell to the ECM. Equal amounts of calcium-(R,R)-tartrate tetrahydrate and of calcium-(S,S)-tartrate tetrahydrate crystals were incubated with rhodamine-conjugated fibronectin (20 $\mu$g/ml), rhodamine-conjugated dextran (50 $\mu$g/ml), or dansyl-conjugated hyaluronan (0.1–0.8 mg/ml). After 2 h of incubation, the crystals were rinsed and examined by fluorescence microscopy.

Dansyl-hyaluronan, irrespective of the specific labeling intensity or concentration (within the range indicated above), was exclusively bound to the (R,R)-tartrate crystals and did not adsorb to the (S,S) counterparts (Fig. 1, *top*).
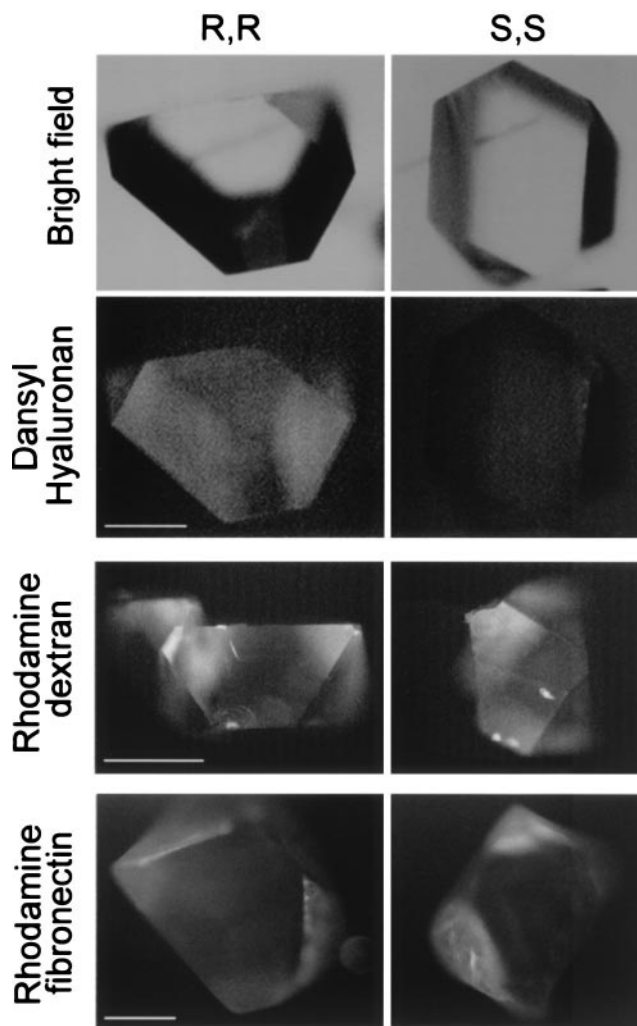


FIGURE 1 Adsorption of dansyl-labeled hyaluronan (*top*), rhodamine-labeled dextran (*center*), and rhodamine-labeled fibronectin (*bottom*) on calcium-(R,R)-tartrate tetrahydrate crystals or calcium-(S,S)-tartrate tetrahydrate crystals (denoted by R,R and S,S, respectively). A bright-field photograph of the dansyl-hyaluronan-labeled crystals is also reported (*topmost row*), to clarify the relation between crystal morphology and the fluorescent micrographs. Bars, 100 $\mu$m.

Control crystals (both enantiomers) incubated with equivalent concentrations of dansylboronic acid in buffer were negative (data not shown). In contrast, dextran (Fig. 1, *center*) and fibronectin (Fig. 1, *bottom*) were comparably bound to both crystals.

Employing standard fluorescence microscopy of dry crystals, the face-specificity of the hyaluronan binding could not be unequivocally resolved due to excessive multiple internal reflections. To overcome this problem, the fluorescent crystals were immersed in a solution of 1:1 anisole:benzyl acetate, which has a refractive index similar to that of the crystal itself. Under such conditions, the fluorescent light is not internally reflected at the surface and propagates only from the original surfaces to which the
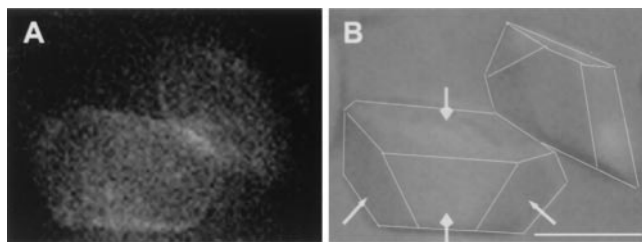
FIGURE 2 Fluorescence (*A*) and bright-field (*B*) micrographs of calcium-(R,R)-tartrate tetrahydrate crystals that were incubated with dansyl hyaluronan. The crystals are viewed while immersed in a solution of 1:1 anisole:benzyl acetate. The crystal profiles are hardly detectable because the refractive index of the medium is similar to that of the crystals. They have been thus enhanced graphically to emphasize their location, and different arrows indicate different face types. The fluorescence is similarly reduced in intensity because of the absence of internal reflection. Bar, 100 $\mu$m.

labeled molecules were adsorbed as in a homogeneous medium. The results (Fig. 2) indicate that dansyl-hyaluronan is adsorbed to a similar extent on both the {011} and {101} faces of calcium-(R,R)-tartrate tetrahydrate.

## Hyaluronidase treatment suppresses adhesion to external surfaces

Hyaluronidases hydrolyze hyaluronan by randomly cleaving the $\beta$-*N*-acetyl-glucosamine-[1–4]glycosidic bonds. To determine the role of hyaluronan in the adhesion to tartrate crystals and conventional tissue culture surfaces, A6 cells were suspended in serum-free medium and incubated for 10 min with 100 units/ml hyaluronidase. After removal of the enzyme, the cells were washed and incubated for 10 min in serum-free medium, with calcium-(R,R)-tartrate tetrahydrate crystals attached to glass slides. Following washing and fixation, the number of cells attached per unit area of the {011} crystal faces, as well as of the surrounding glass, was determined from images taken by SEM. After treatment (Fig. 3 *B*, compare with control, untreated cells in Fig. 3 *A* and Fig. 4 *D*) the number of attached cells decreased by over 90%, from an average of $67 \pm 10$ to $4 \pm 6$ per $10^4$ $\mu$m$^2$.

Close examination by SEM of the {011} faces following incubation with hyaluronidase-treated cells revealed the presence of numerous cell imprints on the crystal surface, suggesting that many transient interactions had occurred between the cells and the crystal, which did not develop into stable adhesions (data not shown). Examination of the glass coverslips in the same cultures revealed a reduction by ~90% of bound cells, following hyaluronidase treatment (Fig. 3, *C* and *D*), similar to the one found with tartrate crystals. Similar suppressive effects of hyaluronidase treatment on substrate adhesion were also noted when cells were plated on glass or tissue culture plastics in regular (serum-containing or serum-free) medium, without calcium tartrate (data not shown). We note that cell attachment to either
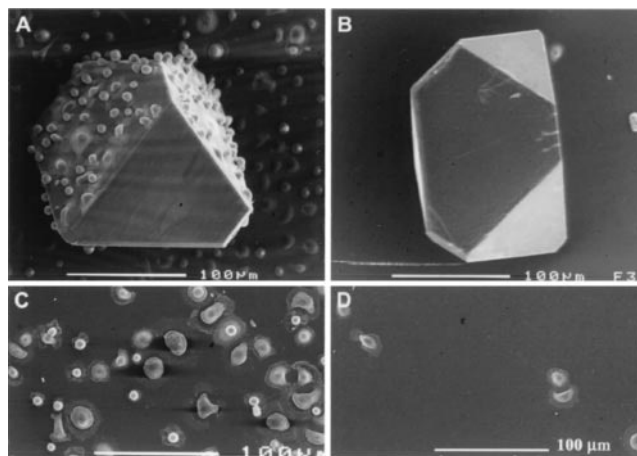


FIGURE 3 Scanning electron micrographs of A6 cells attached to calcium-(R,R)-tartrate tetrahydrate crystals (*A* and *B*) or to glass coverslips (*C* and *D*) after 10 min of incubation in serum-free medium. (*A* and *C*) Untreated cells; (*B* and *D*) Cells treated with hyaluronidase before plating.

tissue culture dishes or to the crystals was decreased when the cells were harvested with EGTA treatment rather than trypsinization before plating (see Fig. 4 in Hanein et al., 1995). Thus, in contrast to hyaluronan, removal of trypsin-sensitive proteins from the cell surface increases early attachment.

To determine whether the loss of cell attachment is directly correlated to the removal of hyaluronan from the cell surface, hyaluronidase-treated A6 cells were incubated in suspension in media containing increasing concentrations (0.01–1 mg/ml) of exogenous hyaluronan. The cells were washed and plated on the glass-attached crystals for 30 min in serum-free medium, and the number of cells attached to both the crystals and the glass coverslips was monitored as above. Following incubation with hyaluronan, the hyaluronidase-treated cells partially regained their ability to adhere to the crystals (Fig. 4, *A* and *D*). The binding to the crystals relative to control untreated cells increased progressively up to a maximum of 60% at 0.5 mg/ml hyaluronan and was somewhat reduced when higher concentrations of hyaluronan were used (Fig. 5 *A*). The recovery of cell attachment to the glass was comparable, and even higher, reaching a maximum of over 80% at 0.2 mg/ml hyaluronan (Figs. 4 *A* and 5 *A*).

To determine whether it is mandatory for cell adhesion that hyaluronan be present on the cell surface, rather than on the substrate, hyaluronidase-treated A6 cells were seeded on crystals that were preincubated with increasing concentrations of hyaluronan. This treatment resulted in complete recovery of cell attachment, suggesting that hyaluronidase-treated cells maintain hyaluronan-binding ability and can efficiently use the crystal-bound hyaluronan for attachment (Figs. 4, *B* and *D*, and 5 *B*). The recovery of adhesion to the crystals was low at low concentrations of hyaluronan and
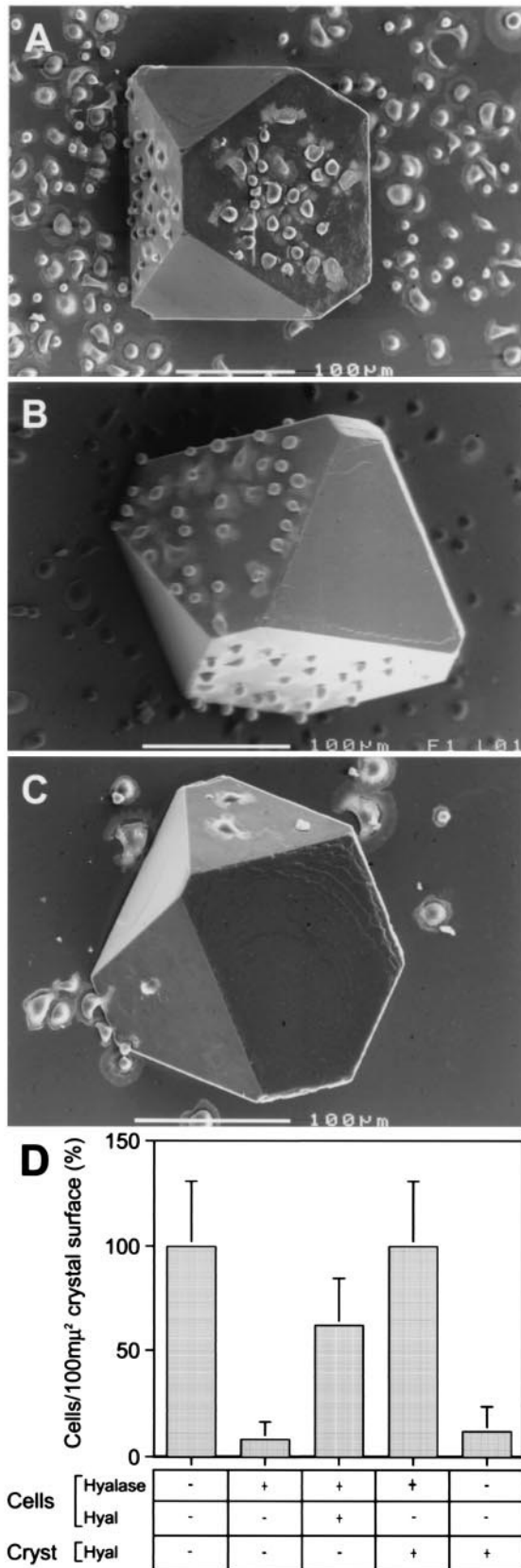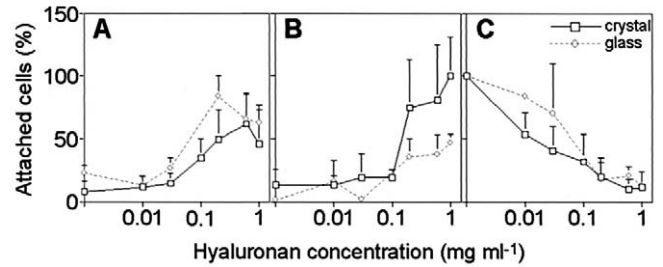
FIGURE 5 Effect of increasing concentrations (0.01–1.0 mg/ml) of hyaluronan on the attachment of hyaluronidase-treated and untreated A6 cells to the {011} faces of calcium-(R,R)-tartrate tetrahydrate crystals (□) or to glass coverslips (◇). Incubation of the cells with the substrates was for 10 min in serum-free medium. (*A*) Hyaluronidase-treated cells attached to the two surfaces after treatment with different concentrations of exogenous hyaluronan; (*B*) Hyaluronidase-treated cells attached to substrates coated with different concentrations of hyaluronan; (*C*) Untreated cells attached after addition of different concentrations of hyaluronan to the substrates.

increased steeply above concentrations of 0.1 mg/ml, reaching a maximum value of 100% at 1 mg/ml (Fig. 5 *B*). The recovery of adhesion to the glass was significant but did not exceed 50%, suggesting that the pattern or extent of hyaluronan adsorption is different on the two substrates (Fig. 5 *B*).

We next determined whether hyaluronan, when present on both the cells and the substrate, can still mediate adhesive interactions. For that purpose, untreated A6 cells were plated on hyaluronan-coated substrate and the number of attached cells determined. As shown, increasing concentrations of hyaluronan dramatically suppressed cell adhesion to both the crystals and the glass surface (Figs. 4, *C* and *D*, and 5 *C*). The inhibition of adhesion was hyaluronan concentration dependent and reached values of over 90% at 1 mg/ml. It is noteworthy that similar hyaluronan-coated crystals showed full adhesive ability toward hyaluronidase-treated cells (compare Fig. 4 *B* with Fig. 4 *C* and Fig. 5 *B* with Fig. 5 *C*). This indicates that although hyaluronan can mediate cell adhesion, excess hyaluronan is anti-adhesive.

In conclusion, following treatment with hyaluronidase, the cells completely lose their ability to form early attachments to crystal and glass surfaces, suggesting that A6 cell adhesion to these faces is both hyaluronan dependent and mediated. This is supported by the ability of hyaluronan,



medium. (*A*) Hyaluronidase-treated cells incubated with 0.5 mg/ml hyaluronan before plating on the crystals; (*B*) Hyaluronidase-treated cells plated on crystals that were preincubated with 0.1 mg/ml hyaluronan; (*C*) Untreated cells plated on crystals that were preincubated with 0.1 mg/ml hyaluronan; (*D*) A histogram showing the binding of A6 cells to the {011} crystal faces of calcium-(R,R)-tartrate tetrahydrate crystals after 10 min of incubation in serum-free medium after the various treatments described above. The values are expressed as percentage of the binding of untreated cells to untreated crystals. Hyal, hyaluronan treatment; Hyalase, hyaluronidase treatment.

FIGURE 4 (*A–C*) Scanning electron micrographs of A6 cells incubated for 10 min with calcium-(R,R)-tartrate tetrahydrate crystals in serum-free
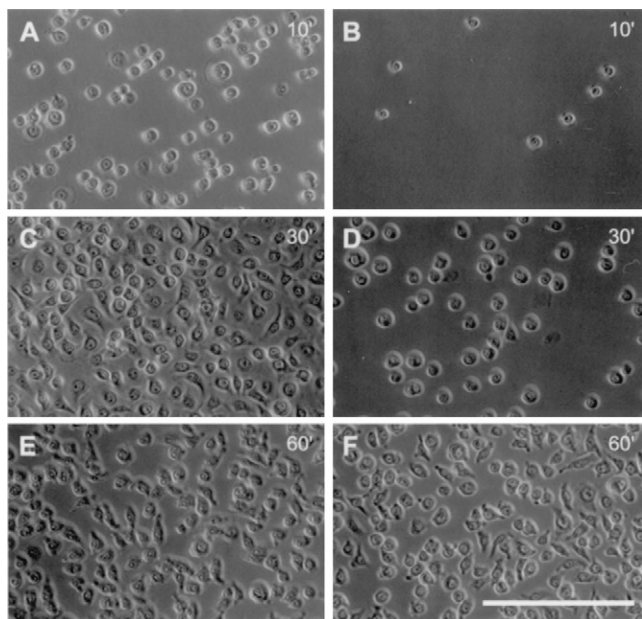
FIGURE 6 Effect of hyaluronidase treatment on the attachment of A6 cells to tissue culture dishes at different times after plating. Phase-contrast photomicrographs of untreated cells (*A*, *C*, and *E*) or hyaluronidase-treated (*B*, *D*, and *F*) A6 cells attached to tissue culture dishes in serum-free medium following incubation for 10 min (*A* and *B*), 30 min (*C* and *D*), or 60 min (*E* and *F*). Bar, 100 $\mu$m.

applied either to the cells or to the crystals, to restore the adhesive activity of hyaluronidase-treated cells. We also show that hyaluronan can act as an adhesion suppressor and, when present on both the cells and the substrate, can block, rather than stimulate, adhesion.

To further explore the generality of the hyaluronan involvement in early adhesion of A6 cells, we checked the effect of hyaluronidase treatment on the time-dependent adhesion and spreading of these cells to tissue culture dishes. A6 cells were incubated with hyaluronidase for 30 min in serum-free medium and plated on Falcon tissue culture dishes. The dishes were washed and fixed after 10, 30, and 60 min, and the number of cells attached was counted by light microscopy (Fig. 6).

Similar to the behavior observed on the crystals and on glass, A6 adhesion to tissue culture dishes was dramatically impaired by hyaluronidase treatment. The most pronounced inhibition (75%, from 440 (untreated) to 110 (hyaluronidase-treated) cells per mm$^2$) was observed after 10 min of incubation of the cells with the culture plates (Fig. 6 *B*, compare with untreated cells in Fig. 6 *A*). The relative reduction in attachment was of 51% after 30 min (Fig. 6, *C* and *D*), suggesting that the cells can recover their adhesive activity over time. We also noted that hyaluronidase treatment had a major effect on cell spreading. Thus, after 30 min of incubation in culture, many of the untreated cells were spread on the surface, whereas the hyaluronidase-treated cells were mostly spherical. After longer incubation

time (60 min and more) there was no significant difference in the attachment and spreading between untreated and treated cells (Fig. 6, *E* and *F*). It was also noted that inhibition of adhesion by hyaluronidase was not affected by the presence or absence of 10% serum in the plating medium (data not shown).

To test the generality of the hyaluronan-mediated adhesion we have tested the effect of hyaluronidase on the adhesion to tissue culture plates of different cell lines. For this purpose we have selected different lymphoid and myeloid lines including Raji, Duadi, and K562 as well as pig aortic endothelial cells. We have detected a dramatic reduction in the short-term adhesion of Raji, Daudi, and pig aortic endothelial cells to the culture plates, whereas the adhesion of K562 was apparently insensitive to hyaluronidase treatment.

Taken together, these results indicate that hyaluronan can serve as a versatile modulator of cell-matrix adhesion, depending on the nature of the cells (e.g., presence or absence of endogenous hyaluronan or hyaluronan-binding receptors) and of the substrate.

## DISCUSSION

In this study we show that hyaluronan is an adhesion modulator molecule, which can mediate early stages of cell-substrate interaction, when presented either on the cell surface or on the substrate, or block adhesion, when present on both surfaces. This mechanism is schematically illustrated in a general model (Fig. 7), based on the following findings. 1) The adhesion of A6 cells to different substrates is drastically reduced (down to 10% or less) by treating cells with hyaluronidase (Fig. 7 *B*, compare with the adhesion of control cells in Fig. 7 *A*). 2) The adhesion of hyaluronidase-treated cells to the substrate can be partially or fully restored by treating either the cells (Fig. 7 *C*) or the external surface (Fig. 7 *D*) with exogenous hyaluronan. 3) The presence of excess hyaluronan on both the cells and the substrate inhibits adhesion (Fig. 7 *E*). 4) Similarly to A6 cells, free hyaluronan (dansyl labeled) binds selectively to calcium-(R,R)-tartrate tetrahydrate crystals and does not bind to the (S,S) enantiomorph (Fig. 1). Surfaces that do not bind hyaluronan, such as the different faces of the (S,S) enantiomorph, fail to engage in rapid adhesion although they bind adhesive proteins such as fibronectin (Hanein et al., 1993, 1995).

These findings, pointing at the involvement of hyaluronan in early and stereoselective cell adhesion, require further discussion, addressing the chemical and physical properties of hyaluronan, its mode of interaction with the cell surface, and the mechanism of its interaction with the external surface. Hyaluronan is a large, linear glycosaminoglycan composed of a repeating disaccharide of glucuronic acid and *N*-acetylglucosamine (Toole, 2001). Its large dimensions render it an excellent candidate for long-range interactions of cells with external surfaces. Each molecule
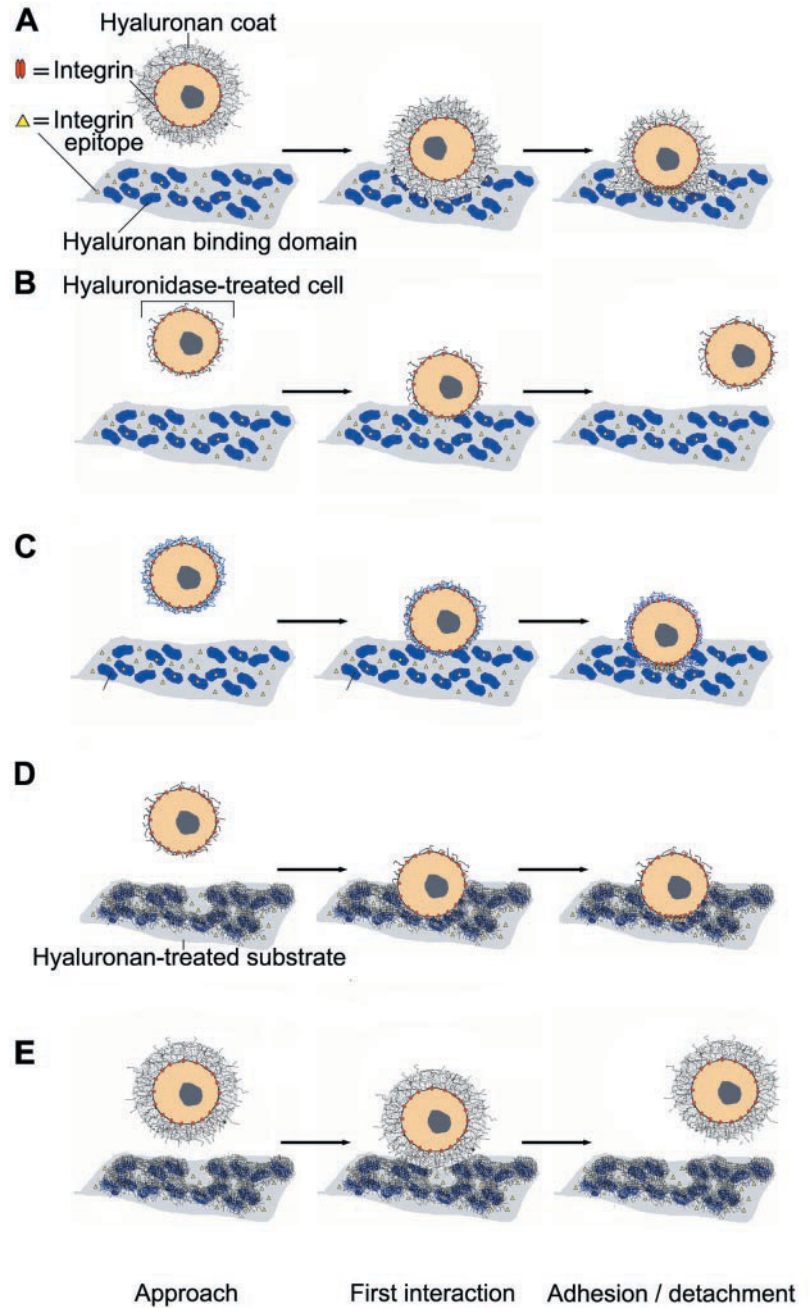
FIGURE 7 Schematic model illustrating the involvement of surface-bound or substrate-bound hyaluronan in cell-substrate adhesion (for details see text).

can reach a mass of several million daltons, and consequently a length of ~10 $\mu$m, when fully extended (Fessler and Fessler, 1966; Ren et al., 1991) (an unlikely situation in physiological environments). It is, however, presumed (Laurent, 1970) that one single molecule may extend to several microns from the surface to which it is tethered or adsorbed. The effective occupied volume and layer thickness depends also on the concentration of neighboring chains, mode of surface attachment, and their charge, such that the excluded volume of each will influence the state and extension of the molecule close by. Thus, although a single molecule may be adsorbed to

a surface at multiple sites along its length, the crowding of chains will force them to extend out into solution in a more or less extended conformation. This is confirmed also by ample evidence on the thickness of the proteoglycan/hyaluronan pericellular coat, mainly in chondrocytes, visualized by the exclusion of fixed particles such as red blood cells (Knudson et al., 1999; Knudson and Knudson, 2001; Toole, 2001). The presence of a thick hyaluronidase-sensitive layer surrounding the cell membrane was reported also for other types of cultured cells (McBride and Bard, 1979; Heldin and Pertoft, 1993; Lee et al., 1993; Evanko et al., 1999), supporting the notion

that hyaluronan is indeed a common long-range adhesion mediator.

Hyaluronan chains interact through multiple intramolecular and intermolecular hydrogen bonding, forming ribbon and ladder structures (Kobayashi et al., 1994; Mikelsaar and Scott, 1994). As there is one charged carboxylate in every alternate ring, hyaluronan behaves in solution as a polyelectrolyte, whose intra-chain and inter-chain repulsion depends on pH and salt composition and concentration (Albersdorfer and Sackmann, 1999). In the synovial fluid and in cartilage, hyaluronan functions as a lubricant by virtue of the repulsion between charges and contributes to the maintenance of osmotic pressure because of its ability to adsorb and hold large amounts of water (Israelachvili and Wennerstrom, 1996). At high concentration, it thus forms swollen gels that can withstand relatively high local pressure without collapse (Levick, 1995).

The abundance of hyaluronan on the cell surface, together with its chemical-physical characteristics, thus suggest that this molecule is forming a viscous coat over the entire plasma membrane, free to interact with external surfaces. Due to the large size of the hyaluronan molecule such interactions may be initiated when the plasma membrane is still located at considerable distance from the adhesive surface, >100-fold larger than the membrane-to-surface gap found, for example, in integrin-mediated focal adhesions.

A single interaction of one functional group with the surface would occur in the time scale of milliseconds. Within seconds the pericellular hyaluronan coat could be anchored to the substrate by multiple cooperative interactions, singularly relatively weak, but collectively sufficient to hold the cells close to the surface until stable contacts of the integrin receptors with their extracellular matrix receptors are established. The latter require time frames of the order of minutes or more, and space-frames of the order of tens of nanometers between the membrane and the substrate. A crucial outstanding question is what is the mechanism of this approach. In principle, at least three different scenarios can be envisaged: 1) the hyaluronan receptors on the membrane could diffuse away from the contact site, removing locally the attached hyaluronan molecules and making space for the integrin receptors and their ECM targets; 2) the physico-chemical microenvironment of the attachment site could be actively changed, either by changing the pH or the counterion concentration, causing dehydration and collapse with consequent condensation of the hyaluronan chains; or 3) hyaluronan could be removed by enzymatic degradation or by endocytosis (Hua et al., 1993).

It might be argued that the scenario of hyaluronan mediation of early interactions is a peculiarity of the experimental system, dictated by the harvesting procedure, i.e., trypsinization. Integrin and surface-bound fibronectin might be removed by this treatment, whereas hyaluronan is not, thus inducing an apparently slower activity of the integrin/ fibronectin-dependent adhesion pathway. It has been, however, shown that early and fast adhesion of A6 cells to crystals as well as to glass and tissue culture dishes is independent not only of the harvesting procedure but also of the presence or absence of serum in the plating medium (Hanein et al., 1995) and of RGD-peptide inhibitors (Hanein et al., 1993). Thus, integrin/fibronectin-dependent pathways do not appear to be involved in these early stages of adhesion.

We do not know yet the molecular nature of hyaluronan binding to the cell surface in A6 cells. In other systems, hyaluronan was shown to associate with the surface of cells via multiple mechanisms. Its most extensively studied transmembrane receptor is CD44, which is involved in many of the physiological effects of hyaluronan, including the interaction, across the membrane, with the actin cytoskeleton (Aruffo et al., 1990; Lesley and Hyman, 1998; Bajorath, 2000) and with transmembrane signaling systems (Entwistle et al., 1996; Ilangumaran et al., 1999; Oliferenko et al., 2000). Some of the surface hyaluronan is also interacting with the submembrane enzymes that are involved in its synthesis, namely, hyaluronan synthases (Weigel et al., 1997). Interestingly, a newly described hyaluronan receptor, namely layilin, was localized to focal contacts and shown to interact, within the cell, with talin (Bono et al., 2001).

Despite the limited information on the exact molecular interactions of hyaluronan with specific components of the cell membrane or the substrate, a number of tentative conclusions can be put forward, based on the observations made here.

If hyaluronan forms an extended gel-like coat surrounding the cell, it is easy to conceive that it would be the first cellular component to encounter any external surface. The molecular moieties being chiral, it is not surprising that this can lead to chiral recognition of the external surfaces provided that they contain complementary chiral moieties, such as those exposed on the {011} surfaces of calcium-(R,R)-tartrate crystals. The binding of free hyaluronan to the calcium-(R,R)-tartrate tetrahydrate (and not to the (S,S) enantiomer) provides further support to the ability of the molecule to mediate specific molecular recognition rather than general charge attraction. In contrast, fibronectin is adsorbed comparably to both (R,R) and (S,S) enantiomers (Fig. 1), further supporting the notion that fibronectin is not involved in these early cell recognition processes. The binding of free hyaluronan to both the {011} and {101} faces of the calcium-(R,R)-tartrate tetrahydrate crystals is, nevertheless, intriguing in view of the fact that the cells show a keen distinction between the two. We have no direct explanation to this apparent discrepancy. Possibly, it may be attributed either to a different mode of interaction of free versus membrane-bound hyaluronan with the crystal surface or to repulsion of the charged carboxylates of cell-bound hyaluronan by the carboxylated surfaces. It is noteworthy that more calcium counterions appear to be exposed per unit

area on the {011} faces, relative to the {101} surfaces. We can only argue that isolated molecules in solution may experience locally less repulsion from the surface than a dense population of polyelectrolytes in a confined volume around the cell. To this same context belongs the observation that adsorption of small amounts of hyaluronan to the otherwise nonadhesive {101} surface enhances its adhesive ability toward A6 cells, whereas large amounts of adsorbed hyaluronan are inhibitory to cell attachment to both crystal face types (data not shown). We surmise that, when present in small amounts, hyaluronan attaches to the surface at multiple sites, thus providing rigid attachment sites. Large amounts, on the other hand, presumably coat the surface with a layer of flexible polyelectrolytes extending in the solution, which become repulsive.

Of particular interest is the dual role of hyaluronan, which can act either as an inhibitor or mediator of cell-substrate adhesion. It appears that, to achieve effective cell adhesion, hyaluronan must act as a direct bridge binding to both the cell surface and the substrate. Excess hyaluronan, on the other hand, was found to be inhibitory, suggesting that hyaluronan-hyaluronan "trans" interactions are repulsive rather than attractive. This is most probably due to steric exclusion and/or electrostatic repulsion between the two juxtaposed hydrated layers (one associated with the cell membrane and the other with the external surface). Although hyaluronan was found to promote adhesion both in its membrane-bound and substrate-bound forms, some lack of symmetry was observed in the above results; Thus, hyaluronan treatment of crystals fully restored the ability of hyaluronidase-treated A6 cells to adhere to surface, whereas hyaluronan treatment of the same cells was only partially (up to 60%) effective. This may be attributed to the fact that some of the surface-associated hyaluronan may not be recovered (for example, molecules that were anchored via hyaluronan synthase). Alternatively, excess hyaluronan added to the cells might have been released into the solution and subsequently adsorbed to the surfaces, thus inhibiting attachment.

The unique physico-chemical properties of hyaluronan, together with the observations reported above, make it an eligible candidate for mediating cell matrix adhesion in general, rather than being a peculiarity of A6 cells. In preliminary studies we have found that some arbitrarily chosen B-cells (Raji and Daudi cell lines) as well as pig aortic endothelial cells also exhibited hyaluronidase-sensitive adhesion, although others did not. The notion of hyaluronan-mediated cell adhesion is also in excellent agreement with reports on carbohydrate and hyaluronan involvement in physiological and pathological adhesion events. In general, interactions mediated by membrane-associated carbohydrates were described in a variety of biological systems, including selectin-dependent attachment of leukocytes to surface carbohydrate molecules of another cell, which precede the integrin-mediated interaction

(Springer, 1994; Rossiter et al., 1997). Involvement in cell-matrix adhesion was also found for other proteoglycans and glycosaminoglycans, which either mediate cell-surface adhesion or modulate it (Laterra et al., 1983; Lark and Culp, 1984; Ruoslahti, 1988; Wight et al., 1992; Bernfield et al., 1999).

Evidently, the notion of involvement of hyaluronan in cell adhesion is not new, yet the present work identifies a unique role for this molecule in the early and long-range engaging interactions of cells with substrates. Furthermore, our findings highlight the ability of hyaluronan to act as a modulator of cell adhesion, inducing or inhibiting adhesive interactions, depending on the specific properties of both the cells and the matrix around them. Finally, this work sheds new light on adhesion processes as a chain of distinct sequential molecular stages and on the involvement of carbohydrates in this complex process.

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