Spatial and Temporal Sequence of Events in Cell Adhesion: From Molecular Recognition to Focal Adhesion Assembly

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The Biological Significance of Cell Adhesion

The adhesive interaction of cells with external surfaces is an ancient biological phenomenon and characteristic of essentially all forms of life, from unicellular to the most complex multicellular organisms (metazoa). In a variety of unicellular organisms, transient adhesion to external surfaces is essential for such processes as locomotion toward food sources or away from repellents, attachment to prey, or binding to another cell during mating.[1] The development of metazoan life, some 600 million years ago, is characterized by the dramatic diversification of cell types forming multicellular organisms and the formation of stable tissues and organs. Concomitantly, new and effective strategies for adhesion emerged that enable individual cells to form higher-order structures. In the slime mold Dictyostelium discoideum, for example, the free-living amoeboid cells can adhere to a variety of surfaces and actively migrate as long as the environmental conditions are favorable. When confronted with starvation, these cells undergo massive aggregation, leading to the formation of a multicellular organism, where cell–cell adhesion and communication take place.[2]

Beginning in the 1960s, ultrastructural studies[3] and subsequent molecular analyses have revealed a rich variety of adhesive structures between neighboring cells or between cells and the extracellular matrix. These, among others, can be linked to different cytoskeletal networks, form intercellular channels, compartmentalize membrane domains, and mediate signaling events. Numerous genes concertedly participate in the formation, maintenance, and regulation of such adhesive interactions.

Molecular Diversity of Cell Adhesions

To illustrate the structural and molecular complexity of cell adhesion let us consider two examples: leukocytes and epithelial cells. The former are short-lived single cells, whose function in protecting the organism from invaders depends on a highly complex adhesive machinery.[4–8] These adhesions involve both protein–protein and protein–carbohydrate interactions, which are regulated by specific signaling factors (chemokines) and their receptors (Figure 1). Epithelial cells, on the other hand, form stable adhesions with their neighbors and with the underlying basement membrane. These include tight junctions that block diffusion of molecules across the epithelial layer, adherens junctions that are associated with the contractile actin cytoskeleton, and desmosomes—linked to the cytokeratin network.[9] Gap junctions are dot-like adhesions forming intercellular channels that allow passage of small molecules from one cell to the next.[10] Attachments to the basement membrane consist of focal adhesions (FA) and hemidesmosomes, each comprised of tens of different proteins, including links to the actin and intermediate filament systems, respectively (Figure 2).[11, 12]

How are these complex, multimolecular adhesive systems regulated in space and time? Does each system function as an independent unit or are they coordinated or even interdependent? How can the cell down-regulate its adhesive interaction to allow, for example, cell migration or division?

In this article we will consider the dynamic molecular events involved in the development of cell adhesions, across a wide range of temporal and spatial scales. While first cell contact and recognition occur on a subsecond timescale, full cell spreading takes tens of minutes to hours. During that time, the distance between the cell membrane and the external surface decreases from micrometers to 10–20 nm. Concomitantly,
The Elementary Modules of Cell Adhesions

Despite the enormous heterogeneity of adhesion systems, there are some common and distinctive features that characterize the molecular interactions frequently found in these sites. Adhesion receptors can mediate direct protein–protein interactions with the external surface. Such receptors are usually single- or multiple-chain transmembrane proteins with an extracellular domain involved in binding to the external surface and an intracellular domain that can interact with the cytoskeleton. Typical examples for such receptors are members of the integrin family, which mediate adhesion to the extracellular matrix or to other cells, and cadherins, which form Ca\(^{2+}\)-dependent cell–cell junctions. In such systems, the characteristic spacing between the plasma membrane and the “adhesive ligand” on the external surface is of the order of 15–20 nm.

Another common mechanism involves interactions between lectin-like protein receptors and their carbohydrate ligands on the external surface. Examples for such receptors include among others different selectins, the hyaluronan receptor CD44, and galectins. Adhesive surface lectins, (e.g. galectin-8) can also be secreted by cells and competitively block, rather than promote, adhesion.

The interactions mediated by adhesive receptors and their ligands can be regulated by a variety of external and internal factors. Cadherins, for example, are activated by ex-
charged group or even for the chemical nature of a charged binding; for example, there is no selectivity for a particular receptor (as opposed to protein, transmembrane receptors) that can interact with molecularly compatible part-
molecular binding sites on the surface. Generally, they show little specificity in the picture. Individually, these form what we shall refer to as the molecular coat. This consists of charged groups, hydrogen-bond donors and acceptors, and lipophilic patches. Counter ions and bound water molecules of varying mobility complete the picture. Individually, these form what we shall refer to as the molecular binding sites (as opposed to protein, transmembrane receptors) that can interact with molecularly compatible partners on the surface. Generally, they show little specificity in binding; for example, there is no selectivity for a particular charged group or even for the chemical nature of a charged group. Occasionally they might, however, be highly specific to certain molecular counterparts, such that stereoselective or even enantioselective interactions (see below) are observed. Individually, the energy involved in each of these interactions is relatively small, of the order of a few kcal mol$^{-1}$ or a few $k_T$ per interaction. Extensive cooperativity may, however, build up to substantial interaction energies. In three-dimensional arrangements, multiple molecular binding sites constitute ligands (epitopes) for highly oriented, localized, and (stereo-) chemically specific recognition and binding by matching receptors on the cell (e.g. integrin-RGD). Recognition in this sense involves binding between matching (e.g. electrostatic, polarity) topographies, and is not limited in complexity to a single receptor-epitope pair. Rather, it can involve any number of simultaneous contacts in a multireceptor cluster. Such receptor-epitope clusters can contribute up to several tens of kcal mol$^{-1}$ in binding energy.$^{[22,23]}$

Adhesive Interactions as a Multistep Process: The Concept

Time-wise, the establishment of receptor–epitope based complexes such as integrin-RGD contacts is observed to occur within a framework of minutes after the first molecular interactions have been established.$^{[25]}$ This lag depends on the surface densities of both receptor (ca. $5 \times 10^5$ $\mu$m$^2$)$^{[26]}$ and epitope, which determine the frequency of mutual encounters. As complex three-dimensional interactions require an appropriate relative orientation between the interacting partners, only a negligible fraction of random encounters would develop into a stable interaction. Binding of pericellular components to the surface, in contrast, may occur within less than a second.

Given these premises, it is reasonable to assume that rapid and transient interactions must ensue first. The transition of a cell in suspension to interacting with a surface could then be based on a subtle interplay of recognition, cooperativity, and kinetic parameters. A great number of simultaneous but rather weak interactions could conceivably lead to a transient bound state, where the multiplicity and cooperativity of weak interactions provide the necessary binding strength. If the arrangement is dynamic, it can both adapt itself to three-dimensionally structured surfaces and quickly accommodate subsequent changes in organization. Such is the interaction between hyaluronan and substrate that we shall consider below.

The adhesion model that derives from the above assumptions would then include at least three consequent steps: 1) Recognition and establishment of contacts between pericellular components and complementary binding domains.

**Figure 3.** Evolution of cell–substrate adhesions in terms of separation distance and contact area as a function of time. Initial cell–surface recognition occurs within less than a second. At this stage the cell membrane is 1–5 $\mu$m from the surface. Within seconds, early attachment ensues, anchoring the cell to the substrate. Reorganization of the pericellular coat enables the cell membrane to approach the surface. At this stage the cells have not yet flattened, but the contact area increases to about 100 $\mu$m$^2$. Transition to the membrane adhesion stage takes on the order of minutes. Integrins begin to interact with RGD epitopes on the surface, initiating the formation of focal adhesions where the separation distance decreases to 15 nm. At the last stage, cell spreading, the cell contact area increases by two orders of magnitude within a few hours.

**Molecular Events Associated with the Formation of new Adhesions**

Unraveling the basic processes of cell adhesion requires an understanding of how multiple adhesive mechanisms are regulated and coordinated in time and space, such that the adhesion is selective, efficient, and dynamic (reversible). Herein we shall examine conceptually an approach to solve this problem, as it has evolved in various cells.

Consider a cell, suspended in aqueous medium, approaching a surface. Such a surface will display varied, repetitive, and sometimes periodic patterns of charged groups, hydrogen-bond donors and acceptors, and lipophilic patches. Counter ions and bound water molecules of varying mobility complete the picture. Individually, these form what we shall refer to as molecular binding sites (as opposed to protein, transmembrane receptors) that can interact with molecularly compatible partners on the surface. Generally, they show little specificity in binding; for example, there is no selectivity for a particular charged group or even for the chemical nature of a charged group. Occasionally they might, however, be highly specific to certain molecular counterparts, such that stereoselective or even enantioselective interactions (see below) are observed. Individually, the energy involved in each of these interactions is relatively small, of the order of a few kcal mol$^{-1}$ or a few $k_T$ per interaction. Extensive cooperativity may, however, build up to substantial interaction energies. In three-dimensional arrangements, multiple molecular binding sites constitute ligands (epitopes) for highly oriented, localized, and (stereo-) chemically specific recognition and binding by matching receptors on the cell (e.g. integrin-RGD). Recognition in this sense involves binding between matching (e.g. electrostatic, polarity) topographies, and is not limited in complexity to a single receptor-epitope pair. Rather, it can involve any number of simultaneous contacts in a multireceptor cluster. Such receptor-epitope clusters can contribute up to several tens of kcal mol$^{-1}$ in binding energy.$^{[22,23]}$

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on the substrate. These contacts involve instantaneous, multiple, cooperative, and sometimes stereoselective molecular recognition.

2) Pericellular component-mediated contacts trigger the approach of the membrane-bound receptors to the substrate.

3) Interactions between integrins in the membrane and their binding domains (e.g. fibronectin) on the surface initiate focal contact development and maturation, including linking to the cytoskeleton.

Although the existence of pericellular components is well documented, their direct participation in cell adhesion events has been firmly established only for few systems, notably selectin–carbohydrate interactions in blood cells.

We report below evidence for and characterization of similar processes for epithelial and chondrocyte (cartilage-forming) cells. This evidence puts the process in a new perspective and suggests that such events might be much more widely spread than suspected so far, and that they might regulate the “decision-making process” of cells vis-à-vis the establishment of stable contacts with substrates.

Adhesive Interactions as a Multistep Process: Experimental Evidence

The possibility of very fast and dense interactions between substrate and some component present on the cell membrane was forcefully brought to our attention in the attachment of certain epithelial cells to one crystal surface type (but not the other) of calcium tartrate tetrahydrate crystals. It was subsequently proven to be operative also in the adhesion of the same cells to more conventional substrates, such as glass and tissue culture dishes. The stereoselectivity of the interaction, manifested in the fast and dense attachment of cells to calcium-(R, R)-tartrate, but not calcium-(S, S)-tartrate crystals (the molecular and structural mirror image), was the give-away evidence for the identity of the component. This had to be a chiral cell-associated biopolymer, presumably a polysaccharide, that was eventually identified as being hyaluronan. Not only did hyaluronan adsorb selectively on the crystal that the cells attach to, and not to the mirror image crystal, but its removal by hyaluronidase hydrolysis substantially reduced cell attachment to the crystal surfaces and to glass and cell culture substrates.

The glycosaminoglycan hyaluronan is a high molecular weight (up to $10^7$ Da), polydisperse linear polysaccharide composed of several thousand repeat units (Figure 4a). In the absence of proteins and at physiological conditions, hyaluronan behaves as a weak polyelectrolyte.

Recognition and immediate establishment of extensive cooperative contacts between hyaluronan and complementary binding domains on the substrate requires that a continuous and relatively thick hyaluronan coat cover the whole surface of the cell in suspension (Figure 5a, b). What are the density and the thickness of the putative hyaluronan coat?

We have demonstrated that the hyaluronan pericellular layer on A6 epithelial cells has a thickness of approximately 2 μm, while it reaches up to 5 μm thickness around chondrocytes in suspension. Furthermore, the 2 μm thick pericellular coat around the epithelial cells establishes a “rigid” nondeformable contact with glass substrates, which is strong enough to resist a shear force of 6.5 dyn cm$^{-2}$ (0.65 pN μm$^{-2}$), applied by flow. Conversely, the 5 μm thick pericellular coat of chondrocytes establishes “soft” contacts, which react to shear by sliding on the glass surface, leaving a hyaluronan “trail” behind the cell. We suggest that the hyaluronan coat consists of one layer of densely packed hyaluronan molecules, directly anchored to the membrane of epithelial cells to form a brush (Figure 5c, e). In contrast, chondrocytes have multiple layers of entangled and cross-linked hyaluronan molecules (Figure 5d, f). Other proteins and glycosaminoglycans such as aggrecan and heparan sulfate most probably contribute to the integrity and to the properties of the coat.
Pericellular Hyaluronan Regulation of Cell Adhesion: Biophysical Considerations

The following theoretical considerations support the above model: hyaluronan deposited on a surface without space constraints forms typically a layer of 200 nm thickness, on the order of magnitude of the characteristic molecular radius (radius of gyration \( R_g \), Figure 4 b). However, if there is a sufficient number of membrane binding sites (e.g. CD44) for hyaluronan such that the distance between individual chains bound on the surface is less than the radius of gyration (ca. 180 nm for a 2 MDa chain), the chains could be forced to stretch out and form an “Alexander-de-Gennes” polymer brush (Figure 4 c, d). This brush is in fact in an equilibrium state in which the osmotic pressure exerted by the tethered polymer in solution drives the stretching of the chain. This is opposed by the elastic energy of the polymer chain, which, for entropic reasons, prefers the coiled state. The brush thickness is primarily a function of molecular weight and grafting density. The molecular weight distribution of hyaluronan in the pericellular coat of chondrocytes or A6 epithelial cells is not known. It seems reasonable, however, to assume an average weight of \( 2 \times 10^5 \) Da (5 \( \mu \)m total length). With a chain density where the distance between chains is between 100 and 10 nm (equivalent to \( 10^2 \)–\( 10^3 \) binding sites per \( \mu \)m\(^2\)), the brush thickness could reach 2 \( \mu \)m.

The presence of such a thick layer of hyaluronan must influence the subsequent fate of the adhesion and of any other signaling process to the cell. In particular, the layer must be modified or removed to make way for receptor–integrin contacts. Any of at least three distinct processes, or combinations thereof, may conceivably foster the evolution of contacts to the second stage. Hyaluronan can be removed from the contact site by: 1) directed (lateral) diffusion or active transport of the hyaluronan and the attached membrane receptors, 2) hyaluronidase-induced hydrolysis or other degradative removal, or 3) collapse of the hyaluronan brush through interaction with the surface or by cell-induced changes of the effective hyaluronan receptor density (Figure 6).

The density and affinity of the molecular contacts will have a determining effect on the process. It may be expected that establishment of a tight network of high affinity and high density contacts will lead to rapid attachment, which may be too strong and essentially irreversible (which is incompatible, for example, with cell motility). This was observed on the (011) faces of (R,R)-calcium tartrate crystals, where the cells attached but did not spread further.

The situation of high affinity but low density receptor contacts is well represented by the integrin interaction, which is consequently slow but stable, once formed. Alone, the time and space scales of these interactions are not sufficient to warrant adhesion. This is demonstrated by the inability of hyaluronidase-treated cells to develop adhesion despite the presence of integrins on their surface. These contacts have to be preceded by the establishment of low-affinity, high-density transient contacts, such as those of hyaluronan. On the other hand, low-affinity and/or low-density contacts will not be efficient. The last case is represented by the inability of hyaluronan-coated cells to attach to hyaluronan-coated surfaces, on the (101) faces of calcium-(R,R)-tartrate, or on any face of calcium-(S,S)-tartrate.

Concluding Remarks

Herein we have developed the concept of a pivotal involvement of the pericellular coat in the early stages of cell adhesion. Quick, adaptable, and transient adhesion through multiple cooperative weak interactions provide the cell with an additional level of modulation in the decision-making process that precedes the commitment to adhesion at a particular site. Hyaluronan emerges thus as a mediator and modulator of cell adhesion, through its properties of electrostatic attraction or...
repulsion due to its polyelectrolyte character, in addition to its chirality and molecular recognition properties.

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Figure 6. Tentative phases leading to adhesion of cells covered with a hyaluronan brush of several microns thickness. The drawing is roughly to scale for epithelial cells (10 μm radius, 1–2 μm pericellular coat). a) Cell in suspension approaches surface. b) Early attachment: Hyaluronan establishes contact to molecular binding sites on the surface (circles). The cell is thus bound, but its membrane is still several microns away from the surface. From this stage, any one (or a combination) of three following processes could lead to the next phase: c1) Receptor-bound hyaluronan diffuses or is actively pushed/pulled away from the developing adhesion sites on the surface (m). The cell is thus bound, but its membrane is still several microns away from the surface. From this stage, any one (or a combination) of three following processes could lead to the next phase: c1) Receptor-bound hyaluronan diffuses or is actively pushed/pulled away from the developing adhesion site. The underlying membrane is exposed, a protrusion can be directed towards the surface, and integrins (double ovals) can now bind to RGD sites (triangles) on the surface. c2) Either by binding to the surface or by cell-induced changes in the medium the brush collapses, the cell is drawn to the surface, and integrin-mediated binding becomes possible. c3) The hyaluronan is either internalized or degraded by hyaluronidases. The cell can thus get closer to the surface and integrins can bind to RGD domains (triangles). Once Integrin-RGD contacts have been established, adhesion sites mature and the cell spreads.

A single integrin–RGD interaction is worth at least 10 kT (6 kcal mol⁻¹) according to ref. [22a] corresponding to a debonding force of ca. 90 pN per interaction as determined in refs. [22b, c]. The interaction between fibronectin and integrin is estimated to be about 50–200 times stronger due to synergistic effects of multiple binding interactions (ref. [22d]).


[35] For neutral polymers, which are good approximations for weak electrolytes at the high salt limit, the scaling law that describes the proportionality between the brush thickness ($L_b$) and the parameters that influence it is:

$$ L_b \propto \left( \frac{L_{ext}}{a/D} \right)^{2/3}. $$

$L_{ext}$ is the total length of the polymer chain, $a$ is the monomer length (1 nm), and $D$ is the distance between graft point. For a treatment of polyelectrolyte brushes at lower ionic strength see refs. [34, 36].