

## Selective interactions of cells with crystal surfaces

### Implications for the mechanism of cell adhesion

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#### SUMMARY

In this study we have characterized the mode of cell adhesion to calcite and calcium (R,R)-tartrate tetrahydrate crystals. The use of crystals as adhesion substrata was motivated by their well-established chemical nature and structurally defined surfaces. We show that calcite binds A6 *Xenopus laevis* epithelial cells rapidly and efficiently, most likely via surface-adsorbed proteins. Surface topology had only a limited effect on the adhesive interactions. Calcium (R,R)-tartrate tetrahydrate crystals exhibits two chemically equivalent, yet structurally distinct faces that differ mainly in the surface distribution of their lattice water molecules and charges. However, despite the gross similarity between the two faces striking differences were noted in their adhesive behavior. One of the faces was highly adhesive for cells, leading to protein-independent attachment and spreading followed by cell death. In contrast, cell adhesion to the

other surface of tartrate was slow (>24 h) and apparently mediated by RGD-containing protein(s). It was further shown that the latter face of tartrate crystals could be "conditioned" by long (24 h) incubation with serum-containing medium, after which it becomes highly adhesive.

The results presented here indicate that crystal surfaces may serve as excellent, structurally defined, substrata for cell adhesion, that cell binding may occur directly or via RGD-containing proteins and that cell adhesion may be dramatically modulated by variations in surface structure. The implications of the results to the mechanism of cell-substratum adhesion are discussed.

Key words: cell adhesion, crystals, epithelial cells, adhesive substrata

#### INTRODUCTION

Adhesion to exogeneous surfaces has profound effects on the structure and behavior of cells. Cellular interactions with suitable substrata apparently trigger a multitude of molecular interactions, which determine and regulate cell morphology, motility, growth and metabolism (Grinnell, 1978; Ben-Ze'ev, 1991). All these physiological activities depend on the accurate molecular orchestration of cell contact formation and on the transmembrane transduction of contact-related signals. The wide variety of structurally defined cell contacts and the molecular diversity of adhesion-related molecules render it difficult to provide a unified model for cell adhesion. Indeed, a variety of approaches have been employed in recent years to characterize the basic requirements for cell-contact formation. These include analysis of cytoskeletal involvement in cell contacts, identification of cellular adhesive proteins and receptors, as well as molecular and structural characterization of adhesive substrata (Geiger, 1989; Geiger et al., 1990; Ruoslahti and Pierschbacher, 1987; Yamada and Kennedy, 1978).

At the molecular level, cell adhesion to substrata requires

a battery of cytoplasmic, transmembranal and extracellular proteins that assemble into stable contact sites (Geiger et al., 1987). The extracellular proteins that together form the various extracellular matrices (ECM) include fibronectin (Grinnell and Feld, 1981; Petersen et al., 1989), laminin (Timpl, 1989), vitronectin (Bale et al., 1989), collagen (Nagata et al., 1985) and different proteoglycans (Guidry and Grinnell, 1987). These proteins may bind both to external substrata and to cell surface receptors, mostly of the integrin superfamily (Akiyama et al., 1990; Hynes, 1992). Those two binding processes, may however be interdependent, since the composition of the substratum-attached protein layer, and the conformation of the adsorbed proteins may be affected by the adsorption to the surface (Hattori et al., 1985; Brynda et al., 1990). Thus, cell adhesion and growth of anchorage-dependent cells, may depend directly or indirectly on the nature of the surface to which ECM proteins adsorb. Although much information is available on the identity and structure of "adhesive proteins", only limited information exists on the precise surface chemistry of adhesion-promoting substrata, which either form direct associations with the plasma membrane or promote ECM- and receptor-mediated cell adhesion.

Characterization of the properties of synthetic substrata that possess adhesive potential is mostly based on chemical modification of polymeric surfaces. These include modulation of hydrophobicity/hydrophilicity (Maroudas, 1977; van Wachen et al., 1985; Pratt et al., 1989; Dekker et al., 1991), charge density (van Wachen et al., 1987; Curtis and McMurray, 1986), physical and chemical heterogeneity (Lydon et al., 1985), and deformability (Guidry and Grinnell, 1987). It is, however, noteworthy that such definitions are macroscopic in nature, insufficiently defined to describe the structural properties of the interface accurately and that most surfaces used for cell culture are highly heterogeneous at the molecular level (Curtis and Clark, 1990).

While the absence of uniform surface organization on common substrata such as glass and tissue culture plastic is evident, the significance of this heterogeneity for cell adhesion is not clear. One possibility is that, since adhesive cellular interactions occur via structures such as focal and close contacts that characteristically have dimensions of several square micrometers, the adhesivity of the substratum might not be significantly affected by angstrom-scale fluctuations in surface structure. Alternatively, surface recognition by cells may depend on highly specific molecular conformations and distributions, and thus chemically equivalent but structurally distinct surfaces may differ in their adhesion potential. To distinguish between these possibilities it appears mandatory to examine cell adhesion to surfaces whose composition and structure are fully defined and which may be experimentally modulated. The approach taken in this work was to use crystal surfaces as adhesive matrices.

Crystals expose to the environment characteristic faces whose structures correspond to that of the bulk, limited by planes corresponding to specific crystallographic directions and defined by the crystal morphology. Crystal surfaces can be regarded as being composed of "active sites" of well-defined and well-known structures, which interact stereospecifically with molecules or macromolecules in solution, at a level of recognition comparable to that of enzyme-substratum or antibody-antigen interactions (Weissbuch et al., 1991; Weiner and Addadi, 1991; Kam et al., 1992). The advantage of these surfaces lies in their highly ordered, repetitive arrangement and the knowledge available on their structure at the atomic scale, which offers accurate means to define structural and chemical parameters. Crystals of different chemical composition can be chosen among a wide variety of known structures. The modulation of the interactions with the same molecules, exposed at different faces of the same crystal in different orientations, adds further to this variety.

We describe here the adhesive response of cultured A6 cells on two selected inorganic crystals, calcite ( $\text{CaCO}_3$ ), and calcium (R,R)-tartrate tetrahydrate [ $\text{Ca}^{+2}(\text{-OOC-CH(OH)-CH(OH)-COO-}) \cdot 4\text{H}_2\text{O}$ ]. These crystals present a diversity of well-characterized surfaces, ranging from highly ionic (calcite) to moderately charged and hydrophilic (tartrate), with different stereochemical characteristics and crystalline water distribution. We report here major differences in the adhesive response of the cells to these crystal surfaces. These differences are particularly intriguing in the tartrate crystals, where the cells distinguish between two

chemically equivalent faces, indicating the fine specificity of adhesive interactions.

## MATERIALS AND METHODS

### Crystallization experiments

For each crystal system optimal conditions for crystallization from aqueous solution were determined, ensuring that the crystals were well formed, homogeneous and reproducible with respect to morphology. All crystallization experiments were carried out at room temperature.

#### Calcite

Crystallization was induced by slow diffusion (over a period of 3 days) of ammonium carbonate vapor into cell culture dishes (Nunc, 3.5 cm) containing 2 ml of 7.5 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (pH 6.5) in a closed desiccator (Addadi and Weiner, 1985). The crystals are typically 100-200  $\mu\text{m}$  in size.

#### Calcium (R,R)-tartrate tetrahydrate

A 10 ml sample of a solution of 3.2 mM sodium hydrogen tartrate was mixed with 10 ml of a solution containing 4.4 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  at pH 6.5 and aliquoted into cell culture dishes (Falcon, 3.5 cm) (Addadi and Weiner, 1985). Crystals of typically 1 mm in size form within one day.

Crystal morphology was determined by X-ray diffraction on a Nonius CAD-4 diffractometer (Addadi et al., 1982). In the scanning electron microscope, the tartrate face types can be further directly distinguished by the distinct morphology of the etch-pits on the different faces, and their different sensitivity to the electron beam.

### Cell culture

The A6 kidney cell line derived from *Xenopus laevis* (ATCC, U.S.A.) was cultured, at 28°C, in 85% DMEM, supplemented with 8.5% fetal calf serum ("complete medium") in a humidified atmosphere of 5%  $\text{CO}_2$  in air. The cells were seeded in saturated complete medium (see below), on the same culture dishes in which the crystals were previously grown, still attached to the dish.

### Electron microscopy (EM)

#### Transmission EM (TEM)

A6 cells seeded on calcite crystals were fixed for 30 min in 2% glutaraldehyde and 3% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2, containing 5 mM  $\text{CaCl}_2$ . (The presence of  $\text{CaCl}_2$  in the fixation buffer, prevented dissolution of the crystals.) The dishes were rinsed four times and embedded in a thin layer of 1.7% agar to prevent the collapse of the cell monolayer grown on the crystals. The calcite crystals were dissolved at pH 4 and 4°C, for three days. After complete dissolution of the crystals, cells were postfixed with 1% osmium tetroxide, 0.5% potassium ferrocyanide and 0.5% potassium dichromate in 0.1 M cacodylate buffer, pH 7.2. The cells were stained *en bloc* with 2% aqueous uranyl acetate, followed by ethanol dehydration. The dishes were embedded in t-Epon 812 (Tuosimis, Maryland, USA). Sections of 500-700 Å were cut using a diamond knife (Diatome, Switzerland), and examined using a Philips CM-12 transmission electron microscope at an accelerating voltage of 100 kV.

The preparation of the cells seeded on tartrate crystals for the TEM followed the same procedure as described above, except that fixation buffer was saturated with respect to tartrate and the crystals were dissolved in 0.2 M EGTA, pH 7.2.

### Scanning EM (SEM)

All the solutions were saturated with respect to the particular crystal used. Fixation was carried out as described above for the TEM, followed by ethanol dehydration using Microporous Spec Cap (Spi Supplies, USA) and critical point drying (Autosamdri-810, Tuosimis, Maryland, USA) with CO<sub>2</sub>. The crystals were placed on carbon-coated stubs (Spi Supplies, USA) and sputter coated with gold for 12 min at 6 mA (S150 Edwards, USA). The specimens were examined at an accelerating voltage of 15-20 kV using a JEOL 6400 scanning electron microscope.

### Cell counting

The number of cells attached to the surface was directly counted for each crystal during SEM observations and the cell density per mm<sup>2</sup> area was calculated.

### Synthetic peptide inhibition

Adhesion to surfaces was also tested in culture medium supplemented with a synthetic peptide containing the Gly-Arg-Gly-Asp-Ser sequence (RGD peptide). A control peptide containing the Gly-Arg-Gly-Glu-Ser (RGE) sequence was also examined. Both peptides were synthesized at the peptide synthesis unit of the Weizmann Institute and purified by high-pressure liquid chromatography.

## RESULTS

### Choice of cells and crystals for adhesion assays

A series of preliminary experiments with cultured A6 cells indicated that calcite and calcium (R,R)-tartrate tetrahydrate crystals provide compatible surfaces upon which the cells may grow. Brushite crystals (calcium hydrogen phosphate dihydrate) were also used in the adhesion assay and the results obtained with them will be described elsewhere. A series of additional crystals was examined, and found unsuitable for adhesion. These included gypsum (calcium sulfate dihydrate) crystals, which were discarded because

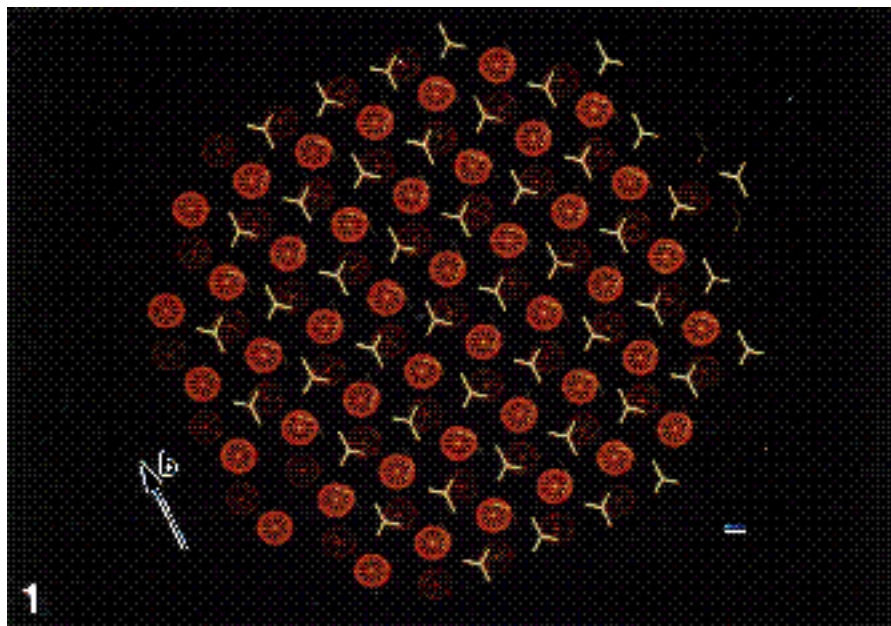
of their roughness, and calcium fumarate trihydrate and calcium creatine monohydrate crystals, which had toxic effects.

A6 cells were chosen because they are optimally cultured at 28°C, a temperature that is lower than that commonly used (37°C) for mammalian cell culture. These conditions prevent drastic temperature changes during sample handling, which may cause crystal dissolution or reprecipitation. Routinely, cell growth was performed in the presence of 8.5% fetal calf serum, saturated with respect to the particular crystals used (complete medium). The salt concentrations after equilibration with the crystals did not significantly exceed the normal values in complete medium (calcite, 1.44 mM Ca<sup>2+</sup>, 1.44 mM CO<sub>2</sub>; tartrate, 2 mM Ca<sup>2+</sup>, 2 mM tartrate). Light microscopy revealed that cell attachment, spreading and growth in calcite- or tartrate-saturated complete medium, is indistinguishable from that observed in non-saturated complete medium (data not shown).

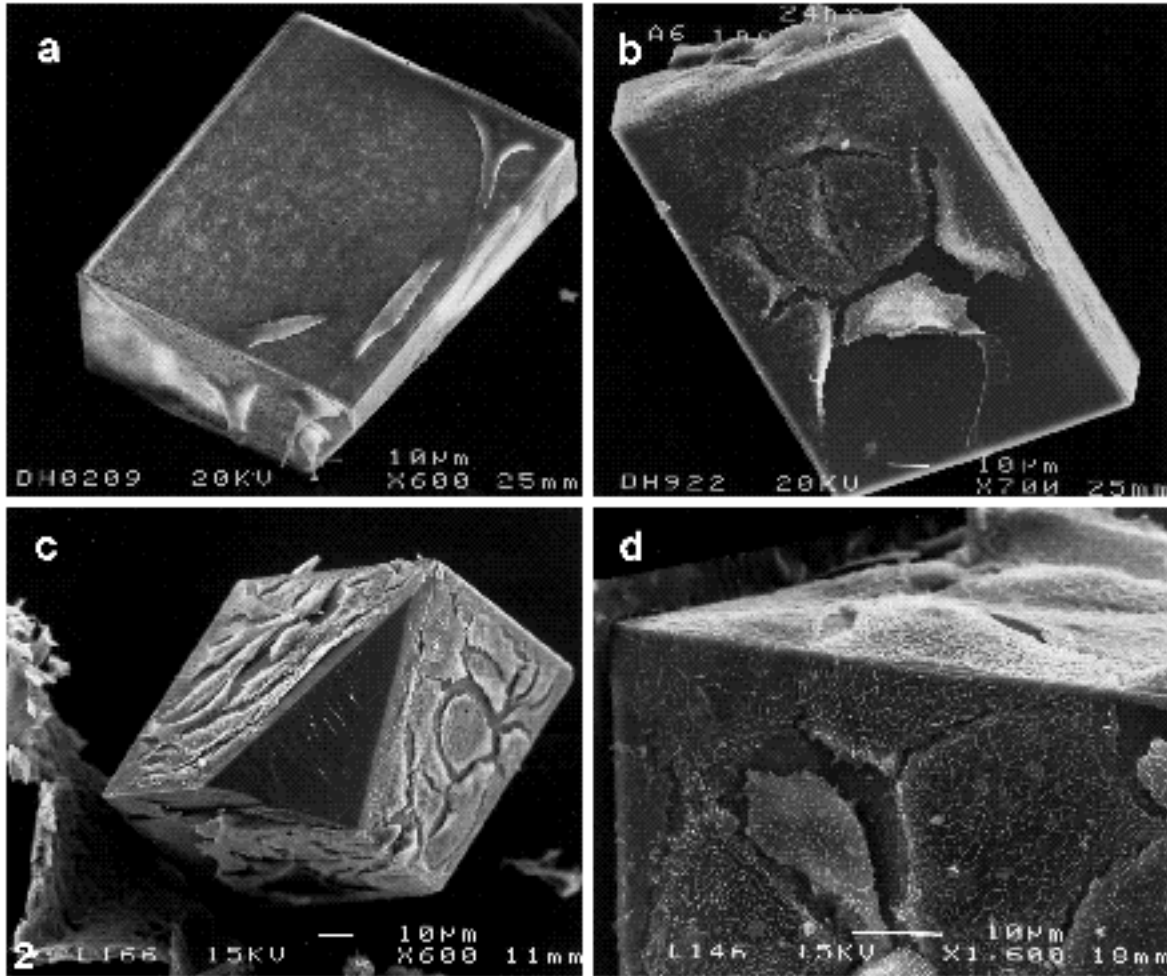
### Adhesion and spreading of A6 cells on calcite crystals

Calcite is a polymorph of calcium carbonate, stable at physiological conditions. The rhombohedral crystals (100-200 μm) are delimited by six equivalent faces, the {104} faces (see note at the end of this section), characterized by a tight packing of calcium and carbonate ions emerging at an oblique angle to the surface (Fig. 1) (Lippmann, 1973).

A6 cells were seeded in complete medium on the culture dishes where the calcite crystals were grown. This procedure allows the crystals to stay attached to the surface of the culture dish, during the experiments. The adhesion of A6 cells to the {104} faces of calcite was monitored directly by optical microscopy (data not shown) and scanning electron microscopy. One hour after seeding only a small percentage of cells were adsorbed to the crystal surfaces and adhesion proceeded for several h (Figs 2a, 4a). The cells were initially moderately spread and were detected on essentially all crystal faces, irrespective of their orientation



**Fig. 1.** Computer graphic representation of the packing arrangement of calcite, viewed on the (104) plane that delineates the developed crystal faces. The calcium ions are represented by the red circles, and the carbonate ions (CO<sub>3</sub><sup>2-</sup>) by yellow lines. The surface ion layer is designated by brighter colours, and the layer underneath by darker colours. *b*, *b*-axis. Bar, 1 Å.



**Fig. 2.** Scanning electron micrographs of A6 cells plated on calcite crystals in complete medium. All crystal faces are equivalent. (a) The cells after 1 h of incubation. The cells on the bottom-left face are probably in contact with both the crystal and the culture dish. (b) The cells after 24 h of incubation. Note the extracellular matrix layer deposited on the bare crystal surface (bottom-right). (c) The cells after 72 h of incubation. The bare triangular crystal face is the face through which the crystal was attached to the culture dish. In the bottom-left the undersurface of a confluent monolayer of flat cells can be detected, which detached from the crystal during CPD treatment. The monolayer preserves the characteristic angles between adjacent crystal faces. (d) After 72 h incubation. Detail of the junction between two adjacent crystal faces. Note the cells that spread across the edge, bending over at an angle of  $70^\circ$ . Extracellular matrix deposition can be detected between cells that shrank during the CPD treatment. Bars, 10  $\mu\text{m}$ .

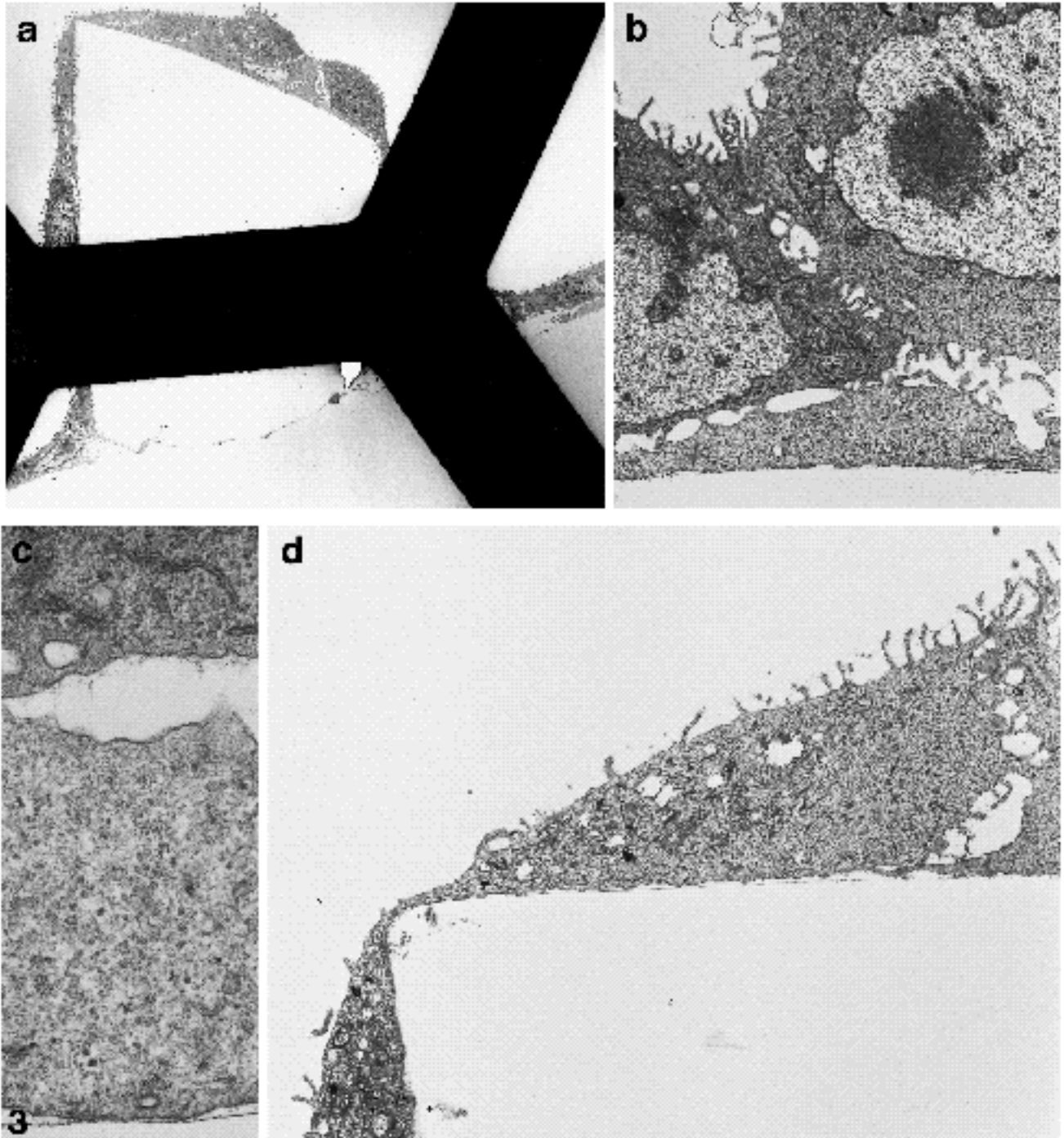
vis-à-vis the plane of the culture dish (with the exception of the face through which the crystal is attached to the dish). Further incubation resulted in extensive spreading of the A6 cells accompanied by the development of a typical rough dorsal surface (Fig. 2b). This process continued and by 72 hours the crystals were mostly covered by a confluent monolayer of flat epithelial cells (Fig. 2c). Careful examination of the crystal edges showed that cells have the capacity to “bend over” the edges, which have characteristic angles of  $70^\circ$  and  $115^\circ$  (Fig. 2d). Interestingly, many cells located near the attachment site of the crystal to the culture dishes were also attached to the dish surfaces (Figs 2a, 3a). Macroscopic surface roughness neither favored cell attachment and spreading nor prevented it.

The SEM observations were confirmed by TEM analysis; in cross-section (relative to the crystal face) tight adhesion to the substratum was observed (Fig. 3b), including

the formation of distinct focal contacts associated with cytoplasmic actin-connecting filaments (Fig. 3c). Ultrathin sections revealed the general morphology of the monolayer, still preserving the overall crystal morphology (Fig. 3a). Furthermore, the cells in the monolayer displayed a typical epithelial morphology with characteristic intercellular junctions (Fig. 3b,c) and often spread over crystal edges (Fig. 3d).

At their ventral surface, cells were apparently associated with a layer of ECM, which could be visualized by SEM, either between spread cells (Fig. 2d) or by examination of the undersurface of cells that occasionally detached (Fig. 2c). Cross-sections examined by TEM showed a distinct and nearly continuous “serum line”, running along the ventral cell membrane. These lines were also continuous across the crystal/plate interfacial junction (Fig. 3b,c).

To determine whether adhesion to calcite surfaces is

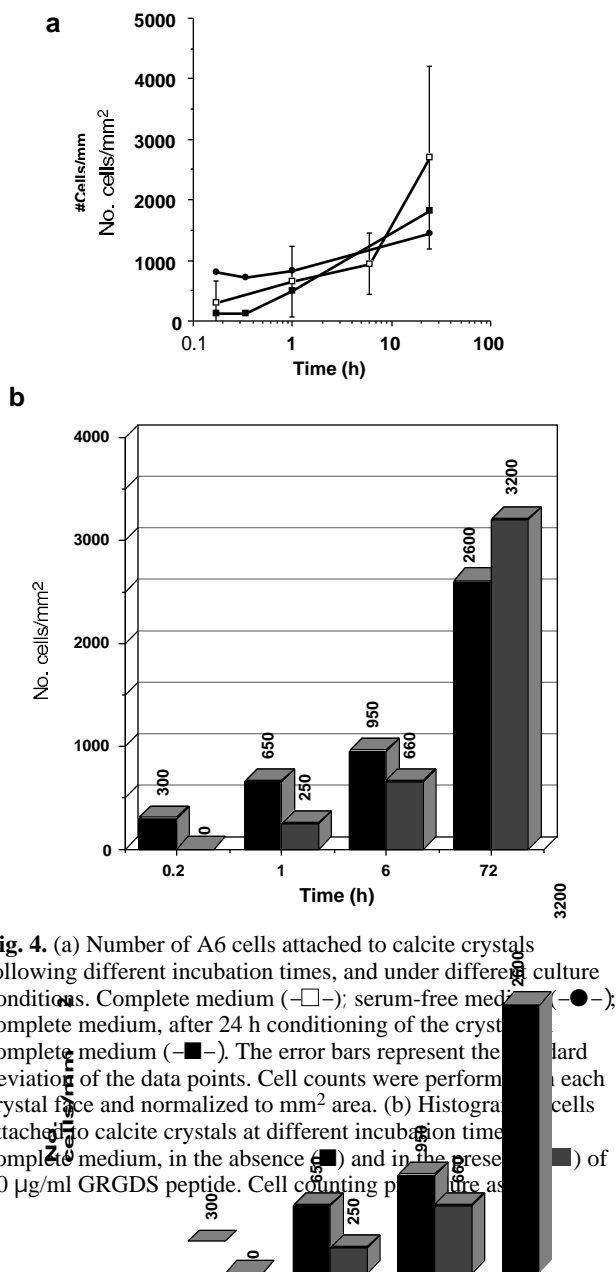


**Fig. 3.** Transmission electron micrographs of A6 cells attached to calcite crystals, following 72 h of incubation in complete medium. (a-d) Cross-section of a whole calcite crystal, attached to the culture dish. The thin line (bottom-center) delineates the site of attachment of the crystal to the culture dish. The fixed embedded cell monolayer preserves the dissolved crystal morphology, with typical angles between adjacent faces. The cell monolayer is continuous across the crystal-plate interfacial junction. a,  $\times 1400$ ; b,  $\times 6800$ ; c,  $\times 34,000$  and d,  $\times 6200$ .

mediated by specific Arg-Gly-Asp (RGD)-containing adhesion proteins such as fibronectin or vitronectin, 50  $\mu\text{g/ml}$  Gly-Arg-Gly-Asp-Ser (GRGDS) synthetic peptide were added to the culture medium. As shown in Fig. 4b, this treatment resulted in an essentially complete inhibition of the attachment when examined up to 1 hour after plating.

The RGD peptide is known to be degraded upon longer incubation in culture (Fok et al., 1991; Ylänne, 1990). In contrast, the RGE peptide, added under the same conditions, had no apparent effect.

To distinguish further between the contributions of exogenous proteins (supplied in the medium) and endogenous



**Fig. 4.** (a) Number of A6 cells attached to calcite crystals following different incubation times, and under different culture conditions. Complete medium (□); serum-free medium (○); complete medium, after 24 h conditioning of the crystals (■). The error bars represent the standard deviation of the data points. Cell counts were performed on each crystal face and normalized to mm<sup>2</sup> area. (b) Histogram of cells attached to calcite crystals at different incubation times in complete medium, in the absence (■) and in the presence (□) of 50 µg/ml GRGDS peptide. Cell counting performed as

described in Materials and Methods. The effect of exogenous proteins (secreted by the cells) to the adhesion process, cell spreading on calcite crystals was examined in serum-free medium (Fig. 4a). Under these conditions, the initial attachment of cells following 1 hour of incubation, was slightly higher than that observed in the experiments using complete medium. On the other hand, after further incubation (>6 h) the adhesion of A6 cells to the calcite surface was lower than that obtained in complete medium.

“Conditioning” of the calcite crystals by incubation with complete medium for 24 hours before the seeding of cells slightly inhibits the short-term adhesion of cells to the crystals (Fig. 4b).

(Note: crystal faces are described by a set of indices (*hkl*) that unequivocally define the orientation of the face relative to the crystallographic axes *a*, *b*, *c* of the structure. The notation {*hkl*}, e.g. {104} or {011}, {101} includes a set of identical, symmetry-related faces.)

### Adhesion and spreading of A6 cells on calcium (R,R)-tartrate tetrahydrate crystals

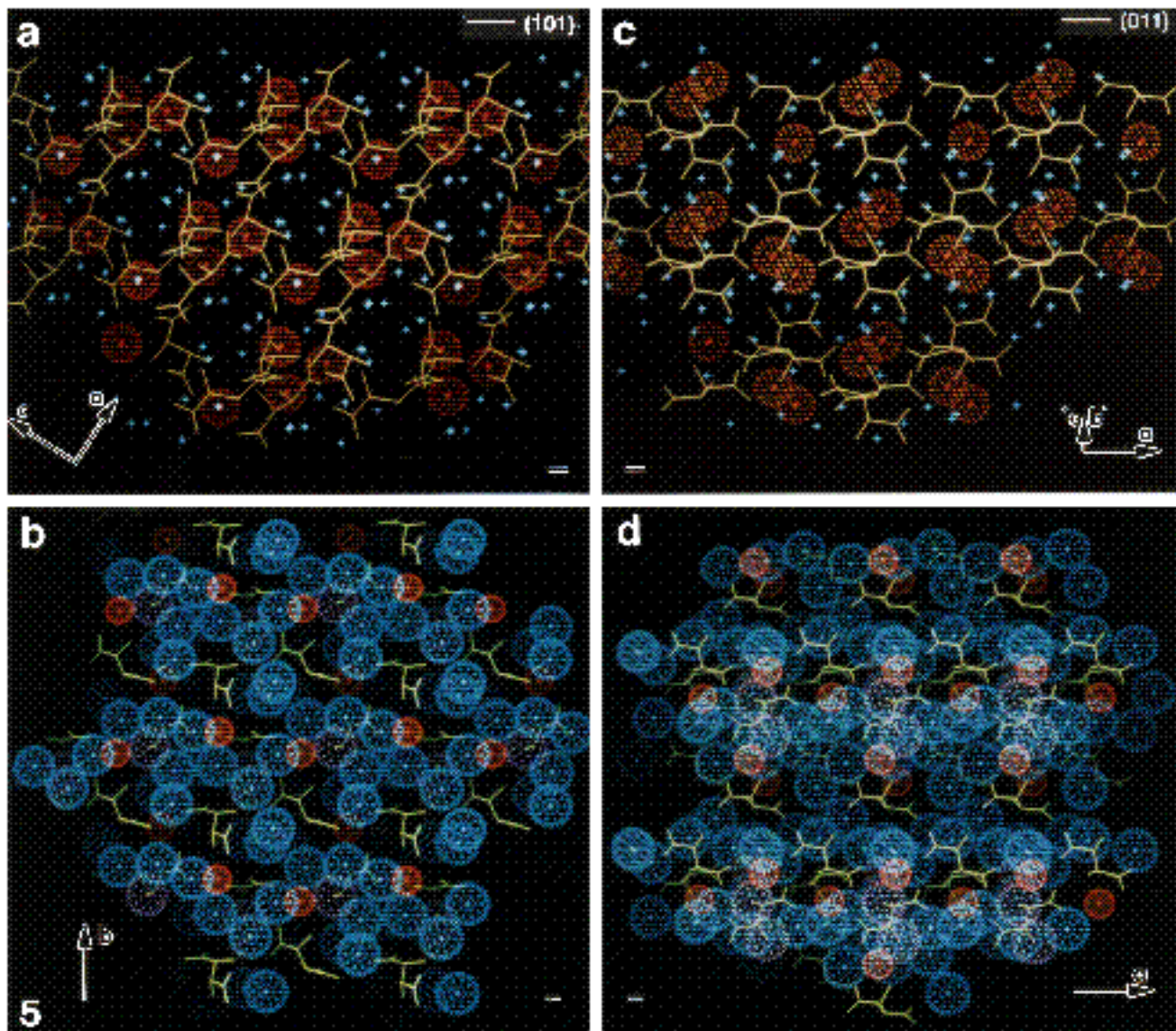
Calcium (R,R)-tartrate crystallizes in a structure containing four molecules of water per molecule of tartrate as a part of the crystal lattice. The structure is thus characterized by a net of combined ionic interactions and hydrogen bonds (Ambady, 1968). The large prismatic crystals (1 mm size) are delimited by two different face types, denoted {011} and {101} (see note above) (Fig. 5a-d). As the two face types delimit the same crystal structure from different directions, by definition they are chemically equivalent, but differ in their structure and stereochemical organization. The orientation of the carboxylate and hydroxyl groups of the tartaric acid, as well as the organization and distribution of calcium and water molecules on these surfaces are different. A more detailed description of the crystal faces and the water molecule organization is reported elsewhere (Hanein et al., 1993).

The cell adhesion processes on the two face types were observed to be drastically different, and will thus be described separately.

### The adhesive behavior of the {011} faces

The attachment of cells to the {011} faces can be described as an exponentially decaying function, with an average of 1350 cells/mm<sup>2</sup> attached to the faces initially (within 10 min), sharply declining to an average of 47 cells/mm<sup>2</sup> observed at 72 hours (Fig. 6). Shortly after plating, a massive attachment of cells to {011} faces was observed. SEM (Fig. 7a) and TEM (Fig. 8a) indicated that the adherent cells were initially spherical and attached to the {011} surface through a foot-like structure. In contrast to cells plated on calcite, cells attached to the {011} faces remained spherical (Fig. 7b), during the first several hours and only after about 6 hours was moderate spreading on the surface apparent (Fig. 8b). During this period the overall morphology was normal, yet upon longer incubation progressive cell death was observed on these faces and at time points of 24–72 hours living cells were rarely detected on it, leaving mainly patches of cell debris and/or extracellular matrix (Figs 7c, 8c). By TEM, a discontinuous serum line was observed.

To determine whether the binding of cells to the {011} surfaces depends on proteins supplied with the complete culture medium, we have plated A6 cells on the crystals in serum-free medium. The results indicate that cell adhesion occurred essentially as with cells plated in complete medium (Fig. 9a) or even faster. This suggests that exogenous serum proteins do not play an essential role in the massive attachment of cells to these faces. After 24 hours in serum-free medium, the number of cells attached to the same faces still increased. This is in agreement with the observation that conditioning of the crystals for 24 hours, prior to cell seeding, resulted in a reduced number of attached cells (Fig. 9a). The two observations together suggest that adsorbed proteins mask, to some extent, the attachment sites located on the crystal {011} faces. The overall morphology of the attached A6 cells was similar to that of cells seeded in complete medium: namely, delayed spreading followed by cell death (Fig. 10c).



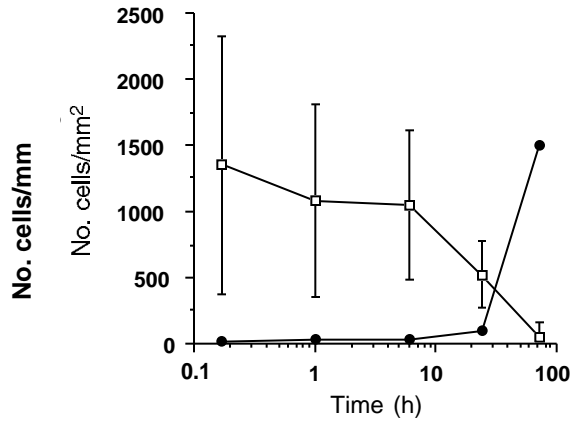
**Fig. 5.** Computer graphic representation of the packing arrangement of calcium (R,R)-tartrate tetrahydrate crystals. The calcium ions are represented by red circles (representing the van der Waals' radius of the ion), the tartrate molecules by yellow lines. The water molecules are represented by blue crosses in a and c and by blue circles in b and d. The purple circles in b and d represent the tartrate hydroxyl groups emerging at the surface. The molecules in the uppermost layer of the picture are shown by brighter colours, and the molecules underneath by darker colours. Bars, 1 Å. (a) Edge-on view of the structure of the (101) face. The interface at the top indicated by (101), shows the molecules exposed at the crystal surface. Note the water molecules organized in deep pools, separated by the emerging carboxylates ( $\text{O}-\text{C}-\text{O}^-$ ) of the tartrate molecules. The direction of the *a* and *b* crystallographic axes are indicated. (b) Top view of the structure on the (101) face, visualizing the water "pools" seen from above. The direction of the crystallographic *b* axis is indicated. (c) Edge-on view of the structure of the (011) face. The interface at the top indicated by (011), shows the molecules exposed at the crystal surface, as in a. Note the sparse but continuous layer of water molecules covering the surface, and the tartrate and calcium ions lying beneath it. (d) Top view of the structure on the (011) face. Note the ridge and channel structure formed by the rows of water and tartrate molecules. The notation *b c* indicates the projection of the *b* and *c* crystallographic axes on the plane of the drawing.

Addition of the GRGDS peptide to the growth medium had only a marginal effect on the cell attachment to the {011} faces (Figs 10a, 11a). Similarly, there was no apparent effect of the GRGDS peptide on cell adhesion to these faces in the "conditioning" experiments (Fig. 10d).

#### Adhesive behavior of the {101} faces

The attachment of cells to the {101} faces can be described

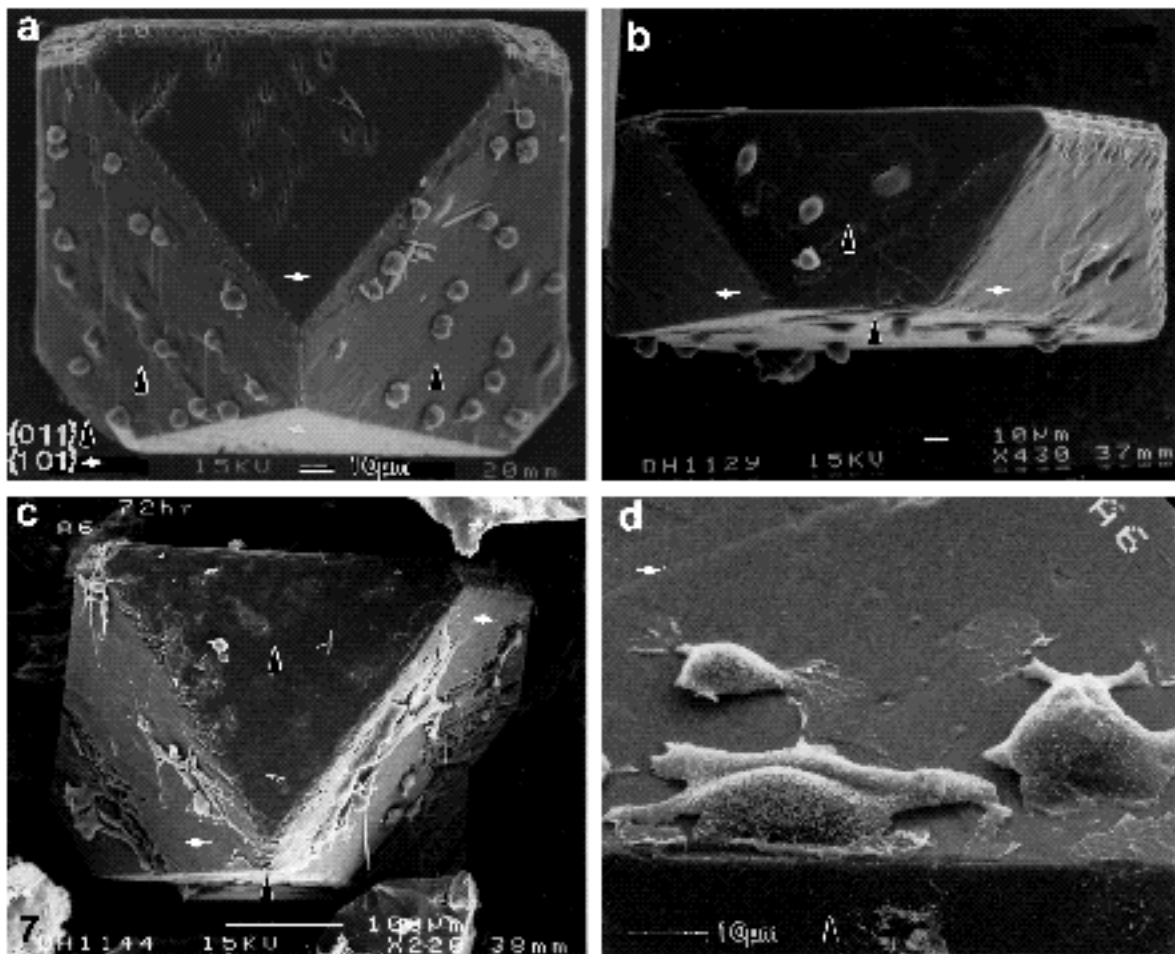
as an exponential function, with an average of 10 cells/mm<sup>2</sup> attached to the faces initially (at 1 h after seeding), up to an average of 1500 cells/mm<sup>2</sup> observed at 72 hours. In contrast to the rapid adhesion of A6 cells to {011} faces, attachment and spreading on the {101} faces proceeded slowly (Fig. 6). Up to 24 hours after seeding, only a small number of cells were detected on these faces. However, after further incubation the {101} faces gradually become



**Fig. 6.** Number of cells attached to the {011} faces (—□—) and {101} faces (—●—) of calcium (R,R)-tartrate tetrahydrate crystals, following different incubation times, in complete medium. Error bars and counts are as described for Fig. 4.

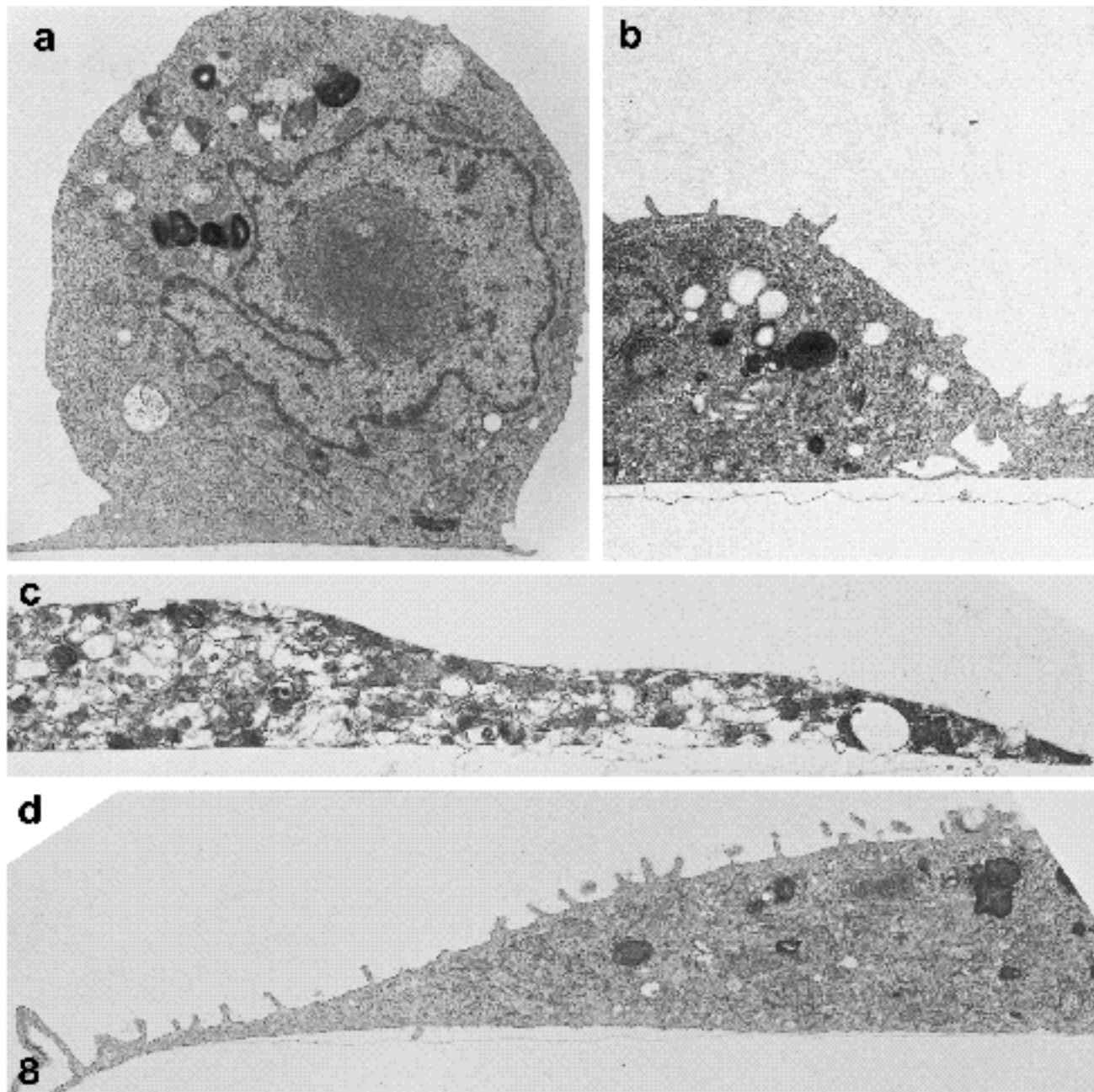
coated with well-spread cells, possessing morphology similar to that of cells growing on calcite, or on tissue culture dishes (Fig. 7c,d). It appears that these cells migrate to the {101} faces from the dish surface and/or from the border of {011} faces. Transmission electron microscopy revealed a continuous “serum line”, focal contacts and well-developed cytoskeletal organization (Fig. 8d).

The mechanism of adhesion to the {101} faces was further investigated by serum-conditioning of the crystals before cell plating. It was found that adhesion of A6 cells to conditioned crystal {101} faces started shortly after seeding (10 min) (Figs 9b, 10b) and within 24 hours these faces were covered by a nearly confluent layer of well-spread cells with normal morphology (Fig. 10c). This is in contrast to the slow adhesion kinetics described for the same crystal faces in either complete medium or serum-free medium. Addition of 50 µg/ml GRGDS peptide to cells plated on conditioned crystals resulted in a complete inhibition of cell adhesion to the {101} faces (Figs 10d and



**Fig. 7.** Scanning electron micrographs of A6 cells plated on calcium (R,R)-tartrate tetrahydrate crystals in complete medium. The two face types, {011} and {101}, are marked by (▲) and (◇), respectively. (a) Cell adhesion to the crystal following 10 min of incubation. Note that cells are attached only to the {011} faces. (b) Cell adhesion to the crystal following 1 h incubation. (c) Cell adhesion to the crystal following 72 hours incubation. Only dead cells and debris are detected on the {011} faces. Well-spread cells coat the {101} faces. The etch pits on the crystal were produced during CPD treatment, after incubation. (d) Cell adhesion to the crystal following 72 h incubation. Well-spread cells are seen on the {101} face while {011} contains only cell debris.



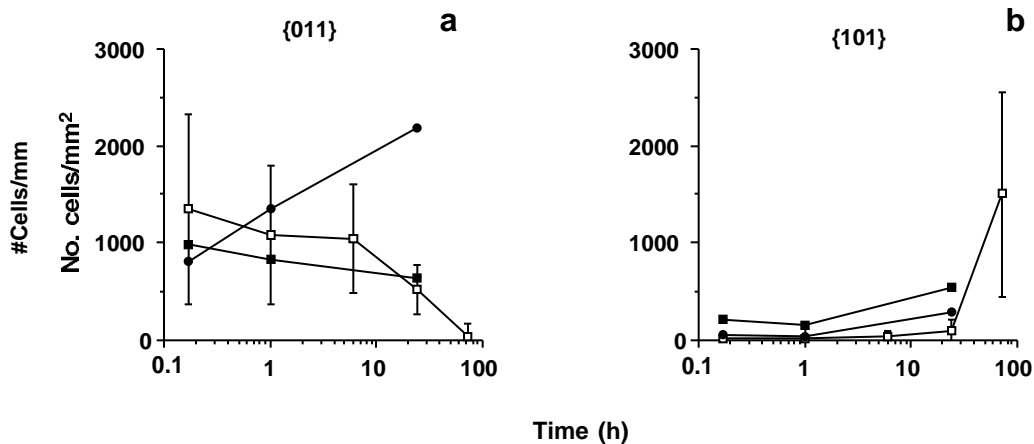


**Fig. 8.** Transmission electron micrographs of A6 cells attached to the two different faces of calcium (R,R)-tartrate tetrahydrate crystals, after different incubation times in complete medium. (a) An A6 cell attached to the (011) face, following 1 h of incubation. (b) A partially spread cell, attached to the (011) face, following 6 h of incubation. (c) Dead cell on the (011) face, following 72 h of incubation. (d) Well-spread cell on the (101) face, following 72 h of incubation.  $\times 9,000$ .

11b). These results indicate that the adsorption and organization of RGD-containing adhesion proteins on {101} faces is essential for cell attachment. It is noteworthy that the conditioning period needed to convert the {101} faces to being highly adhesive is quite long, relative to the time scale that is normally considered to be required for protein adsorption and reorganization (Norde and Lyklema, 1979; Andrade and Hlady, 1987).

## DISCUSSION

Adhesion of cells to extracellular substrata presumably involves two distinct and complementary mechanisms: receptor-mediated binding to specific extracellular matrix (ECM) molecules and generalized “non-specific” adhesion of the plasma membrane to the surface of solid substrata. The present study addresses these two processes using crys-



**Fig. 9.** The number of cells attached to the {011} (a) and {101} (b) faces of calcium (R,R)-tartrate tetrahydrate crystals following different incubation times and under different culture conditions. Incubation conducted in complete medium (□—); serum-free medium (○—); complete medium after 24 h conditioning of the crystals in complete medium (■—).

tals both as direct adhesive surfaces and as binding surfaces for “adhesive proteins”. One of our initial observations, made while screening a variety of crystals to serve as substrata for cell growth, was the high variability between crystals. Some of the crystal systems were apparently toxic to the cells while others were compatible with cell growth or even stimulated it (data not shown). These findings raised the possibility that selected crystals, in addition to their capacity to serve as defined adhesion substrata, may also serve as useful substrata for cultivation of cells.

Cell adhesion is known to be profoundly affected by the chemical properties of the underlying substratum. Modifications of substratum charge or hydrophobicity/hydrophilicity were shown to markedly alter its capacity to promote adhesion (Lewandowska et al., 1989; Schakenraad et al., 1989). Although it has been shown that cells (and proteins) interact more readily with surfaces of mixed hydrophobic and hydrophilic character (van Wachem et al., 1987; Golander et al., 1990), the structural requirements of an adhesive substratum at the microscopic level are still largely unknown. The most common substrata used for cell culture consist of a hydrophobic polymer with hydrophilic, polar and ionic groups such as hydroxyl, carbonyl, carboxylate and sometimes sulfate groups (Chinn et al., 1989; Lewandowska et al., 1989; Maroudas, 1977; van Wachem et al., 1987). The organization of these groups on the surfaces is, however, unknown. They may be clustered or dispersed, generating segregated patches or a uniform mixed surface layer, respectively.

The level at which the surface must be defined for understanding its cell binding activity is also not clear. Cell adhesion is typically mediated by cellular structures that measure several micrometers and one may wonder whether adhesive interactions may be significantly affected by minor (angstrom-scale) variations in surface atomic structure. A cell approaching a substratum may, initially, “sense” only the general chemical potential of the surface, but once contact is established the specificity of the interactions may shift to the molecular scale. Obviously, if the interactions are essentially non-specific and averaged over a large area, overall adhesion will not be affected by angstrom-scale fluctuations in surface structure. In contrast, if interactions are highly specific, cells may bind only to specific epitopes on the substratum or only to the adsorbed extracellular matrix macromolecules.

The question of specificity of cell adhesion to the substratum surface chemistry and organization was addressed here using rather unorthodox surfaces as adhesive matrices, namely crystals. The primary advantages of this choice are the level of atomic definition of the surface, and the possibility of distinguishing between chemical and structural parameters, by comparing different faces of the same crystal.

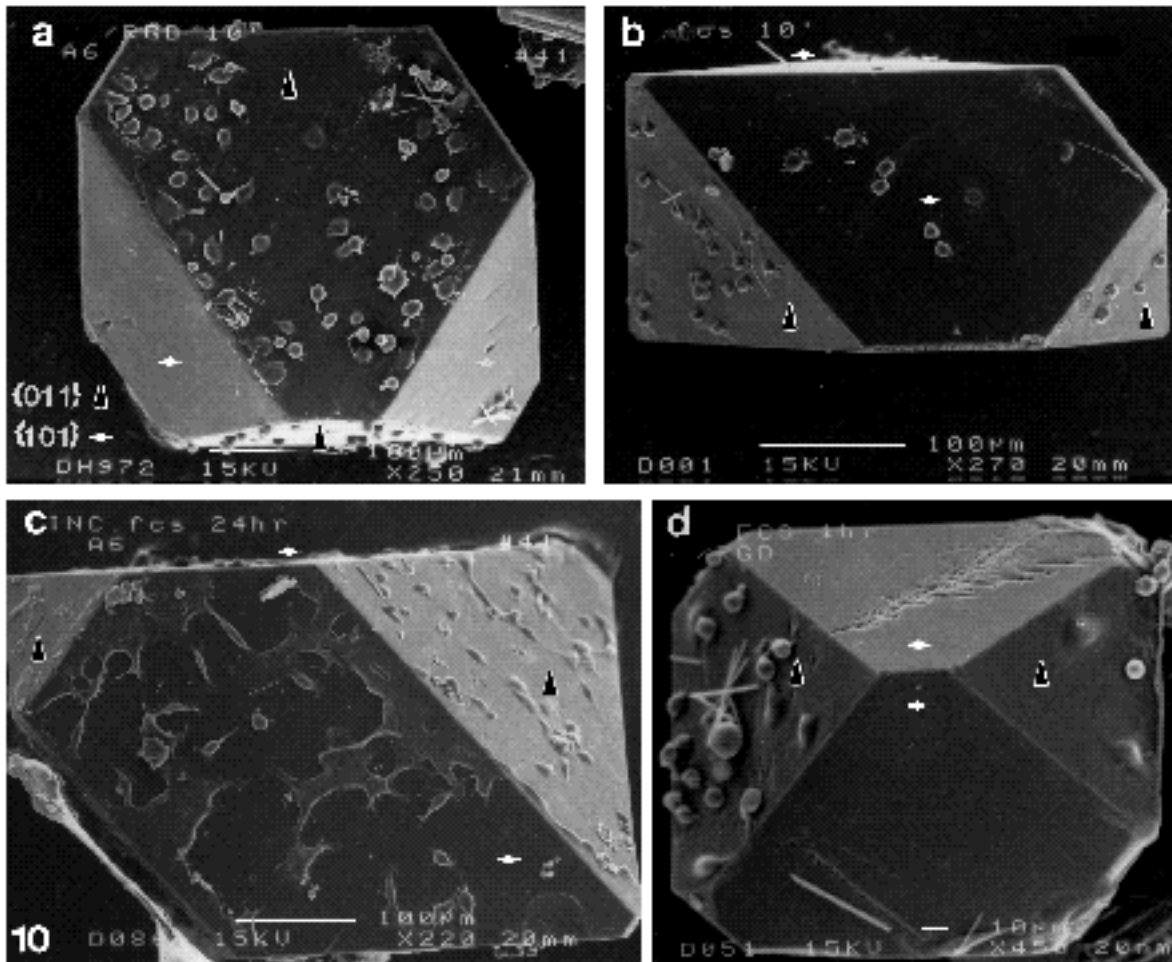
The results presented in this study support the notion that even limited variations in surface atomic structure may have profound effects on its capacity to induce cell adhesion and spreading. We have shown that the two distinct faces of the same crystal, calcium (R,R)-tartrate tetrahydrate, differ greatly in their capacity to serve as adhesive substrata, despite the fact that they are built of chemically identical components. The {011} faces are highly adhesive to A6 cells. This binding is apparently independent of exogenous proteins and cannot be inhibited by RGD peptides. Adhesion to the {101} faces, on the other hand, is relatively slow, is promoted by serum proteins and is RGD-inhibitable.

This distinct behavior of the two faces of tartrate vis-à-vis cell adhesion raises two related questions. (a) What are the structural differences that may specifically account for the distinct adhesive activity? (b) Are the differences in adhesion-promoting activity attributable to direct crystal-membrane interactions or to differential binding of adhesion-promoting or -inhibiting proteins to the surface?

In order to answer these questions, a brief comparison of the two surface types of calcium (R,R)-tartrate tetrahydrate crystals is required.

First, we must stress that the differential behavior of the cells indicates a resolution in the interactions at the angstrom level at least in thickness. The two surfaces delimit the same crystal structure in different directions. Thus a layer of unit cell thickness (7.1 Å for the {011} faces and 6.7 Å for the {101} faces) contains exactly the same molecules, irrespective of the surface considered. This means that the approaching cell “senses” only the chemical potential of groups directly exposed at the surface or within the first few (3-4 or less) angstrom units.

With these considerations in mind it is useful to examine the surface moieties exposed on the two faces. Fig. 5a,c and b,d shows the structures of the {011} and {101} faces, viewed edge-on and face-on. Both surfaces contain water,



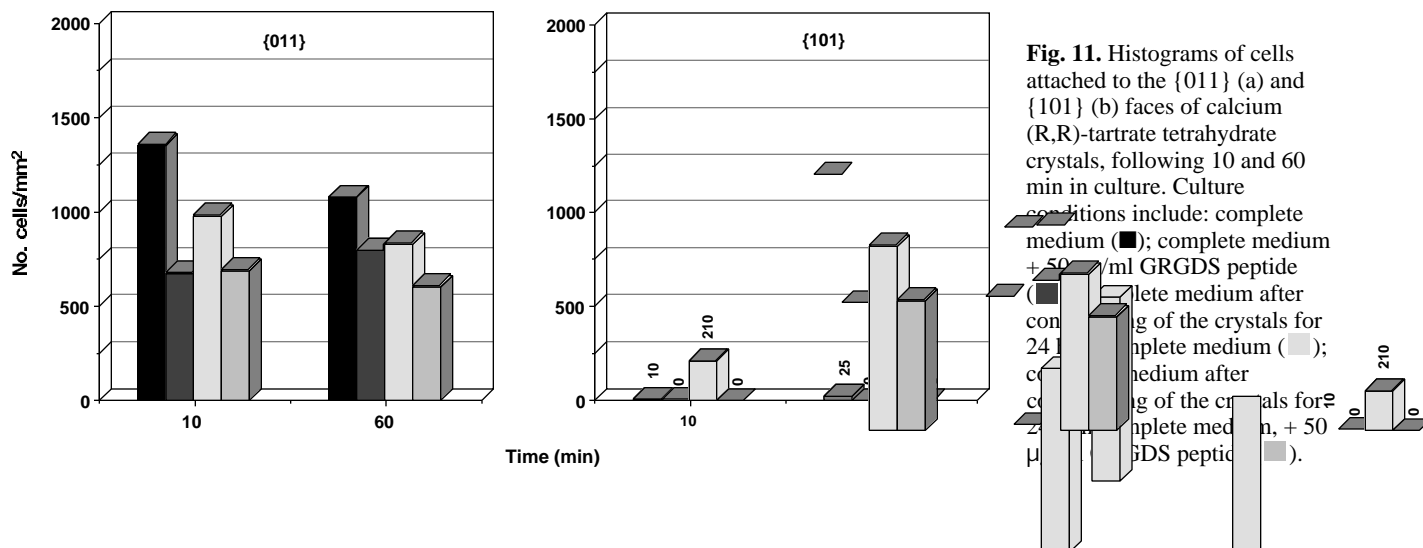
**Fig. 10.** Scanning electron micrographs of A6 cells plated on calcium (R,R)-tartrate tetrahydrate crystals under different culture conditions and times. The two faces of the crystals, {011} ( $\blacktriangle$ ) and {101} ( $\diamond$ ), are indicated. (a) Cell adhesion to the crystal following 10 min of incubation in complete medium, in the presence of 50  $\mu\text{g/ml}$  GRGDS peptide (compare with Fig. 7a). (b) Cell adhesion to the crystal following 10 min of incubation in complete medium with crystals preconditioned 24 h in complete medium. Note that under these conditions cells also attached to the {101} face. (c) Cell adhesion to the crystal following 24 h of incubation in complete medium with crystals preconditioned for 24 h in complete medium. Note that the cells are well spread on the {101} face, whereas only partially spread cells and dead cells appear on the {011} faces. (d) Cell adhesion to the crystal following 1 h of incubation in complete medium in the presence of 50  $\mu\text{g/ml}$  GRGDS peptide, on crystals preconditioned for 24 h in complete medium. Note that, in contrast to b cells are not observed on the {101} faces, indicating that adhesion to that surface is RGD-dependent.

calcium and tartrate molecules. In both faces the calcium ions that are part of the crystal lattice are located at a lower level, masked by either water or tartrate molecules. It is important to realize that the water molecules represented in the figures are an intrinsic part of the crystal lattice, and not part of the bulk water of hydration that surrounds both crystal and cell surfaces in solution. This water is strongly bound to the surface at precise sites, and presumably plays an essential role in the interactions between the two surfaces. Indeed we have shown elsewhere (Hanein et al., 1993) that this kind of “crystal water” has a crucial importance in the adsorption of proteins to surfaces, because energy must be invested in order to remove or rearrange it upon adsorption.

A number of differences can be detected between the tartrate and water molecule organization and orientation at the {011} and {101} faces. The {011} face is covered by bound

water molecules organized in parallel rows, giving rise to channels and ridges on the surface. Of the tartrate molecule, hydroxyl groups are exposed at the surface, and one carboxylate moiety, in an oblique orientation. The face has thus an overall hydrogen-bonding character, with very little charge separation. On the {101} face, on the other hand, the lattice water molecules are organized in “pools”. In between the water pools carboxylate groups emerge directly, exposed to the face. This surface has thus a more-charged character. Experiments in progress are being conducted to determine, mechanistically, which of these surface properties has a dominant effect on cell adhesion and whether the surface interacts directly with the membrane proper or affects adhesion indirectly, via surface-bound “adhesive” proteins.

It is noteworthy that in experiments performed on adsorption of fibronectin and other serum proteins to tartrate crys-



**Fig. 11.** Histograms of cells attached to the {011} (a) and {101} (b) faces of calcium (R,R)-tartrate tetrahydrate crystals, following 10 and 60 min in culture. Culture conditions include: complete medium (■); complete medium + 50 μg/ml GRGDS peptide (▒); complete medium after 24 h of the crystals for complete medium (□); complete medium after 24 h of the crystals for complete medium, + 50 μg/ml GRGDS peptide (▒).

tals, Hanein et al. (1993) showed that those globular proteins are adsorbed on both face types to a comparable extent with only a modest quantitative preference for the {101} faces. Thus if differential protein adsorption accounts for the differences in cell adhesion to the two surfaces, these differences might be qualitative (namely, affecting the conformation or orientation of the molecules) rather than quantitative.

Another interesting phenomenon is the fact that the rapid, serum-independent adhesion to the tartrate {011} faces is invariably fatal to the cells within 24 hours. It appears that this effect is maximal when cells attach to bare {011} faces and spread on them. This is based on the observation that surface-bound proteins could somewhat moderate this effect, but did not abolish it, probably due to partial masking of the cell-binding sites at the crystal surface. It has long been recognized that while the physical properties of the substratum may influence cell attachment, only substrata coated with specific adhesive matrix proteins will promote normal spreading of the cells (Grinnell et al., 1977; Schakenraad et al., 1989; Culp, 1983). In the light of the above observations, it appears likely that the extensive attachment to the {011} faces is due to direct binding of the cell membrane to the surface at multiple sites. This attachment is apparently incompatible with cell survival because of either excessive mechanical deformation of the plasma membrane proper or perturbations of specific surface proteins. The former mechanism might also account for the differences observed between cell and protein adsorption on the two face types, because the interaction between, for example, membrane sugars and surface hydrogen-bonding groups would establish strong molecular contacts, which are particularly favored on the {011} faces. Additional mechanistic studies must be performed in order to verify this hypothesis, including modulation of metal ions, addition of non-adhesive proteins, etc.

In contrast to the {011} faces, on the {101} faces of tartrate, as on the calcite crystals, cell adhesion and spreading appear to be predominantly indirect, mediated by extracellular matrix macromolecules. There are, however, significant differences in the binding kinetics and/or mode of protein adsorption by the former two surfaces. Calcite was shown to bind serum proteins avidly to its charged surfaces

(Hanein et al., 1993), to an extent much greater than that observed at the tartrate {101} surface. Consequently, RGD-inhibitable cell attachment and spreading on calcite occurred within ten minutes to one hour after plating, even without extensive conditioning. On the tartrate {101} faces, on the other hand, the adhesion kinetics are rather slow and depend upon long conditioning (~24 h). In fact, these results suggest that the delay in cell adhesion may be mainly attributed to a slow conditioning of the crystals when the cells are plated in serum-containing medium. It is unlikely that this long period is required just for binding of the protein(s) to the crystals, or even for competitive protein exchange. Such processes were extensively examined, and are believed to take place over time scales ranging from minutes to one hour (Norde and Lyklema, 1979; Brynda et al., 1990; Golander et al., 1990). A more likely explanation is that the acquisition of specific adhesiveness to {101} faces requires reorganization and/or assembly of the proteins, accompanied by conformational changes that are stabilized by the crystal. In this respect the {101} surface may differ from the {011} face or from the calcite surfaces. This hypothesis is being examined.

It is well known that surface topology affects overall cell shape and cytoskeletal organization (see Curtis and Clark, 1990). The results described here stress the predominance of surface chemistry over macroscopic topology in affecting adhesive interactions of A6 epithelial cells. This was especially noticeable in the calcite crystals, in which cells are often spread across rough surfaces containing steps and ridges. Apparently, the presence of such surface irregularities neither favored nor interfered with cell adhesion. Moreover, we have frequently noted that cells extended thin lamellipodia even over sharp edges to adjacent surfaces. It is also noteworthy that the site of attachment was not significantly affected by the orientation of the surface relative to the plane of the culture dish. These observations are in agreement with those of Brunette (1986), who observed that spreading and orientation of epithelial (E) cells are not markedly affected by vertical-walled grooves and V-shaped grooves. On the other hand, Dunn and Heath (1976) observed that the shape of the substratum imposes mechanical restrictions on the formation of certain linear elements of the locomotory system, thus preventing normal spreading

and locomotion of chick heart fibroblasts. It is still not clear whether the differences between the results reported here and by Brunette, and those of Dunn and Heath, are attributable to the differences in cell types used or to some unique properties of the different substrata.

In conclusion, the application of crystal surfaces as adhesive substrata appears to shed light on alternative molecular mechanisms affecting cell adhesion and the fine interplay between the atomic structure of the surface, the binding specificity of the cells and adhesion-mediating proteins. It remains for future experiments to define systematically the precise structural patterns responsible for specific selective surface binding of cell and proteins.

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## REFERENCES

- Addadi, L., Berkovitch-Yellin, Z., Domb, N., Gati, E., Lahav, M. and Leizerowitz, L.** (1982). Resolution of conglomerates by stereoselective habit modifications. *Nature* **296**, 21-26.
- Addadi, L. and Weiner, S.** (1985). Interactions between acidic proteins and crystals: Stereochemical requirements in biomineralization. *Proc. Nat. Acad. Sci. USA* **82**, 4110-4114.
- Akiyama, S.K., Nagata, K. and Yamada, K.M.** (1990). Cell surface receptors for extracellular matrix components. *Biochim. Biophys. Acta* **1031**, 91-110.
- Ambady, G.K.** (1968). The crystal and molecular structures of strontium tartrate trihydrate and calcium tartrate tetrahydrate. *Acta Crystallogr. sect. B* **24**, 1548-1557.
- Andrade, J.D. and Hlady, V.** (1987). Plasma protein adsorption: The big twelve. *Ann. N. Y. Acad. Sci.* vol. **576**, 158-172.
- Bale, M.D., Wolfahrt, L.A., Mosher, D.F., Tomasini, B. and Sutton, R.C.** (1989). Identification of vitronectin as a major plasma protein adsorbed on polymer surfaces of different copolymer composition. *Blood* **2698-2706**.
- Ben-Ze'ev, A.** (1991). Animal cell shape changes and gene expression. *BioEssays* **13**, 207-212.
- Brunette, D.M.** (1986). Spreading and orientation of epithelial cells on grooved substrata. *Exp. Cell Res.* **167**, 203-217.
- Brynda, E., Hlady, V. and Andrade, J.D.** (1990). Protein packing in adsorbed layers studied by excitation energy transfer. *J. Coll. Int. Sci.* **139**, 374-380.
- Chinn, J.A., Horbett, T.A., Ratner, B.D., Schway, M.B., Haque, Y. and Hauschka, S.D.** (1989). Enhancement of serum fibronectin adsorption and clonal plating efficiencies of swiss mouse 3T3 fibroblast and MM14 mouse myoblast cells on polymer substrata modified by radiofrequency plasma deposition. *J. Coll. Int. Sci.* **127**, 67-87.
- Culp, L.A.** (1983). Adhesion of fibroblasts to artificial substrata. In *Biocompatible Polymers, Metals and Composites* (ed. M. Szycher), pp.701-720. Technomic, USA.
- Curtis, A.S.G. and Clark, P.** (1990). The effects of topographic and mechanical properties of materials on cell behavior. *Crit. Rev. Biocomp.* **5**, 343-363.
- Curtis, A.S.G. and McMurray, H.** (1986). Conditions for fibroblast adhesion without fibronectin. *J. Cell Sci.* **86**, 25-33.
- Dekker, A., Reitsma, K., Beugeling, T., Bantjes, A., Feijen, J. and van Aken, W.G.** (1991). Adhesion of endothelial cells and adsorption of serum proteins on gas plasma-treated polytetrafluoroethylene. *Biomaterials* **12**, 130-138.
- Dunn, G.A. and Heath, J.P.** (1976). A new hypothesis of contact guidance in tissue cells. *Exp. Cell Res.* **101**, 1-14.
- Fok, K.F., Panzer-Knodle, S.G., Nicholson, N.S., Tjoeng, F.S., Feigen, L.P. and Adams, S.P.** (1991). Aminopeptidase resistant Arg-Gly-Asp analogs are stable in plasma and inhibit platelet aggregation. *Int. J. Peptide Protein Res.* **38**, 124-130.
- Geiger, B.** (1989). Cytoskeleton-associated cell contacts. *Cur. Opin. Cell Biol.* **1**, 103-109.
- Geiger, B., Ginsberg, D., Salomon, D. and Volberg, T.** (1990). The molecular basis for the assembly and modulation of adherens type junctions. *Cell Differ. Dev.* **32**, 343-354.
- Geiger, B., Volk, T., Volberg, T. and Bendori, R.** (1987). Molecular interactions in adherens-type contacts. *J. Cell Sci. Suppl.* **8**, 251-272.
- Golander, C-G., Lin, Y-S., Hlady, V. and Andrade, J.D.** (1990). Wetting and plasma-protein adsorption studies using surfaces with hydrophobicity gradient. *Coll. Surf.* **49**, 280-302.
- Grinnell, F.** (1978). Cellular adhesiveness and extracellular substrata. *Int. Rev. Cytol.* **53**, 65.
- Grinnell, F. and Feld, M.K.** (1981). Adsorption characteristics of plasma fibronectin in relationship to biological activity. *J. Biomed. Mat. Res.* **15**, 363-381.
- Grinnell, F., Hays, D.G. and Minter, D.** (1977). Cell adhesion and spreading factor. *Exp. Cell Res.* **110**, 175-190.
- Guidry, C. and Grinnell, F.** (1987). Heparin modulates the organization of hydrated collagen gels and inhibits gel contraction by fibroblasts. *J. Cell Biol.* **104**, 1987-1097.
- Hanein, D., Geiger, B. and Addadi, L.** (1993). Fibronectin adsorption to surfaces of hydrated crystals. An analysis of the importance of bound water in protein-substrate interactions. *Langmuir* (in press).
- Hattori, S., Andrade, J.D., Hibbs, J.B., Gregonis, D.E. and King, R.N.** (1985). Fibroblast cell proliferation of charged hydroxyethyl methacrylate copolymers. *J. Coll. Int. Sci.* **104**, 73-78.
- Hynes, R.O.** (1992). Integrins: Versatility, modulation, and signaling in cell adhesion. *Cell* **69**, 11-25.
- Kam M., Perl-Treves, D., Caspi, D. and Addadi L.** (1992). Antibodies against crystals. *FASEB* **6**, 2608-2613.
- Lewandowska, K., Balachander, N., Sukenik, C.N. and Culp, L.A.** (1989). Modulation of fibronectin adhesive functions for fibroblasts and neural cells by chemically derivatized substrata. *J. Cell. Physiol.* **141**, 334-345.
- Lippmann, F.** (1973). *Sedimentary Carbonate Minerals*, pp. 6-16. Springer, Berlin.
- Lydon, M.J., Minett, T.W. and Tighe, B.J.** (1985). Cellular interactions with synthetic polymer surfaces in culture. *Biomaterials* **6**, 396-402.
- Lyklema, J.** (1984). Proteins at solid-liquid interfaces. *Coll. Surf.* **10**, 33-42.
- Maroudas, N.G.** (1977). Sulphonated polystyrene as an optimal substrate for the adhesion and spreading of cells in monovalent and divalent saline solutions. *J. Cell. Physiol.* **90**, 511.
- Nagata, K., Humphries, M.J., Olden, K. and Yamada, K.M.** (1985). Collagen can modulate cell interactions with fibronectin. *J. Cell Biol.* **101**, 386-394.
- Norde, W. and Lyklema, J.** (1979). Thermodynamics of protein adsorption. *J. Coll. Int. Sci.* **71**, 350-365.
- Petersen, T.E., Skortenggaard, K. and Vide-Pedersen K.** (1989). Primary structure of fibronectin. In *Fibronectin* (ed. Mosher, D.F.), pp. 47-121. Academic Press Inc., New York.
- Pratt, K.J., Williams, S.K. and Jarrel, B.E.** (1989). Enhanced adherence of human adult endothelial cells to plasma discharge modified polyethylene terephthalate. *J. Biomed. Mat. Res.* **23**, 1131-1147.
- Ruoslathi, E. and Pierschbacher, M.D.** (1987). New perspectives in cell adhesion: RGD and integrins. *Science* **238**, 491-497.
- Schakenraad, J.M., Arends, J., Busscher, H.J., Dijk, F., van Wachem and Wildevuur, C.R.H.** (1989). Kinetics of cell spreading on protein precoated substrata: A study of interfacial aspects. *Biomaterials* **10**, 43-50.
- Timpl, R.** (1989). Structure and biological activity of basement membrane proteins. *Eur. J. Biochem.* **180**, 207-212.
- van Wachem, P.B., Beugeling, T., Feijen, J., Bantjes, A., Detmers, J.P. and van Aken, W.G.** (1985). Interaction of cultured human endothelial cells with polymeric surfaces of different wettabilities. *Biomaterials* **6**, 403-408.
- van Wachem, P.B., Hogt, A.H., Beugeling, T., Feijen, J., Bantjes, A., Detmers, J.P. and van Aken, W.G.** (1987). Adhesion of cultured human

- endothelial cells onto methacrylate polymers with varying surface wettability and charge. *Biomaterials* **8**, 323-328.
- Weiner, S. and Addadi, L.** (1991). Acidic macromolecules of mineralized tissues: the controllers of crystal formation. *Trends Biochem. Sci.* **16**, 252-256.
- Weissbuch, I., Addadi, L., Lahav, M. and Leiserowitz, L.** (1991). Molecular recognition at crystal interfaces. *Science* **253**, 637-645.
- Yamada, K.M. and Kennedy, D.W.** (1978). Dualistic nature of adhesive protein function: Fibronectin and its biologically active peptide fragments can autoinhibit fibronectin function. *J. Cell Biol.* **99**, 29-36.
- Ylanne, J.** (1990). RGD peptides may only temporarily inhibit cell adhesion to fibronectin. *FEBS Lett.* **267**, 43-45.

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