

Morphological alterations of T24 cells on flat and nanotubular TiO₂ surfaces

Aim To investigate morphological alterations of malignant cancer cells (T24) of urothelial origin seeded on flat titanium (Ti) and nanotubular titanium dioxide (TiO₂) nanostructures.

Methods Using anodization method, TiO₂ surfaces composed of vertically aligned nanotubes of 50-100 nm diameters were produced. The flat Ti surface was used as a reference. The alteration in the morphology of cancer cells was evaluated using scanning electron microscopy (SEM). A computational model, based on the theory of membrane elasticity, was constructed to shed light on the biophysical mechanisms responsible for the observed changes in the contact area of adhesion.

Results Large diameter TiO₂ nanotubes exhibited a significantly smaller contact area of adhesion ($P < 0.0001$) and had more membrane protrusions (eg, microvilli and intercellular membrane nanotubes) than on flat Ti surface. Numerical membrane dynamics simulations revealed that the low adhesion energy per unit area would hinder the cell spreading on the large diameter TiO₂ nanotubular surface, thus explaining the small contact area.

Conclusion The reduction in the cell contact area in the case of large diameter TiO₂ nanotube surface, which does not enable formation of the large enough number of the focal adhesion points, prevents spreading of urothelial cells.

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Received: July 13, 2012

Accepted: December 1, 2012

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The material and topographical characteristics of the contact surface affect the functional activity of cells (1-7). Titanium is the most widely used material in numerous medical applications, because it is non-toxic. The titanium surface has recently been modified by a self-assembled layer of vertically oriented titanium oxide (TiO_2) nanotubes with diameters between 15 nm and 100 nm (8-10). It was revealed that cell adhesion, spreading, growth, and differentiation was maximally induced on 15 nm nanotubes, but hindered on 100 nm nanotubes, which facilitated cell death (8,9). These results suggest that magnitude of TiO_2 nanotube diameter has an important role in cell adhesion and cell growth, and that the mechanics of the formation of focal adhesions is similar between different types of cells. The aim of the present study is to analyze effects of flat titanium and nanotubular TiO_2 surfaces on the morphology of malignant cancer cells (T24) of urothelial origin.

Using anodization method, vertically aligned TiO_2 nanotubular surfaces were produced (Figure 1). The produced nanotubes had a large diameter (50-100 nm). The T24 cells

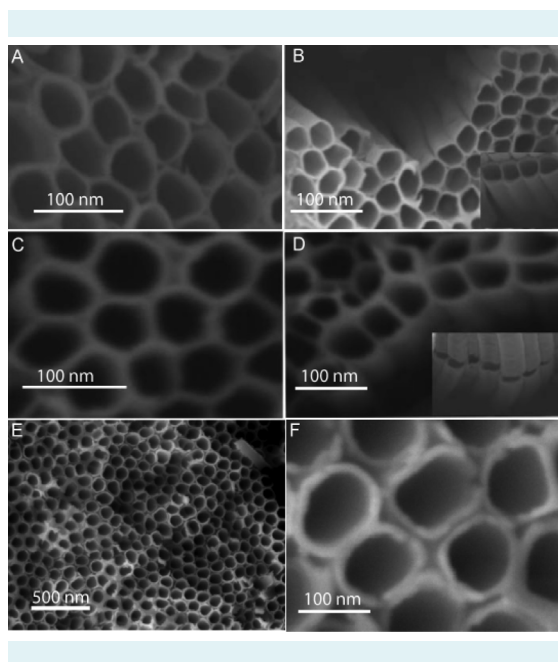


FIGURE 1. Scanning electron microscopy images of surface layers of self-assembled vertically aligned TiO_2 nanotubes synthesized by anodization method. Ethylene glycol solution with 0.3 wt % NH_4F and 1% volume water was used in preparation. High-resolution scanning electron microscopy FEG-SEM 7600F from JEOL was used. Images were taken at 100,000 \times magnitude using low accelerating voltage of 2kV. The internal TiO_2 tube diameter was around 50-60 nm (**A-D**) and 100 nm (**E-F**) while the length could be up to 10 micrometers (**B**).

grown on the nanotubular surface had a smaller top view diameter and more membrane protrusions than the counterpart on the flat titanium surface. A computational model was constructed to shed light on the biophysical mechanism underlying the observed morphologic changes. The underlying hypothesis is that the low density of TiO_2 nanotube edges could not facilitate the cell adhesion, ie, the formation of large enough number of focal adhesion points. Mathematically, the density of negative charges is predicted to be greatest at sharp edges (7), which would then facilitate the mediated electrostatic interactions between the TiO_2 nanotube surface and membrane proteins at the focal contact. Using numerical simulations of membrane dynamics, it is revealed that low adhesion of the membrane to the large diameter nanotubular surface is not sufficient to counterbalance the loss of entropic energy during the clustering of proteins at a focal contact, and consequently, the increased membrane bending energy does not favor intensive spread of cancer cells on the large diameter TiO_2 nanotube surface underneath.

MATERIAL AND METHODS

TiO_2 nanotube synthesis

TiO_2 nanotubes were synthesized by anodization of Ti foil (thickness 0.25 mm; purity 99.5%; Sigma-Aldrich, St. Luis, MO, USA) in a two electrode electrochemical cell. Prior to nanotube synthesis, titanium foils were degreased by sonicating in acetone, methanol, and ethanol, then rinsed with deionized water and dried in a nitrogen stream. The electrolyte was ethylene glycol containing 0.2 vol% H_2O and 0.3 w% NH_4F . Anodization was done at room temperature with titanium foil as anode and platinum foil as cathode. A DC power supply was used as the voltage source. The Ti foil was biased at H 60V and 80 V for 1 hour to grow a nanotubular TiO_2 layer. After the electrochemical treatment, the samples were rinsed with deionized water and dried in air. A field-emission SEM was used for morphological characterization of samples (Figure 1).

Urothelial cell culture

Human urothelial T24 cell line was cultured in a 1:1 mixture of Advanced-Dulbecco's modified essential medium (Invitrogen, Gibco, Paisley, UK) and Ham's F-12 medium (Sigma-Aldrich Corp.), supplemented with 10% fetal bovine serum (FBS, Gibco, Invitrogen, Carlsbad, CA, USA), 5 $\mu\text{g}/\text{ml}$ insulin-transferrin-selenium supplement (Gibco), 100 $\mu\text{g}/\text{ml}$ streptomycin, and 100 units/mL penicillin. Just at confluence,

the cells were incubated in TrypLE Select (Gibco) at 37°C for 5 minutes, resuspended in the medium, centrifuged at 200 g for 5 minutes, and plated at seeding density of 5×10^4 cells/cm² on TiO₂ nanotube and flat surfaces. The urothelial-TiO₂ constructs were maintained for a week at 37°C in a humidified atmosphere of 5% CO₂ (v/v) in air. After a week, the ultra-structural status of the urothelial cells growing on the TiO₂ nanotube and flat surfaces was analyzed.

Scanning electron microscopy

A scanning electron microscope was employed for morphological characterization of urothelial cells adhered to TiO₂ nanotubular and flat titanium surfaces. Urothelial-titanium/TiO₂ constructs were prepared for scanning electron microscopy (SEM) as described previously (11). In brief, after a week of culturing, the urothelial-titanium/TiO₂ constructs were fixed in 4% (w/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 for 2 hours 45 minutes. The fixation was followed by overnight rinsing in 0.1 M cacodylate buffer. The samples were then postfixed in 1% (w/v) osmium tetroxide for 1 hour at 4°C. After dehydration through a graded series of acetone, the samples were dried at the critical point, gold sputtered with, and observed in a Jeol 840A (Jeol, Tokyo, Japan) scanning electron microscope.

Statistical analysis

The surface area of T24 cells was measured using surface area tool in ImageJ. A total of eleven SEM micrographs

were analyzed for each of the cell groups in a single micrograph. The two images in Figure 2 have a resolution of 1200 dpi (472.441 pixels/cm), while the rest of the images have a resolution 600 dpi (236.22 pixels/cm). The figures were 6.8 cm in width and 4.87 cm in height. The statistical analysis between T24 cells grown on the nanotubular surface and T24 cells grown on the flat TiO₂ surface was done using a *t* test for independent samples. Statistical significance was set at the $P < 0.0001$. Data are reported as means \pm standard deviation.

The model

To investigate the dynamics of membrane growth on nanotubular TiO₂ scaffolds and flat titanium surface, we constructed a simple model based on the membrane free energy. The idea behind the model is that the membrane attains the shape that corresponds to the minimum free energy. This quasi-equilibrium shape is derived by the minimization of the membrane free energy. The equation of motion (Equation 1) describes the interplay of the adhesion, bending, and cross-interaction energies. The modeled membrane constituent is an integrin molecule that facilitates (mediates) membrane adhesion, and due to the large extracellular part, it has a positive spontaneous curvature. In the model, the overall number of integrins was conserved, moving laterally over the cell membrane. The theoretical model is an extension of our previous theoretical models (7,10,12-14). The investigated shape was a segment of the cell (membrane) outer contour, which was initially flat (Figure 3). The membrane shape was under

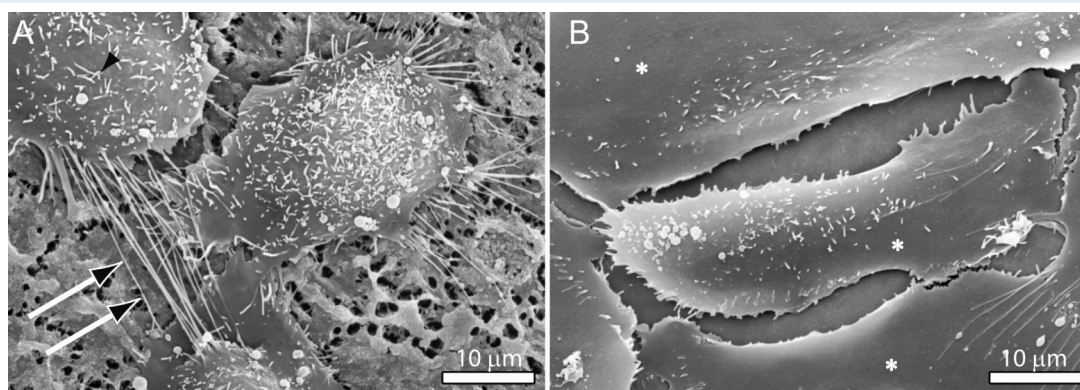


FIGURE 2. Urothelial T24 cells on (A) large diameter (of 50-100 nm diameter) TiO₂ nanotubes and on (B) flat titanium surfaces. The surface topography of urothelial cells reveals smaller cell diameter and numerous membrane protrusions (eg, nanotubular structures – thin arrows and pleomorphic microvilli – arrowheads) on the large diameter TiO₂ nanotubes (A), whereas, on the flat titanium surface, the cells are considerably larger in size and the cell membrane is smoother (asterisks) (B). Scale bars: 10 μ m.

the constraint of translational symmetry. Moreover, we assumed that the membrane curvature along the direction perpendicular to the contour was roughly constant, and thus, it entered our calculation as a modified membrane tension. The types of surfaces were: 1) a flat titanium surface; 2) a small diameter (15 nm) TiO₂ nanotubular surface; and 3) a large diameter (100 nm) nanotubular surface. The TiO₂ nanotubular surfaces were modeled explicitly by including a vertical intersection of the nanotubes.

Equations of membrane dynamics

Our model investigated the observed differences in the cell diameter between two different types of TiO₂ nanotube surfaces. The membrane free energy expression was

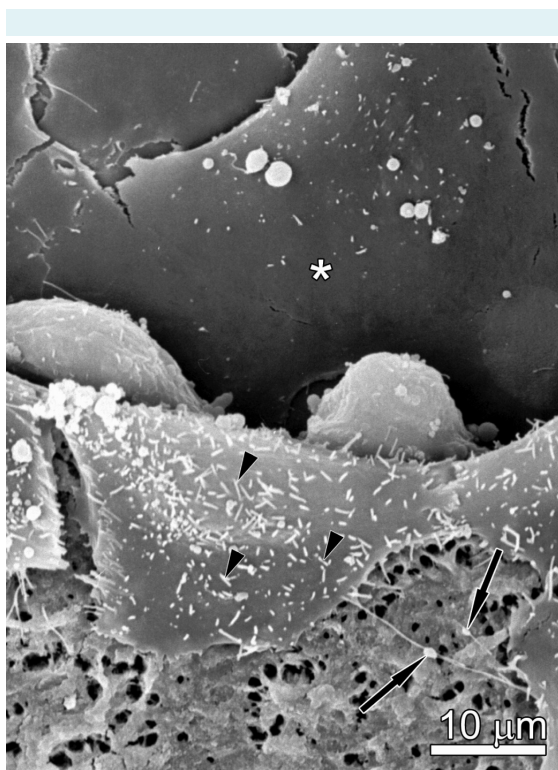


FIGURE 3. Urothelial T24 cells on TiO₂ and Ti scaffold. One half of the scaffold was flat titanium and another half was constructed into TiO₂ nanotubes. Urothelial cells growing on the TiO₂ nanotube surface are smaller, more spherical (ie, less flattened) and have numerous long membrane exvaginations. Their apical surface is covered by pleomorphic microvilli (arrowheads). The gondola-like structures are indicated by arrows. The urothelial cells on the flat titanium surface are flatter than cells on the large diameter TiO₂ nanotube surface. Their apical surface is smooth (asterisk), only few microvilli are seen. Scale bar: 10 μ m.

employed to derive the equations of motion of a cell membrane contour and the density distribution of integrins. The membrane free energy in our dynamical model was:

$$F = \int \left(\frac{1}{2} \kappa (H - \bar{H}n)^2 + \sigma_t + \varphi_s h^2 - \omega n_s n - n_s \frac{c\Omega}{2} n^2 + k_b T n_s n \ln(n) + k_b T n_s (1-n) \ln(1-n) \right) dA \quad (1)$$

where the first term gives the bending energy due to the mismatch between the membrane curvature and the membrane spontaneous curvature of integrins, κ is the bending modulus, H is the local membrane mean curvature, \bar{H} is the intrinsic mean curvature due to embedded integrin, and n is the area fraction density of integrins (relative density) (7). The second term σ_t is Lagrange multiplier (having the units of membrane surface tension) for the conservation constraint of the total membrane area. The third term gives the energy due to force of the cytoskeleton inside cells and φ_s is the restoring cytoskeleton spring constant. The fourth term is the negative binding potential of integrins ($\omega > 0$). The fifth term describes direct interaction Bragg-Williams term between neighboring integrins. The sixth and seventh terms are the entropic energies, taking into account the finite areas of integrins. Ω is the direct-interaction Bragg-Williams constant ($\Omega > 0$), c is the number of the nearest neighbor integrins, $h = h(s)$ describes the magnitudes of small deformations from the flat membrane, $n_s = 1/a_0$ is the saturation area density of integrins, where a_0 is the cross-section area of a single integrin, $k_b T$ is thermal energy and dA is the area element.

Next, we derived the equations of motion of the membrane contour using the differentiation of the free energy (Equation 1) with respect to the membrane amplitude ($h(s)$) and integrin concentration (n). To take into account the drag due to viscous forces, we assumed, for simplicity, only local friction forces with coefficient ξ . For the nearly flat membrane segment, the equation of motion of the membrane was given by

$$\xi \frac{\partial h(s)}{\partial t} = - \frac{\delta f}{\delta h(s)} \quad (2)$$

where t is time and f is F/dA . Note that the force density $\frac{\delta f}{\delta h(s)}$ equals the membrane shape velocity times friction coefficient, but opposite in direction. Since the relative change in the y (ie, vertical, $y = h(s)$) direction is considerably greater than the change along the x (ie, horizontal) direction, we only consider the changes along the vertical y direction. We now calculate the dynamics of the integrin density, using the following conservation equation:

$$\frac{\partial n}{\partial t} = - \nabla \cdot \vec{J} = \frac{\Lambda}{n_s} \nabla (n \nabla \frac{\delta F}{\delta n}) - \frac{n}{ds} \frac{\partial ds}{\partial t} \quad (3)$$

where Λ is the mobility of filaments, $\Lambda = D/k_b T$, D is the diffusion coefficient, and J is the total current of integrins on the membrane. For details of forces and fluxes derived from the differentiation of the free energy see our previous studies (12-14).

The results in the present study were calculated using numerical simulations of model system dynamics beyond linear limit. For simplicity, the boundary conditions on the nearly flat membrane were considered to be periodic. The following is the list of parameter values incorporated in our numerical simulations: $\varphi_s = 0.008 \text{ g}\mu\text{m}^{-2} \text{ s}^{-2}$, $\kappa = 100 k_b T$, $\bar{H} = 10 \mu\text{m}^{-1}$, $\sigma_t = 0.001 \text{ g s}^{-2}$, $\omega = 1 \cdot 10^{-3} \text{ g}\mu\text{m}^2 \text{ s}^{-2}$, $c = 4$, $\Omega = 3.5 \cdot 10^{-6} \text{ g}\mu\text{m}^2 \text{ s}^{-2}$, $n_s = 100 \mu\text{m}^{-2}$, $\xi = 125 \text{ s}^{-1} \text{ g}$, $D = 0.005 \mu\text{m}^2 \text{ s}^{-1}$. The binding coefficient ω was nonzero only above the nanotube edges, which was over segments of 20 nm in the case of the large diameter (100 nm) surface and over segments of 3 nm in the case of the small diameter (15 nm) surface, whereas, on the corresponding 100 nm and 15 nm void regions, the binding coefficient was zero. The binding coefficient was nonzero and uniformly distributed throughout the flat surface. The direct interaction term was nonzero only on the flat and the 15 nm nanotubular surface. The values of the diffusion constant, and bending modulus, were selected according to experimental data (15,16). The restoring cytoskeleton spring constant φ_s was chosen arbitrarily to prevent vertical drift of the membrane segment during the simulation. The ω and direct interaction Bragg-Williams term (Ω) were chosen in the range that facilitates dynamic instability (ie, the growth of membrane protrusions). The range of stability and dynamic instability for these parameters was explored as described in previous theoretical studies (13,14,17).

RESULTS

Under anodization conditions used in this study the internal TiO₂ tube diameter was 50 nm and 100 nm, while the length of the TiO₂ nanotubes was up to 10 μm (Figure 1)

The morphological alterations following the growth of cells on the large diameter TiO₂ nanotubular and flat Ti surfaces were imaged using scanning electron microscope (Figures. 2-3). TiO₂ nanotubular surface cells exhibited a small cell diameter and numerous membrane protrusions, whereas the cells grown on the flat Ti surface had a large diameter and few membrane protrusions (Figure 4). The same experimental procedure was repeated on a surface that was one half Ti flat and one half constructed into TiO₂ nanotubes (Figure 3). The boundary between these two re-

gions also marked the boundary for the above mentioned morphological alterations. The cells growing on the flat part of the surface were larger in size and had fewer membrane exvaginations than the cells growing on the nanotubular part of the surface.

To evaluate the differences in the geometry, the surface area of the cells in the two cell groups were measured using ImageJ. The number of analyzed cells was 54 from the nanotubular group and 15 from the flat titanium group. Roughly the same number of micrographs were taken for both types of samples at the same magnification. Thus, there were more smaller cells on the same number of figures in comparison to larger cells. Summary statistics for the differences in the surface area is shown in Figure 4. The difference in the surface area was highly significant ($P < 0.0001$). The average surface area of T24 cells on nanotubes was only 487 μm^2 , whereas the average surface area of T24 cells on the flat titanium surface was 2744 μm^2 (standard deviations, 191 μm and 1303 μm , respectively).

The vertical intersection of TiO₂ nanotubular surfaces was modeled explicitly as rectangular edge-like profiles. The widths of the repeating nanotube edges were 3 nm and 20 nm for the small and large diameter nanotubular surfaces, respectively. According to the diameter of the nanotubes, the corresponding void regions between the edges were

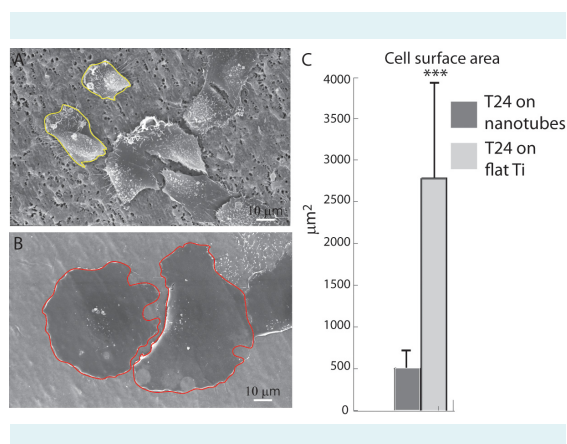


FIGURE 4. Summary statistics of changes in surface area of T24 cells grown on large diameter (50-100 nm) nanotubular TiO₂ surfaces and on flat titanium surfaces. Note the clear difference in size between the traced cells (examples in yellow) on the nanotubular surface (A) in comparison to the cells (examples in red) on the flat titanium surface (B). Statistical analysis showed a significant ($P < 0.0001$ ***) increase in the surface area of T24 cells on flat surface when compared to T24 on 50-100 nm nanotubes (C). Data are reported as mean \pm standard deviation.

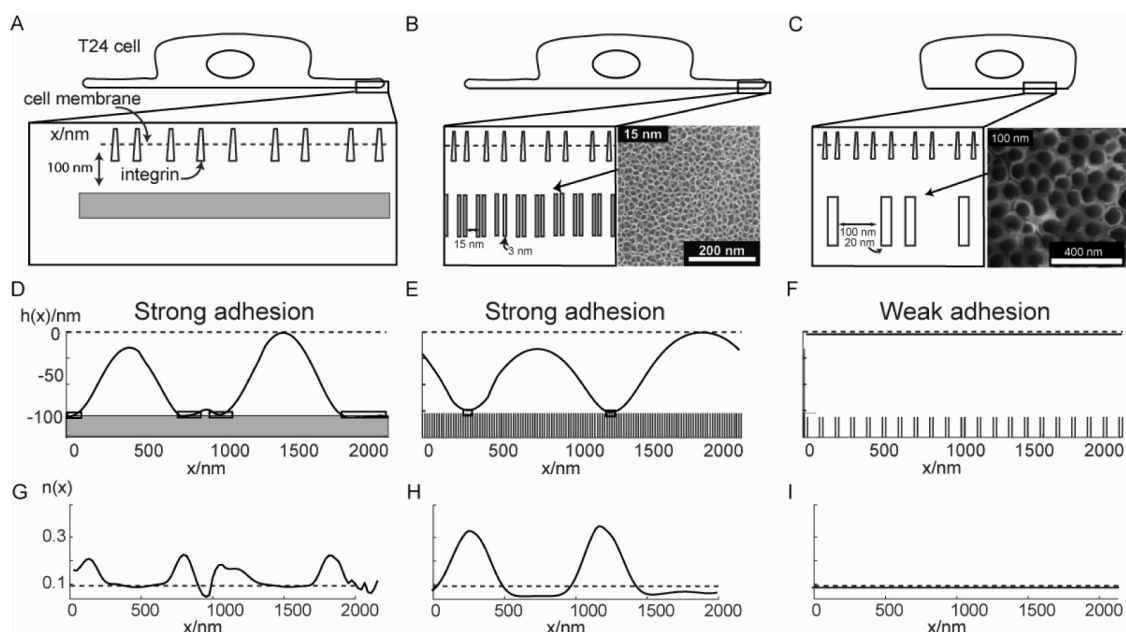


FIGURE 5. The effects of small diameter (15 nm) and large diameter (100 nm) nanotubular TiO₂ surfaces (10), as well as flat (amorphous) titanium surfaces, on cell membrane adhesion. The modeled membrane segment distributed with integrins of positive spontaneous curvature is initially positioned 100 nm above the flat surface (A), the 15 nm diameter nanotube surface (B); see right inset), and the 100 nm diameter nanotube surface (C); see right inset). The membrane adhesion is driven by the negative adhesion energy and the positive spontaneous curvature of integrins. The membrane shapes and integrin density distributions are obtained by the minimization of the membrane free energy. Steady state membrane shapes $h(x)$ (D-F) and integrin density distributions $n(x)$ (G-I) of the cell membrane adhered to the corresponding surfaces are plotted. Note the cell membrane adhesion to the flat titanium surface and to the 15 nm TiO₂ nanotube surface, in comparison to the hindered adhesion to the 100 nm TiO₂ nanotube surface.

15 nm and 100 nm. Since the adhesion of integrins could occur at the nanotube edges, the binding (adhesion) coefficient was nonzero only at the membrane regions directly above the edges. On the other hand, in the case of the flat surface, the binding coefficient was nonzero throughout the cell membrane. The initial condition of the following numerical simulations is a small random perturbation in the integrin density. The membrane regions that grew and reached titanium surface were trapped to the end of the simulation. This membrane trapping could be due to strong electrostatic interactions of the nanotube surface with the cell membrane and due to the binding of membrane integrins to fibronectin molecules attached at the nanotube edges (7).

Numerical simulations demonstrated that the cell membrane adhered strongly to small diameter and flat surfaces, whereas a weak adhesion was observed to the large diameter surface (Figure 3). The steady state shapes reached a steady state in the time scale of minutes.

The cell membrane adhered more to the flat surface and small diameter TiO₂ nanotube surface than to the large diameter surface (see rectangular boxes in Figure 5A-C). The attraction of integrins toward positive membrane curvatures was responsible for a positive feedback loop, facilitating the membrane protrusive growth toward the underlying surface. In addition, the dynamic instability that was responsible for the membrane growth was driven by the negative binding energy of the integrins to the titanium surface. The density of integrins was highest at the regions making contact with the titanium surface.

DISCUSSION

The growth of T24 cells on large diameter (50-100 nm) nanotubular TiO₂ surfaces revealed a significant reduction in the cell contact area as well as the enhanced growth of membrane exvaginations, in comparison to T24 cells grown on flat titanium surface. A simple computational model was constructed to explain the difference in the cell

contact area. The model assumption was that low density of TiO₂ nanotube edges on the large diameter TiO₂ nanotubular surface presented less adhesion area than the one on the flat surface. The model investigated cell membrane adhesion to TiO₂ surfaces of small diameter (15 nm) TiO₂ nanotubes, large diameter (100 nm) TiO₂ nanotubes, and a flat Ti surface. The membrane constituent was an integrin molecule, which could adhere and induce a positive membrane curvature. Numerical simulations demonstrated dynamic instability-driven adhesion mostly for the small diameter TiO₂ nanotube and flat titanium surfaces, whereas the adhesion to the large diameter TiO₂ nanotube surface was not facilitated (Figure 5). The integrin density was highest at the adhesion contacts of concave geometry and lowest at the indented membrane regions in between. To conclude, the smaller contact area of adhesion of the cell membrane to the large diameter TiO₂ nanotube surface could be explained by the smaller density of TiO₂ nanotube edges, which does not facilitate the adhesion-driven membrane growth. In the case of small diameter TiO₂ nanotube surface, the voids are completely compensated by the increased strength of adhesion on TiO₂ nanotube edges in comparison to the flat Ti surface (10).

The present experimental results illustrate morphological changes of human urothelial T24 cells on different TiO₂ nanotubes compared to on Ti scaffolds. Imaging of cell morphology with SEM revealed nanotubular intercellular membrane connections on large diameter nanotubular surface, in contrast to a Ti flat surface where intercellular membrane nanotubes were nearly completely absent. Similar intercellular membrane nanotubular structures were found previously also in weakly connected cells, such as immune cells and astrocytes, keratinocytes after long term TiO₂ or ZnO treatment, or in cells that actively migrate, for example, cancer cells (18,19). It is possible that the weak adhesion of T24 cells to the large diameter TiO₂ nanotubular surface might be accompanied by smaller contact adhesion area, and more spherical shape of the cells (ie, less flattened). Furthermore, the weak adhesion to the surface could be compensated by the growth of long membrane protrusions and intercellular membrane connections that increase the cell anchoring to the neighboring cells.

Previous studies analyzed the effects of convex and concave surface edges of nanostructured TiO₂ surface on the electric surface charge density and electrical field (7,10). Mathematically, convex edges of finite curvature would aggregate electrical charges and provoke larger electrical field, which is decreased with increasing distance from the

edge (7). In contrast, the surface charge density and the electric field strength were smallest at the concave edges (7). Consequently, we proposed that the sharp edges of TiO₂ nanotube surface aggregate electrical charges, which facilitate the strong binding of T24 cells to the sharp convex edges of small TiO₂ diameter nanotubes (7-9). On the other hand, the area density of sharp edges of TiO₂ nanotubes walls is smaller in the case of large diameter TiO₂ nanotube surface, and thus does not facilitate the formation of focal adhesions and the cell flattening to the same extent as is the case with small diameter TiO₂ nanotube surface (Figure 6).

To conclude, the observed small cell contact area on the large diameter (50-100 nm) TiO₂ nanotube surface could be due to the weak adhesion to the TiO₂ nanotube surface, whereas, the strong cell adhesion to the flat titanium surface might drive the cell flattening. More detailed studies of the growth of benign RT4 cancer cells should be performed on different titanium nanostructured scaffolds. Us-

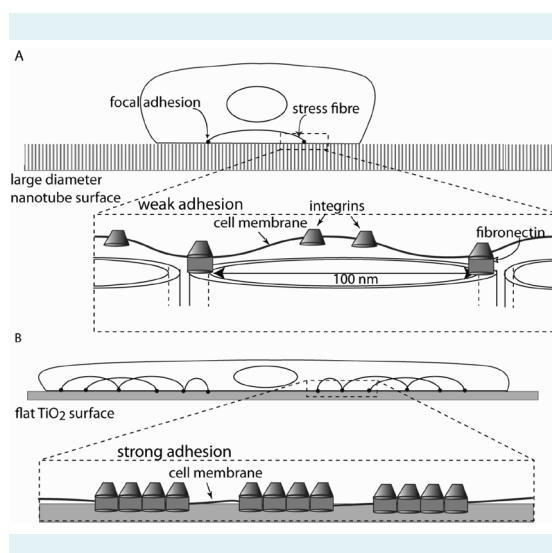


FIGURE 6. A possible mechanism underlying the weak adhesion of a T24 cell membrane to large diameter (100 nm) TiO₂ nanotubular surfaces. The low density of TiO₂ nanotube edges in the case of large diameter TiO₂ surface presents less adhesion areas for integrins and fibronectins, and thus, the adhesion to 100 nm nanotubes is weak (A). Furthermore, the long distance (100 nm) between neighboring nanotubes may not facilitate the cross-interaction between integrins, which may be crucial for the formation of a focal adhesion (in close-up). On the flat titanium surface, the even distribution of integrins that make contact with the substrate surface may facilitate the formation of focal adhesions, and thus, explaining the observed increase in cell diameter and cell flattening (in close-up) (B).

ing fluorescent labels for actin filaments and integrins, the predicted changes in the adhesion strength can be investigated. The aggregation of negative electrical charges at highly curved edges (7), such as the nanotubular edges of TiO₂ nanotube surface, should be analyzed using positively charged fluorescent dyes. We hope that the study of shape transformation of malignant cancer cells on different nanostructured surface may set the ground for the development of the novel intervention strategies in cancer treatment (20,21).

Acknowledgement The authors thank Dr Raheleh Mahammadpour for help in synthesis of TiO₂ surfaces.

Funding This work was supported by ARRS grants J3-9219-0381 and P2-0232-1538.

Ethical approval Not required.

Declaration of authorship RI did the experimental part, synthesized and characterized TiO₂ nanotubes, and helped with cancer cell cultures. DK performed simulations of cell membrane adhesion and the analysis of the morphologic changes of T24 cells on titanium and nanopatterned surfaces. MEK took an active part in acquisition, analysis, and interpretation of data, drafting the manuscript, and critical revision of the manuscript. EG based on the results of her previous work, helped to define the study and to plan (design) the experiments, contributed to the physical and biological interpretation of the results, and helped to define the basis of the theoretical model. SP collaborated in fabrication of TiO₂ nanotubes, helped to design the device and program the potentiostat to control the electric parameters during the fabrication of the nanotubes. KE performed SEM images; helped to design the biological part of the experiment. AK helped with the construction of the device for TiO₂ nanotube fabrication. PV performed microscopy and micrograph analysis, and participated in writing of the results and discussion. RZ interpreted the results of the experiments with cancer cells. AI led and coordinated the work, designed the mathematical model, helped with the design of experiments with cells, and wrote main conclusions of the work.

Competing interests All authors have completed the Unified Competing Interest form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare: no support from any organization for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work.

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