Substrate flexibility regulates growth and apoptosis of normal but not transformed cells

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Wang, Hong-Bei, Micah Dembo, and Yu-Li Wang. Substrate flexibility regulates growth and apoptosis of normal but not transformed cells. Am J Physiol Cell Physiol 279: C1345–C1350, 2000.—One of the hallmarks of oncogenic transformation is anchorage-independent growth (27). Here we demonstrate that responses to substrate rigidity play a major role in distinguishing the growth behavior of normal cells from that of transformed cells. We cultured normal or H-ras-transformed NIH 3T3 cells on flexible collagen-coated polyacrylamide substrates with similar chemical properties but different rigidity. Compared with cells cultured on stiff substrates, nontransformed cells on flexible substrates showed a decrease in the rate of DNA synthesis and an increase in the rate of apoptosis. These responses on flexible substrates are coupled to decreases in cell spreading area and traction forces. In contrast, transformed cells maintained their growth and apoptotic characteristics regardless of substrate flexibility. The responses in cell spreading area and traction forces to substrate flexibility were similarly diminished. Our results suggest that normal cells are capable of probing substrate rigidity and that proper mechanical feedback is required for regulating cell shape, cell growth, and survival. The loss of this response can explain the unregulated growth of transformed cells.

mechanical signaling; cell cycle; cell shape; traction force; cancer

Although it has been recognized for many years that adhesions with substrates or other cells regulate the growth of normal but not transformed cells, little is known about the nature of such anchorage-dependent regulation. To date most emphasis has been placed on characterizing the integrin receptor complexes (14), the extracellular matrix (ECM)-integrin binding interactions (19), and the downstream signaling events (10). However, physical forces at adhesion sites may play an equally important role, as evidenced by the responses of cell morphology, growth, apoptosis, and gene expression to mechanical forces such as fluid shear stress (6) or substrate stretching (1, 11, 20). It is possible that the main function of receptor binding is to establish a physical linkage between the cytoplasm and the outside environment, whereas subsequent physical interactions through such linkages elicit the actual enzymatic reactions on the cytoplasmic side of adhesion sites.

Our recent observations further indicate that normal cells not only respond to mechanical forces but actively probe substrate flexibility, most likely by applying contractile forces to the substrate and responding to the feedback of counterforces (21, 23). When plated on flexible substrates, normal cells show enhanced motility and reduced tyrosine phosphorylation (21). Thus an attractive hypothesis is that normal cells may regulate their motility, growth, and apoptosis according to mechanical properties of the substrate, whereas transformed cells may be defective in either detecting or responding to such mechanical signals.

Although previous observations have provided strong evidence that cell-substrate adhesions play a key role in regulating cell growth (14), it has not been possible to identify specifically the role of physical parameters because of the use of substrates that differ in both chemical and physical properties. To address this problem, we recently developed ECM-coated polyacrylamide substrates, which, through minor changes in the concentration of cross-linkers, allowed the regulation of flexibility over a wide range without altering their chemical properties. In this study, we cultured normal or H-ras-transformed 3T3 cells on substrates of different flexibility and compared their rates of growth and apoptosis, as well as cell spreading area and traction forces exerted on the substrate. We found that normal cells are much more sensitive to substrate flexibility than transformed cells. Our data can explain the growth advantage of transformed cells in vivo, where they are able to survive and grow independent of mechanical input from the surrounding tissues.

METHODS

Preparation of polyacrylamide substrate. Polyacrylamide substrates coated with collagen I were prepared essentially as described previously (23, 28). The flexibility of the substrate was manipulated by maintaining the total acrylamide concentration while varying the bis-acrylamide concentration. Experiments with growth and apoptosis were performed with substrates of 5% total acrylamide and bis-acrylamide between 0.012 and 0.06%. Measurements of cell spreading...
and traction forces were performed with substrates of 8% total acrylamide and either 0.03 or 0.06% bis-acrylamide. This change was necessary for generating high-quality substrate deformation vectors appropriate for computer analysis (too soft a substrate causes surface fluorescent beads to lie on different planes of focus as cells exert forces). Before cells were plated, the acrylamide gels were soaked for 30 min in DMEM at 37°C.

Cell culture and microscopy. NIH 3T3 cells (ATCC, Rockville, MD) and H-ras-transformed NIH 3T3 cells (PAP2) were kindly supplied by Dr. Ann Chambers (London Regional Cancer Center, London, Ontario, Canada; Refs. 2, 3), and were cultured in DMEM (Sigma, St. Louis, MO), supplemented with 10% donor calf serum (JRH Biosciences, Lenexa, KS), 2 mM L-glutamine, 50 µg/ml streptomycin, 50 U/ml penicillin, and 250 µg/ml amphotericin B (GIBCO-BRL, Gaithersburg, MD). Images were recorded with a Zeiss ×40, NA 0.75 Plan-Neofluar objective on a Zeiss Axiovert 10 microscope, using a cooled charge-coupled device camera (Series 200, Photometrics, Tucson, AZ).

Assay of DNA synthesis. Twenty-four or forty-eight hours after plating, NIH 3T3 and PAP2 cells were incubated with 100 µM 5-bromodeoxyuridine (BrdU; Sigma) in DMEM for 1.5 h at 37°C. Cells were then processed for immunofluorescence staining for BrdU as follows: cellular DNA was digested with DNase I (1 mg/ml in 10 mM Tris-HCl, 20 mM MgCl₂, pHe 7.5) for 20 min at 37°C before incubation with primary monoclonal antibodies against BrdU (1:200; Clone BU-33, Sigma). The secondary antibody was Alexa 546-labeled goat anti-mouse IgG (H+L) antibody (1:200; Molecular Probes, Eugene, OR). Nuclei were counterstained with Hoechst 33258 (10 µg/ml, Sigma) for 10 min at room temperature before observation with fluorescence microscopy. The percentage of BrdU (+) cells was calculated by counting 200 cells in multiple fields in each experiment. The results are shown as means ± SE from five independent experiments.

Assay of apoptosis. Quantitation of apoptotic cells by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was performed using the In Situ Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN) at 24 or 48 h after cells were plated on collagen-coated polyacrylamide substrates. Briefly, cells were fixed with 4% formaldehyde in PBS, pH 7.4, for 30 min at room temperature, then permeabilized with 0.1% Triton X-100, 0.1% sodium citrate for 2 min on ice. After incubation with the TUNEL reaction mixture for 60 min at 37°C, cells were counterstained with Hoechst 33258 (10 µg/ml, Sigma) for 10 min at room temperature before observation with fluorescence microscopy. The percentage of apoptotic cells was calculated by counting 200 cells in multiple fields in each experiment. The results are shown as means ± SE from five independent experiments.

Calculation of traction forces and cell spreading area. Traction forces generated by the cell were determined as described previously (7, 17). The deformation of the substrate due to cell-generated stresses was detected based on the displacement of embedded fluorescent beads near the substrate surface. Young’s modulus of the substrate was estimated based on the depression created by the weight of a 0.65-mm steel ball, using an equation derived from the Hertz theory as was applied previously in atomic force microscopy (17, 25). Calculation of traction was carried out on a supercomputer, using the displacement vectors, the cell boundary, Young’s modulus, and the Poisson’s ratio as the input. Average traction was calculated within regions that generated significant tractions. Briefly, the uncertainty of calculation at each pixel (7) was compared with the magnitude of traction. Pixels with a corresponding traction below the estimated uncertainty were excluded from averaging. This procedure minimizes the impact of cell spreading on average traction. Otherwise, because tractions were usually confined in limited peripheral areas while most regions near the nucleus generated no significant force (7), global averaging would cause peripheral exerted tractions to be dilated according to the degree of cell spreading. Cell spreading area was measured by counting the number of image pixels within the cell boundary using custom software.

RESULTS

To test the hypothesis that normal cells respond not only to the occupancy of the integrins by anchored ECM molecules but also to the rigidity of the substrate, we cultured 3T3 cells on flexible collagen I-coated polyacrylamide sheets. Substrates of similar chemical property but different rigidity were prepared by maintaining a constant total concentration of acrylamide while varying the concentration of bis-acrylamide (23, 28). The substrates used for the measurement of cell growth and apoptosis rates have a Young’s modulus varying between 4.7 and 14 kN/m². In the following discussion, these substrates will be referred to as flexible and stiff substrates, respectively.

To study the effects of substrate flexibility on cellular responses to substrate flexibility, we compared normal NIH 3T3 cells with PAP2 cells, a line of H-ras-transformed NIH 3T3 cells selected for their ability to metastasize in chick embryos (2, 3). Unlike some highly transformed cells, PAP2 cells require some ECM interactions for their growth, as they did not multiply or survive on bare polyacrylamide substrate without collagen I coating. Thus the following results on growth and apoptosis reflect differential downstream responses to integrins bound to collagen-coated surfaces of different flexibility.

Effects of substrate flexibility on cell growth and apoptosis. We first studied the effects of substrate flexibility on cell growth by measuring the percentage of cells incorporating BrdU (Fig. 1). Cells were plated at a density of 1,000/cm² onto either stiff or soft substrates. For normal cells, an approximately twofold difference in the rate of BrdU incorporation was observed 24 h after plating on flexible versus stiff substrates (Fig. 1B). This difference became even more pronounced at 48 h (≈4-fold). For H-ras-transformed cells, the rate of growth was not affected significantly by the substrate flexibility. It is also worth noting that, on stiff substrates at a limited cell density, H-ras-transformed 3T3 cells showed no growth advantage over nontransformed cells. However, on flexible substrates, transformed cells grew at a rate approximately twofold higher than that for nontransformed cells after 48 h of plating (Fig. 1B).

To minimize the effects of cell-cell interactions, which may affect the rate of DNA synthesis, the experiments were also performed with a low cell density of 250/cm². At this density, most normal and transformed cells remained as single cells 24 h after plating. However, the response of DNA synthesis to substrate flex-
ibility was similar to that seen at higher densities (Fig. 1B). From these experiments, we conclude that cell cycle of normal cells is regulated by the rigidity of the substrate and that this regulation is lost in transformed cells.

To complement the measurements of cell cycle rate, the rate of apoptosis was measured with a fluorescent TUNEL assay (9). We observed an approximately two-fold increase in apoptotic rate for nontransformed cells cultured on flexible (0.012% bis-acrylamide; a, b, e, f) or stiff (0.06% bis-acrylamide; c, d, g, h) polyacrylamide substrates for 48 h before incubation with 5-bromodeoxyuridine (BrdU) for 1.5 h. The cells were then processed for immunofluorescence staining with antibodies against BrdU (b, d, f, h) and counterstained with Hoechst 33258 (a, c, e, g). Cells undergoing DNA synthesis were stained with both agents. The percentage of cells undergoing DNA synthesis at 24 h (at 2 different cell densities) and 48 h after plating is shown in B as means ± SE from 5 independent experiments.

When cells were plated on soft substrates at high densities or allowed to grow to a high density, both normal and transformed cells formed highly packed, tissue-like aggregates (Fig. 3A). Time-lapse recording indicated that these aggregates were formed by active migration and incorporation of cells into the mass. However, only normal cells responded to aggregation in their growth and apoptosis rates; those within aggregates showed lower rates in both cell growth and apoptosis, reminiscent of changes during tissue formation (Fig. 3, B and C). Transformed cells, on the other hand, showed no difference in the rates of DNA synthesis or apoptosis within the aggregates (Fig. 3, B and C).

To rule out the possibility that the reduced growth and increased apoptosis of normal cells on flexible substrates were due to deficiency in collagen I coating, we first performed RIA to determine the relative amount of collagen I coupled to the polyacrylamide sheets. We found that flexible substrates (0.012% bis-acrylamide) had about twofold higher total collagen...
than did stiff substrates (0.06% bis-acrylamide), possibly reflecting a greater ability of collagen to penetrate through the less cross-linked surface. A second assay, based on fluorescence measurements using antibodies conjugated to fluorescent particles (and unable to penetrate into the substrate), indicated that the surface density of collagen was similar between stiff and soft substrates (17). Even if there were more collagen on soft substrates, the difference should promote, rather than inhibit, cell growth and survival (18). Thus flexibility played a dominant role over receptor occupancy in regulating DNA synthesis and apoptosis.

**Effects of substrate flexibility on cell spreading and traction forces.** The effects of substrate flexibility are likely linked to changes in cell morphology, spreading, and/or mechanical interactions with the substrate. Previous studies have correlated the rate of cell growth with the degree of cell spreading on stiff substrates of different chemical properties (8). We found that when cultured on polyacrylamide substrates, normal cells showed a significant decrease in spreading area when the Young’s modulus decreased from 33 to 14 kN/m² (Table 1). H-ras-transformed cells showed no apparent decrease in spreading area over this range of flexibility (Table 1) and became less spread only upon further decrease in Young’s modulus to <10 kN/m².

Our polyacrylamide substrates also allowed us to measure traction forces exerted by cells on the substrate. The method is based on mathematical analysis of the pattern of deformation of the flexible substrate, as detected by the movement of embedded fluorescent beads (7). We found that tractions generated by normal cells were significantly stronger on stiff substrates than on soft substrates. Traction forces generated by transformed cells, on the other hand, showed no significant difference on stiff or flexible substrates (Table 1).

**DISCUSSION**

To address specifically the impact of mechanical input on cell growth, we cultured cells on flexible polyacrylamide substrates that have similar chemical
properties but differ dramatically in their flexibility. The growth and motile behaviors of cells on stiff substrates are generally similar to those on glass or plastic surfaces. However, striking differences are observed as the flexibility increases, indicating that normal cells respond to not only the occupancy of the ECM receptors but mechanical signals transmitted through these receptors.

It has long been recognized that the degree of cell spreading is correlated with the rate of cell growth. Folkman and Moscona (8) first demonstrated that DNA synthesis in nontransformed cells increases as the cell becomes increasingly spread on more adhesive substrates, whereas transformed cells grew independently of cell shape or substrate adhesiveness. This is supported by a subsequent study indicating a progressive loss of shape-responsive metabolic control in transformed cells (29). Recent experiments by Ingber and coworkers (4, 13, 15, 26) further demonstrated changes in growth and apoptosis rates of normal cells under equivalent integrin occupancy but different geometry. However, although most previous studies have emphasized the importance of cell shape (4, 8, 12, 15), little is known about the physical/chemical parameters linking cell spreading/cell shape with the progression of cell cycle.

From both previous and the present studies (21), we propose that it is the mechanical input associated with shape change that regulates the cell cycle. To detect chemically identical but physically different surfaces, cells must rely on an active probing mechanism using contractile forces. Mechanical input from the substrate, transmitted through adhesion receptors, then activates downstream signals that regulate both the degree of cell spreading and the rate of growth. On stiff substrates, resistance to mechanical probing may lead to protein conformational changes and activation of signaling enzymes at the adhesion sites (5, 22). The response in turn causes an increase in traction forces and in cell spreading (Table 1). Activation of downstream chemical events may follow as a result of the direct activation of regulatory pathways or as a consequence of changes in cell shape or surface-to-volume ratio (4), leading to increased DNA synthesis and decreased apoptosis (Figs. 1 and 2). In contrast to normal cells, H-ras-transformed cells appear to be locked in a nonresponsive state in terms of substrate flexibility. They exert a similar magnitude of traction forces irre-

<table>
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<tr>
<th>Young’s Modulus, kN/m²</th>
<th>Traction Forces, kdyn/cm²</th>
<th>Spreading Area, 10⁻⁵ μm²</th>
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<tr>
<td></td>
<td>NIH 3T3</td>
<td>PAP2</td>
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<td>14</td>
<td>10.20 ± 3.63 (n = 8)</td>
<td>14.63 ± 1.63 (n = 5)</td>
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<tr>
<td>33</td>
<td>15.46 ± 2.13 (n = 7)</td>
<td>13.08 ± 4.06 (n = 6)</td>
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Values are means ± SD. Traction forces and cell spreading area of NIH 3T3 cells on stiff substrates were significantly larger than those on soft substrates (P = 0.027 and 0.014, respectively). No statistically significant difference was found for PAP2 cells on different substrates.
spective of the mechanical input from the substrate, which may then lead to a sustained mechanical stimulation of cell growth. Therefore, although additional defects in signal transduction are likely, the lack of response of traction forces to substrate flexibility in PAP2 cells may be sufficient to cause defects in the regulation of cell growth. For other types of transformed cells, the regulatory mechanism may break down at other levels, for example, through degradation of the ECM (3), deactivation of the force-sensing mechanism, or changes in the expression of integrin receptors (23).

The defects in mechanical response of H-ras-transformed cells may lead to significant growth advantages in the body, which consists largely of flexible tissues. At low cell densities on flexible substrates, normal cells suffered from both increased apoptotic death and reduced growth, whereas transformed cells maintained their rates of growth and apoptosis as on stiff substrates. At high densities, cell-cell contacts and aggregation became dominant. It protects normal cells from apoptosis but also inhibits their growth, whereas transformed cells grow independently of aggregate formation (Fig. 3). Thus transformed cells show a clear growth advantage over normal cells on flexible substrates at both low and high cell densities.

Cellular responses to substrate flexibility, stretching, and fluid shear may share a common mechanism involving active probing the environment and responses to mechanical forces. For nontransformed cells, this mechanism likely plays an important role during embryonic development and wound healing, allowing cellular proliferation or apoptosis to be regulated in response to changes in physical properties of the environment. Conversely, defects in mechanical signals may allow transformed cells to survive through the blood stream and to proliferate in diverse tissue environment.

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