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Nuclear-Cytoskeletal Interactions: Evidence for Physical Connections Between the Nucleus and Cell Periphery and Their Alteration by Transformation

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Abstract The overall coordination of cell structure and function that results in gene expression requires a spatial and temporal precision that would be unobtainable in the absence of structural order within the cell. Cells contain extensive and elaborate three-dimensional skeletal networks that form integral structural components of the plasma membrane, cytoplasm, and nucleus. These skeletal networks form a dynamic tissue matrix and are composed of the nuclear matrix, cytoskeleton, and extracellular matrix. The tissue matrix is an interactive network which undergoes dynamic changes as cells move and change shape.

Pathologists have long recognized cancer in pathologic specimens based on the altered morphology of tumor cells compared to their normal counterparts. The structural order of cells appears to be altered in transformed cells. This structural order is reflected in the altered morphology and motility observed in transformed cells compared to their normal counterparts, however, it is unclear whether the structural changes observed in cancer cells have any functional significance. We report here on the nature of the physical connections between the nucleus and cell periphery in nontransformed cells and demonstrate that the nucleus is dynamically coupled to the cell periphery via actin microfilaments. We also demonstrate that the dynamic coupling of the nucleus to the cell periphery via actin microfilaments is altered in Kirsten-ras transformed rat kidney epithelial cells. This loss of structure-function relationship may be an important factor in the process of cell transformation.

Key words: tissue matrix, actin, nuclear matrix, cytoskeleton, cancer

Several investigators have proposed that a tissue matrix system forms a structural and functional bridge from the cell periphery to the DNA [Isaacs et al., 1981; Bissell et al., 1982; Fey et al., 1984; Fujita et al., 1986; Getzenberg et al., 1990; Getzenberg et al., 1991a; Pienta et al., 1991a]. This cell matrix system consists of dynamic linkages between the skeletal networks of the nucleus (the nuclear matrix), the cytoplasm (the cytoskeleton), and the extracellular environment (the extracellular matrix), and must be functionally intact for the coordination of normal cell function [Pienta et al., 1991b; Ingber and Folkman, 1989; Ingber and Jamieson, 1985; Dennerll et al., 1988]. Fey and Penman [1981] have demonstrated the physical connections between the cell periphery and nuclear matrix and have further shown how this scaffold system is altered in tumor cells and by tumor promoters [Fey et al., 1984]. Several investigators have demonstrated that cell metabolism, including DNA, RNA, and protein synthesis, is modulated by cell shape and external surface contact [Benecke et al., 1978; Gospodarowitz et al., 1978; Folkman and Moscana, 1978; Wittlesberger et al., 1981; Ben-Ze'ev, 1985; Murphy et al., 1992; Boyd et al., 1991; Ben-Ze'ev et al., 1980; Zambetti et al., 1991]. Ben-Ze'ev [1985] has demonstrated alterations in the cytoskeleton of cancer cells and has proposed that growth-related cellular functions are regulated by signals which are transmitted through an organized cytoskeletal system that has been disrupted by the carcinogenic process. These disruptions in the structural networks of cancer cells are reflected by

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the altered static morphology (shape) and altered dynamic morphology (motility) that can be observed in transformed cells.

These observations emphasize the need for better definition of the functional connections between the nucleus and cell periphery in the normal cell as well as the cancer cell. Each subcomponent system of the cytoskeleton has been implicated to some degree in nuclear-cytoskeletal interaction and transport [for review, see Pienta et al., 1991a], but the relationships of these networks to the nucleus are unclear. This paper presents evidence that for nontransformed cells in vitro, dynamic nuclear morphology is controlled to a large extent by the actin microfilament network. Furthermore, this nucleus-actin network system is functionally altered in at least one tumorigenic cell line, the Kirsten-ras transformed rat kidney epithelial cell. This loss of shape-responsive control may be an important factor in cell transformation.

MATERIALS AND METHODS

Materials

Cytochalasin D and colchicine were obtained from Sigma Chemical Co. (St. Louis, MO). Acrylamide was obtained from Hoeffer Scientific (San Francisco, CA). Trypsin/EDTA was obtained from Gibco BRL (Grand Island, NY).

Cell Culture

The normal rat kidney epithelial cell line (NRK-52E, CRL 1571) and its Kirsten-ras transformed counterpart the KNRK (ATCC CRL 1569) were obtained from the American Type Culture Collection (Rockville, MD) and were maintained in Dulbecco's modified essential medium supplemented with 5% fetal bovine serum.

Immunofluorescence

Cells were plated in double well chamber slides (Nunc, Inc., Naperville, IL) at a concentration of 1×10^4 cells/well. Cells were incubated at 37°C for 24 h. Cells were then treated with drug for 30 min to 1 h. Cells were then washed twice with phosphate buffered saline (PBS) and fixed in 3.75% formaldehyde/PBS for 10 min at room temperature. Cells were then washed twice with PBS and extracted with −20°C acetone for 5 min. The slides were then allowed to air dry after which the cells were stained and counterstained for 20 min, each at room temperature. Slides were then washed twice with PBS and mounted with 1:1 PBS/glycerol. Slides were viewed with a Zeiss fluorescent microscope equipped with 10× and 40× objectives (Zeiss, Inc., Thornwood, NY).

Cell Motility Assay

NRK and KNRK cells were filmed using time-lapse videomicroscopy in 2 h segments. After seeding with 2×10^4 cells/35 mm single well chamber slide, cells were incubated at 37°C for 24 h in standard media. Cells were viewed with a high resolution black and white video camera (DAGE MTI, Michigan City, IN, Series 66) at ×400 magnification with an inverted Zeiss (IM35) microscope (Hoffman optics) with heated stage as previously described [Pienta et al., 1991c]. Cells were videotaped with a time-lapse video recorder (JVC Br9000) every 15 s. Translational motility of each cell was measured from the TV monitor over 2 h by directly measuring the total path distance each cell traveled. A minimum of 25 cells was measured for each surface.

Morphometric Assay

Cells were plated in double well chamber slides (Nunc, Inc., Naperville, IL) at a concentration of 1×10^4 cells/well. Cells were incubated at 37°C for 24 h. Cells were then treated with drug for 30 min to 1 h. After incubation, chambers were transferred to a 37°C heated microscope stage (Zeiss TRZ 2700, Thornwood, NY) for analysis. Images of control and treated cells were captured by a Compac Deskpro 386/25 using the Zeiss Dynacell program. One hundred cells and nuclei for each treatment group and controls were captured and then digitized and the X-Y coordinates of the cell and nuclear boundaries were stored using the Dynacell software and a digitizer tablet (SummaSketch Model MM-1201) as previously described [Boyd et al., 1991; Pienta and Coffey, 1991a]. Cell and nuclear area in square microns and perimeter in microns were measured for each cell and nucleus. After compilation of baseline data in control and treated cells, trypsin/EDTA was added to the chamber. Cells and nuclei were monitored as they rounded up by capturing an image of the microscope field every 5 s. Cells and their nuclear boundaries were digitized at each time point and rates of rounding up were calculated. After rounding up and release from the substratum, 100 cells and their respective nuclei were then captured and analyzed as outlined above.

Statistics

Statistical analysis was performed using Statgraphics v4.0 (STSC, Inc. Rockville, MO). Statistical significance was determined using the Student's t-test.

RESULTS

Cancer cells exhibit a wide variation in size and shape (morphologic heterogeneity) that is accompanied by wide variations in cellular functions (functional heterogeneity) compared to normal cells. In addition, a consistently observed property of epithelial cancer cells is their increased ability to metastasize. This increase in in vivo metastatic ability is accompanied by an increase in in vitro motility [Partin et al., 1988]. For example, the immortal but nontumorigenic normal rat kidney epithelial cell has an average translational motility in vitro of 3 ± 1 μm/h (Fig. 1). When these cells are transformed by the Kirsten-ras oncogene, in vitro cell motility increases to 11 ± 2 μm/h, and the cells simultaneously lose their contact inhibition and acquire the ability to become tumorigenic (Table I). Concomitant with these changes in motility and tumorigenicity, the transformed rat kidney cells become more varigated in shape and assume a smaller cell and nuclear area compared to the normal cells, which is reflected in an increase in their nuclear/cytoplasmic ratio [Pienta et al., 1991b].

In an attempt to define the structural systems involved in these alterations in static structure (cell and nuclear morphology) as well as dynamic structure (motility), normal and transformed rat kidney epithelial cells were analyzed utilizing immunofluorescent stains and/or antibodies. The major skeletal systems of the cytoplasm were visualized with phalloidin or actin antibody to stain the microfilaments, colchicine or alpha and beta tubulin antibodies to stain the microtubules, and vimentin and keratin antibodies to stain the intermediate filaments. The results of these studies are presented in Figure 2 and Table II and demonstrate a remarkable alteration in the microfilaments of the transformed cells. Normal rat kidney cells demonstrate stress fibers when stained with phalloidin (Fig. 2A), but the transformed kidney cells do not appear to contain actin stress fibers (Fig. 2B). Since actin is thought to play a major role in cell motility [Pollard and Cooper, 1986; Pienta

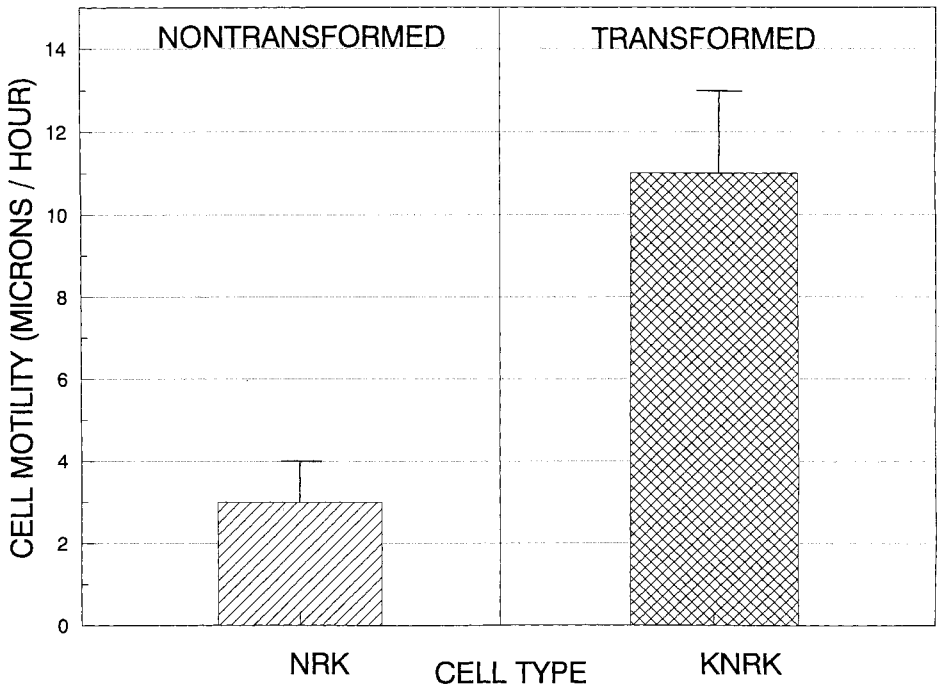

Fig. 1. Translational motility of normal rat kidney epithelial cells (NRK) and their Kirsten-ras transformed counterparts (KNRK). Twenty-five individual cells were filmed by time-lapse videomicroscopy as outlined in Materials and Methods. Translational motility is measured as the total path length a cell travels in 1 h and is significantly different between normal and transformed cells, $P < 0.001$. Error bars represent ± standard error.

TABLE I. Morphologic and Functional Characteristics of Normal Rat Kidney (NRK) Cells and Their Transformed Counterparts, the Kirsten-ras Normal Rat Kidney (KNRK) Cells[a]

Cell type	Phenotype	Cell area (μm^2)	Cell shape[b]	Nuclear area (μm^2)	N/C ratio[c]	Motility ($\mu m/h$)[d]	Contact inhibition	Tumors in athymic mice[e]
NRK	Immortal	2,504 ± 201	1,205 ± 186	171 ± 3	7 ± 1	3 ± 1	+	−
KNRK	Transformed	712 ± 62	2,842 ± 263	102 ± 4	19 ± 1	11 ± 2	−	+

[a]All values for cell area, cell shape, nuclear area, N/C ratio, and motility are represented as mean ± standard error. All values are significantly different, NRK compared to KNRK, $P < 0.001$.
[b]Shape is measured as a form factor: $\{(\text{perimeter}/2\text{xpi}/(\text{areaxpi})^{1/2}) - 1\} \times 1{,}000$. A perfect circle has a value of zero.
[c]*N/C ratio = (projected nuclear area/projected cytoplasmic area) × 100.*
[d]Motility is measured as the total path length a cell travels in 1 h utilizing a time-lapse video assay.
[e]500,000 cells were injected into athymic mice on day 0. NRK cells did not form tumors in 30 days. KNRK cells formed large tumors leading to animal sacrifice by day 14.

and Coffey, 1991b], this alteration in microfilament ultrastructure may reflect an underlying mechanism of the increased in vitro motility observed in the ras transformed cells (see Fig. 1). Both the microtubule networks as well as the intermediate filament networks of both the normal and transformed cells appear to be similar by immunofluorescent staining (see Table II). These data suggest that the microfilament network of transformed rat kidney cells may be structurally and therefore possibly functionally different than their normal kidney cell counterparts.

To test this hypothesis, normal and transformed cells were observed as they attached and spread in vitro as a function of time (Table III). Normal and transformed rat kidney epithelial cells in suspension exhibit similar sizes for their cells (506 ± 22 μm^2 vs. 456 ± 16 μm^2) and nuclei (89 ± 4 μm^2 vs. 96 ± 4 μm^2). Both the normal and transformed cells begin to attach and spread within 4 h of plating in glass tissue culture chambers. Individual normal rat kidney cells which are not contact inhibited continued to spread over the next 24 h, achieving average areas of 2,504 ± 201 μm^2, a 5-fold increase in area. Concomitantly, normal nuclei spread out and increased in area to an average size of 171 μm^2. The Kirsten-ras transformed rat kidney cells, however, do not attach and spread out in the same manner as the normal cells. The transformed cells only increase their projected area by approximately 50%, from 456 ± 16 μm^2 to 712 ± 52 μm^2, over 24 h. Simultaneously, the nuclear projected area of the transformed cells did not increase. This may account for the increase in the nuclear/cytoplasmic ratio observed in many cancer cell lines in vitro [Murphy et al., 1992]. These data combined with the immunofluorescence data suggest that a mechanism of increased motility present in transformed rat kidney cells may be a result of altered cell attachment to the substratum in combination with alteration in actin stress fiber formation. These data further suggest that the relationship between the nucleus and cell periphery may be significantly altered in transformed cells.

To test this hypothesis, the relationship of the nucleus to each of the cytoskeletal networks was investigated. Releasing or detaching normal cells from the substratum by trypsin/EDTA allows cells to contract and round up [Ingber and Folkman, 1989]. This contraction process requires approximately 150 s for the normal rat kidney cells to complete, i.e., for the cells to break their bonds with the substratum and return to a rounded-up state in suspension. During the contraction process, both the normal rat kidney cell and nucleus can be reproducibly monitored and measured as they round up (Fig. 3). Figure 3 demonstrates that the normal rat kidney cells contract at a constant rate of 0.5% cell area per second and the nuclei contract at a constant rate of 0.3% nuclear area per second. For nontransformed epithelial cells the contraction slope is essentially size independent for cells whose areas are 500 to 4,000 μm^2 (data not shown).

As outlined in Table IV, the cellular networks involved in the contraction process can be dissected through the use of cytoskeletal inhibitors. Table IV demonstrates that cell contraction requires an intact microfilament system and can be reversibly blocked by treating the cells with 10 $\mu g/ml$ cytochalasin D for 30 min. These cells take on a fractured appearance but maintain their projected area. Cells treated with trypsin/EDTA after cytochalasin treatment did not round up. Inhibition of microtubules by

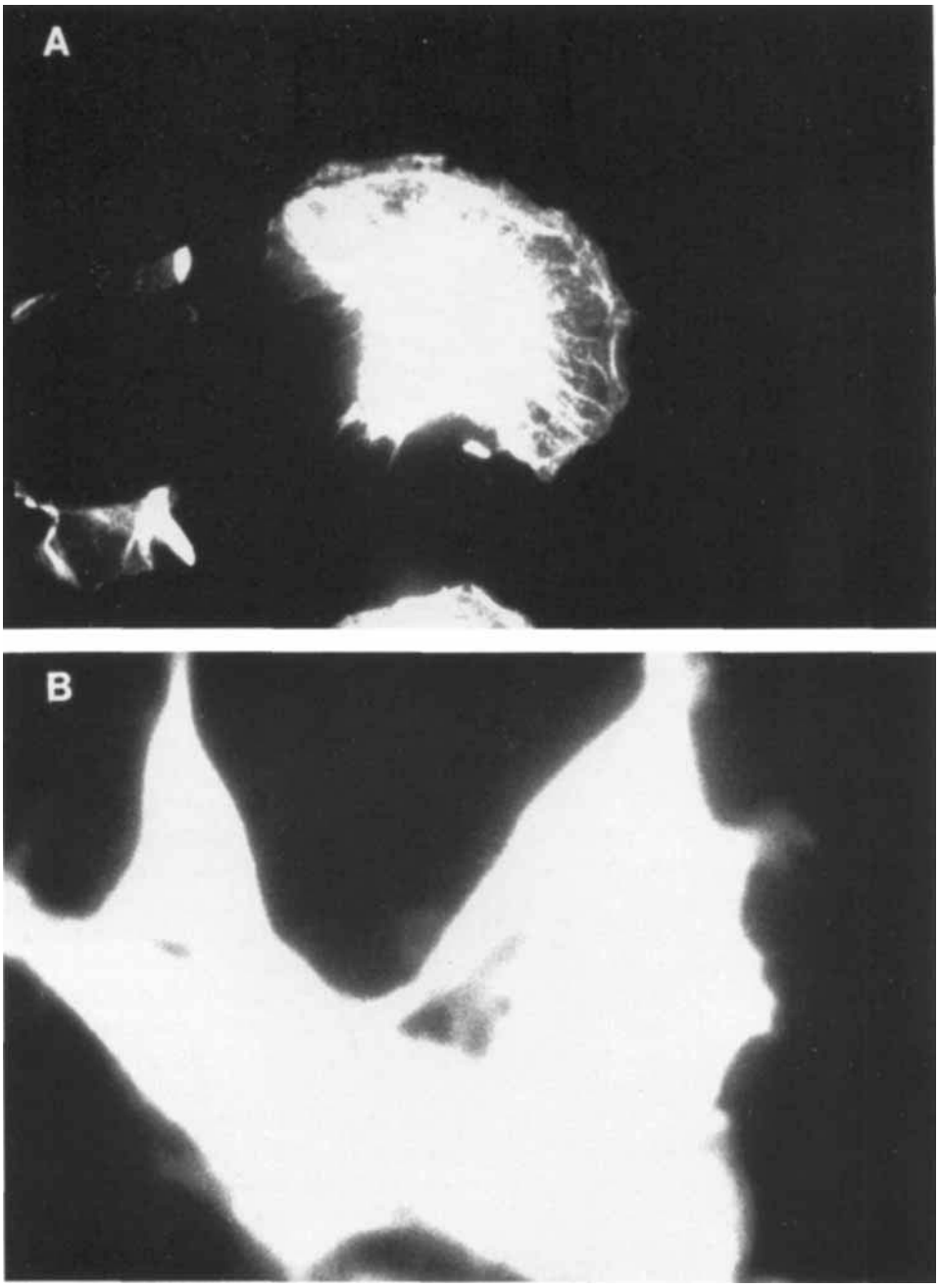

Fig. 2. **A:** Normal rat kidney cells stained with phalloidin demonstrate actin stress fibers. **B:** Kirsten-ras transformed rat kidney cells stained with phalloidin do not demonstrate actin stress fibers and have a different morphology than the normal rat kidney cells.

colchicine or intermediate filaments by acrylamide resulted in loss of cell area to 60–66% of control size secondary to uniform contraction of the peripheral cytoplasmic zone of the epithelial cells (Table IV). Cells treated with colchicine or acrylamide, however, were still able to contract normally upon treatment with trypsin/EDTA, indicating that microtubules and intermediate filaments appear to have a passive role in the process of cell rounding (Table IV). The transformed rat kidney cells exhibit similar but less dramatic results compared to their normal counterparts (Table IV). Colchicine caused a decrease in cell area, but acrylamide had no effect. Cytochalasin D was able to inhibit cell rounding while inhibition of the microtubule or intermedi-

ate filament networks did not appear to alter the ability of the transformed cells to contract in response to trypsin/EDTA.

The normal rat kidney epithelial cell nucleus appears to spread in area in relation to the cell area increases resulting from spreading (see Table III) and this relationship appears to be dependent on an intact actin microfilament network (Table V). Treating normal cells with cytochalasin D results in a decrease of projected nuclear area that appears to be secondary to the nucleus rounding up and that is equivalent to that seen when the cell is released from its substratum by trypsin/EDTA (nuclear projected area 49% vs. 52% of control, respectively). This effect is not observed when the normal kidney cells are treated with colchicine or acrylamide. Thus, it appears that the nucleus is tethered to the cell periphery through actin microfilaments. When the cell spreads, the actin microfilaments stretch the nucleus, and when the cell is released from its substratum or its microfilament network is disrupted, the nucleus rounds up.

This nuclear to cell periphery relationship appears to be altered in the transformed cells (Table V), where nuclear area remains constant despite the alterations in cell area/volume which occur with trypsin/EDTA treatment or manipulation of the cytoskeleton through the use of inhibitors. Thus, the relationship of the nucleus to the cell periphery appears to be fundamentally altered in the Kirsten-ras transformed rat kidney epithelial cells.

TABLE II. Cytoskeletal Networks in Normal and Cancer Cells

Fiber	Protein	Normal cells (NRK)	Cancer cells (KNRK)
Microfilament	Actin	Fibers	Collapsed
Microtubule	Tubulin	Filaments	Filaments
Intermediate filaments	Keratin	Filaments	Filaments

DISCUSSION

The data presented here indicate that the nucleus of the cell is connected to the cell periphery in a dynamic manner. Table I and Figure 1, as well as Table II and Figure 2, demonstrate that both the static and dynamic morphology of the Kirsten-ras transformed kidney cell is dramatically different than its normal rat kidney counterpart. Table III demonstrates that as a nontransformed cell alters its shape by spreading on a substratum, the nucleus mirrors these changes by spreading out within the cytoplasm. This physical alteration in nuclear shape has been previously demonstrated to contribute to the control of DNA synthesis and gene expression [Benecke et al., 1978; Gospodarowitz et al., 1978; Folkman and Moscona, 1978; Wittlesberger et al., 1981; Ben-Ze'ev et al., 1980]. The data presented in Tables IV and V indicate that this control of nuclear shape appears to be mediated in part by interactions with intermediate filaments and actin microfilaments. Previously, Fey et al. [1984], using electron microscopy, demonstrated physical connections between the intermediate filaments and the nuclear matrix. Furthermore, Zambetti and colleagues [1991] demonstrated that disruption of the cytoskeleton with the actin microfilament inhibitor cytochalasin D induced specific gene expression and suggested that the nucleus can respond to signals related to the structural organization of the cytoskeleton. The rounding up of the cell nucleus in response to cytochalasin treatment

TABLE III. Demonstration That as Cells Spread, Cell and Nuclear Shape Are Connected in a Normal Cell and Altered in Transformed Cells

Time (hours post seeding)	Normal kidney cell area (μm^2)[a]	Normal kidney nuclear area (μm^2)[a]	Transformed cell area (μm^2)[a]	Transformed nuclear area (μm^2)[a]
0	506 ± 22	89 ± 4	456 ± 16	96 ± 4
4	600 ± 25	95 ± 5	536 ± 20	98 ± 4
8	905 ± 48	102 ± 5	639 ± 37	101 ± 5
16	1,835 ± 160	145 ± 8	656 ± 41	100 ± 6
24[b]	2,504 ± 201	171 ± 3	712 ± 62*	102 ± 4*
48	4,000 ± 282	220 ± 11	745 ± 58	105 ± 4

[a]Area values ± standard error.

[b]All subsequent experiments done at 24 h.

*Significantly different from nontransformed cell values, $P < 0.001$.

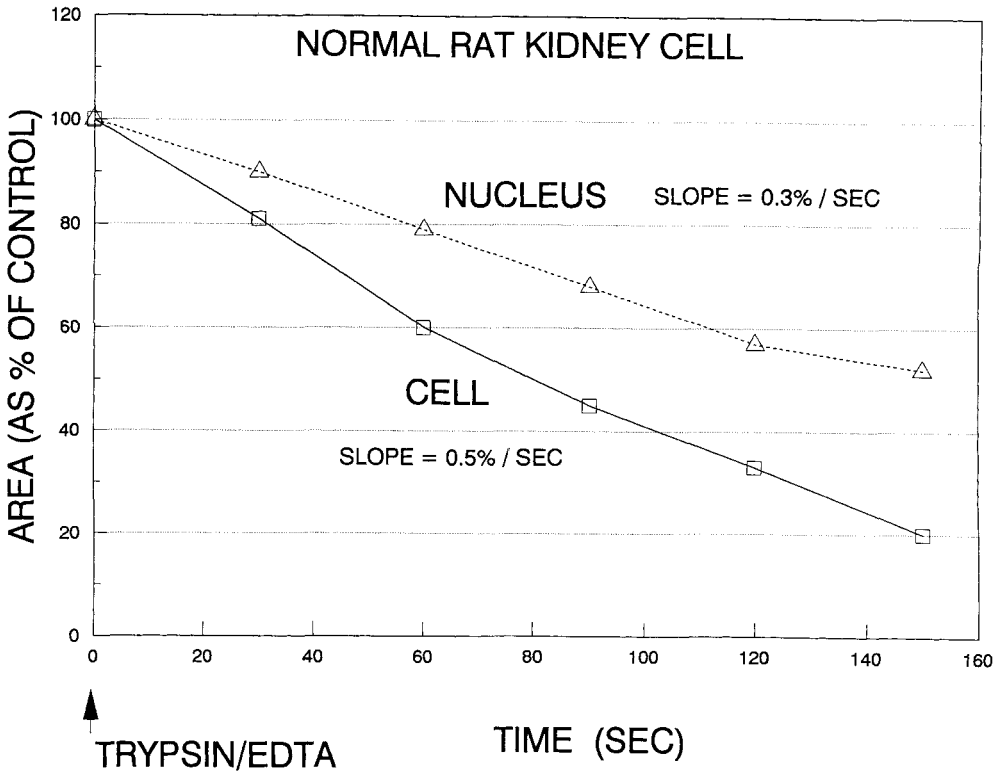

Fig. 3. Dynamic morphometric microscopic assay of cells and their nuclei. Cells were plated in double well chamber slides at a concentration of 1×10^4 cells/well. Cells were incubated at 37°C for 24 h. Cells were then treated with drug for 30 min to 1 h. After incubation, chambers were transferred to a heated microscope stage for analysis as outlined in Materials and Methods. One hundred cells and nuclei for each treatment group and controls were captured and then analyzed. Cell and nuclear area in square microns and perimeter in microns were measured for each cell and nucleus. After compilation of baseline data in control and treated cells, trypsin/EDTA was added to the chamber. Cells and nuclei were monitored as they rounded up by capturing an image of the microscope field every 5 s. Cells and their nuclear boundaries were digitized at each time point and rates of rounding up were calculated. After rounding up and release from the substratum, 100 cells and their respective nuclei were then captured and analyzed as outlined.

TABLE IV. Response of Normal and Transformed Cells to Cytoskeletal Network Inhibitors

Treatment	NRK cell area (% of control)[a]	NRK cell slope (% area/s)[b]	Transformed cell area (% of control)[c]	Transformed cell slope (% area/s)
Control	100 ± 8	0.5	100 ± 6	0.4
Colchicine	66 ± 9*	0.5	72 ± 5*	0.2
Acrylamide	60 ± 8*	0.5	103 ± 8	0.2
Cytochalasin D	96 ± 8	—[d]	86 ± 7	—[d]

[a]Area presented as mean percent ± standard error. Control normal rat kidney epithelial cell (NRK) area of 2,504 μm^2 = 100%.
[b]% Area/s slope represents the rate of cell area loss per second as cell rounds up in response to trypsin/EDTA.
[c]Transformed cell = the Kirsten-ras transformed rat kidney cell. Control transformed cell area of 712 μm^2 = 100%.
[d]Cells did not round up in response to trypsin/EDTA.
*Significantly different from control ($P < 0.01$).

suggests that the physical interactions of the nucleus with the rest of the cell are mainly mediated by the actin microfilaments. The data (Table IV) that cells are prevented from rounding up by disruption of the microfilament system but not the disruption of microtubules or intermediate filaments further suggest that actin microfilaments are critical mediators of nucleus-cell interactions.

Several investigators have previously demonstrated the altered morphology and structure of cancer cells, but the significance of these find-

TABLE V. Response of Normal and Transformed Nuclei to Cytoskeletal Network Inhibitors

Treatment	NRK nuclear area[a] (% of control)[b]	NRK nuclear slope (% area/s)[c]	Transformed nuclear area (% of control)[d]	Transformed nuclear slope (% area/s)
Control	100 ± 8	0.3	100 ± 3	—[d]
Colchicine	98 ± 7	0.3	100 ± 5	—[d]
Acrylamide	71 ± 7*	0.3	107 ± 6	—[d]
Cytochalasin D	49 ± 5*	—[d]	102 ± 4	—[d]

[a]Area presented as mean percent ± standard error. Control normal rat kidney epithelial cell (NRK) nuclear area of 171 μm^2 = 100%.
[b]% Area/s slope represents the rate of nuclear area loss per second as cell rounds up in response to trypsin/EDTA.
[c]Transformed cell = the Kirsten-ras transformed rat kidney cell. Control transformed cell area of 102 μm^2 = 100%.
[d]Nuclei did not round up in response to trypsin/EDTA.
*Significantly different from control ($P < 0.01$).

ings was unclear, i.e., does altered cell structure contribute to the process of cell transformation or is it simply an innocent bystander in the functional alterations which occur during carcinogenesis [Pienta et al., 1989; Ben-Ze'ev, 1985; Fey and Penman, 1988; Getzenberg et al., 1991a,b]. Transformation is accompanied by morphological changes at the cell and nuclear level and alterations in nuclear morphology have been a pathologic hallmark of neoplasia for over a century and have been correlated with phenotypically aggressive tumors [Pienta et al., 1989]. Virtually every part of the tissue matrix system including the cytoskeleton has been reported to be biochemically or structurally altered in transformed cells but the functional significance of these findings has not been well defined [Ben-Ze'ev, 1985; Getzenberg et al., 1991b; Bissell et al., 1981].

This paper presents evidence that the physical connections between the nucleus and the cytoskeleton are functionally altered in a transformed cell. The nuclei of the Kirsten-ras transformed rat kidney epithelial cells do not respond to alterations in cell shape in the same manner that nontransformed cells do. This loss of shape-responsive control may be functionally significant in the process of cell transformation.

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