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Increased Cell-Substrate Adhesion Accompanies Conditional Reversion to the Normal Phenotype in Ras-Oncogene-Transformed NIH-3T3 Cells

DALE K. SHUMAKER,* MARSHALL D. SKLAR,† EDWARD V. PROCHOWNIK,‡ AND JAMES VARANI*,[1]

*Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan; ‡Section of Hematology and Oncology, Children's Hospital of Pittsburgh, and the Department of Molecular Genetics and Biochemistry, The University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania; and †Department of Radiation Oncology, University of Miami School of Medicine, Miami, Florida

We recently reported (1991, *Mol. Cell Biol.* 11, 3699–3710) that depletion of c-myc protein by myc antisense sequences in ras-transformed NIH-3T3 cells reverses several of the malignant characteristics of these cells. These include transformed morphology, growth in soft agar, and ability to form tumors in athymic mice. In the present study we examined the same cells for *in vitro* adhesive behavior. Cells depleted of c-myc protein by antisense transfection showed no change in attachment to fibronectin-coated dishes as compared to ras-transformed NIH-3T3 cells but had greatly increased resistance to trypsin/EDTA-mediated release from the substratum after attachment. In concomitant studies, the cells were examined for fibronectin biosynthesis and cell surface fibronectin. There was no overall change in fibronectin biosynthesis in the c-myc antisense transfected cells as compared to the ras-transformed NIH-3T3. However, immunofluorescence staining revealed increased amount of surface fibronectin associated with the antisense c-myc-expressing transfectants. Taken together, these data indicate that the conditional reacquisition of the nonmalignant phenotype in ras-transformed NIH-3T3 cells by selected depletion of c-myc protein is associated with an increase in cell-substrate adhesion. This, in turn, is associated with an increase in surface fibronectin.

INTRODUCTION

Decreased cell surface fibronectin has been shown to accompany the transformation of a wide variety of cells [1, 2, 4, 5, 7, 9, 14, 15, 17, 23, 34, 35, 40, 42]. Induction of differentiation in many tumor cell types by chemical agents, with a concomitant loss of malignant behavior, is also associated with deposition of surface fibronectin [5, 6, 13, 20, 30, 32, 39, 40]. What accounts for the loss of fibronectin during the transformation process is not fully understood. Although in some cases there is a clear reduction in fibronectin-binding integrins on the cell surface [22, 24], decreased biosynthesis of fibronectin has also been noted [2, 23]. Our own studies have shown that fibronectin production and fibronectin receptor expression are coordinately downregulated [5, 7, 15, 40]. Likewise, agents that induce differentiation often have an effect on both the biosynthesis of fibronectin and the expression of its receptor [5, 6, 13, 18, 20, 25, 28, 30, 32, 40]. These studies unambiguously demonstrate an inverse relationship between surface fibronectin and maintenance of the transformed phenotype. They do not, however, address the mechanism by which surface fibronectin helps to maintain the normal phenotype or the molecular steps that occur following the initial transforming event and which lead to the alterations in surface fibronectin.

Recently, we reported that maintenance of the transformed phenotype in v-Ki-ras oncogene-transformed NIH-3T3 cells (referred to as DT cells) required the concomitant expression of the c-myc oncogene [29]. This was demonstrated by transfecting DT cells with a plasmid bearing the c-myc cDNA sequence in antisense orientation relative to a glucocorticoid inducible murine mammary tumor virus (MMTV) promoter. In several clones, expression of c-myc antisense transcripts resulted in depletion of c-myc protein and in a corresponding loss of several properties associated with transformation. Specifically, the c-myc antisense cells demonstrated reversion of the transformed morphology, reduced capacity for growth in soft agar and attenuation of the ability to form tumors in athymic mice compared to the parental DT cells. The conditional nature of the changes was established by demonstrating the reacquisition of the transformed phenotype upon withdrawal of the hormone and replenishment of endogenous c-myc oncogene. We have made use of these cells in the present study to examine the relationship between alterations in cell–substrate adhesion which ac-

[1] To whom reprint requests should be addressed at Department of Pathology, The University of Michigan Medical School, 1301 Catherine Road/Box 0602, Ann Arbor, MI 48109.

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company reversion of the transformed phenotype and alterations in fibronectin synthesis and cell surface deposition.

MATERIALS AND METHODS

Cells. Cells utilized in this study included untransformed NIH-3T3 cells, DT cells, and two clonal isolates of DT cells transfected with the MMTV c-myc antisense plasmid vector (referred to as B2C4 and D6AD2). The source of the parental NIH-3T3 cells and the derivation of the clones has been described previously [29]. The DT cells contain two copies of the v-Ki-ras oncogene, making spontaneous reversion a rare event. Unlike untransformed NIH-3T3 cells, which grow as flat, fibroblast-like cells, the DT cells grow as round clusters of loosely attached cells in monolayer. DT cells also grow in suspension in soft agar and form tumors in athymic mice [29]. In the absence of dexamethasone, the c-myc antisense clones are similar to the parental DT cells in terms of morphology, growth in soft agar, and tumorigenicity in athymic mice. Within 24 h of dexamethasone addition, however, antisense cells express the morphological features and acquire the biological properties of untransformed NIH-3T3 cells. We have previously shown that this reversion is accompanied by the induction of high levels of c-myc antisense transcripts and by a profound reduction in c-myc protein.

For the present study, all of the cell lines were maintained in Eagle's Minimal Essential Medium with Earle's salts (MEM) supplemented with nonessential amino acids, 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. When dexamethasone was used, it was added to the culture medium at 10^{-6} *M* (final concentration). Growth of all lines was at 37°C in a humidified atmosphere containing 5% CO_2/95% air. Cells were passaged by exposure to trypsin as required.

Fibronectin and anti-fibronectin. Human plasma fibronectin was obtained from Collaborative Research (Boston, MA). Analysis by SDS–PAGE under reducing conditions showed a single band or closely spaced doublet with an apparent molecular weight of 200 kDa. The fibronectin reacted with anti-fibronectin antibodies in enzyme-linked immunosorbant assay (ELISA) but did not react with anti-laminin or anti-thrombospondin antibodies. Rabbit polyclonal anti-fibronectin was purchased from Accurate Scientific and Chemical Co. (Westbury, NY). The anti-fibronectin reacted with fibronectin by ELISA (undilute to 10^6 dilution) but did not react with laminin or thrombospondin.

Biosynthetic labeling. Cells were grown in 60-mm (diameter) culture dishes to an approximate density of 1×10^6 cells per dish. The cells were washed and incubated for 30 min in methionine-free, serum-free minimum essential medium (GIBCO, Grand Island, NY) followed by a 4-h incubation in the same medium supplemented with 100 μCi per dish of [^{35}S]methionine (1000–1400 μCi/μmol: NEN, Boston, MA). The 4-h cells were lysed in a solution of phosphate-buffered saline (PBS) containing three detergents (1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS, all obtained from Sigma Chemical Co., St. Louis, MO) and protease inhibitors including 20 m*M* EDTA, 5 m*M* *N*-ethylmaleimide, 2 m*M* phenylmethylsulfonyl fluoride (PMSF), and 10 μl/10 ml of a protease inhibitor cocktail containing 1 mg/ml leupeptin, 2 mg/ml antipain, 10 mg/ml benzamidine, 10,000 kallikrein-inactivating units/ml aprotinin, 1 mg/ml chymostatin, and 1 mg/ml pepstatin, as described by Ronnett *et al.* [26] in studies on the insulin receptor. All of the protease inhibitors were obtained from Sigma Chemical Co. The cell lysates were frozen at −80°C, thawed, and clarified by ultracentrifugation (37,000*g* for 60 min). Immunoreactive fibronectin was precipitated with a 1:100 dilution of rabbit polyclonal anti-fibronectin antibody and protein A–Sepharose (Sigma Chemical Co.) according to the protocol of Ruddon *et al.* [27]. Normal rabbit serum served as a control. The washed immunoprecipitates were eluted with boiling (5 min) in twofold concentrated Laemmli SDS–PAGE sample buffer [19] with 2% 2-mercaptoethanol. The immunoprecipitated material was fractionated on a 5% polyacrylamide gel employing the Laemmli system [19]. Radioactive bands were visualized by fluorography with En^{3}Hance (NEN), exposing the dried gels to X-ray film (Kodak XAR-2) for 2 days.

ELISA. A solid-phase ELISA, with purified fibronectin as standard, was used to quantitate the amount of fibronectin secreted into the culture medium by the cells. Briefly, 2-h serum-free cell culture fluids were collected and clarified by low-speed centrifugation. The culture fluids (200 μl) were added to the wells of a 96-well plate (Falcon, Oxnard, CA) from lots which had been prescreened for acceptability in ELISA. Authentic fibronectin served as standard and culture medium alone served as a negative control. The plates were incubated for 4 h at 37°C. After the 4-h incubation, the culture medium from the cells and the control culture medium were removed and the ELISA were then run as described by Varani *et al.* [36]. The alkaline phosphatase-conjugated goat anti-rabbit IgG (Fab fraction) used in the ELISAs was obtained from Cappel (Malvern, PA) and the alkaline phosphatase substrate was obtained from Sigma.

Immunofluorescence. Cells were seeded onto sterile glass coverslips and cultured 1 day before processing for immunofluorescence. The procedure for fixing cells and immunostaining for fibronectin was exactly as described by Chakrabarty *et al.* [5]. Briefly, at the time of staining the cells were washed in PBS. The cells were then incubated in a 1:50 dilution of the primary antibody solution for 1 h at 37°C. Cells were washed three times for 10 min each in PBS and then incubated in a fluorescein-conjugated goat anti-rabbit immunoglobulin solution (1:50) (Cappel) for 1 h at 37°C. After treatment with the second antibody, the cells were again washed three times for 10 min each in PBS and mounted in 50% glycerol–50% PBS (pH 8.0). Localization of antigen was assessed and images recorded with a Zeiss fluorescence microscope equipped with a Nikon automatic camera and epifluorescence illumination. In all cases normal rabbit serum was used as a control and gave only minimal background fluorescence.

Adhesion assays. Two different assay procedures were used to assess adhesion. The first measured the ability of the cells to attach to fibronectin-coated plastic culture dishes. Wells of a 24-well plastic cell culture dish were treated with 0.5 ml of serum-free MEM containing either 25 or 2 μg of fibronectin and incubated for 2 h at 37°C. Following this, the wells were washed twice to remove unbound protein and treated for 30 min with 0.5 ml of serum-free MEM containing 200 μg/ml bovine serum albumin (BSA). After the second incubation, the wells were again washed. Freshly trypsinized cells were resuspended in MEM containing 200 μg/ml BSA, added to the wells, and incubated at 37°C for 1 h. At the end of the incubation period, nonattached cells were removed and counted. The cells remaining in the wells were fixed by the addition of 0.5 ml of 2% glutaraldehyde and the percentage of cells that had spread was assessed microscopically. By combining the two values the percentage of the originally added cells that were attached and spread at the end of the incubation period was determined.

Sensitivity of the cells to release from the substratum in the presence of 0.05% trypsin/0.02% EDTA was used as a second measure of adhesiveness. Cells were grown for 1 day in wells of a 24-well culture dish using MEM with 10% fetal bovine serum as culture medium. At the end of the incubation period, the culture medium was removed and the cells were washed once in serum-free MEM. They were then exposed to the trypsin/EDTA solution for 10 min. Cells which detached from the substratum during the 10-min incubation period were harvested and counted. Fresh trypsin/EDTA was added to the wells and left in contact until all of the cells had detached. Cell counts made from these wells were combined with the initial counts to determine the total number of cells per well and the percentage of cells which had detached during the first 10-min exposure to trypsin/EDTA was determined from this.

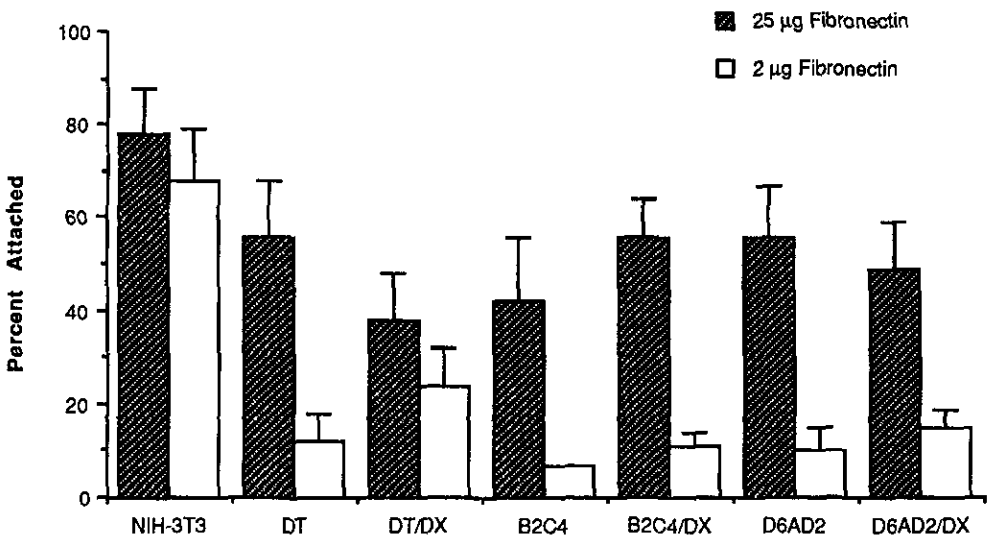

FIG. 1. Attachment and spreading of NIH-3T3 cells, DT cells, and c-myc antisense-expressing cells on plastic culture dishes coated with either 2 or 25 µg per well of human plasma fibronectin. The assay was carried out as described under Materials and Methods. Values shown represent average percentages of added cells (±standard deviations) that were attached and spread 1 h after the start of the assay in a single experiment. Statistical significance levels were determined using analysis of variance (ANOVA). At 2 µg of fibronectin, all of the transformed lines were statistically different from the parental NIH-3T3 cells at the $P < 0.01$ level. The experiment was repeated three times with similar results.

RESULTS

Cell-substrate adhesion. Two different aspects of cell–substrate adhesion were assessed with the NIH-3T3 cell lines. First, we measured the ability of the cells to attach and spread on fibronectin-coated plastic culture dishes. As seen in Fig. 1, parental NIH-3T3 cells and all of the transformants attached and spread rapidly on dishes coated with 25 µg of fibronectin per well. NIH-3T3 cells also attached and spread rapidly on wells coated with 2 µg of fibronectin. However, the DT cells and the fully transformed c-myc antisense cells attached and spread more slowly on dishes coated with the lesser amount of fibronectin. Treatment of these cells with dexamethasone failed to increase significantly the attachment rate on dishes coated with the low amount of fibronectin (Fig. 1). Although all of the data shown in this figure were obtained after incubation for 1 day in the presence or the absence of dexamethasone, other experiments were conducted in which attachment to fibronectin was assessed at different time points. At times ranging from 4 to 48 h, data similar to those shown here were obtained.

As a second approach to assessing changes in adhesion, we measured the ability of the cells to detach from the substratum in the presence of trypsin/EDTA. As shown in Fig. 2, NIH-3T3 cells were much more resistant to removal from the substratum with trypsin/EDTA than were the DT cells. In the absence of dexamethasone, the c-myc antisense clones behaved like DT cells in this regard. However, when these cells were treated for 1 day with dexamethasone, their resistance to trypsin/EDTA-mediated release from the substratum increased significantly and they became virtually as adhesive as NIH-3T3 cells. In contrast, treatment of DT cells with dexamethasone had only a minimal effect on sensitivity to trypsin/EDTA-mediated release from the substratum (Fig. 2). The data shown in this figure were obtained after incubation for 1 day in the presence or the absence of dexamethasone. Shorter exposure (4 h) of the B2C4 and D6AD2 cells to dexamethasone failed to induce a strong increase in adhesion while the adhesion response after a longer exposure (48 h) was similar to that seen at 24 h.

Fibronectin production, secretion, and cell surface deposition. Fibronectin biosynthesis and secretion were measured in the parental NIH-3T3 cells, in DT cells, and in two c-myc antisense DT clones. As seen in Fig. 3, NIH-3T3 cells synthesized a much greater amount of immunoreactive fibronectin than did DT cells. It can be seen in Fig. 3 that the two c-myc antisense clones (B2C4 and D6AD2) also synthesized lesser amounts of fibronectin than NIH-3T3 cells, consistent with their highly transformed phenotype. Further, exposure of these two clones to dexamethasone for 1 day did not induce an appreciable increase in fibronectin biosynthesis (Fig. 3).

An ELISA was used to quantitate the secretion of fibronectin into the culture fluid. Parental NIH-3T3 cells secreted significantly more fibronectin into the culture medium than did DT cells or the c-myc antisense transfectants grown in the absence of dexametha-

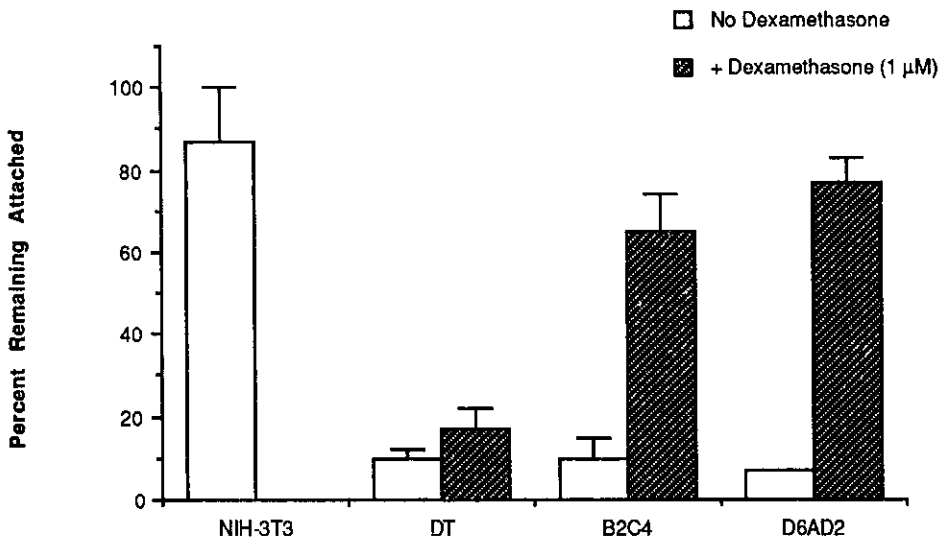

FIG. 2. Sensitivity of cells to trypsin/EDTA-mediated release from the substratum. NIH-3T3 cells, DT cells, and c-myc antisense-expressing cells were examined for sensitivity to trypsin/EDTA-mediated release from the substratum as described under Materials and Methods. Values shown represent average percentages of cells (±standard deviations) remaining attached to the substrate after exposure to trypsin/EDTA for a 10-min period in a single experiment. Statistical significance levels were determined using a series of *t* tests. In each case, the percentage of cells remaining attached in the presence of dexamethasone was compared to the percentage of cells remaining attached in the absence of dexamethasone. With both B2C4 and D6AD2 cells, values obtained in the presence of dexamethasone were significantly different from control values at the $P < 0.01$ level. The experiment was repeated three times with similar results.

sone (Table 1). Treatment of both antisense clones with dexamethasone did not result in increased secretion. If anything, glucocorticoid treatment was associated with decreased secretion of fibronectin.

The surprisingly low level of fibronectin production and secretion by the glucocorticoid-treated antisense clones in the face of morphological reversion prompted us to examine for cell surface fibronectin by immunofluorescence. As anticipated, there was intense staining of viable NIH-3T3 cells for fibronectin but essentially no staining of their ras-transformed counterparts (Fig. 4). There was also minimal staining of the two antisense clones in the absence of dexamethasone. However, there was an increase in surface staining after exposure for 1 day to dexamethasone. D6AD2 cells are shown in Fig. 4. B2C4 cells demonstrated a similar pattern in the presence of dexamethasone (not shown). It should be noted that treatment of the parental NIH-3T3 cells or control DT cells with dexamethasone had no significant effect on surface fibronectin in these cells.

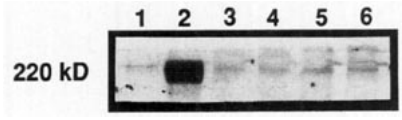

FIG. 3. Fibronectin synthesis in NIH-3T3 cells, ras-transformed NIH-3T3 cells, and c-myc antisense-expressing cells. Fibronectin synthesis was measured by biosynthetic labeling/immunoprecipitation as described under Materials and Methods. Lane 1, DT cells; lane 2, NIH-3T3 cells; lanes 3 and 4, B2C4 clone of c-myc antisense-expressing cells under control conditions and in the presence of 10^{-6} *M* dexamethasone, respectively. Lanes 5 and 6, D6AD2 clone of c-myc antisense-expressing cells under control conditions and in the presence of 10^{-6} *M* dexamethasone, respectively.

DISCUSSION

A number of prior studies have demonstrated that transformation of fibroblasts by ras oncogenes gives

TABLE 1

Fibronectin Secretion into the Culture Fluid by NIH-3T3, DT, B2C4, and D6AD2 Cells

Cell line	Fibronectin (ng/100 μl)
NIH-3T3	150 ± 35
DT	
No dexamethasone	10 ± 2
10^{-6} *M* dexamethasone	7 ± 5
B2C4	
No dexamethasone	27 ± 5
10^{-6} *M* dexamethasone	5 ± 1*
D6AD2	
No dexamethasone	7 ± 1
10^{-6} *M* dexamethasone	<5

Note. Immunoreactive fibronectin secreted into serum-free culture medium during a 2-h period (1 day after treatment) was assessed by ELISA as indicated under Materials and Methods. Values shown represent means and standard deviations based on quadruplicate samples in a single experiment.

* Statistical difference from control value at the $p < 0.01$ level by *t* test. The experiment was repeated three times with similar results.

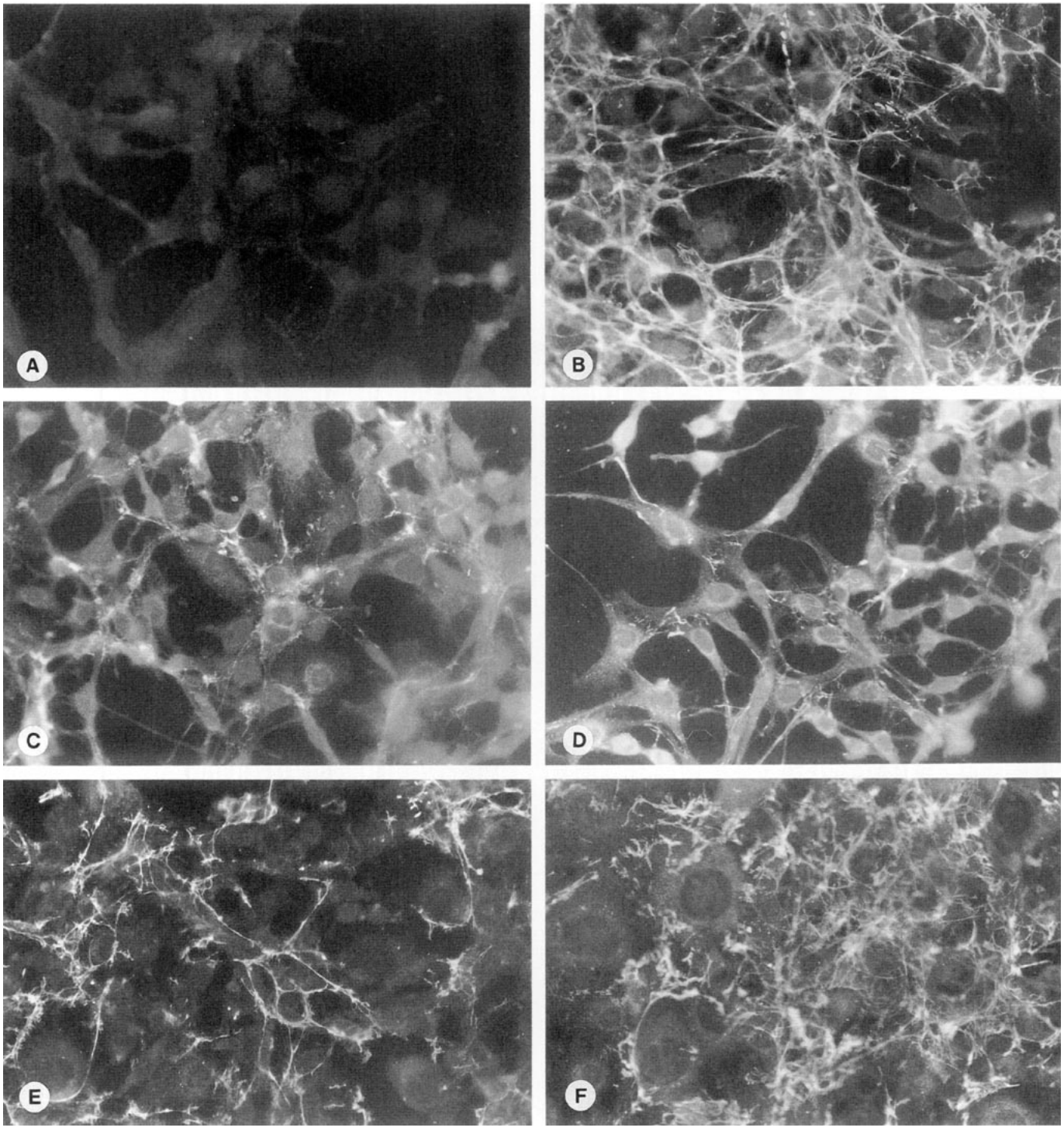

FIG. 4. Indirect immunofluorescence staining of viable NIH-3T3, DT, and D6AD2 cells for surface fibronectin. Staining was done as described under Materials and Methods. (A) NIH-3T3 cells stained with normal rabbit serum as the primary antibody. (B) NIH-3T3 cells stained with anti-fibronectin. (C) DT cells stained with anti-fibronectin under control conditions. (D) DT cells stained with anti-fibronectin 1 day after treatment with dexamethasone. (E) D6AD2 cells stained with anti-fibronectin under control conditions. (F) D6AD2 cells stained with anti-fibronectin 1 day after treatment with dexamethasone.

rise to cells which are both tumorigenic in appropriate hosts and often invasive [3, 21, 33, 37]. Thus, these cells express the full malignant phenotype and are different in this regard from some transformed cells, which have lost growth control without gaining capacity for metastasis [31]. What additional phenotypic characteristics are required for a cell which is tumorigenic to metastasize is not fully understood. Highly malignant cells tend to be more motile than their nonmetastatic counterparts and usually have the capacity to degrade extracellular matrix more efficiently than nonmetastatic cells. Transformation by ras oncogenes has been shown to confer both characteristics [3, 21, 33, 37]. Another characteristic associated with the transformed phenotype is a loss of adhesiveness. This is seen as a reduction in both cell–cell adhesion and cell–matrix adhesion in highly malignant cells as compared to their normal counterparts. The loss of cell–cell adhesion can be explained, at least in part, by altered expression of molecules such as E-cadherin [11].

In the present study we assessed two different parameters of cell–substrate adhesion in a series of NIH-3T3 cell lines. One of these was ability to attach to the surface of plastic culture dishes coated with a low concentration of fibronectin. Consistent with previous findings from several laboratories including our own [5, 7, 9, 22, 42], all of the transformed lines were deficient in their ability to attach to the fibronectin substrate relative to the parental control cells. Interestingly, expression of antisense c-myc transcripts in the B2C4 and D6AD2 cells did not induce a reversion in this characteristic. Thus, while the ability of cells to attach to low concentrations of fibronectin is a characteristic of the normal phenotype, it does not appear to be a prerequisite.

Release of cells from the substratum in the presence of trypsin/EDTA was assessed as a second parameter of adhesion. All of the cell lines expressing the transformed phenotype were significantly more sensitive to release from the substratum in the presence of trypsin/EDTA than were the parental NIH-3T3 cells. Further, sensitivity to trypsin/EDTA was downregulated in the antisense c-myc-expressing clones. Thus, unlike capacity to attach to low concentrations of fibronectin, resistance to trypsin/EDTA-mediated release from the substratum correlates strongly with expression of the normal phenotype in these cells. Conversely, high sensitivity to trypsin/EDTA correlates with expression of c-myc in the fully transformed cells.

The molecular basis for the changes in adherence properties noted here is not fully understood. Previous studies from our laboratory have shown that resistance to removal from the substratum with enzymes and/or cation chelators is associated with a high level of fibronectin production and cell surface expression [5, 7, 40]. Consistent with this, immunofluorescence staining of the transformed NIH-3T3 cells demonstrated a lack of fibrillar fibronectin on the surface of the cells while intense staining of the parental cells was seen under the same conditions. Upon transfection with antisense c-myc and glucocorticoid induction of expression, a fibrillar pattern of surface fibronectin was restored to the surface of the B2C4 and D6AD2 cells. We speculate that deposition of fibronectin (as well as other matrix components) by cells allows the cells to attach firmly to the substratum and to take on the flattened morphology that is characteristic of the normal phenotype. This thesis is difficult to prove, however, because once the cells have attached and spread, use of antibodies to individual matrix components is generally ineffective in dislodging the cells. We attempted this in the present study but without effect (unpublished observation). The failure to dislodge attached cells with specific antibodies may reflect an inability of the antibody to reach critical target sites. Alternatively, cells utilize multiple adhesion moieties in parallel and blocking interactions with individual ones may be doomed to failure.

What accounts for the differences in surface fibronectin between the parental NIH-3T3 cells, the ras-transformed NIH-3T3 cells and the antisense c-myc revertants is not known. Past studies have suggested that expression of fibronectin-binding integrins is critical. Plantefaber and Hynes [22] showed that expression of $\alpha 5\beta 1$ correlated most strongly with capacity of rodent fibroblastic cells to attach rapidly and spread on culture dishes coated with a limited amount of fibronectin. In a variety of transformed cell lines, this moiety was missing or present in reduced amounts relative to amounts present on their untransformed counterparts. In contrast, other $\beta 1$ integrins (e.g., $\alpha 3\beta 1$) were present in equal amounts on both normal and transformed cells. Consistent with these past results, the parental NIH-3T3 cells used in the present study rapidly attached to dishes coated with either a high or low concentration of fibronectin, while the transformed lines attached much less well in the presence of the low fibronectin concentration. Interestingly, however, the induction of c-myc expression with glucocorticoids did not significantly enhance the ability of either the B2C4 or D6AD2 cells to attach to the dishes coated with the low fibronectin concentration. This might imply that c-myc expression is not directly responsible for regulation of fibronectin-binding integrins. This conclusion will need to remain tentative until an analysis of surface integrin expression on these cell lines can be carried out. It should be pointed out in this regard that fibronectin is capable of binding to a number of moieties (for example, to collagen, proteoglycans, and certain glycolipids) in addition to integrin receptors [8, 10, 12, 16]. Changes in expression of one or more of these surface components could account for the alterations in surface fibronectin noted here.

In summary, these studies demonstrate that rever-

sion of the transformed phenotype in c-myc antisense-expressing, v-Ki-ras-transformed cells is accompanied by enhanced cell–substrate adhesion and that this occurs in conjunction with appearance of fibronectin on the cell surface. Elucidation of the mechanism by which this occurs may help us to understand at the biochemical and molecular levels how the processes of malignant transformation and reversion to the normal phenotype are controlled.

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