Role of E-cadherin in the response of tumor cell aggregates to lymphatic, venous and arterial flow: measurement of cell-cell adhesion strength

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SUMMARY

Defects in the expression or function of the calcium dependent cell-cell adhesion molecule E-cadherin are common in invasive, metastatic carcinomas. In the present study the response of aggregates of breast epithelial cells and breast and colon carcinoma cells to forces imposed by laminar flow in a parallel plate flow channel was examined. Although E-cadherin negative tumor cells formed cell aggregates in the presence of calcium, these were significantly more likely than E-cadherin positive cell aggregates to disaggregate in response to low shear forces, such as those found in a lymphatic vessel or venule (<3.5 dyn/cm^2). E-cadherin positive normal breast epithelial cells and E-cadherin positive breast tumor cell aggregates could not be disaggregated when exposed to shear forces in excess of those found in arteries (>100 dyn/cm^2). E-cadherin negative cancer cells which had been transfected with E-cadherin exhibited large increases in adhesion strength only if the expressed protein was appropriately linked to the cytoskeleton. These results show that E-cadherin negative tumor cells, or cells in which the adhesion molecule is present but is inefficiently linked to the cytoskeleton, are far more likely than E-cadherin positive cells to detach from a tumor mass in response to low shear forces, such as those found in a lymphatic vessel or venule. Since a primary route of dissemination of many carcinoma cells is to the local lymph nodes these results point to a novel mechanism whereby defects in cell-cell adhesion could lead to carcinoma cell dissemination

Key words: cadherin, tumor, adhesion

INTRODUCTION

Alterations in cell-cell and cell-extracellular matrix adhesion properties are consistently associated with the progression of carcinoma from a non-invasive to an invasive, metastatic phenotype (Liotta and Stetler Stevenson, 1991; Liotta, 1992; Takeichi, 1991; Albelda and Buck, 1990; Hynes, 1992). Several families of adhesion molecules have been implicated in these changes including the cadherins and integrins (Schipper et al., 1991; Frixen et al., 1991; Hynes, 1992). The expression of the calcium-dependent cell-cell adhesion molecule E-cadherin is reduced or completely lost in some invasive carcinomas and carcinoma cell lines (Schipper et al., 1991; Shimoyama et al., 1989; Shiozaki et al., 1991). Although these cells are likely to be capable of forming aggregates using alternative adhesion pathways, the loss of E-cadherin expression and/or function is generally thought to aid the local invasion process (Frixen et al., 1991; Behrens et al., 1989). Nevertheless, in other studies we found that most E-cadherin negative breast cancer cell lines were not more invasive than E-cadherin positive cells (Sommers et al., 1991). Instead, the highly invasive phenotype was invariably associated with expression of the mesenchymal intermediate filament protein

vimentin. Disruption of E-cadherin function in E-cadherin positive breast cancer cells resulted in the loss of cell-cell contact but did not result in the cells becoming more invasive (Sommers et al., 1991). Similarly, transfection of E-cadherin into invasive vimentin positive cells did not reverse the invasive phenotype even though it allowed the transfected cells to aggregate specifically with E-cadherin transfected fibroblasts (Sommers et al., 1994). Clearly, in this system loss of functional E-cadherin expression does not necessarily lead to an invasive phenotype. Although it is possible, in certain circumstances, that complete cell-cell detachment might be a required step in local invasion, in many developmental and clinical examples and in other instances of tissue remodeling, invasion of a surrounding tissue or matrix is not necessarily accompanied by complete loss of cell-cell contact. Rather, cohorts of cells migrate as cohesive sheets or as linked cells in single file (the so called 'Native American file'). This is certainly the case in malignant neoplasia of the breast (Pierson and Wilkinson, 1990). Why then should so many breast cancer cells and tumors have lost E-cadherin mediated adhesion if it may not be absolutely required for local invasion? Another important step in carcinoma progression is the movement of tumor cells or emboli to local lymph nodes or distant sites via

the lymphatic or venous circulation. In some situations it is possible that, in order to enter the fluid in a lymph vessel or vein, tumor cells must be detached from the primary tumor mass either as individuals or as aggregates by the laminar flow imposed by the circulatory system. In this case, the physical strength of homotypic cell adhesion may be a significant determinant in the ability of a tumor cell to enter the circulation.

In the present study we test the hypothesis that defects in E-cadherin-mediated adhesion result in a reduction in cell-cell adhesion strength which in turn leads to an increased likelihood of cells detaching from a tumor mass when exposed to lymphatic, venous or arterial flow. In order to exert tensile and shear forces on cell-cell contact sites, laminar flow was imposed on cell aggregates which were adherent to a planar substratum. The time course of the deformation and disaggregation response of the aggregates was recorded at a wide range of flow rates. The cells used in these assays included E-cadherin positive epithelial cells, cells with known defects in E-cadherin expression or function as well as cell lines transfected with E-cadherin. The assays showed that defects in the expression or function of E-cadherin or associated molecules significantly reduces the physical strength of homotypic cell-cell adhesion. We measure for the first time the strength of E-cadherin mediated adhesion and, importantly, show that the shear stresses required to disaggregate E-cadherin negative cells correspond closely to those found in a lymphatic vessel or capillary.

MATERIALS AND METHODS

Cells

The cell lines used in the experiments were E-cadherin positive normal human breast epithelial cells (MCF-10A) (Soule et al., 1990), E-cadherin positive weakly invasive human breast carcinoma cells (MCF-7), E-cadherin negative poorly invasive human breast cancer cell line SKBR3, E-cadherin negative highly invasive human breast carcinoma cells (HS578T, BT549; Sommers et al., 1991), E-cadherin and control transfected HS578T, BT549 cells (HS-Ecad, BT-Ecad; Sommers et al., 1994), E-cadherin transfected mouse L-cells (L-Ecad; Sommers et al., 1994), E-cadherin positive, α-catenin negative human colon cancer cell clone A (Breen et al., 1993), E-cadherin negative human colon cancer cell RKO, and E-cadherin and control transfected RKO cells (RKO-Ecad, Breen et al., 1995). Following 3 days in culture confluent cultures of cells were trypsinized with 0.025% trypsin in the presence of 5 mM Ca^{2+}. The resulting suspension of single cells and small aggregates was washed and resuspended in 5 ml of DMEM containing 5% FBS at a concentration of 2×10^6 cells/ml and maintained at 37°C in a humidified CO_2 incubator for 2-4 hours to regenerate cell surface proteins. All experiments were performed at 32°C within 4 hours of trypsinization.

Flow chamber

A parallel flow chamber of uniform width was used in the laminar flow assays (Chien and Sung, 1987). The chamber consists of (a) an upper plate having appropriate openings for the delivery of the fluid into and out of the channel, (b) a gasket with an opening in the form of a channel, (c) a transparent bottom plate (grade no. 1 coverslip) and (d) top and bottom stainless steel cover plates with observation slots. The bottom plate, the gasket, and the base plate are fastened between the cover plates. The entry port of the chamber is connected through a valve and teflon tubing to two syringes, one filled with cell suspension and the other filled with suspending medium. Before use

in the flow chamber glass coverslips were coated with laminin (10 μg/cm^2) or collagen type I (10 μg/cm^2) as described earlier (Tozeren et al., 1994).

A syringe pump (Harvard Apparatus) was used to pump medium into the chamber at specified flow rates. The shear stress on the bottom plate of the chamber along the direction of flow, τ (dyn/cm^2), was evaluated using the following equation, assuming Poiseuille flow:

$$\tau = 6\mu Q/h^2 w, \qquad (1)$$

where μ (0.01 dyn-s/cm^2) is the viscosity of the medium, Q (cm^3/s) is the flow rate, h is the gap thickness of the channel (0.012 cm) and w (1 cm) is the width of the chamber (Chien and Sung, 1987).

Laminar flow assays

Laminar flow assays were inititated by placing the flow chamber on the stage of an inverted microscope (Diaphot, Nikon Inc., Garden City, NJ) equipped with 10× and 40× Hoffman and brightfield objective lenses. The cell suspension was gently infused into the flow channel and cell aggregates allowed to interact with the matrix protein-coated glass coverslip for 20 minutes under static conditions. Flow was then initiated at $\tau = 1.75$ dyn/cm^2 and the flow rate increased at 30 second or one minute intervals up to a maximum value of $\tau = 100$ dyn/cm^2.

A video camera (DAGE-MTI) was attached to the side port of the microscope to record the deformation/disaggregation response of cell aggregates to imposed laminar flow. The times were displayed on the video monitor with a data mixer (Vista Electronics, La Mesa, CA) and the length and width of the cell aggregates before and during flow were determined using a position analyzer mixer (Vista Electronics, La Mesa, CA) that provided a digital readout proportional to the distance between two sets of vertical and horizontal lines.

Flow-induced disaggregation of both small (2-6) cells and large aggregates were recorded. Large aggregates were defined as those whose largest dimension before the imposition of flow was 70-140 μm. Large carcinoma cell aggregates typically contained multiple layers of cells with many cells adherent to neighboring cells but not to the planar substratum.

A detachment event was said to occur when a single cell or a small cell aggregate detached from the parent aggregate in response to the imposed flow. In each experiment with a large aggregate, the number of detachment events during a one minute interval of infusion at a constant level of shear stress was determined. The total number of disaggregation events performed with each cell type varied between 8 and 14. The mean and standard deviation of the number of detachment events were computed as a function of the fluid shear stress imposed on the laminin or collagen-coated glass coverslip. The mean value was denoted as the frequency of disaggregation.

RESULTS

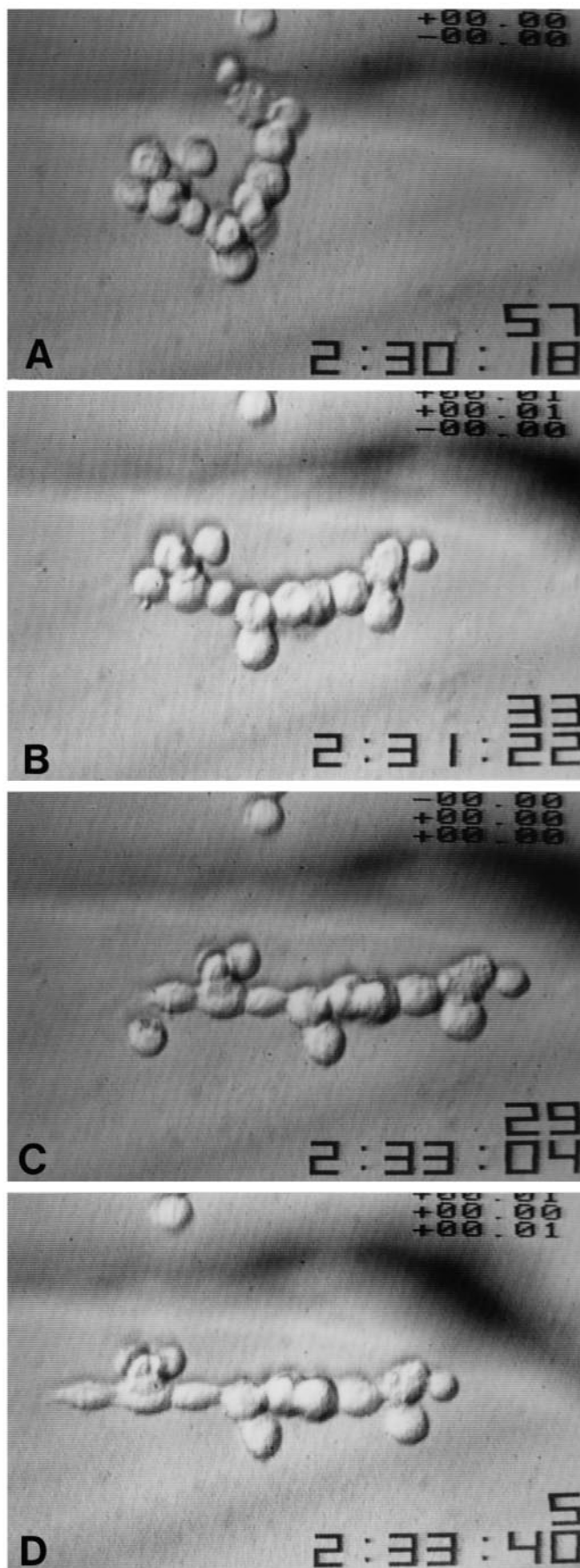### Flow-induced disaggregation of large cell aggregates

Laminar flow was imposed on large aggregates which were incubated on the bottom plate of the flow channel for 20 minutes under static conditions. The coverslip was coated with either laminin or collagen depending on the cell type. Pilot experiments showed that MCF-10A and clone A cells attached more firmly to laminin and that MCF-7, HS578T and BT549 cells attached better to collagen type 1. Following the attachment period, a few cells at the bottom of the aggregates formed adhesive contacts that were strong enough to resist detachment by flow. However, most of the cells in the aggregates were not in contact with the substratum and it is these cells that could
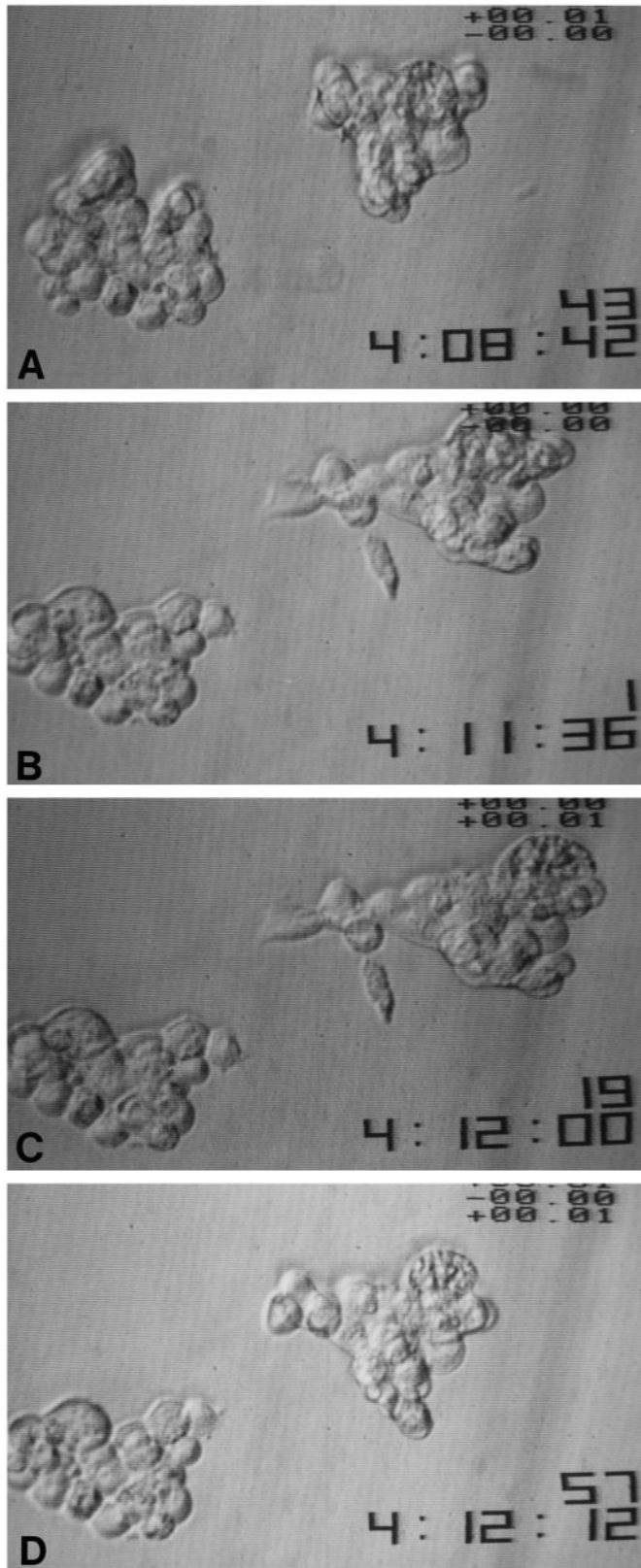
be detached (or could not be detached) from one another by flow. In this system, laminar flow will impose force on cell-cell contact sites only if some cells in the aggregate are anchored to the substratum. In this case it is clear that strength of cell-substratum adhesion of those cells which are in contact with the substratum must be strong enough to allow disaggregation without the aggregate detaching from the substrate as a whole. The videomicrographs (particularly Figs 1, 2 and 7) clearly show that even the small aggregates reoriented rapidly in response to the imposition of flow showing that cell-substratum attachment is not involved in the stretching response of the cells to shear forces. Generally, aggregates were anchored to the substrate through one or two cells, which remained attached to the substratum even after the rest of the aggregate completely disaggregated in response to flow. Cells that did become detached from one another were instantly swept away by the flow without any interaction with the substratum, indicating that cell-matrix adhesion did not contribute significantly to the observed phenomena.

MCF-7 and MCF10A cells which were pre-treated for several hours with low calcium medium (50 µM) or with antibodies to E-cadherin prior to laminar flow assays did not form aggregates of measurable adhesive strength (not shown). In other studies using some of the same cells we demonstrate that for those cells with functional E-cadherin-mediated adhesion the ability to form aggregates is lost when cells are exposed to low calcium medium or antibodies to E-cadherin (Sommers et al., 1991, 1994). The E-cadherin positive cells used in these experiments do not form strong aggregates under these conditions, consequently cell-cell adhesion strength is very low and cell aggregates disaggregate as they are being infused into the flow chamber. Preformed aggregates exposed to low calcium medium in the flow chamber exhibited much weaker cell-substratum adhesion and many of the aggregates detached from the substratum as a whole at low shear forces. In two other cell types (L-cells and RKO cells) transfection of E-cadherin restored strong cell cell adhesion whereas neo transfectants behaved as controls. These E-cadherin transfected cells also do not form aggregates in low calcium medium or in the presence of antibodies to E-cadherin (see also results in Sommers et al., 1994: Breen et al, 1995).

The fluid shear stress applied to the coverslip ranged from 2.5 dyn/cm^2 to 100 dyn/cm^2. At low flow rates, aggregates of E-cadherin positive MCF-10A or MCF-7 cells rapidly aligned in the direction of flow in order to reduce fluid drag (Fig. 1). At high flow rates, these aggregates deformed extensively, physically straining cell-cell contact sites (Figs 1, 2). However, cells or small aggregates could not be detached from the aggregates of MCF-10A or MCF-7 cells despite the imposition of high flow rates ($\tau = 100$ dyn/cm^2; Table 1). In a few instances aggregates detached as a whole from the coverslip at high flow rates.

Fig. 1. Sequence of video-micrographs showing the typical deformation response of MCF-10A cells to imposed laminar flow. The numbers at the bottom of the screen represent, hour, minute, second, and tens of milliseconds. Flow was initiated at 2:30:00 at τ – 1.75 dyn/cm^2 and was incrementally increased every 30 seconds such that the shear stress τ took the values 1.75 (A), 3.5, 7.0 (B), 10.5, 14, 21, 35 (C) and 50 dyn/cm^2 (D). The figure shows that the string of MCF-10A cells orient in the direction of flow and deform extensively but do not detach from each other.

Fig. 2. Sequence of video-micrographs showing the typical deformation response of large aggregates of MCF-7 cells to imposed laminar flow. Flow was initiated at 4:08:00 at $\tau = 1.75$ dyn/cm^2 and was increased every 30 seconds such that the shear stress τ took the values 1.75, 3.5 (A), 7.0, 10.5, 14, 21, 35 dyn/cm^2 (B,C). The flow ceased at 4:12:02 and the aggregate returned to its original configuration in 10 seconds (D).

Table 1. Relationship between E-cadherin protein expression and E-cadherin function

Cell	E-cadherin	E-cad function	τ for disagg.	Invasiveness
MCF-10A	+	+	>100 dyn/cm^2	−
MCF-7	+	+	>100 dyn/cm^2	+
T47D	+	+	>100 dyn/cm^2	+
SKBR3	−	−	<7 dyn/cm^2	+
HS578T	−	−	<7 dyn/cm^2	+++
BT549	−	−	<7 dyn/cm^2	+++
RKO	−	−	<7 dyn/cm^2	+++
L949	−	−	<7 dyn/cm^2	++
HS578T-Ecad	+	−	<7 dyn/cm^2	+++
BT549-Ecad	+	−	<7 dyn/cm^2	+++
L949-Ecad	+	+	7-100 dyn/cm^2	+
RKO-Ecad	+	+	>100 dyn/cm^2	+
Clone A	+	−	>7 dyn/cm^2	++

The relationship was assessed by the presence or absence of Triton-insoluble E-cadherin at points of cell-cell contact and/or the ability of transfected E-cadherin to mediate a morphological change (Sommers et al., 1991, 1994; Breen et al., 1993, 1995), shear stress forces for disaggregation, and invasiveness. The invasive characteristics of these cells have been described previously (Sommers et al., 1991, 1994; Breen et al., 1993, 1995).

detached from the parent aggregate at low levels of fluid shear stress ($\tau = 2.5$ dyn/cm^2).

The flow-induced detachment of cells and cell aggregates from the parent aggregate is a stochastic process that not only depends on the applied fluid shear stress, the geometry of the cell aggregate and its orientation with respect to flow, but also on the number density and the physical strength of the bonds which act to keep the cells together. For these reasons a large number of disaggregation experiments were performed on cell aggregates of comparable size (70-140 μm) for each cell type. The frequency of detachment events from parent aggregates as a function of applied fluid shear stress (τ) for HS-578T and BT-549 cells is presented in Fig. 4. Both these cell types began to disaggregate at fluid shear stress levels found in lymphatics and in the circulation (2.5-15 dyn/cm^2). The frequency of detachment events decreased with increasing shear stress because the number of cells available for detachment was reduced during the course of the experiments. Thus, although the E-cadherin negative highly invasive breast carcinoma cells used in the present study form large aggregates in the presence of calcium the shear forces required to disaggregate these cell aggregates are quite low.

In similar experiments we found that all cells which were E-cadherin negative exhibited a similar detachment response to flow (Table 1). However, the inability of cell aggregates to remain intact in laminar flow was not restricted to aggregates of E-cadherin negative carcinoma cells. Table 1 shows that the E-cadherin positive colon carcinoma cell line clone A also disaggregated in response to low shear stresses. This cell is known not to express the E-cadherin-associated molecule α-catenin, a
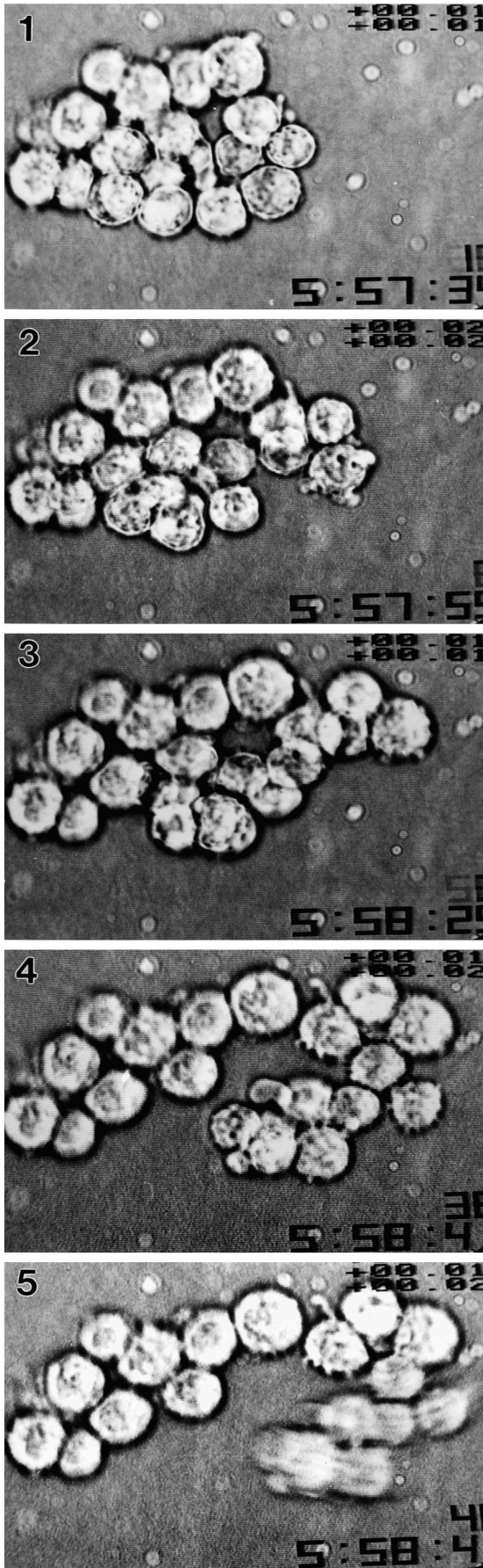
In contrast, cells from large aggregates of E-cadherin negative HS578T and BT549 breast carcinoma cells disaggregated in response to the imposed laminar flow at low to moderate flow rates (2.5 dyn/cm$^2 \leq \tau \leq 15$ dyn/cm^2). Fig. 3 shows that individual cells and small BT549 cell aggregates

Fig. 3. Sequence of video-micrographs showing the flow-induced disaggregation of a large aggregate of E-cadherin-negative BT-549 cells. Flow was initiated at 5:57:00 at $\tau = 1.75$ dyn/cm^2 and was increased every 60 seconds such that the shear stress τ took the values 2.5 dyn/cm^2 (1, 2), and 5.0 dyn/cm^2 (3, 4, 5).

defect that is likely responsible for the failure of aggregates of these cells to resist low shear stresses (Breen et al., 1993).

E-cadherin transfection prevents flow induced-disaggregation only when it is restricted to cell-cell contact sites

Transfection of BT-549 and HS-578T cells with E-cadherin cDNA did not change their flow-induced aggregation properties (Fig. 4) even though we had demonstrated previously that it could mediate specific aggregation with E-cadherin transfected fibroblasts (Sommers et al., 1994). Expression levels of E-cadherin protein in the transfected cells was similar to those of MCF-7 cells as judged by immunocytochemistry, western analysis and immunoprecipitation (Sommers et al., 1994). In contrast, aggregates of E-cadherin transfected RKO cells remained intact when exposed to high shear stresses (Fig. 5). Similarly, E-cadherin transfected L-cells acquired calcium-dependent cell-cell adhesion properties and their frequency of detachment was much lower at all shear stress levels than E-cadherin negative breast tumor cells (Fig. 5). As shown on several occasions by others, untransfected L-cells did not form aggregates in the presence or absence of calcium (Sommers et al., 1991, 1992, 1994). Similarly, E-cadherin transfected RKO cells do not form aggregates in low calcium medium or in the presence of E-cadherin antibodies (Breen et al., 1995). These results indicate that whereas E-cadherin expression is required for cell aggregates to resist high shear stress forces other factors also contribute to the ability of E-cadherin to mediate strong cell-cell adhesion. It is well known that E-cadherin is linked to the cell cytoskeleton through other molecules, β-catenin, α-catenin, γ-catenin and/or plakoglobin (see for review, Kemler, 1993). Alterations in the expression or phosphorylation state of these E-cadherin-associated molecules have previously been demonstrated to modulate E-cadherin mediated adhesion (Shimoyama et al., 1992; Hirano et al., 1992; Matsuyoshi et al., 1992). The two E-cadherin transfected invasive breast cancer cell lines used in the present study have elevated levels of tyrosine phosphorylated β-catenin and reduced plakoglobin levels (Sommers et al., 1994). In these cells the transfected E-cadherin is not restricted to cell-cell contact sites and is largely Triton soluble. In contrast, exogenous E-cadherin expressed in MCF-7 cells and in L-cells becomes restricted to cell-cell contact sites and is Triton insoluble in these areas (Sommers et al., 1994). Similarly, Triton insoluble E-cadherin is expressed at cell-cell contact sites in the E-cadherin transfected colon carcinoma cell line RKO (Fig. 6). Therefore the ability of cell aggregates to remain intact in laminar flow not only depends upon E-cadherin expression but also on the presence of a Triton-insoluble form of E-cadherin at cell-cell contact sites.

The role of E-cadherin expression in the physical strength of cell-cell contact sites

The capacity of cell-cell contact sites to resist external tensile forces was investigated by determining the deformation response of string-shaped aggregates to imposed laminar flow. Laminar flow imposed on MCF-10A chains led to extensive
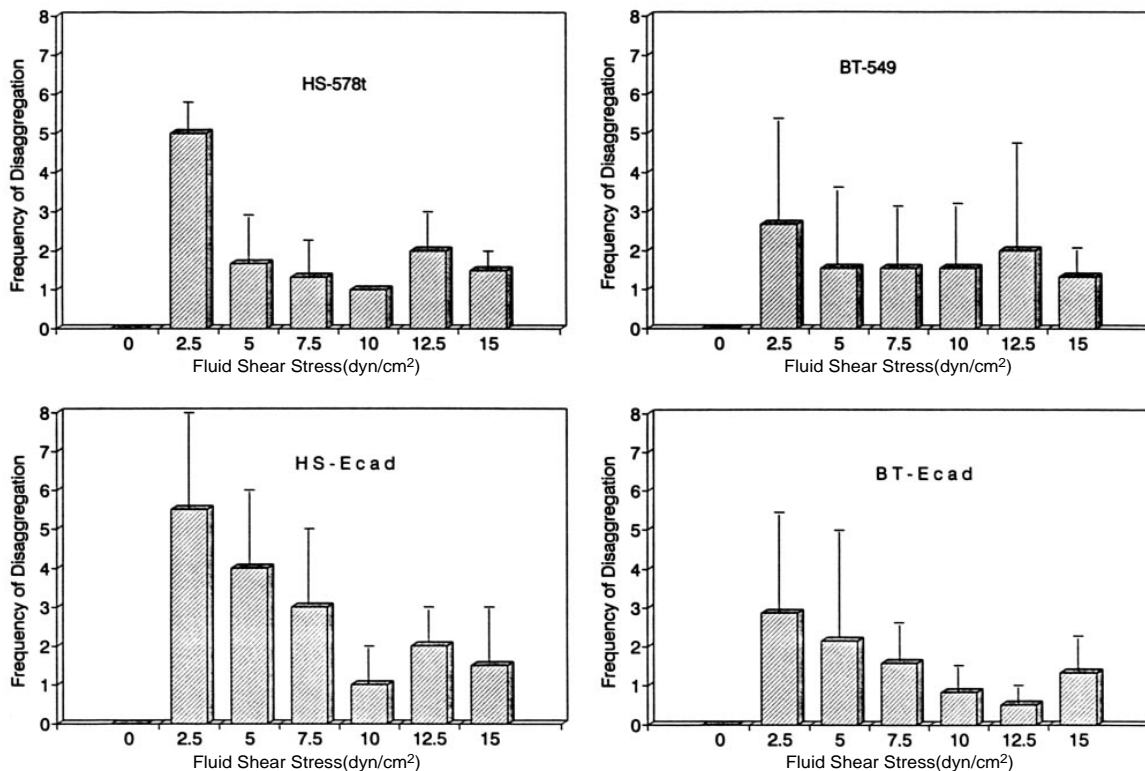
Fig. 4. Frequency of disaggregation of BT-549, BT-Ecad, HS-578T and HS-Ecad cell aggregates in response to applied fluid shear stress. The bars and vertical lines indicate the mean values and the standard deviation of the number of detachment events observed during 60 seconds of flow at a specified shear stress. The total number of experiments was 12. The disaggregation response of aggregates of control (BT-hyg and HS-hyg) cells was similar to that of untransfected cells (not shown).

cell elongation in the direction of flow (Figs 1, 7). As shown in the free body diagram (see Fig. 10) these cells were approximately under uniaxial tension loading. The cell aggregates remained attached to the substratum through a single cell at a few focal contacts and the adhesion contacts between cells could not be broken at levels of fluid shear stress greater than those found in arteries ($\tau = 100$ dyn/cm^2). Flow-induced cell elongation became more pronounced with increasing shear stress and with relative position within the string of cells.

A measure for the extent of cell deformation in the direction of flow is the ratio of instantaneous cell length in the direction of flow (L) to the corresponding length before the imposition of flow (L_0). The deformation index (L/L_0) for three individual cells in different MCF-10A strings was plotted in Fig. 8 as a function of the tensile force exerted on each cell (F_{jm}, see the free body diagram in Appendix). This tensile force was estimated by using the known mathematical solutions of flow past strings of spheres or spheroids (Gluckman et al., 1971) as described in Appendix 1. Fig. 8 shows that MCF-10A cells elongated in the direction of flow as much as 60% under the
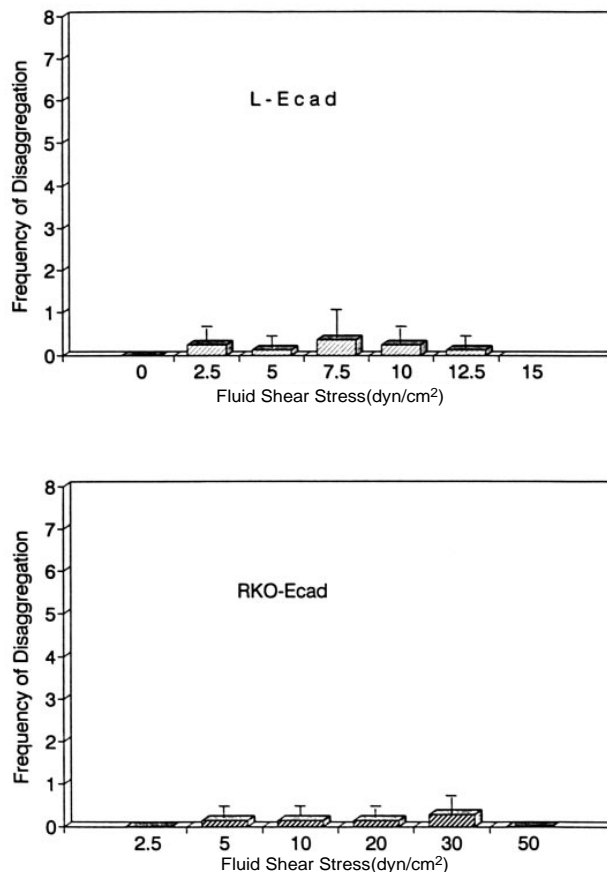
Fig. 5. Frequency of disaggregation of RKO-Ecad and L-Ecad cell aggregates in response to applied fluid shear stress. The bars and vertical lines indicate the mean values and the standard deviation of the number of detachment events observed during 60 seconds of flow at a specified shear stress. The total number of experiments in each case was seven. Aggregates of non-transfected and control RKO transfectants (RKO-neo) disaggregated as they were infused into the flow channel. Untransfected and control (L-neo) L-cells did not form aggregates under the conditions used in the experiments.
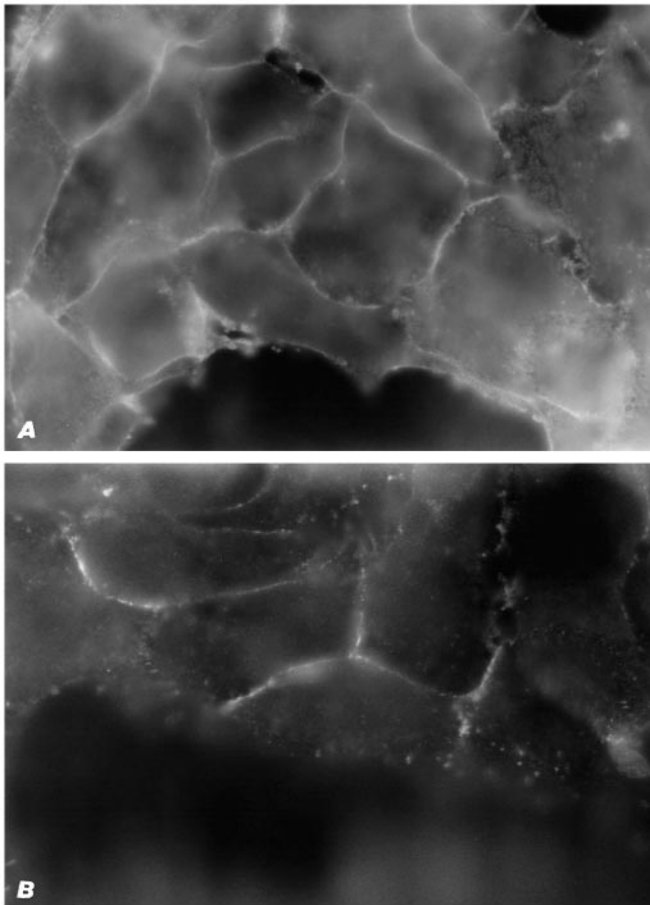
Fig. 6. Triton insoluble E-cadherin is present at cell-cell contact sites in E-cadherin-transfected RKO cells. RKO cells transfected with E-cadherin were immunostained for E-cadherin before (A), or after (B) extraction with Triton X-100 as described previously (Sommers et al., 1994).

Table 2. Biophysical parameters of homotypic cell-cell adhesion

Cell type	Flow (dyn/cm²)*	Tensile contact force (dyn)†	Longitudinal stretching‡
MCF-10A	>100	>5×10⁻³	>60%
MCF-7	>70	>2×10⁻³	>60%
HS-578T	>2.5	≅10⁻⁵	Tethered
BT-549	>2.5	≅10⁻⁵	Tethered

*Fluid shear stress that leads to disaggregation.
†Tensile force resisted by contact sites.
‡Longitudinal stretching before cell detachment.

was imposed on aggregates of cells that were adherent to a laminin or collagen-coated coverslip. The shear flow past aggregates exerted large forces on some of the cell-cell contact sites in the aggregate. The results indicated that cell-cell adhesion strength is severely compromised in E-cadherin negative carcinoma cells and that E-cadherin expression is a necessary but not sufficient condition for firm cell-cell adhesion. Cells which expressed E-cadherin in a Triton-insoluble form at cell-cell contact sites resisted disaggregation when exposed to shear stress forces in excess of 100 dyn/cm². In contrast, E-cadherin negative cells or cells in which E-cadherin was present as a diffusely distributed Triton-soluble form detached from one another at values of fluid shear stress comparable to those found in lymphatic and post-capillary blood venules. Consistent with these experimental observations the external forces resisted by adhesive contacts between E-cadherin positive MCF-10A cells were at least two orders of magnitude larger than those between E-cadherin negative breast carcinoma cells.

Transfection of E-cadherin into HS-578T and BT-549 cells does not alter their morphology or invasive properties (Sommers et al., 1994) and we show in this study that it has no effect on the disaggregation response of these cells to flow. However, the absence of a Triton-insoluble pool of E-cadherin in the transfected carcinoma cells points to a defect in E-cadherin interaction with the cytoskeleton (Sommers et al., 1994; Ozawa et al., 1990; Nelson et al., 1990). It is known that these particular invasive breast carcinoma cells have a defect in the expression or function of the cadherin associated molecules β-catenin and plakoglobin (Hirano et al., 1992; Matsuyoshi et al., 1992; Shimoyama et al., 1992; Sommers et al., 1994). The inability of these cells to link transfected E-cadherin to the cytoskeleton probably explains the failure of E-cadherin transfection to alter the disaggregation response of these cells in the present study. In order to rigorously test the contribution of E-cadherin-mediated adhesion to the resistance to disaggregation forces we transfected the mouse fibroblast cell line L-949 with E-cadherin. This line has previously been demonstrated to express several cadherin-associated molecules and to link the transfected cadherin to the cytoskeleton (Ozawa et al., 1990; McNeill et al., 1990). E-cadherin transfected L-cells acquired calcium-dependent cell-cell adhesion and had disaggregation properties in response to shear, similar to those of E-cadherin positive normal breast and non-invasive breast tumor cells (Fig. 4). Although a small number of E-cadherin transfected L-cells could be detached by shear forces the frequency of detachment was 20 fold less than E-cadherin negative tumor cells. As shown on several occasions by others,
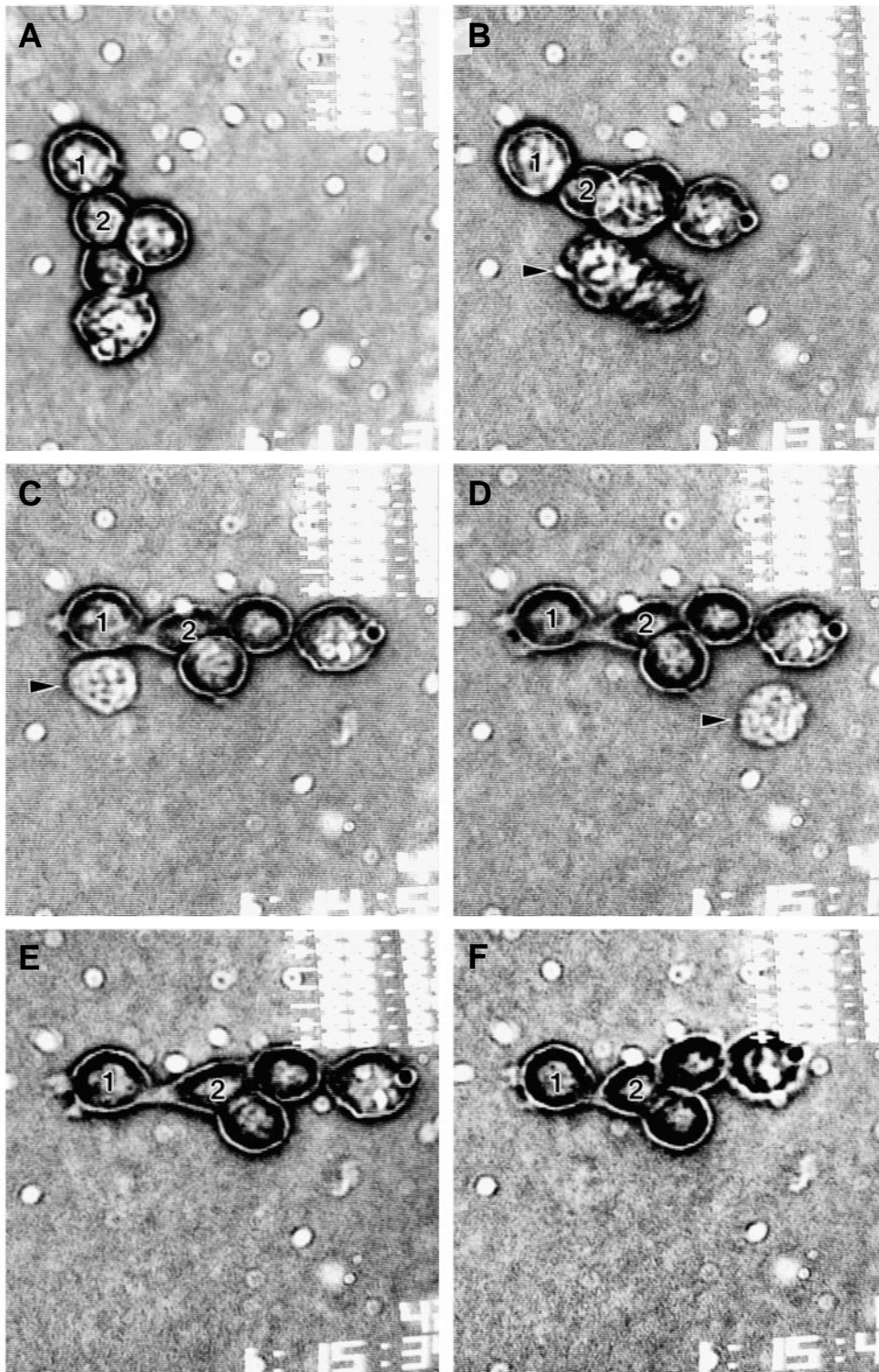
action of tensile forces in the order of 10^{-3} dyn. The deformation response of these cells is elastic, as the string of cells returned to their undeformed configuration within a few seconds following the cessation of flow (Fig. 7). The force-deformation response of MCF-7 breast carcinoma cells was similar to that of MCF-10A cells (Table 2).

Fig. 9 shows the deformation and disaggregation response of a typical HS-578T doublet exposed to laminar flow. The cells started to detach from each other at $\tau = 7$ dyn/cm². The tensile force that caused detachment in this case (F_{22}) was estimated, using equations (2) to (5) in Appendix, as 10^{-5} dyn, a value that is two orders of magnitude smaller than the external force resisted by MCF-10A cell-cell contact sites. BT549 cells also exhibited a weak adhesive contact (Table 2). Fig. 9 also shows that the HS-578T cell disaggregation was preceded by the formation of a tether between the two cells, a phenomenon that was also frequently observed during the flow-induced detachment of clone A and BT-549 cells.

DISCUSSION

In this study laminar flow assays were used to investigate the forces involved in homotypic cell-cell adhesion. Laminar flow

Fig. 7. The effect of shear stress on the orientation and deformation of a small aggregate of E-cadherin positive MCF-10A cells. The fluid shear stress on the laminin-coated coverslip corresponding to micrographs A-F was 0, 7, 35, 70, 100 and 0 dyn/cm^2, respectively. Note the longitudinal stretching between cells 1 and 2. The arrowhead indicates a cell transiently interacting with the substratum.

untransfected L-cells did not aggregate significantly in the presence or absence of calcium (McNeill et al., 1990; Ozawa et al., 1990; not shown). Immunocytochemistry revealed a Triton-insoluble pool of E-cadherin at points of cell-cell contact in aggregates of non-invasive breast tumor cells and E-cadherin transfected L-cells indicating that a strong linkage had been established with the cytoskeleton (Sommers et al., 1994; Ozawa et al., 1990; Nelson et al., 1990). Another E-cadherin

negative carcinoma cell line that responds to E-cadherin transfection by a marked change in morphology and motility properties was also used to investigate the role of E-cadherin in adhesion strength (RKO-Ecad; Breen et al., 1995). These cells form few aggregates of low adhesive strength before E-cadherin transfection (Table 1). Following transfection of E-cadherin into these cells they acquired disaggregation properties similar to those of E-cadherin positive epithelial cells such
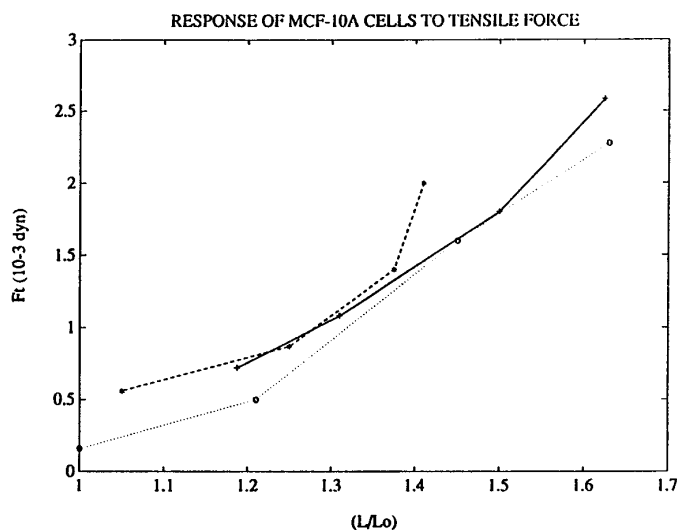
RESPONSE OF MCF-10A CELLS TO TENSILE FORCE

Fig. 8. The deformation response of three typical MCF-10A cells to tensile force. The data shown were obtained in laminar flow assays on MCF-10 cell aggregates in the form of strings of cells. The tensile fluid force (Ft) acting on a cell was computed as described in the appendix. The deformation index (L/L0) denotes the ratio of the cell length at a specified shear stress and time to that before the imposition of flow. The parameter L was measured 28 seconds after the imposition of flow at a given fluid shear stress.

as MCF-10A (Table 1). These results indicate that E-cadherin-mediated adhesion is largely responsible for the disaggregation properties of cells which express this molecule on the cell surface and which are able to link it appropriately to the cell cytoskeleton. It is possible that further strengthening of

adhesion may require the assembly of other epithelial cell-specific junctions such as desmosomes.

The physical strength of adhesion between two cells is likely to be dependent upon a number of factors, including the number of adhesion bonds per contact area, their spatial distribution, and linkage to the cytoskeleton. In epithelial cells E-cadherin is generally restricted to the actin-associated adherens junction which forms a belt within which the E-cadherin is presumably present at a high local density and linked to the underlying actin cytoskeleton. The physical strength of MCF-10A cell-cell adhesion is comparable to that between T-lymphocytes and their specific target cells and between phorbol-12-myristate-13-acetate-stimulated T-lymphocytes and planar membranes containing intercellular adhesion molecule-1 (Tozeren et al., 1992a,b; Sung et al., 1986). In these experi-
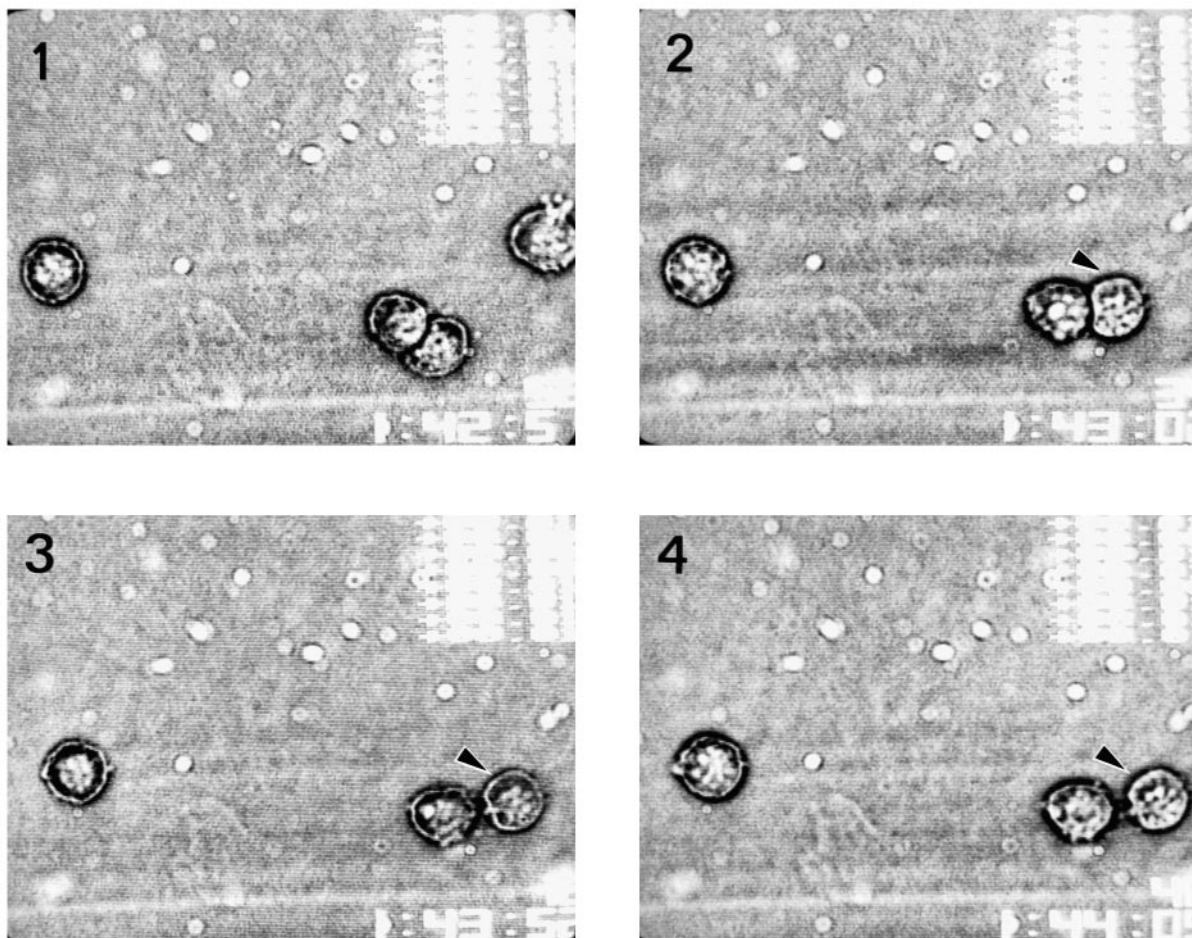

Fig. 9. The effect of fluid shear stress on the deformation and disaggregation of a HS-578T breast carcinoma cell doublet. The flow was imposed on the doublet adherent to a laminin-coated coverslip and was increased incrementally every 30 seconds. In micrographs 1-4 the fluid shear stress on the coverslip was 0, 7, 14 and 21 dyn/cm^2. Note that the cell indicated by the arrowhead detaches from the adjacent cell without appreciable longitudinal stretching.

ments, the tensile forces acting on the adhesion sites were evaluated using a micromanipulation procedure in which cell couples were detached from each other using a micropipette attached to a pressure control system. The extent of MCF-10A elongation in response to tensile force is also comparable to that of T-lymphocytes under similar loading conditions suggesting similar bulk rheological properties (Tozeren et al., 1992a,b; Sung et al., 1986). MCF-10A cells retracted to their undeformed spherical configuration rapidly after the cessation of flow. This elastic behaviour may be due to metabolically regulated tension in the actin-rich submembrane cortical shell (Stossel, 1993). Alternatively, such elastic properties are inherent in the tensegrity model of cytoskeletal organization proposed by Ingber and co-workers (Wang et al., 1993).

The deformation response of E-cadherin-negative breast carcinoma cells to fluid forces is quite different from that observed with MCF-10A cells. At low to moderate flow rates tensile forces acting on contact sites between two cells typically results in the formation of tethers without appreciable change in cell shape. This suggests that although cell-cell adhesion does occur between these cells, the adhesive molecules which mediate it are not linked strongly to the cell cytoskeleton.

To our knowledge the present study is the first to use laminar flow assays to measure epithelial cell-cell adhesion strength. The use of such direct mechanical measurements coupled with the techniques of molecular and cellular biology may lead to a new understanding of the mechanochemical processes which govern many cellular events (Ingber, 1994). In this study such a combination of molecular and cellular manipulations together with biophysical approaches was used to investigate the role of E-cadherin in cell-cell adhesion. The results suggest that E-cadherin negative tumor cells, or cells in which the adhesion molecule is present but is inefficiently linked to the cytoskeleton, are far more likely than E-cadherin positive cells to detach from a tumor mass in response to low shear forces, such as those found in a lymphatic vessel or venule. In these cells the relative strength of cell-substratum (endothelial cells

or extracellular matrix) and cell-cell adhesion is likely to determine whether a particular tumor would disaggregate in response to flow. This permits the speculation that once a breast or colon tumor infiltrates a blood or lymphatic vessel, E-cadherin negative cells may be detached by the flow and subsequently be captured by local lymph nodes or become lodged in distant capillary beds. These data, taken together with recent evidence that the E-cadherin-associated molecule β-catenin interacts directly with the tumor suppressor gene APC (Su et al., 1993), strongly suggest that alterations in the cadherin-based adhesion and signalling system not only affect the invasive capacity of tumor cells but may also influence contact-dependent growth control and metastasis.

APPENDIX

Evaluation of external forces acting on cell-cell boundaries

External forces acting on cell-cell contact sites in the direction perpendicular (tensile force) to the contact surface were computed by using data obtained from laminar flow assays on cell aggregates that were composed of a string of cells. Mathematical solution of axisymmetric flow past a finite chain of spheres each having radius R was considered (Fig. 10). Let m denote the number of spheres in the chain and integer j indicate the relative position of spheres along the chain such that $j = 1$ is the free end of the chain and $j = m$ is the end fixed to the substratum (Fig. 10). The drag force f_{jm} exerted by the surrounding fluid on the j sphere in the chain can be computed using the following equation:

$$f_{jm} = 6\pi\mu(U)(R)\lambda_{jm}, \tag{2}$$

where μ is the coefficient of viscosity of the surrounding fluid, U is the velocity of the uniform flow far from the chain of spheres, R is the radius of the identical spheres in the chain and the drag correction factor λ_{jm} is a function of the total number of spheres in a chain and the relative position of the sphere
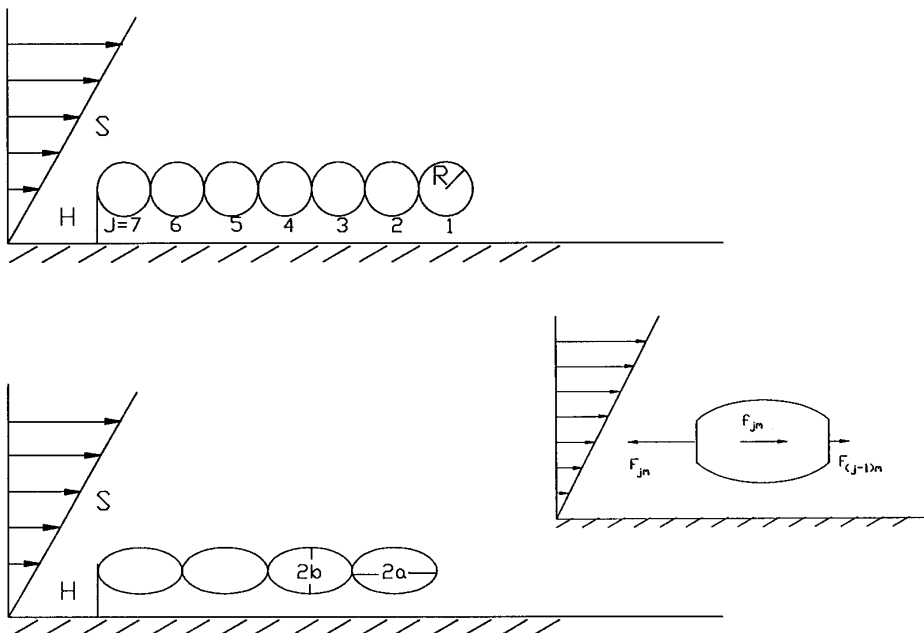

Fig. 10. Schematic diagram showing chains of spheres and spheroids fixed in simple shear flow near a planar boundary. Also shown is a free body diagram of a cell within the chain which indicates the forces exerted on the cell.

within the chain (Gluckman et al., 1971). $\lambda_{jm} = 1$ when $j = m = 1$, and $\lambda_{jm} = 0.65$ for $m = 2$ and $j = 1$ or 2. The solution for five or more chains indicates that the drag on the spheres located in the central portion of the chain changes little as the number of spheres in the chain is increased. As the end of the chain is approached, the drag on the spheres increases rapidly indicating a shielding effect. For example, in a seven sphere chain $\lambda_{17} = \lambda_{77} = 0.56$, $\lambda_{27} = \lambda_{67} = 0.28$, $\lambda_{37} = \lambda_{57} = 0.26$ and $\lambda_{47} = 0.25$ (Gluckman et al., 1971).

Let F_{jm} denote the total external force exerted by the surrounding fluid on the contact area between spheres j and $j + 1$ in a chain composed of m spheres (Fig. 10). The condition of the force balance indicates that:

$$F_{jm} = \sum_{1}^{j+1} (f_{jm}). \qquad (3)$$

This force is tensile in nature, that is, it will tend to stretch the bonds connecting the two cells in the direction perpendicular to the contact surface.

Gluckman et al. (1971) also provided Stokes flow solutions involving chains of prolate spheroids. A prolate spheroid is an axisymmetric body obtained by rotation of an ellipse along its long axis (Fig. 10). In this case of strings of prolate spheroids equation (2) is replaced by the following equation:

$$f_{jm} = 6\pi\mu(U)(b)\lambda_{jm}, \qquad (4)$$

where b is the maximal radial distance along the minor axis of the prolate spheroid (Gluckman et al., 1971). In this case, the drag correction factor λ_{jm} depends not only on j and m but also on the ratio of the maximal length of the spheroid ($2a$) to its maximal diameter ($2b$). For two touching spheroids with $a/b = 2$, $\lambda_{12} = \lambda_{22} = 0.85$ the total drag force on a chain of spheroids is approximately equal to that of a chain of spheres having the same length and maximal radial distance (Gluckman et al., 1971).

In the experimental investigation presented here the cell chains were stationary not in uniform flow but in simple shear flow near a planar boundary. In order to use equations (2) and (4) in the actual experimental case, the velocity parameter U appearing in these equations was taken to be equal to the velocity of simple shear flow at the central axis of the string of cells:

$$U = (H)(S) = H\tau/\mu, \qquad (5)$$

where H is the distance from the central axis of the cell chain to the planar membrane, S is the shear gradient of the simple shear flow and the wall shear stress τ is equal to the coefficient of friction m times the shear gradient S (Hsu and Ganatos, 1989). In this approximation, the effect of the planar wall on the drag coefficient factor λ_{jm} is not taken into account. The known solution concerning a sphere near a plane wall in simple shear flow indicates that wall effects may result in an increase in the drag force by as much as 60% (Goldman et al., 1967). Thus, the values provided here underestimate the external force resisted by MCF-10A cell-cell contact sites.

In actual computations, the wall shear stress τ was determined from the specified flow rate with the use of equation (1). The geometric parameters a and b were determined from the videotapes of the time course of deformation of the string of cells. The distance from the string center to the glass coverslip (H) was assumed for all cases to be equal to 1.5 times the maximal cell radius (b). The measured distance variations

required for the inverted microscope to clearly focus on the strings of cells with a 40× objective indicated that the assumed value of H cannot be different from the actual value by more than 50%. Because the tensile force is proportional to H, the order of magnitude of the estimate for the total drag force must be correct.

The string of cells attached at one end to the laminin-coated glass coverslip also had cells attached to the side of the chain (see Figs 1, 6). In such instances the contact forces between cells positioned upstream of the side-wise attached cell increased by the amount equal to the drag force acting on the side-wise attached cell. This force was computed by using the drag force coefficient factor for two equal spheres whose line of centers is perpendicular to the direction of imposed flow (Ganatos et al., 1978).

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