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# E-cadherin distribution in interleukin 6-induced cell–cell separation of ductal breast carcinoma cells

(cell adhesion/ZR-75-1 cells/antimorphogenetic cytokine action/cancer cell dyshesion)

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ABSTRACT **E-cadherin is expressed in both the ZR-75-1-Tx and the ZR-75-1-Ro sublines of ductal breast carcinoma cells and is concentrated at cell–cell borders as shown by immunocytochemical examination. Free cell borders generally show no or little staining. The localized decrease in E-cadherin expression observed after interleukin 6 (IL-6) treatment of either subline correlates with the increase in free cell borders as IL-6 causes cell–cell separation. As we previously reported, many IL-6-treated ZR-75-1-Tx cells round up and detach from the substratum while ZR-75-1-Ro cells remain adherent and display prominent processes. The results are consistent with the view that E-cadherin expression is not responsible for the marked difference in the IL-6-induced phenotypes in these cell lines, although the localized decrease may play a role in cell–cell separation. ZR-75-1-Tx cells are deficient in desmosomes and show a wider intercellular space than ZR-75-1-Ro cells. Alternative mechanisms involving different aspects of the interlinked cytoskeletal and cell adhesion structures are considered to account for the IL-6-induced antimorphogenetic effect.**

Although dyshesiveness has been recognized as a cardinal feature of ductal breast carcinoma cells of escalating malignancy (1), the underlying molecular mechanisms remain to be elucidated. An outstanding feature of the action of interleukin 6 (IL-6) on human ductal breast carcinoma cells in culture is loss of adhesiveness (2–5). We have therefore investigated the effect of IL-6 on the distribution of E-cadherin, a calcium-dependent cell adhesion molecule (reviewed in ref. 6), in breast carcinoma cells. Two epithelioid sublines of ZR-75-1 cells in which IL-6 causes cell–cell separation were examined. Treatment of the Tx subline with IL-6 leads to cell rounding and detachment not only from neighbors but also from the substratum (5). In contrast, the Ro subline cells become angular/stellate, remain adherent to the substratum, and translocate directionally (2).

The E-cadherin discovered as CAM 120/80 in the MCF-7 line of breast carcinoma cells was found by immunostaining to be localized in cell borders at sites of cell–cell interaction (7). That E-cadherin functions as an adhesion molecule in breast carcinoma cells was established by the findings that the 80-kDa fragment of the 120-kDa glycoprotein disrupts MCF-7 cell–cell adhesion, as does antibody against the fragment (8). These findings posed the question of whether there is a relationship between E-cadherin expression and the malignant state of carcinoma cells. High levels of E-cadherin expression have been reported to correlate with a low invasive potential of cells, whereas low levels of expression are associated with increased invasiveness (9). Poorly differentiated carcinoma cell lines which display a fibroblastoid phenotype in culture have completely lost their E-cadherin expression (10). Such poorly differentiated fibroblastoid lines are more invasive in the collagen assay (11) than the E-cadherin-expressing carcinoma lines with an epithelioid phenotype (10). The invasiveness of the dedifferentiated carcinoma cells can be prevented by transfection with E-cadherin cDNA and it can again be induced by treatment of the transfected cells with anti-E-cadherin monoclonal antibodies (10). Evidence for an inverse relationship between the amount of E-cadherin and tumorigenicity has been obtained in the mouse epidermal carcinogenesis model (12). Loss of E-cadherin expression is not the only mechanism whereby invasiveness can be acquired; scatter factor induces invasiveness in MDCK epithelial cells without affecting E-cadherin expression (13).

E-cadherin is one of several possible targets that may be critically involved in the cell adhesion-disrupting action of IL-6. We show that both ZR-75-1-Tx and ZR-75-1-Ro cells express E-cadherin. While IL-6 does not shut off E-cadherin expression, the down-regulation of this molecule at free cell borders in IL-6-treated cells correlates with dissociation of epithelial aggregates as ZR-75-1-Tx cells round up and ZR-75-1-Ro cells undergo fibroblastoid conversion. Thus, while E-cadherin down-regulation may play a role in IL-6-induced cell–cell separation in both sublines, it cannot *per se* explain the two ultimate cell phenotypes, which differ strikingly in adhesiveness to the substratum.

## MATERIALS AND METHODS

**Cells.** The ZR-75-1 cell line obtained in January 1992 from C. Kent Osborne (The University of Texas Health Science Center, San Antonio) was designated the Tx subline (5). The ZR-75-1 cell line obtained from the American Type Culture Collection in September 1988 was designated the Ro subline (5).

**Culture Conditions.** Both the Tx and Ro sublines of the ZR-75-1 line of cells were grown in RPMI 1640 medium containing 10% fetal bovine serum and 10 nM 17 $\beta$-estradiol. Stock cultures were split 1:5 by trypsinization once a week and refed once during the week. Alternatively, cultures were passaged by trypsinization and seeding at $10^4$ cells per $cm^2$ once a week with one change of medium, or at $2 \times 10^4$ cells per $cm^2$ twice a week.

**Reagents.** E9 monoclonal antibody, directed against E-cadherin, was a gift of Caroline H. Damsky (University of California, San Francisco). It was raised in rat against human antigen. Dichlorotriazinylaminofluorescein-conjugated affinity-purified goat anti-rat IgG (heavy and light chains) was purchased from Jackson ImmunoResearch. Other reagents used were described previously (5).

**Photomicrography.** Colonies in six-well plates (Falcon) (2) were photographed with a ×16 objective after staining with the Diff-Quik stain (Scientific Products).

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Abbreviations: aFGF, acidic fibroblast growth factor; PMA, phorbol 12-myristate 13-acetate; IL-6, interleukin 6.

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**Electron Microscopy.** Cells were prepared for electron microscopy as described (14, 15) and were viewed with a JEOL 100 CX electron microscope.

**Immunofluorescence Microscopy.** For immunocytochemical localization of E-cadherin, cells were grown on sterile glass coverslips in six-well dishes in 1.5 ml of medium. Cells were fixed with 4% formalin in phosphate-buffered saline (PBS, pH 7.2). The primary antibody was rat E9 monoclonal antibody against E-cadherin (8), diluted 1:5 in PBS, 2 hr, and the secondary antibody was dichlorotriazinylaminofluorescein-conjugated goat anti-rat IgG, 1:25 in PBS, 1 hr. Photomicrographs were taken with a ×40 or ×63 objective.

## RESULTS

**Contrasting Effects of Prolonged Treatment with IL-6 on ZR-75-1-Tx and ZR-75-1-Ro Cells.** We previously described the development of IL-6-induced morphological changes in cultures of ZR-75-1-Tx and ZR-75-1-Ro cells over the course of days (2, 3, 5). Fig. 1 compares ZR-75-1-Tx and ZR-75-1-Ro cells after prolonged incubation with IL-6. Control ZR-75-1-Tx cells are pleiomorphic and form irregularly shaped colonies (Fig. 1*A*). There are some elongated cells present and also round cells on the surface of the colonies, which, however, generally consist of a single layer of cells. In contrast to ZR-75-1-Tx cells, the control ZR-75-1-Ro cells form colonies that are commonly multilayered and consist of tightly packed cuboidal or polygonal cells of a typical epithelial character (Fig. 1*B*).

After prolonged treatment with IL-6 at 5 ng/ml, most ZR-71-1-Tx cells are dispersed as rounded singlets or doublets with only some small clusters of epithelioid or fibroblastoid cells remaining (Fig. 1*C*). Many of the rounded cells lack processes that can be resolved by low-power microscopy; however, some show evidence of filopodia of variable length. The IL-6-treated rounded cells may be either adherent or nonadherent (5). The rounded cells seen in Fig. 1*C* represent the adherent subfraction, as they withstood medium aspiration and a PBS rinse prior to staining. The essentially random distribution suggests that as cells proliferated over the long period of incubation, the daughter cells were passively scattered. Time-lapse cinemicrography has shown that IL-6-treated ZR-75-1-Tx cells do not translocate directionally (5). In striking contrast, the scatter of IL-6-treated ZR-75-1-Ro cells is mostly limited to the vicinity of colonies (Fig. 1*D*) and takes place through active translocation (2). IL-6 causes cell–cell separation in ZR-75-1-Ro cells, but many of the cells remain connected via processes and they do not detach from the substratum (Fig. 1*D*). Only very rare cells are nearly completely round.

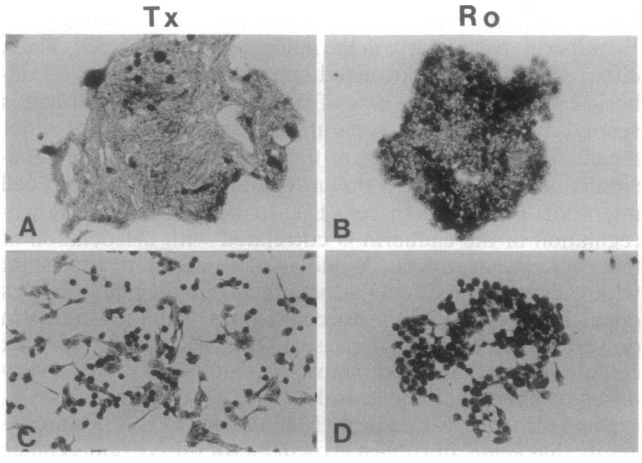


FIG. 1. Effects of prolonged treatment with IL-6 on the morphology of ZR-75-1-Tx (*A* and *C*) and ZR-75-1-Ro (*B* and *D*) cell colonies. Cells were seeded at 420 cells per $cm^2$ in six-well plates in a volume of 1 ml (0.104 ml/$cm^2$). Medium was changed and treatment began 3 days after planting. Eleven days later the colonies were stained and photographed with a ×16 objective. (*A* and *B*) Control. (*C* and *D*) IL-6 at 5 ng/ml. (×48.)

**E-Cadherin Distribution.** Several carcinoma lines with an epithelioid phenotype possess E-cadherin at cell–cell borders (7, 8, 10). Most intense immunofluorescence staining is detected in the apical and subapical regions of the cells, where intercellular boundaries appear as sharp lines (16).

Staining of ZR-75-1-Tx cells for E-cadherin reemphasizes the fact that these pleiomorphic cells are commonly angular and elongated (Fig. 2*A*). E-cadherin is concentrated at cell–cell borders. This also applies to adjoining nearly round cells. Free cell borders generally do not stain. After IL-6 treatment many cells have separated from each other and a considerable number have rounded up, but at contact regions the presence of E-cadherin is clearly evident, whereas there is little or no staining of free cell borders (Fig. 2*B*). PMA causes some flattening of cells (5), and again E-cadherin is characteristically present at cell–cell borders (Fig. 2*C*). PMA enhances the antimorphogenetic effect of IL-6 in breast carcinoma cells (5). In cells treated with IL-6 plus PMA, the E-cadherin distribution conforms to the general pattern observed after treatment with IL-6 alone (Fig. 2*D*).

With ZR-75-1-Tx cells we also observed some isolated round cells whose periphery unexpectedly stained for E-cadherin regardless of whether they were control or treated cells. Whether these cells represent genetic variants or whether they happen to have been stained just before loss of peripheral E-cadherin or whether some other explanation applies cannot be decided. In some instances we also observed irregular staining patterns of free edges of cells in the extended configuration, as previously noted (16).

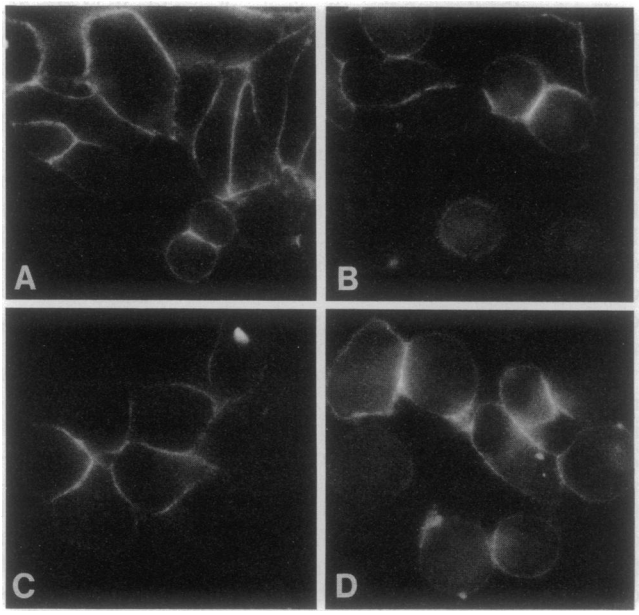


FIG. 2. Immunofluorescence staining of ZR-75-1-Tx cells with anti-E-cadherin antibody. ZR-75-1-Tx cells were seeded at $2 \times 10^4$ cells per $cm^2$ in six-well plates containing glass coverslips (22 mm × 22 mm). Three days later the medium was changed and treatment was begun, as follows: control (*A*), IL-6 at 5 ng/ml (*B*), 5 nM phorbol 12-myristate 13-acetate (PMA) (*C*), or IL-6 plus PMA (*D*). Two days later the cultures were fixed and processed for immunofluorescence staining. Cells were photographed with a ×63 objective. Without the primary antibody or without both the primary and secondary antibodies there was only faint diffuse fluorescence over the cytoplasm (data not shown). (×301.)

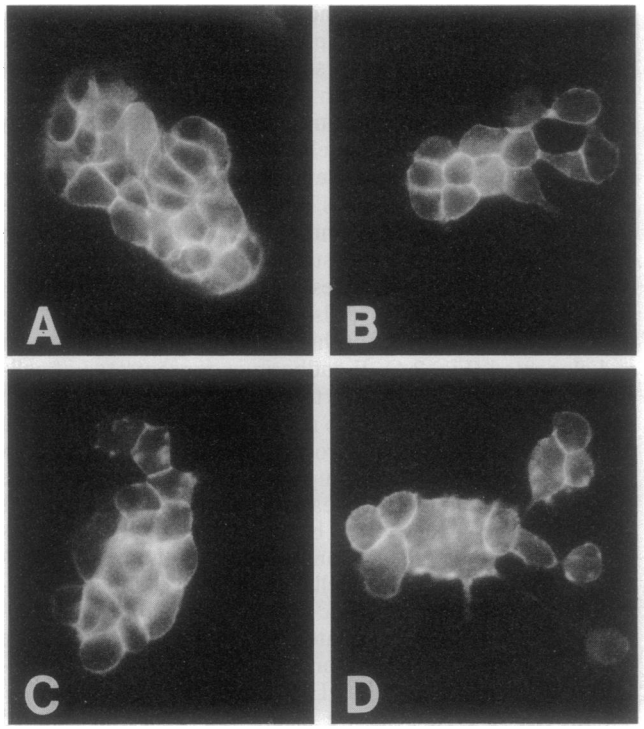


FIG. 3. Immunofluorescence staining of ZR-75-1-Ro cells with anti-E-cadherin antibody. ZR-75-1-Ro cells were seeded at $1 \times 10^4$ cells per $cm^2$ in six-well plates containing glass coverslips (22 mm × 22 mm). Four days later the medium was changed and treatment was begun, as follows: control (*A*), IL-6 at 5 ng/ml (*B*), 5 nM PMA (*C*), or IL-6 plus PMA (*D*). The following day the cultures were fixed and processed for immunofluorescence staining. A ×40 objective was used for photography. (×214.)

The localization of E-cadherin in the epithelioid ZR-75-1-Ro cells, which is illustrated in Fig. 3*A*, is consistent with previous reports on other carcinoma lines and our findings in ZR-75-1-Tx cells, as cell–cell borders stain for E-cadherin and free cell borders generally do not. IL-6 increases free cell borders which do not stain but has little if any effect on the staining of cell–cell borders (Fig. 3*B*). The free cell borders in PMA-treated cultures also do not stain, or stain very little for E-cadherin, whereas the cell–cell borders are brightly stained (Fig. 3*C*). Treatment with IL-6 plus PMA causes pronounced cell scatter (3), but the cells remaining in aggregates still display marked E-cadherin staining at cell–cell borders, although free cell borders show little staining (Fig. 3*D*).

**Intercellular Space and Abundance of Desmosomes.** As noted, in the absence of IL-6, ZR-75-1-Ro cells form exceedingly compact, tightly packed colonies whereas colonies of ZR-75-1-Tx cells have a looser organization. Electron microscopic examination of control cells shows that the intercellular space is wider in ZR-75-1-Tx than in ZR-75-1-Ro cell colonies (Fig. 4). In ZR-75-1-Tx cells no desmosomes were found in the viewed cells. Immunofluorescence staining of control ZR-75-1-Tx cells with anti-desmoplakin antibodies showed some punctate staining at the periphery of only a few cells (data not shown). In contrast, desmosomes were found by electron microscopic examination of ZR-75-1-Ro cells (Fig. 4*B*). Cellular substructures were not well resolved by immunofluorescence staining of the cuboidal control ZR-75-1-Ro cells because of multilayering.

## DISCUSSION

Our results in ZR-75-1-Tx and ZR-75-1-Ro cells contribute further evidence that in epithelioid lines of breast carcinoma

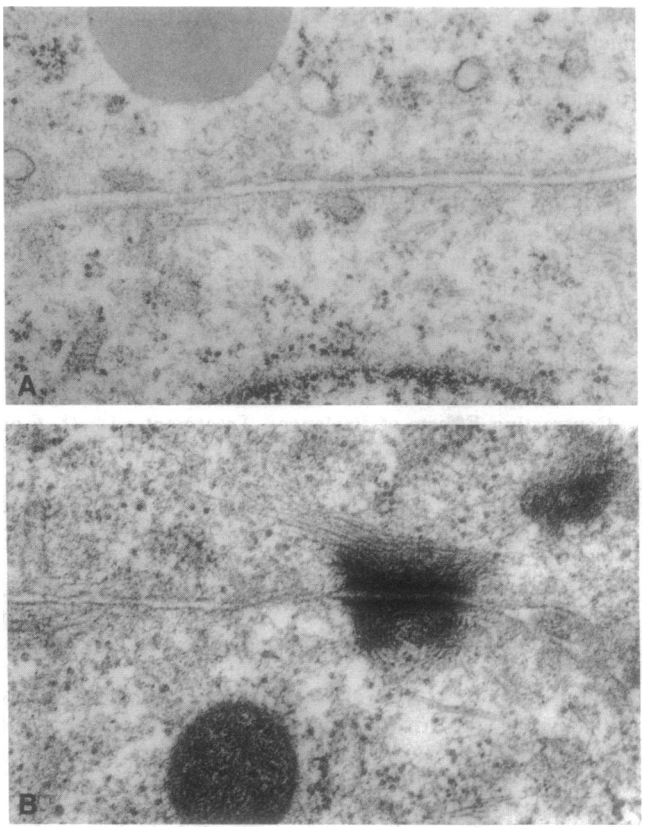


FIG. 4. The intercellular space between ZR-75-1-Tx cells is wider than that between ZR-75-1-Ro cells. ZR-75-1-Tx cells (*A*) were seeded at $1 \times 10^4$ cells per $cm^2$ in 25-$cm^2$ flasks in 5 ml of growth medium and incubated for 7 days. Then the medium was changed and incubation was continued for an additional 4 days. Cells were fixed in 2% glutaraldehyde in sodium cacodylate buffer. In the same experiment ZR-75-1-Ro cells (*B*), which grow slower than ZR-75-1-Tx cells, were seeded at $4 \times 10^4$ cells per $cm^2$ and then processed along with ZR-75-1-Tx cells. Electron photomicrographs were taken at a magnification of 8000. (×18,000.)

cells, E-cadherin is localized at cell–cell borders and is not present at free cell borders. In IL-6-treated cultures of either ZR-75-1-Tx or ZR-75-1-Ro cells, there are more free cell borders and such borders commonly show no or little staining for E-cadherin. Remaining cell junctions stain strongly for E-cadherin, particularly in the apical region of the cell. Thus, either IL-6 causes a localized suppressive effect on E-cadherin expression or the lack of staining of free cell borders is secondary to cell–cell separation brought about by other means. In the neural crest of the chicken embryo the adhesion molecule A-CAM is down-regulated at the onset of cell migration (reviewed in ref. 17). In both situations down-regulation of the cadherin is correlated with dissociation of the epithelial aggregate. While such evidence is consistent with cadherin functioning as an adhesive molecule, it leaves open the question of the mechanism of localized cadherin down-regulation and, in the case of ZR-75-Ro vs. ZR-75-1-Tx cells, the question of the pathways leading to the two very different phenotypic end results.

**Mechanism of E-Cadherin Modulation.** We reported recently that acidic fibroblast growth factor (aFGF) can replace serum as a required cofactor in IL-6-induced disruption of ZR-75-1-Tx cell adhesion (5). aFGF causes NBT-II rat bladder carcinoma cells to undergo changes resembling those of embryonic epithelial–mesenchymal transformation, and the cells disperse (18). NBT-II cells express E-cadherin along cell junctions, with free cell borders being devoid of immunostaining. After incubation with aFGF, E-cadherin immuno-

reactivity persists at remaining cell junctions, although it is lacking almost completely from free cell borders (18). Our results after IL-6 treatment of either the Tx or the Ro subline of ZR-75-1 cells parallel those findings. In NBT-II cells that had undergone aFGF-induced morphological transformation and separation from neighbors, a fraction of E-cadherin is redistributed over the entire cell surface, with the remainder found in an intracellular compartment (18). Cortical expression is reinduced upon cell contact. These changes occur in the absence of a change in the steady-state level of E-cadherin as determined by immunoblot analysis using antibodies against the extracellular part of E-cadherin.

E-cadherin mediates cell–cell adhesion only when complexed with the cytoplasmic proteins called catenins (19). The complexes associate with cortical actin bundles at adherens junctions (16), of which there are two kinds, the zonula adherens and the lateral adhesion plaques (reviewed in refs. 20 and 21). The E-cadherin-mediated adherens junctions can be disrupted in MDCK epithelial cells by increasing tyrosine phosphorylation either by inhibiting tyrosine phosphatases with vanadate or $H_2O_2$ or by introducing the v-*src*-encoded protein-tyrosine kinase (ref. 22 and 23; reviewed in ref. 24). Activity of $pp60^{v\text{-}src}$ in MDCK cells leads rapidly to cell–cell separation, acquisition of fibroblast-like morphology, and increased invasiveness. There is increased tyrosine phosphorylation of the E-cadherin/catenin complex and in particular of β-catenin (25). Phosphorylation of E-cadherin itself is also increased. These findings support the proposal that disturbance of intercellular adhesion may result from post-transcriptional modification of the E-cadherin–catenin complex (25).

Some of the findings in the aFGF–NBT-II cell system fit this model, whereas others do not. Inhibition of protein-tyrosine kinases with genistein or tyrphostin does suppress the scattering action of aFGF on NBT-II cells and, conversely, inhibition of protein-tyrosine phosphatases with sodium orthovanadate reproduces the aFGF effect (26). However, in aFGF-stimulated NBT-II cells E-cadherin remained linked to catenins and the phosphorylation pattern of E-cadherin–catenin complexes, in which phosphorylation on tyrosine residues was not detected, remained unchanged in the morphologically altered cells.

Several studies of E-cadherin expression in human tumors have been carried out. Examination of human tissue sections from squamous carcinomas of the head and neck established that (*i*) well-differentiated carcinomas often expressed E-cadherin as strongly as normal stratified epithelium, (*ii*) moderately differentiated carcinomas expressed intermediate amounts of E-cadherin or were heterogeneous, and (*iii*) poorly differentiated carcinomas were all negative for E-cadherin (27). Evidence for a correlation was also found in the expression of E-cadherin relative to the state of differentiation/dedifferentiation in cases of ovarian carcinomas (28) and lobular breast carcinomas (reviewed in refs. 10 and 29). However, the invasive forms of ductal breast carcinoma were found to express E-cadherin (reviewed in refs. 10 and 29). This may be characteristic of the particular tumors that were examined or it may be representative of ductal breast carcinomas as a whole. Other instances have been reported in which E-cadherin expression in dedifferentiated tumors was apparently normal, although the tumor cells exhibited reduced intercellular adhesiveness (30).

Our results in cell culture indicate some heterogeneity in the E-cadherin response of individual ZR-75-1-Tx and ZR-75-1-Ro cells to IL-6, which suggests that IL-6-induced cell–cell separation may be occurring via E-cadherin but through more than one mechanism, and possibly also through a mechanism not involving E-cadherin. The biologically important fact is that infiltrating ductal breast carcinoma is characterized by disordered cell adhesion (1).

**Desmosomes.** That ZR-75-1-Tx cells are deficient in desmosomes suggests that their absence contributes to profound antimorphogenetic action of IL-6 in these cells, as a result of which cells become spherical and nonadherent. In previous studies it was shown that IL-6 treatment leads to a decrease in desmosomes and focal adhesions in the T-47D line of ductal breast carcinoma cells (2). IL-6 treatment converts these cells from epithelial to fibroblastoid, with the predominant shape changing from polygonal to fusiform, and it greatly increases their motility (2, 3). Corresponding to the decrease in desmosomes, there is perinuclear retraction of cytokeratin filaments and the intercellular keratin filament connections are greatly diminished (2). The decrease in focal adhesions is associated with a marked diminution in the number of prominent F-actin stress fibers. It has been proposed that desmosomes and not E-cadherin molecules are the primary targets in the aFGF-induced dissociation process in the rat bladder carcinoma line NBT-II (18, 31). Desmosomes are disassembled relatively early in aFGF-induced NBT-II cell dissociation and epithelial–mesenchymal conversion, and this is accompanied by an increase in soluble desmoplakins (31). The cytokeratin intermediate filaments decrease progressively and there is appearance of vimentin intermediate filaments (31). Vimentin expression has been found in a higher proportion of estrogen receptor-negative breast carcinomas than in estrogen receptor-positive tumors (32, 33). Thus, vimentin expression generally predicts aggressive behavior (34). Breast cancer lines expressing only cytokeratin appear epithelioid, whereas those expressing vimentin are fibroblast-like (33).

**Integrins and Hemidesmosomes.** In the cell rounding and detachment induced by IL-6 in ZR-75-1-Tx cells the role of integrins deserves special attention because most integrins are involved in cell attachment to the substratum (21). In breast cancer, $\alpha_6\beta_4$ integrin is down-regulated and its distribution is altered (35, 36). Variations in integrin expression of carcinomas reflect disruption of the extracellular matrix (37). Transfection and antisense RNA experiments have suggested that integrin can play a role suppressing the transformed phenotype (38–40). Hemidesmosomes mediate attachment of cells to the underlying basal lamina (reviewed in ref. 21). They contain a cytoplasmic plaque associated with intermediate filaments and they also possess anchoring filaments. $\alpha_6\beta_4$ integrin localizes at hemidesmosomes and appears to be involved in hemidesmosomal attachment (reviewed in ref. 21).

**Microtubules.** Studies of microtubules in cellular morphogenesis have indicated that in the morphogenetic process microtubules are stabilized selectively and at particular cellular locations (reviewed in ref. 41). In ZR-75-1-Tx cells, IL-6 has an antimorphogenetic effect, which raises the possibility that it may be mediated via a massive destabilization of microtubules.

**Transmission of IL-6 Signal.** The IL-6 receptor and the receptors for several other cytokines that operate in the immune and the hematopoietic systems do not contain a tyrosine kinase domain (reviewed in ref. 42). This distinguishes them from numerous growth factor receptors. The binding of IL-6 to its receptor leads to the formation of a complex with homodimerized IL-6 signal-transducer gp130 molecules and association with cytoplasmic tyrosine kinases (43). The dimerized gp130 is phosphorylated on tyrosine as well as on serine and threonine residues. Much remains to be learned about target proteins for the tyrosine kinases that become associated with the IL-6–receptor–signal transducer complex. It is not known whether any proteins of the cytoskeletal–cell adhesion structures are direct targets for these tyrosine kinases. The major IL-6-induced changes in cell structure and adhesiveness may also involve activation of transcription factors through phosphorylation. Cascades of

phosphorylation and transcription may operate (42). IL-6 was recently shown to cause marked stimulation of the production of endothelin 1, a basal constrictive peptide, by ZR-75-1 cells (44). Whether this protein has cell–cell separating activity for ZR-75-1 cells remains to be determined.

It is evident that the available information regarding E-cadherin and carcinoma cell dispersion cannot be fitted into one simple and consistent scheme. This is not surprising, as cells coexpress cadherins and other classes of cell–cell adhesion molecules, and there is evidence for differential roles for these molecules. There appear to be mechanisms that can override E-cadherin function or function in place of E-cadherin in cell adhesion. The evidence is consistent with the possibility, however, that under certain circumstances E-cadherin may be a predominant and effective cell–cell adhesion molecule in epithelioid carcinoma cells and that loss of its expression or function can play a role in the loss of the epithelioid phenotype in the progression of cells to a more advanced stage of malignancy.

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