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Cell-adhesion-disrupting action of interleukin 6 in human ductal breast carcinoma cells

IGOR TAMM, TOYOKO KIKUCHI, IRMA CARDINALE, AND JAMES G. KRUEGER

Laboratories of Cell Physiology and Virology and of Investigative Dermatology, The Rockefeller University, 1230 York Avenue, New York, NY 10021

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ABSTRACT **Recombinant baculovirus-derived interleukin 6 (IL-6) disrupts the attachment of human ductal breast carcinoma subline ZR-75-1-Tx cells to neighbors and the substratum in culture without inhibiting the proliferation of the cells. The nonadherent cells lack pseudopodia and do not translocate directionally. These findings stand in contrast to the earlier observations in the Ro subline of ZR-75-1 cells in which IL-6 induces cell–cell separation without detachment of the cells from the substratum, with the cells displaying pseudopodia, increased motility, and decreased proliferation. The IL-6-induced ZR-75-1-Tx cell detachment and rounding are reversible by incubation of the treated cells in IL-6-free medium for several days. The distinctive changes induced by IL-6 in ZR-75-1-Ro cells are similarly reversible. Either acidic fibroblast growth factor or phorbol 12-myristate 13-acetate can replace serum as a cofactor in IL-6-induced ZR-75-1-Tx cell detachment. Our findings indicate that genetic changes can occur in breast carcinoma cells that through cytokine action markedly affect cell structure, adhesiveness, and motility.**

Infiltrating ductal breast carcinoma is characterized by disordered cell adherence (1). Cell distribution in cytological preparations varies from minimally dyshesive multilayered sheets to small piled groups and isolated cells. Both the degree of dyshesion and extent of nuclear alteration depend on the differentiation of the carcinoma (1). Poorly differentiated tumors are characterized by the presence of many dyshesive groups and isolated cells. The other cardinal feature of ductal breast carcinoma is that once obvious neoplasia develops genetic defects multiply and accumulate (2).

Earlier studies identified interleukin 6 (IL-6) as a cytokine that can act on epithelioid breast carcinoma cells in culture to induce conversion to a fibroblastoid phenotype accompanied by cell–cell separation and increased cell motility with cells in preformed colonies moving apart (3–5). These studies were carried out with T-47D (6) and ZR-75-1 (7) (henceforth called ZR-75-1-Ro subline) cells, in which IL-6 suppressed cell proliferation. An examination of IL-6 action on the clone B subline of ZR-75-1 cells (8, 9) revealed a modified response: IL-6 did not cause cell–cell separation in preformed colonies or inhibit the proliferation of clone B cells, but it did prolong the interval between mitosis and readherence of daughter cells, presumably because of somewhat decreased adhesivity of the IL-6-treated cells (10).

These findings led to the investigation of a third subline of ZR-75-1 cells (11, 12), henceforth called ZR-75-1-Tx cells. We now report that in these cells IL-6 disrupts both cell–cell and cell–substratum adhesion: cells round up and are unable to translocate directionally; however, they continue to proliferate at an undiminished rate.

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MATERIALS AND METHODS

Cells. The ZR-75-1 cell line was obtained from C. Kent Osborne (The University of Texas Health Science Center at San Antonio) (11, 12) in January 1992 and designated ZR-75-1-Tx to distinguish it from the ZR-75-1 cell line that we obtained from the American Type Culture Collection (Rockville, MD) in September 1988 (3, 7) and which we now designate ZR-75-1-Ro.

Karyotype analysis by the Cell Culture Laboratory, Children's Hospital of Michigan, has shown that both ZR-75-1-Tx and ZR-75-1-Ro are aneuploid human female, with chromosome counts in the triploid range. Both are bona fide subpopulations of ZR-75-1 since the majority of chromosome markers are common in both subpopulations and the isozyme phenotypic profiles of ZR-75-1-Tx and of ZR-75-1-Ro are concordant with that of other ZR-75-1 cultures. Cells other than those of line ZR-75-1 were not detected in either subline.

Chromosome counts gave the following ploidy distributions per 100 metaphases: ZR-75-1-Tx, 87 metaphases with 61–68 chromosomes and 13 metaphases with 120+ chromosomes; ZR-75-1-Ro, 94 metaphases with 63–75 chromosomes and 6 metaphases with 120+ chromosomes.

In the ZR-75-1-Tx line, the marker chromosome distribution among the metaphases examined did not indicate the presence of distinguishable subpopulations of cells. In the ZR-75-1-Ro line, a lower-ploidy (63–66 chromosomes) subpopulation had a marker chromosome array that was quite similar to that of the marker chromosome distribution in the ZR-75-1-Tx line. A higher-ploidy (74 chromosomes) subpopulation in the ZR-75-1-Ro line had a marker chromosome distribution that was essentially the same as that in yet another ZR-75-1 subline that we now designate as ZR-75-1-Rs subline, which we obtained from the American Type Culture Collection in April 1989. The ZR-75-1-Rs subline, received subsequent to obtaining what we designate as the ZR-75-1-Ro subline (see above), is distinct in its morphology and responsiveness to IL-6 from the ZR-75-1-Ro subline and the ZR-75-1-Tx subline (unpublished data). Clonal variation and selection are common in established cell lines (13, 14).

The ZR-75-1-Tx subline and the lower-ploidy ZR-75-1-Ro subpopulation have the following 13 marker chromosomes: M1, M1A, M2A, M3A, M4, M5, M5A, M6, M7A, M8, M9, M10, and M11. M7 was detected in only two out of eight karyotypes examined in the ZR-75-1-Tx subline and it was not present in the lower-ploidy ZR-75-1-Ro subpopulation. The higher-ploidy subpopulation in the ZR-75-1-Ro subline and the ZR-75-1-Rs subline has the following 12 marker chromosomes: M1, M2, M3, M4, M5, M6, M7, M8, M9, M10, M10A, and M11.

The Tx, Ro, and Rs sublines of ZR-75-1 cells lack normal chromosome 11. There are some differences among the cell lines in the copy number of chromosomes 2, 5, 8, 10, 18, 20, and X.

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

Culture Conditions. Cells of both the Tx and Ro sublines of the ZR-75-1 line were grown in RPMI 1640 medium containing 10% (vol/vol) fetal calf serum. The medium was supplemented with 10 nM 17β-estradiol. Stock cultures were split 1:5 by trypsinization in 0.25% trypsin/0.05% EDTA at 37°C once a week and refed once during the week. Cultures were also passaged by trypsinization and seeding at 1×10^4 cells per cm^2 once a week with one change of medium or at 2×10^4 cells per cm^2 twice a week.

Cytokines and Chemicals. Human recombinant IL-6 was kindly provided by Masayoshi Kohase (National Institute of Health, Tokyo). It was prepared in a baculovirus system and, after extensive purification, had a specific activity of 1×10^8 units/mg of protein as determined by stimulation of [^{3}H]thymidine incorporation in the IL-6-dependent murine hybridoma line, MH60, BSF-2 (15). In some experiments baculovirus-derived IL-6 kindly provided by Lester T. May (New York Medical College, Valhalla, NY) was used. The specific activity of this very highly purified IL-6 preparation was 1.7×10^8 units per mg of protein as measured by the B9 cell assay (16). Human platelet-derived growth factor was purchased from R & D Systems, epidermal growth factor was from Collaborative Research, and insulin-like growth factor I was from Amgen. Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma. Acidic fibroblast growth factor (aFGF) was purchased from Biosource International (Westlake Village, CA), and heparin was from Organon.

Cell Enumeration and Viability Determination. In most experiments, cells were planted in 25-cm^2 flasks (no. 3012; Falcon) in 4 ml of growth medium. The seeding density, the interval between planting and medium change with the addition of reagents, and the subsequent treatment period varied. Nonadherent cells were collected by pipette and adherent cells were collected by trypsinization. Cells were enumerated by Coulter and hemocytometer counting. Viability was evaluated by erythrocin B exclusion.

Photomicrography. Unstained cultures in 25-cm^2 flasks were photographed with Kodak TMY 400 film in an IM35 Zeiss inverted photomicroscope using a ×16 or ×25 phase-contrast objective.

Time-Lapse Cinemicrography. Experiments were performed as described (7). Films were analyzed with an L-W International (Athena, Woodland Hills, CA) projector.

RESULTS

IL-6 Decreases the Adherence of ZR-75-1-Tx Cells. The control colonies of ZR-75-1-Tx cells consisted of pleiomorphic epithelial cells with an angular and somewhat elongated appearance (Fig. 1 *A* and *B*). In contrast, most of the cells that had been treated with IL-6 at 5 ng/ml for 3 days appeared round and were generally lacking processes (Fig. 1 *C* and *D*). Only some treated cells still showed an epithelial phenotype. Of the rounded cells, a fraction had completely detached from neighbors and the substratum (see below). In a set of three time-lapse cinemicrographic experiments, ZR-75-1-Tx cells were seeded at 1×10^4 cells per cm^2, 3 or 4 days later the medium was changed, and treatment with IL-6 at 0.2, 1, or 5 ng/ml was begun. The control cultures received no IL-6. Filming was started shortly after medium change and continued for 1 week. In the control cultures, divisions were observed both in the well-spread cells, and also in round cells, the proportion of which decreased with time. In IL-6-treated ZR-75-1-Tx cultures, rounded cells increased with time in a dose-dependent manner and, in parallel, the patches of flat cells decreased. As would be expected, round cells not anchored to the substratum did not migrate directionally. The IL-6-induced rounding effect on ZR-75-1-Tx cells became apparent ≈6 h after the beginning of treatment and peaked in 2–3 days. IL-6-treated rounded cells divided actively and generally remained round and detached. Cytokinesis was sometimes incomplete and sometimes followed by division into three cells.

The time course of the IL-6-induced morphological changes in ZR-75-1-Tx cells is illustrated in Fig. 2. After 1 day of IL-6 treatment, many of the ZR-75-1-Tx cells became round and separated from neighboring cells, but not necessarily from the substratum. After a 2-day exposure to IL-6, the proportion of rounded cells exceeded that of cells remaining in small patches in which the cells did not appear to have separated from each other or from the substratum. After

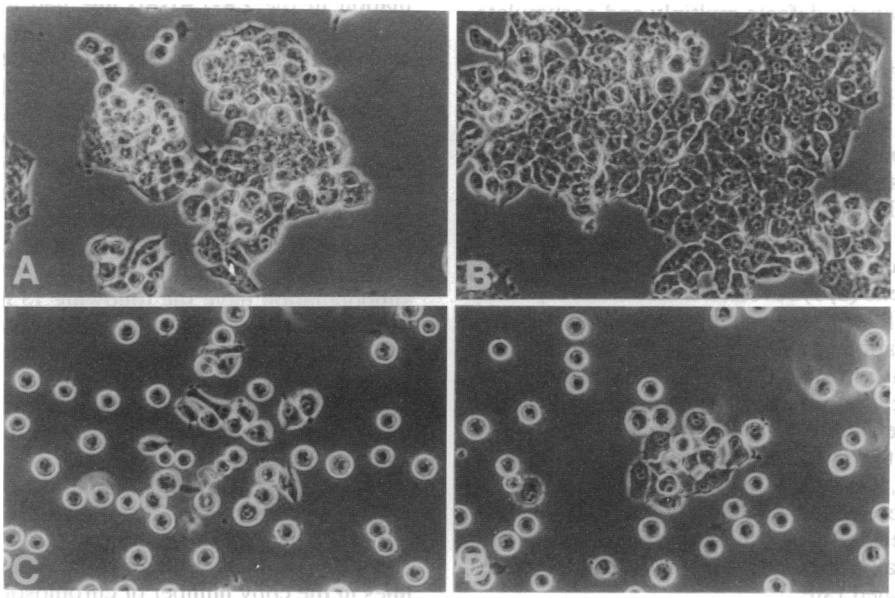

FIG. 1. IL-6 induces rounding of ZR-75-1-Tx cells. ZR-75-1-Tx cells were seeded in 25-cm^2 flasks at 2×10^4 cells per cm^2 in 4 ml of growth medium per flask (0.16 ml/cm^2). Two flasks were used per variable. The medium was changed and treatment was begun the following day. Cultures were photographed 3 days later using a ×25 phase-contrast objective, two frames each of control (*A* and *B*) and IL-6-treated (5 ng/ml) (*C* and *D*) cells. (×106.)

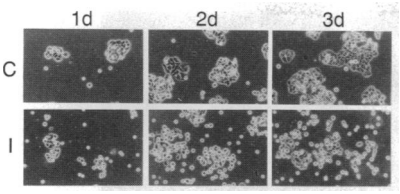

FIG. 2. Increase with time in IL-6-induced ZR-75-1-Tx cell rounding. Culture samples were from the same experiment as described in Fig. 1. Cultures were photographed 1, 2, or 3 days (d) after the beginning of treatment using a ×16 phase-contrast objective. C, Control; I, IL-6 at 5 ng/ml. (×20.)

a 3-day treatment, the IL-6-treated cells remained round and scattered.

Viability determinations on nonadherent cells showed that 1 or 2 days after medium change, 98–100% of control as well as IL-6-treated cells were viable (data not shown). However, an even longer total incubation (and in a smaller volume of medium) resulted in considerable mortality of the nonadherent control cells, but not of the nonadherent IL-6-treated cells (see below).

IL-6 Does Not Suppress or Enhance the Proliferation of ZR-75-1-Tx Cells. After planting of ZR-75-1-Tx cells at 2 × 10^4 cells per cm^2, the medium was changed and treatment with IL-6 at 5 ng/ml was begun the following day. Fig. 3*B* documents the marked time-dependent increase in the number of nonadherent cells in IL-6-treated cultures and lack of such an increase in control cultures. Correspondingly, the increase in adherent cells was somewhat greater in control as compared to IL-6-treated cultures (Fig. 3*A*), as many more cells became detached in IL-6-treated cultures (Fig. 3*B*). The increases in total cells in 2 days were closely similar in control and IL-6-treated cultures, i.e., 2.54-fold and 2.67-fold, respectively (Fig. 3*C*). Similar results were obtained in other experiments. Thus, IL-6 did not either suppress or enhance ZR-75-1-Tx cell proliferation. The nonadherent cells, expressed as a percentage of the total number of cells present at 1, 2, and 3 days, represented 2.8, 2.6, and 1.4% in controls and 11, 28, and 35% in IL-6-treated cultures in the experiment illustrated in Fig. 3. IL-6-treated nonadherent cells were competent in the soft agar assay (data not shown).

IL-6 Effects on Cell Morphology Are Reversible. Two kinds of cell enumeration and morphologic evaluation experiments were done, as follows: after IL-6 treatment the detached cells

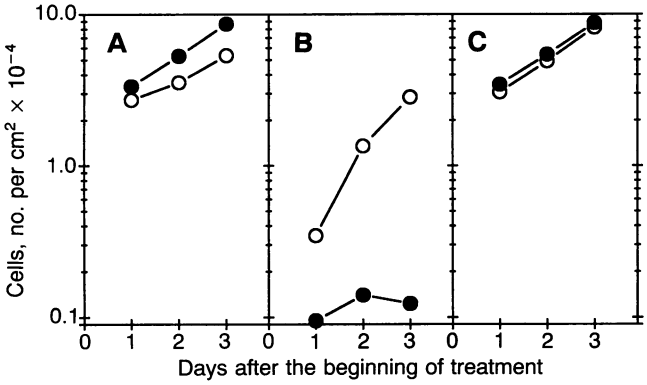

FIG. 3. IL-6 does not suppress or enhance ZR-75-1-Tx cell growth rate. ZR-75-1-Tx cells were seeded at 2 × 10^4 cells per cm^2 in 25-cm^2 flasks in 4 ml of growth medium. Three flasks were used per variable. One day later the medium was changed and treatment with IL-6 at 5 ng/ml was begun. One, 2, and 3 days later, nonadherent cells were collected with the medium and adherent cells were collected by trypsinization. Nonadherent cells and adherent cells were enumerated in a hemocytometer. (*A*) Adherent. (*B*) Nonadherent. (*C*) Total. ●, Control; ○, IL-6.

Table 1. Reversibility of IL-6-induced detachment and enhanced survival of ZR-75-1-Tx cells

Treatment (period)	Nonadherent cells, no. per cm^2 ($\times 10^{-4}$)	% control	% viable
Control (4 days)	0.317	100	39
IL-6 at 5 ng/ml (4 days)	2.950	931	92
Control (4 days) + control (3 days)*	0.603	100	29
IL-6 at 5 ng/ml (4 days) + IL-6 (3 days)*	8.130	1369	97
IL-6 at 5 ng/ml (4 days) + control (3 days)*	5.220	879	85

ZR-75-1-Tx cells were seeded at 1 × 10^5 cells per cm^2 in 25-cm^2 flasks in 4 ml of medium per flask and the experiment was begun the following day.
*Nonadherent cells were collected by centrifugation and readded to cultures as indicated.

were collected by centrifugation and either added back to the cultures from which they came or used to start new cultures.

In the first type of experiment, enumeration of nonadherent cells, summarized in Table 1, indicated that only partial reversal of the IL-6-treated cells to the adherent epithelioid

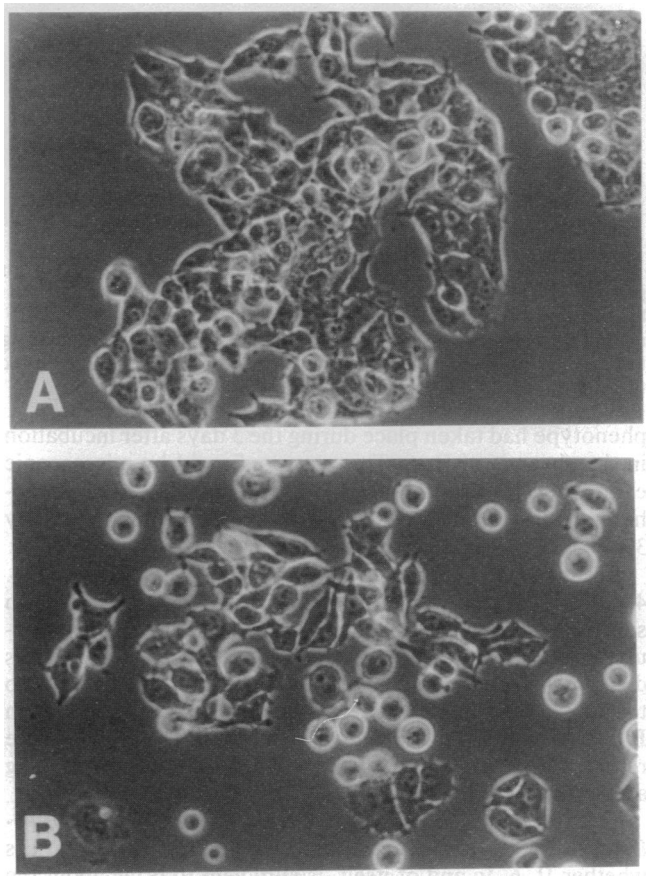

FIG. 4. Reversal of IL-6-induced phenotypic conversion by passage in control medium (*A*) and retention of IL-6 sensitivity by revertant cells (*B*). ZR-75-1-Tx cells were seeded at 2 × 10^4 cells per cm^2 in 25-cm^2 flasks in 4 ml of medium. The following day the medium was changed and treatment with IL-6 at 15 ng/ml was begun. Three days later the nonadherent cells from IL-6-treated cultures were collected by centrifugation, washed once, and seeded in new flasks in control medium at 2 × 10^4 cells per cm^2. Four days later the adherent cells were collected and seeded in new flasks at 2 × 10^4 cells per cm^2 either in control medium (*A*) or in medium containing IL-6 at 15 ng/ml (*B*). The cultures were photographed 3 days later using a ×25 phase-contrast objective. (×152.)

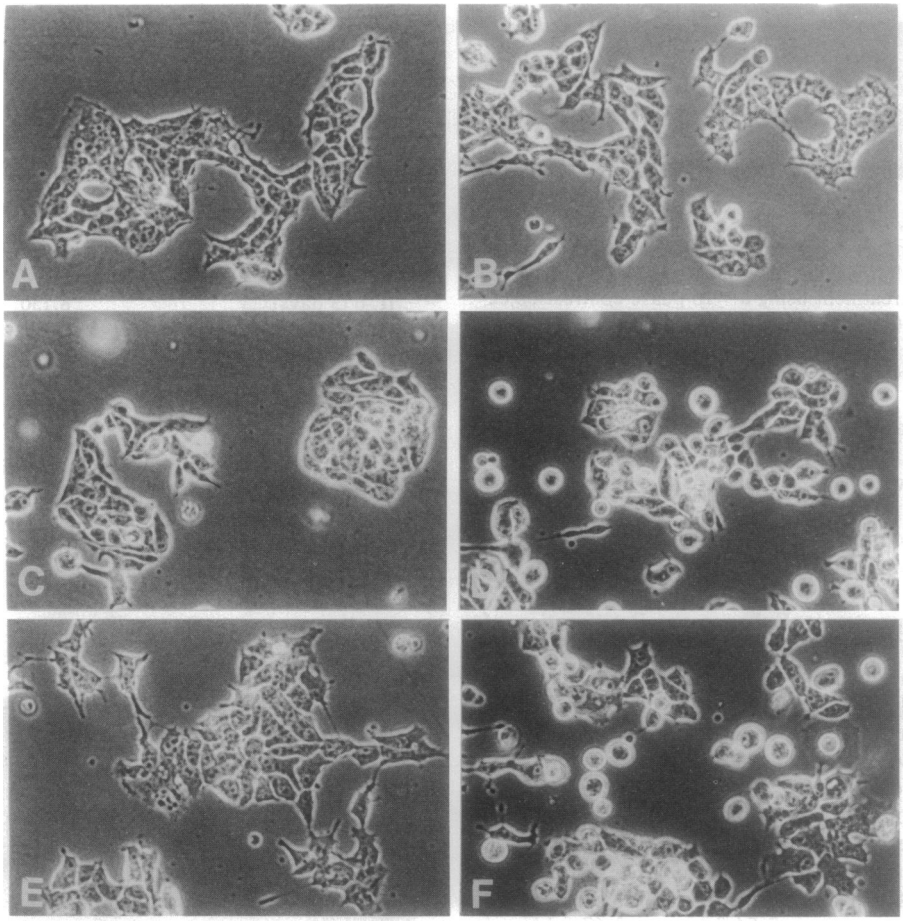

FIG. 5. aFGF or PMA can supplant serum as a cofactor in IL-6-induced cell rounding of ZR-75-1-Tx cells. Cells were seeded at 2×10^4 cells per cm^2 in 25-cm^2 flasks in 4 ml of medium. The following day the medium was changed to serum-free medium and the cultures were treated for 3 days as follows: (*A*) Control. (*B*) IL-6 at 15 ng/ml. (*C*) aFGF at 20 ng/ml plus heparin at 100 μg/ml. (*D*) IL-6 at 15 ng/ml, aFGF at 20 ng/ml, plus heparin at 100 μg/ml. (*E*) PMA at 5 nM. (*F*) IL-6 at 15 ng/ml plus PMA at 5 nM. Heparin was used with aFGF because it potentiates the activity of aFGF; used alone heparin has no cell rounding effect nor does it affect IL-6 action (data not shown). Photomicrographs were taken with a ×25 phase-contrast objective. (×107.)

phenotype had taken place during the 3 days after incubation in the absence of IL-6. This was confirmed by microscopic examination (data not shown). Close to 100% of the nonadherent cells were viable in the IL-6-treated cultures, but only 30–40% were viable in control cultures.

Results of the second type of experiment, illustrated in Fig. 4, show that after cultivation in control medium for two successive 3-day periods, the initially nonadherent cell population from the supernatant fraction of IL-6-treated cultures displayed an epithelioid phenotype that was closely similar to that of the original ZR-75-1-Tx cells (compare Fig. 4*A* with Fig. 1 *A* and *B*). Fig. 4*B* shows that the revertant cells have retained their sensitivity to the cell-separating and rounding action of IL-6.

aFGF or PMA Can Supplant Serum as a Cofactor in IL-6-Induced Cell Rounding and Detachment. The question arises whether IL-6, in and of itself, is sufficient to bring about the major changes in the breast carcinoma cell phenotype or whether it requires the presence of one or more additional factors in serum. Preliminary experiments showed that in the absence of serum, the ability of IL-6 to cause cellular detachment was markedly decreased although not completely abolished. Supplementation of the serum-free medium with platelet-derived growth factor, epidermal growth factor, and insulin-like growth factor 1 had little or no effect.

Fig. 5 shows that combined treatment of ZR-75-1-Tx cells with IL-6 (15 ng/ml) plus aFGF (20 ng/ml) resulted in marked cell rounding and separation in serum-free medium (Fig. 5*D*), whereas treatment with either agent alone did not (Fig. 5 *B* and *C*). Similar findings were obtained in two other experiments. In serum-containing controls, IL-6 used alone caused the usual effect of extensive cell–cell separation and rounding, whereas aFGF had only a slight cell-separating and -rounding effect (data not shown).

PMA treatment of ZR-75-1-Tx cells in serum-containing medium leads to flattening of the cells as the predominant change, with little cell–cell separation and cell rounding (unpublished observations). Fig. 5 shows that in serum-free medium, PMA used alone caused some curving of cell borders and an increase in cellular processes (Fig. 5*E*), but when used together with IL-6, there was considerable cell rounding (compare Fig. 5 *F* with *B* and *E*). Enumeration of nonadherent cells in serum-free culture medium confirmed these findings. The fold increases, treated/control, were as follows: IL-6, 2–3; aFGF, 2–3; PMA, <2; IL-6 plus aFGF, 8–10; IL-6 plus PMA, 8–10.

DISCUSSION

Disrupted cellular cohesiveness is a property of ductal breast carcinomas that correlates with dedifferentiation and with invasiveness and metastatic potential. In poorly differentiated tumors, there are numerous small groups of dyshesive cells as well as isolated cancer cells (1). Little is known as yet of the role of cytokines and of cellular properties that confer disordered cohesiveness. We have identified IL-6 as a factor that can convert a breast carcinoma cell from an adherent/

cohesive phenotype into a phenotype that proliferates independent of substratum attachment and can reestablish the adherent/cohesive phenotype upon removal of IL-6. This has implications for factors that might regulate tumor metastasis.

Comparison of sublines of ZR-75-1 cells has revealed remarkable differences in the responsiveness of the sublines to the cell-adhesion- and cell-growth-modulating activities of IL-6. The present results show that ZR-75-1-Tx cells respond to IL-6 with rapid rounding and detachment of cells from neighbors and the substratum. These effects reach a maximum in 2–3 days, with the nonadherent IL-6-treated ZR-75-1-Tx cells remaining viable and continuing to divide, while not being able to translocate directionally. The ZR-75-1-Ro cells investigated previously are predominantly cuboidal and undergo epithelial–fibroblastoid conversion associated with increased motility and down-regulation of replication in response to IL-6 (3, 4). In the clone B subline of ZR-75-1 cells, IL-6 does not cause scattering of cells in preformed colonies but readherence of newly divided cells is delayed (10).

These findings lead to two hypotheses. IL-6 modulates the expression of genes that determine adhesiveness, with changes in responses to the cytokine resulting from mutations or other changes in these genes, their regulator genes, or genes whose products are involved in the signal transduction pathway through which IL-6 acts. Alternatively, IL-6 may affect cell adhesion through a posttranslational modification in one or more proteins playing critical roles in the adhesion mechanism; this could involve the cellular localization or phosphorylation of adhesion proteins. The underlying assumption again is that the gene(s) for such molecules undergo mutational changes reflected in altered responses to IL-6.

The cell-adhesion-disrupting activity of IL-6 is markedly decreased when treatment of ZR-75-1-Tx cells is carried out in serum-free medium. However, when combined with aFGF or PMA, IL-6 causes rapid and marked changes in cell shape and adhesiveness in serum-free medium. The ability of aFGF and PMA to have a similar cofactor effect in the IL-6/ZR-75-1-Tx cell system may be explained by the fact that aFGF, like PMA, activates protein kinase C (17–19). Such a cofactor requirement is not unique. For example, estradiol has no enhancing effect on the proliferation of estrogen-receptor-positive human breast cancer cells in serum-free or growth factor-deprived media but does stimulate growth in serum-containing medium (20). The stimulation is potentiated by insulin-like growth factor 1 and epidermal growth factor.

Several mechanisms need to be considered to explain IL-6-induced separation of breast carcinoma cells from neighbors and the substratum. We have recently found that the cell adhesion molecule E-cadherin is expressed in both ZR-75-1-Tx and ZR-75-1-Ro cells and is concentrated at cell–cell borders as determined by immunocytochemical examination (I.T., I.C., T.K., and J.G.K., unpublished data). The localized decrease in E-cadherin expression observed after IL-6 treatment of either subline generally correlates with the increase in free cell borders caused by IL-6-induced cell–cell separation. However, it cannot explain the observation that whereas ZR-75-1-Ro cells remain attached to the substratum, many IL-6-treated ZR-75-1-Tx cells detach. Further studies are needed to elucidate the mechanisms underlying IL-6-induced dyshesion. It will be of special interest to determine whether IL-6 effects on the different sublines of ZR-75-1 cells vary because of mutations in one or more protooncogenes (2, 21) whose products may affect the responsiveness of the cells to the cell-shape- and adhesiveness-modulating actions of IL-6. As an example, the receptor for scatter factor/hepatocyte growth factor is the c-*met* protooncogene product, a tyrosine protein kinase (22).

As pointed out (3), time-lapse cinemicrography has shown that even in the absence of added IL-6, a colony of ductal breast carcinoma cells is not static. Cells change shape and some may separate from neighbors and subsequently rejoin the colony or move a distance away. Dyshesion (i.e., disordered cell adherence) characterizes ductal breast carcinomas, which constitute ≈80% of all malignant tumors of the breast (1). Is the complex pattern of cell distribution in these neoplasms a dynamic one that may be undergoing continual change in response to cytokines with the action of IL-6? Does the presence of cell multilayers vs. isolated cells indicate genetic diversity in the carcinoma cell population with respect to the adhesive properties of the cells?

We thank Dr. C. Kent Osborne for the ZR-75-1 line of human ductal breast carcinoma cells that in the present study has been named the ZR-75-1-Tx subline. We thank Dr. Masayoshi Kohase and Dr. Lester T. May for generously providing baculovirus-derived human recombinant IL-6. We thank Dr. James S. Murphy for assistance in the evaluation of time-lapse cinemicrographs, and Drs. James S. Murphy and Samuel D. Wright for discussions. This work was supported by National Institutes of Health Grants CA-18608 and CA-54215 and a grant from the American Skin Association.

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