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Phorbol ester-induced scattering of HT-29 human intestinal cancer cells is associated with down-modulation of E-cadherin

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SUMMARY

The effects of tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) on the growth characteristics of the colon cancer cell line HT-29 M6 were studied. TPA induced the scattering of proliferative HT-29 M6 cells: in the presence of the phorbol ester, HT-29 M6 colonies scattered and the cells acquired a flatter aspect with diminished cell-cell contacts. This effect of TPA required a persistent activation of PK-C and was acompanied by a slight decrease (30%) in the growth rate. Modifications by TPA of two scattering associated properties of these cells were also detected: TPA decreased cell-to-cell aggregation and enhanced the cellular attachment to matrix substrata (collagen, laminin). The decrease in cell-to-cell adhesion was correlated with a loss of cellular E-cadherin as evidenced by immunofluorescence or immunoblotting with a specific monoclonal antibody. Cell scattering was dependent on the extracellular concentration of Ca^{2+}; an increase from 1.6 to 10 mM in the concentration of this ion completely blocked the morphological effects of TPA as well as its action on cell aggregation. This high concentration of Ca^{2+} also prevented the down modulation of E-cadherin as determined by immunofluorescence. However, the TPA-induced increase in cell attachment to the matrix was not affected by high calcium. These findings support the importance of altered cell-cell adhesion in the process of scattering and provide a good system for the study of down modulation of E-cadherin, a protein involved in the control of cell growth, differentiation and invasion of epithelial cells.

Key words: HT-29 cells, TPA tumor promoter, E-cadherin, cell scattering, calcium

INTRODUCTION

Colon cancer is a consequence of the accumulation of genetic alterations that lead to an abnormal balance in the events that regulate intestinal epithelial cell growth and differentiation (Fearon and Vogelstein, 1990). In vitro, the study of these processes has been hampered by the lack of reproducible methods for the culture of normal intestinal epithelium and the undifferentiated phenotype of most colorectal cancer cell lines (Neutra and Louvard, 1991; Zweibaum et al., 1991). Recently, intestinal epithelial cell lines that are able to differentiate in culture into goblet or absorptive cells have been described (Neutra and Louvard, 1991; Zweibaum et al., 1991). HT-29 M6 is a subpopulation of the HT-29 cell line that is isolated by its ability to grow in medium supplemented with 10^{-6} methotrexate (Lesuffleur et al., 1990)*. After confluence, HT-29 M6 cells develop a polarized phenotype, with a moderately well defined apical brush border and a cytoplasm essentially full of mucous droplets (Lesuffleur et al., 1990, 1991). Proliferating HT-29 M6 cells resemble pre-differentiated colon crypt cells that are committed to differentiate into goblet cells (Lesuffleur et al., 1990, 1991). Thus, although HT-29 M6 cells are derived from a tumour, the characteristics described above make them a suitable model with which to identify the molecular mechanisms controlling epithelial cell proliferation and differentiation.

The phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is a potent activator of protein kinase C (PK-C), its specific cellular receptor (Blumberg, 1988; Jaken, 1990). Great interest in this phorbol ester has evolved from its use in carcinogenesis models, in particular in skin and in the gastrointestinal tract (Boutwell, 1974). In vivo carcinogenesis models have shown that tumour development proceeds in two distinct stages: initiation, which involves a genetic change caused by a mutagen; and promotion, an amplification of the mutated cell population as the result of the stimulation of cell proliferation by a 'promoter'. TPA falls in this second category of molecules (Goerttler et al., 1979). Moreover, a role for other activators of PK-C in the development and/or progression of colon cancer has been suggested (Weinstein, 1991). To characterize further the role of PK-C in intestinal epithelial cells, we have investi-

*For the sake of brevity we have given the name of HT-29 M6 to the cell line previously named HT-29 (10-6 MTX) (Lesuffleur et al., 1990)

gated the effects of TPA on the growth and morphological properties of the well-established model of HT-29 M6 cells.

MATERIALS AND METHODS

Materials

TPA was purchased from Sigma Chemical Co. (St. Louis, MO). PK-C inhibitor GF 109203X (GF), a gift from Dr Jorge Kirilovsky (Glaxo, Paris, France), was provided by Dr Jorge Moscat (CSIC, Madrid, Spain); it will be referred as GF. [^{3}H]thymidine was from New England Nuclear. HECD-1 anti-E-cadherin antibody, prepared and characterized by Takeichi and coworkers (Shimoyama et al., 1989), was kindly provided by Dr Amparo Cano (Facultad de Medicina, Universidad Autónoma de Madrid). Anti-E-cadherin rat mAb DECMA-1 was purchased from Sigma. All other chemicals used were commercial products of the highest grade available.

Cell culture

The HT-29 cell line, established by J.Fogh (Fogh and Trempe, 1975) was supplied by Dr Alain Zweibaum (INSERM, Villejuif, France). HT-29 cells selected by adaptation to 10^{-6} M methotrexate (HT-29 M6) (Lesuffleur et al., 1990) were also provided by Dr Zweibaum. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco) in a humidified atmosphere of 5% CO_2, 95% air. Cells were seeded at approximately 2×10^4 cells/cm^2, so that confluence was reached at days 6-7. Culture medium was changed every other day to avoid nutrient depletion.

Cell attachment assay

Cells were labelled overnight with [^{3}H]thymidine (1 μCi/ml), washed three times with phosphate-buffered saline (PBS), trypsinized and seeded at a density of 2×10^4 to 5×10^4 cells/cm^2 on collagen, fibronectin, laminin or complete matrices (see below). Polystyrene 24-well plates (Corning Glass Works, Corning, NY) were coated with 250 μl/well of bovine type I collagen (Collaborative Research, Bedford, MA), mouse fibronectin (Sigma) or mouse laminin (Collaborative Research) (10 μg/ml) and left uncovered in a laminar flow hood overnight to allow evaporation. The plates were then rinsed with PBS and used for the attachment assays. To obtain a complete matrix, HT-29 M6 cells were grown to confluence in 24-well plates, then lysed with distilled water until no cells were visible. The wells were rinsed with PBS and used. Binding assays were performed in 0.5 ml of DME medium (DMEM) supplemented with 0.5% FBS or in PBS plus 5 mg/ml of BSA. After 1 hour of incubation at 37°C, the wells were rinsed three times with PBS, attached cells were lysed in 1% SDS and the radioactivity released was determined in a scintillation counter.

Trypsin 'lift off' assay

Labelled cells were allowed to attach to plastic or to the matrix for at least 3 hours and incubated with the indicated stimulus for 4-12 hours. Cells were washed three times with PBS and incubated with 1 ml of trypsin (Biological Industries, Beth Haemek, Israel; 2.5 mg/ml in Puck's saline solution supplemented with 1 mM EDTA). At the indicated times, samples (100 μl) of the medium were withdrawn and the radioactivity released was determined.

Cell-cell adhesion assay

The assay was performed as described by Pignatelli et al. (1992) with some modifications. Preconfluent HT-29 M6 cells were washed with PBS and trypsinized by 15 minutes of incubation at 37°C with 0.12% trypsin in DMEM supplemented with Ca^{2+} (5 mM). After centrifugation, the cells were passed through a 30-gauge syringe several times in DMEM plus 1% FBS until a single-cell suspension was obtained. One hundred thousand cells were stirred at 80 rpm at 37°C in 2.0 ml of DMEM plus 10% FBS for 2 hours and the number of single cells was determined using a haemocytometer. Assays were performed in triplicate.

Indirect immunofluorescence

Immunofluorescence staining was performed on cells grown on glass coverslips as described (García de Herreros and Birnbaum, 1989). Cells were fixed with paraformaldehyde, permeabilized, and incubated with the mouse mAb anti-E-cadherin HECD-1 (1:100 dilution in HMF buffer: 10 mM Hepes, pH 7.6, 150 mM NaCl, 10 mM $CaCl_2$, 1% BSA) or the rat mAb anti-E-cadherin DECMA-1 (1:500 dilution in HMF buffer) for 1 hour. After washing, coverslips were incubated with rabbit anti-mouse IgG (Vector, Burlingham, CA) or rabbit anti-rat IgG (Malvern Biochemicals, West Chester PA), respectively, for HECD-1 or DECMA-1, and with rhodamine-labelled goat anti-rabbit IgG (Vector).

Western blot analysis

Cells were washed with HMF buffer and scraped into 1 ml of this buffer. Cells were pelleted, solubilized in 350 μl of Laemmli's sample and sonicated for 30 seconds (twice) in a Branson Ultrasonic sonicator. After centrifugation (15,000 ***g***, 10 minutes) 100 μg of supernatant proteins were separated by SDS-PAGE, transferred electrophoretically onto nitrocellulose and incubated for 1 hour with anti E-cadherin antibodies HECD-1 (1:400 in HMF) or DECMA-1 (1:500 in HMF). After washing with PBS, membranes were incubated with a rabbit anti-mouse IgG (Vector) or rabbit anti-rat IgG (Malvern), respectively, for HECD-1 or DECMA-1, and with peroxidase-conjugated goat anti-rabbit IgG (Dakopatts, Copenhague, Denmark), and reacting antigens were visualized using diaminobenzidine and H_2O_2.

DNA synthesis

Subconfluent cells were made quiescent by incubation in DMEM supplemented with 0.5% FBS for 48 hours before the corresponding stimulants were added for 22 hours. Afterwards, [^{3}H]thymidine (2 μCi/ml) was added for 4 hours and de novo DNA synthesis was determined as described (Hiraki et al., 1988).

RESULTS

TPA promotes the scattering of proliferative HT-29 cell cultures

Under standard culture conditions, HT-29 M6 cells grew as compact cell clusters with intercellular cysts, as described (Lesuffleur et al., 1990) (Fig. 1A). The addition of TPA (200 nM) consistently induced morphological changes in HT-29 M6 cells. After 4 hours of culture in TPA, cells at the edges of the colonies became looser, acquired a flatter aspect, and cell-to-cell contacts diminished (Fig. 1B). The cultures acquired a characteristic appearance, with cells protruding from the clusters (Fig. 1B). After 18 hours, TPA-treated cultures appeared almost confluent (Fig. 1C) unlike control cultures. At higher magnifications, control cultures contained tight colonies in which individual cell limits could not be delineated (Fig. 1E). In contrast, cultures treated with TPA showed wider intercellular spaces, and individual cell limits were clearly identified (Fig. 1F). The

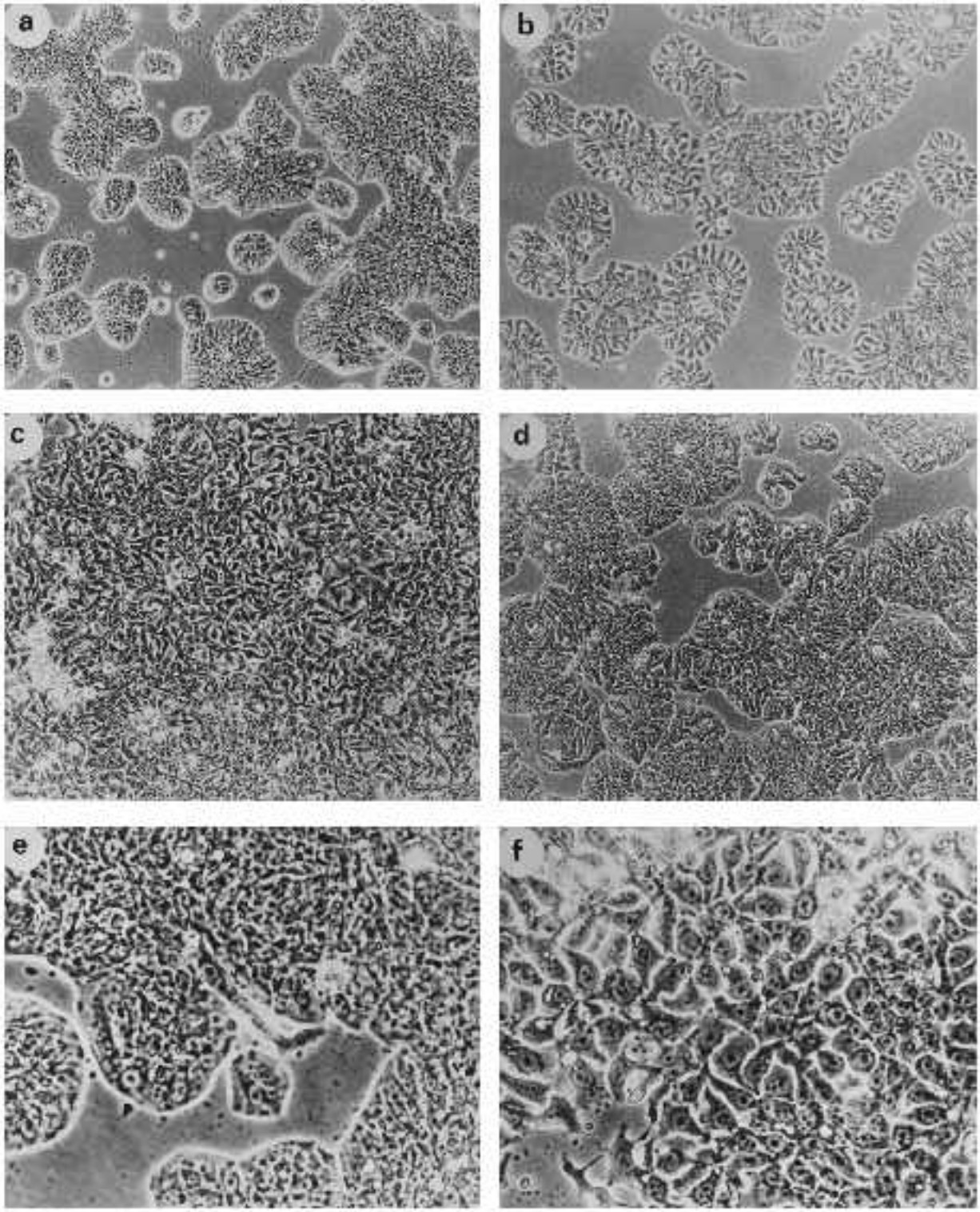


Fig. 1. Morphological changes induced by TPA in HT-29 M6 cells. Subconfluent cells were incubated in complete medium (DMEM plus 10% FBS) supplemented with TPA (200 nM). Pictures were taken under a phase-contrast microscope at ×100 (A-D) or ×350 magnification (E-F), after 0 (A and E), 4 (B) or 20 (C and F) hours of incubation. In the experiment shown in (D) cells were incubated in the presence of TPA for 4 hours; the medium was then supplemented with the PK-C inhibitor GF (5 μM) and the cells were incubated for another 16 hours. No morphological difference from the control (A) was observed when GF, or GF plus TPA, was added to the cells at the beginning of the experiment (not shown).

lowest concentration of TPA at which this effect was observed was 10 nM. DMSO (0.1%), the vehicle in which TPA was dissolved, had no effect on the cellular morphology (not shown). These effects of TPA on HT-29 M6 cell morphology were detected independently of the presence of FBS in the cell culture and were not affected by cycloheximide (2 μg/ml) (not shown).

To determine if the morphological changes induced by TPA were due to PK-C activation, a specific inhibitor of this enzyme was used. GF has been shown to be a potent and selective inhibitor of PK-C, used to block PK-C-mediated processes in platelets and Swiss 3T3 cells (Toullec et al., 1991). The addition of GF (5 μM) completely inhibited the morphological changes induced by TPA; cells treated with both TPA and GF were morphologically indistinguishable from untreated cells. GF addition not only prevented but reverted the action of TPA as well; when HT-29 M6 cells were incubated with TPA for 4 hours and exposed to GF for 12 additional hours, an almost complete reversion to the original aspect was observed (notice the absence of scattered cells at the edge of the colonies in Fig. 1D). These results demonstrate that TPA-induced scattering of HT-29 M6 cells is due to activation of PK-C, not to

Table 1. Effects of FBS and TPA on DNA synthesis in HT-29 M6 cells

Experimental conditions	[^{3}H]thymidine incorporation (cpm×10^3 per 10^6 cells)
Control	13.1 ± 0.8
FBS	90.8 ± 6.4
TPA	7.7 ± 1.3
FBS + TPA	62.4 ± 7.6

Subconfluent cultures (two days after seeding) were made quiescent by incubation in medium containing 0.5% FBS. After two days, cells were incubated for 22 hours in DMEM containing 0.1% BSA (control), supplemented with FBS (10%), TPA (200 nM), or both, and de novo DNA synthesis was determined as described in Materials and Methods. Values are averages ± range, of five assays performed in duplicate.

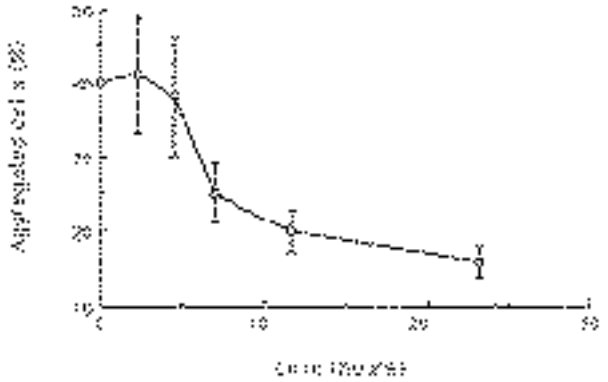
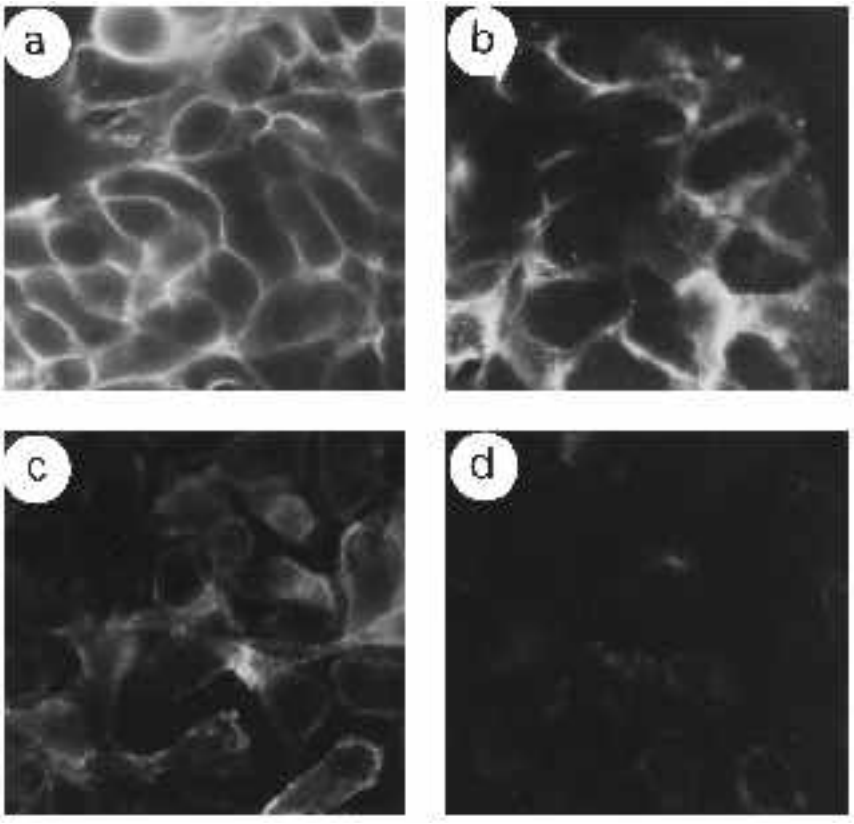


Fig. 2. TPA decreases homotypic aggregation of HT-29 M6 cells. Subconfluent cells were incubated in DMEM supplemented with TPA (200 nM) for the indicated times. After trypsinization, a single cell suspension was made putting the cells several times through a 30-gauge needle; 5×10^5 cells were resuspended in 2 ml of DMEM (supplemented with 10% FBS), rotated at 80 rpm at 37°C and the number of single cells was determined in a haemocytometer immediatly and after 2 hours. The percentage of aggregated cells ($N_{initial} - N_{final}$)/ $N_{initial}$ is represented, where N is the number of isolated cells counted in a haemocytometer. The figure shows the results (average ± range) of two experiments performed in duplicate.



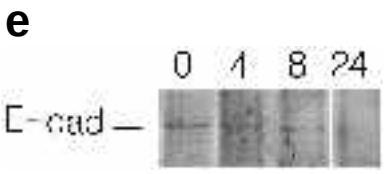


Fig. 3. TPA decreases the levels of E-cadherin in HT-29 M6 cells. Subconfluent cells, cultured over coverslips, were incubated in complete medium (DMEM plus 10% FBS) (a), or complete medium supplemented with TPA for 4 (b), 8 (c) or 24 (d) hours. Cells were washed, fixed, permeabilized and analyzed by immunofluorescence with the HECD-1 anti-E-cadherin antibody. Results shown (1000-fold magnification) are representative of three independent experiments (e). Similar results were obtained with another anti-E-cadherin antibody DECMA-1. M6 cells were incubated with TPA (200 nM) for 0, 4, 8 or 24 hours, cell extracts were prepared as described, analyzed by SDS-PAGE in 9% polyacrylamide gels and immunoblotted using a 1:500 dilution of DECMA-1 anti-E-cadherin monoclonal antibody.

down-regulation of this enzyme, as has been suggested in other systems (Rosen et al., 1991a).

TPA-induced scattering is accompanied by a decrease in the growth rate

The confluent appearance of TPA-treated cultures could result from the morphological changes as well as from a proliferative stimulus of TPA. To examine these possibilities, direct cell counts and cell proliferation assays were performed. Proliferative HT-29 M6 cultures (day 2 after seeding) were fed with complete medium (DMEM plus 10% FBS) or with complete medium supplemented with TPA (200 nM). Three days later, the total cell number was 35 (± 8)% (s.d.) lower in TPA-treated cells than in control cultures. This growth-inhibitory effect of TPA was confirmed by thymidine incorporation experiments. Table 1 summarizes the results of five DNA synthesis experiments. In the absence of FBS, TPA did not stimulate the incorporation of [^{3}H]thymidine into DNA. In complete medium, TPA decreased DNA synthesis by 32% in HT-29 M6 cells.

Therefore, the confluent appearance of the cell culture treated with TPA in Fig. 1C was not a consequence of increased cell proliferation in the presence of TPA, but of cell scattering. Actually, TPA-treated HT-29 M6 cultures showed a lower confluence density (3.0 (± 0.5)×10^5 cells/cm^2) than control cultures (6.5 (± 0.2)×10^5 cells/cm^2) (average ± s.d. of three independent experiments). However, saturation densities were similar in both cultures (approximately 7×10^5 cells/cm^2), since TPA-treated cells continued to proliferate after reaching confluence, unlike control HT-29 M6 cells.

Scattering induced by TPA is associated with a loss in intercellular contacts and an increased affinity for the cell matrix

Next, we studied in greater detail the changes promoted by TPA, focusing on the loss of cell-to-cell contacts and the alterations in the affinity for cell matrix, which is the presumed reason for the TPA-induced scattered and flatter shape.

To study the cell-to-cell interactions, homotypic aggregation assays were performed. Preconfluent HT-29 M6 cells were mildly trypsinized in the presence of Ca^{2+}, washed, resuspended in complete DME medium and rotated for 2 hours at 37°C. In control cells, the number of single cells decreased by 40 (± 5)% after a 2-hour incubation; in cells pretreated with TPA for 15 hours this decrease was only 16 (± 2)%. No effect of TPA was observed when this phorbol ester was added during the 2-hour incubation (single cells decreased by 42 (± 4)%. A significative loss of homotypic aggregation was not seen unless cells were pretreated with TPA for at least 6 hours (Fig. 2).

Since E-cadherin plays a major role in homotypic aggregation of epithelial cells (Takeichi, 1990), the expression of this molecule was studied. HT-29 M6 cells were grown on coverslips and, at different times after TPA addition, fixed, permeabilized and incubated with an antibody detecting E-cadherin. Control HT-29 M6 cells grew as compact colonies that were labelled by the anti-E-cadherin antibody in the cell-to-cell contacts (Fig. 3a). After 4 hours of incubation with TPA, some cells at the edges of the colonies showed a flatter aspect with a more diffuse staining, although no appreciable change in the cellular levels of E-cadherin was detected (Fig. 3b). After 8 hours, a considerable proportion of cells presented a scattered mor-

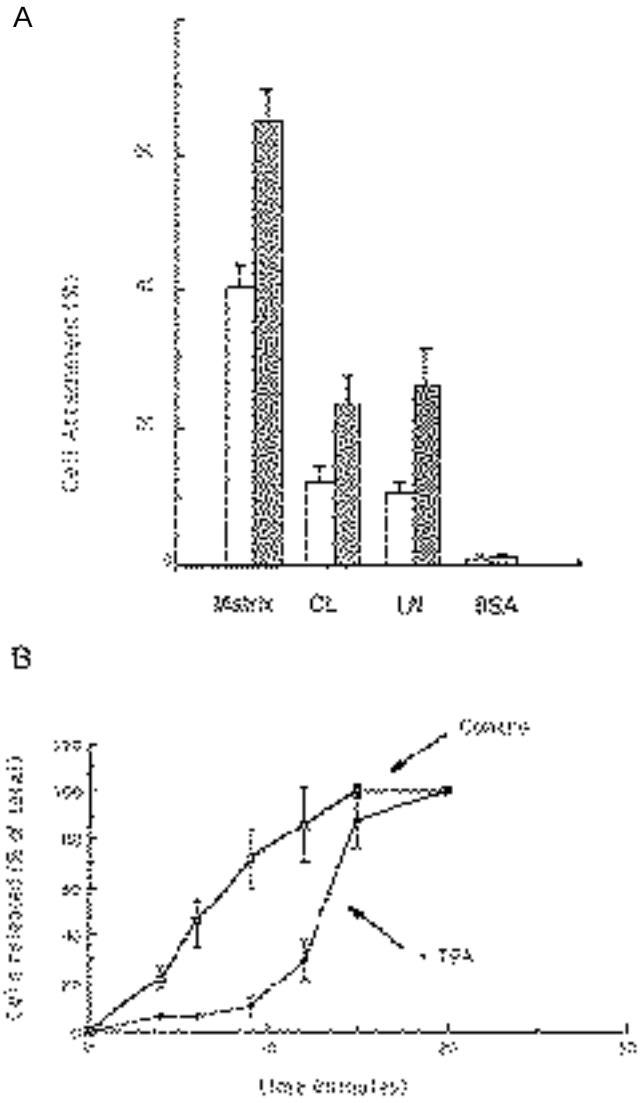


Fig. 4. TPA increases the attachment of HT-29 M6 cells to cellular matrix proteins and induces a higher resistance to trypsin 'lift off'. (A) Trypsinized labelled cells were resuspended in DMEM (plus 10% FBS) with the addition of 200 nM TPA when indicated and plated into 24-well plates previously coated with one of the extracellular matrix proteins (CL, collagen; LN, laminin; BSA, bovine serum albumin) or containing the total HT-29 M6 cellular matrix. After 60 minutes at 37°C, non-attached cells were washed away with PBS, and attached cells were lysed with SDS (1%) and the supernatant was counted. Attachment data shown in A represent the mean ± standard deviations of three determinations. (B) TPA promotes an increased resistance to trypsin 'lift off'. Labelled cells were incubated in control medium or in medium supplemented with TPA (200 nM) for 15 hours and a controlled trypsin digestion was performed as described in Materials and Methods. The kinetics of cell release to the medium is represented in (B). The average ± s.d. of the results of four experiments is shown.

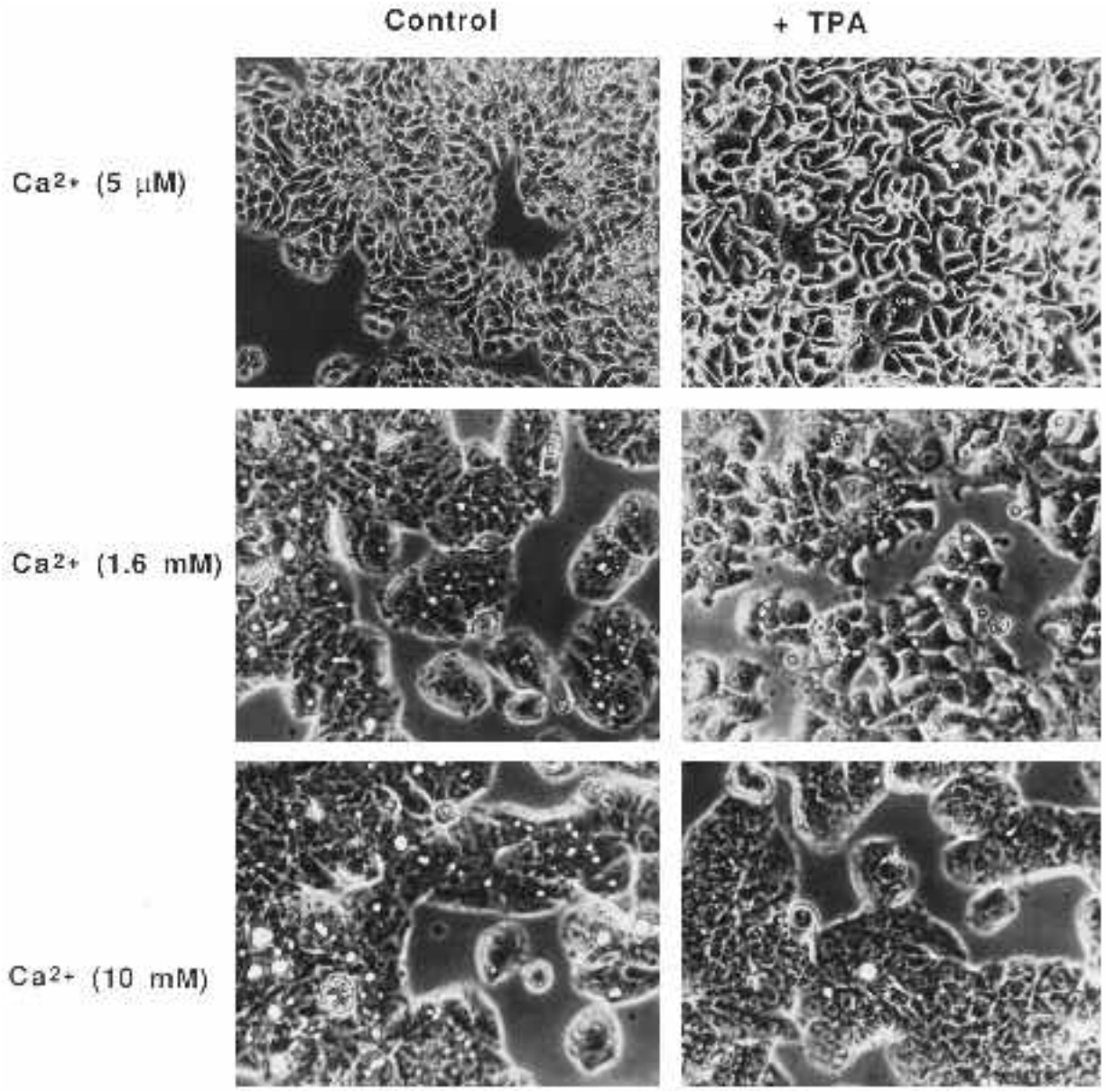


Fig. 5. Extracellular Ca^{2+} regulates TPA-induced HT-29 M6 cell scattering. Cells were incubated in complete medium (DMEM plus 10% FBS) (middle panels) or in complete medium supplemented with EGTA (2 mM) (upper panels) or with $CaCl_2$ (8.5 mM) (lower panels). Considering that the concentration of Ca^{2+} in complete medium is 1.6 mM and that the apparent association constant of the EGTA-Ca^{2+} complex is 2×10^6 M^{-1}, the final concentrations of free Ca^{2+} were 5 μM (upper), 1.6 mM (middle) and 10 mM (lower). After 1 hour of incubation, TPA (200 nM) was added (right panels) and a picture (×320 magnification, upper; ×350, middle and lower) was taken 6 hours later. The figure shows representative cell fields of one of the four experiments performed, which gave similar results.

phology and very few cell-to-cell contacts were observed; these cells still showed a faint staining with E-cadherin antibody, which was considerably reduced with respect to the control (Fig. 3c). After longer periods of incubation, E-cadherin reactivity became almost undetectable in these cells (Fig. 3d). This down-modulation of the total levels of E-cadherin was also detected by immunoblotting; as shown in Fig. 3e, the decrease in the amount of 120 kDa polypeptide, detected at 8 hours of incubation with TPA, was practically complete after 24 hours of treatment with this phorbol ester.

TPA also induced an alteration in the ability of HT-29 M6 cells to attach to different extracellular matrix components. TPA addition quickly increased the rate of attachment of HT-29 M6 cells to a complete matrix (prepared as described in Materials and Methods) by 55% (Fig. 4A). The attachment to components of the matrix was also studied: TPA induced 92% and 135% increases in the attachment to collagen and laminin matrices, respectively.

Similar effects of TPA have been reported in NIH 3T3 fibroblasts (Kato et al., 1988). Probably as a consequence of this increased affinity for the cell matrix, cells treated with TPA adhered better to the plate and showed a higher resistance to trypsin 'lift off' than control cells (Fig. 4B).

This increased resistance to trypsin 'lift off' was correlated with the appearance of the morphological changes induced by TPA; the maximal stimulation in this assay required 12-15 hours of incubation with TPA, which is also the time required to induce the complete scattering of HT-29 M6 colonies (see above).

An increase in extracellular Ca^{2+} blocks the TPA-induced scattering of HT-29 M6 cells

It has been reported that extracellular Ca^{2+} concentration modulates the interactions of cells with their substrata (Kirchhofer et al., 1991; Grzesiak et al., 1992) as well as cell-to-cell adhesion (Takeichi, 1990). The influence of extracellular Ca^{2+} concentration on cell scattering was examined. As shown in Fig. 5, the characteristic scatter effect of TPA on HT-29 M6 cells (Fig. 5, middle panels) was not observed when the medium was supplemented with $CaCl_2$ to a final Ca^{2+} concentration of 10 mM (Fig. 5, lower panels). When cells were grown on medium depleted of Ca^{2+}, HT-29 M6 colonies lost their compact aspect; even in these conditions the addition of TPA induced a flatter and more scattered phenotype (Fig. 5, upper panels). The inhibitory effect of Ca^{2+} on TPA-induced scattering was reflected in the cell confluence density. Cells cultured in the presence of 10 mM Ca^{2+} showed a confluence density of 7.1 (± 0.4)×10^5 cells/cm^2 (average ± range of two experiments), not significantly different from the value obtained in control conditions (6.5 (± 0.2)×10^5 cells/cm^2; see above). In contrast, confluence densities in TPA-treated cultures were 2.0 (± 0.5) in medium containing 1.5 mM Ca^{2+} and 6.0 (± 0.2) in medium containing 10 mM Ca^{2+} (×10^5 cells/cm^2, average ± range of three and two experiments, respectively). Cell viability was not affected by a 3-day incubation in high Ca^{2+} medium.

Table 2. Effects of extracellular Ca^{2+} on HT-29 M6 cell-cell adhesion and cell attachment to matrix: influence on TPA-induced scattering

Experimental conditions	Scattering*	Cell-cell adhesion (% of aggregated cells)†	Cells released by trypsin (%)‡
Control§	−	40 ± 5	69 ± 10
TPA	+	16 ± 2	24 ± 8
Ca^{2+} (10 mM)	−	68 ± 10	72 ± 11
Ca^{2+} (10 mM) + TPA	−	70 ± 12	9 ± 7

*Scattering was considered positive when cell spreading was detected in the cell clusters examined after 8 hours of incubation with TPA in the conditions specified. The morphology of the cultures is shown in Fig. 5.

†Cell-cell adhesion was measured as described in Materials and Methods. Cells were incubated, under the experimental conditions described for 20 hours. Cultures were trypsinized and single cells were counted immediately and after a 2 hour incubation; the decrease in this number reflects cell-cell adhesion (see Fig. 2). The results show the average ± s.d. of two experiments performed.

‡Labelled cells were incubated with TPA for 8 hours, washed, and incubated with trypsin as described. After 10 minutes, the percentage of cells released was determined. Average ± s.d. of the results of two experiments are shown.

§The concentration of Ca^{2+} in the control medium was 1.6 mM. In the samples described, $CaCl_2$ was added to obtain a final concentration of 10 mM. TPA was always used at a final concentration of 200 nM.

The influence of Ca^{2+} on the two scatter-associated events (decreased cell aggregation and increased substratum attachment) was also investigated. High [Ca^{2+}] (10 mM) significantly enhanced cell-to-cell aggregation and prevented the effects of a 20-hour incubation with TPA (Table 2). The effects of this phorbol ester on E-cadherin down-modulation were also prevented. As shown in Fig. 6, in the presence of high concentrations of extracellular Ca^{2+}, very little, if any, decrease in E-cadherin activity was observed after TPA addition. This result is probably associated with the fact that very few of the cells from the HT-29 M6 colonies scattered; the colonies retained their compact morphology in the presence of TPA only when the culture medium was supplemented with 10 mM Ca^{2+} (Fig. 6). However, TPA-increased cell attachment to the plate, measured as resistance to trypsin 'lift off', was not apreciably modified by Ca^{2+} (Table 2). This increase in the concentration of Ca^{2+} did not affect the cellular attachment to complete matrix, collagen or laminin, in either the presence or the absence of TPA (data not shown).

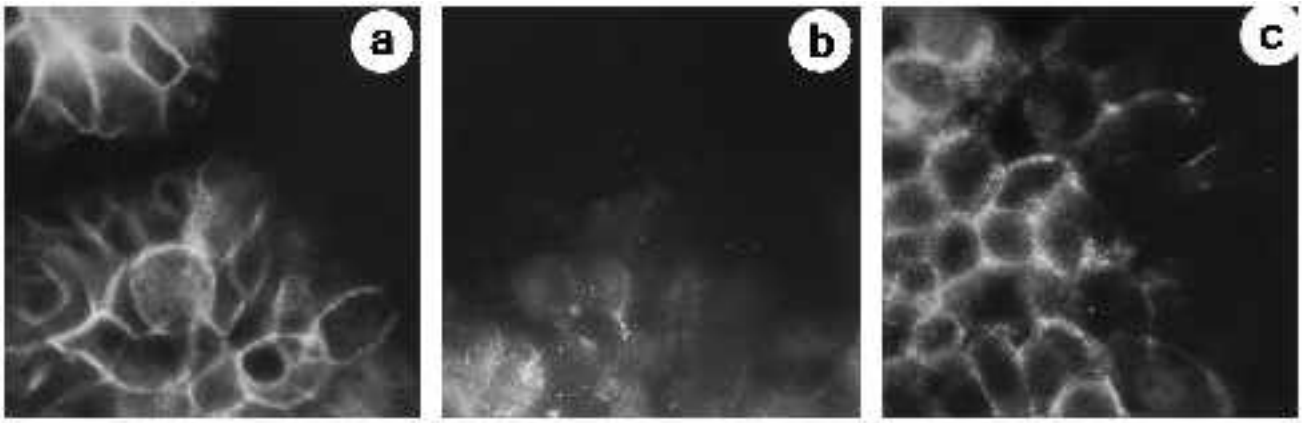


Fig. 6. Extracellular Ca^{2+} controls TPA-induced E-cadherin down modulation. Subconfluent HT-29 M6 cells were incubated in complete medium supplemented with TPA (200 nM) (b) or TPA and $CaCl_2$ (10 mM final concentration) (c) for 20 hours. Immunofluorescence studies were performed using anti E-cadherin mAb DECMA-1 as described for Fig. 4A. (a) corresponds to the E-cadherin staining of control cells incubated in complete medium without additions. The result shown is from a representative experiment of the two performed.

DISCUSSION

The results presented here show that PK-C activation by TPA promoted quick and reproducible morphological changes in HT-29 M6 cultures. These changes (colony dispersion, lower cell confluence density, fibroblastic cell shape) have been usually defined as 'scattering' (Rosen et al., 1991b). Therefore, it is possible to conclude that TPA exerts a 'scatter' activity on the HT-29 M6 cells. The main scatter factor of epithelial cells has recently been shown to be the hepatocyte growth factor (HGF) (Rosen et al., 1991b; Cooper, 1992), the ligand for the *met* proto-oncogene (Bottaro et al., 1991). It is, thus, possible that TPA might be inducing scattering of HT-29 M6 cells through changes in the secretion of HGF or in the activity of the c-*met* protein kinase; these two possibilities are being studied in our laboratory.

The scatter activity of TPA was associated with a loss of intercellular contacts and an increase in the cellular attachment to the matrix. As described for NIH fibroblasts (Kato et al., 1989), TPA increased the attachment of the HT-29 M6 epithelial cells to the matrix components, collagen and laminin. Collagen and laminin interact with different members of the integrin family, all composed of the same β subunit (β_1) and different α subunits (Hynes, 1992). Changes in collagen affinity of $\alpha\beta_1$ integrins after TPA addition are being studied using chromatography in collagen-Sepharose columns.

HT-29 M6 cell scattering induced by TPA was blocked when cells were cultured in medium supplemented with Ca^{2+} (10 mM final concentration). In these conditions colony dispersion was not observed and confluence cell density was not significantly altered by TPA. The main effect of the rise in extracellular Ca^{2+} was to increase the cell-cell adhesion, measured in the presence or absence of TPA. However, the attachment to cell matrix was not altered. These results suggest that a decrease in the cell-cell adhesion is a critical event in the process of scattering; when cell-cell adhesion was artificially enhanced, the process of colony dispersion was blocked.

The TPA-induced loss of intercellular aggregation was correlated with a decrease in the total levels of E-cadherin present in the cell. The expression of this molecule has been reported to be very low in poorly differentiated colorectal cancers, as well as other malignant epithelial tumors (Shimoyama et al., 1989; Shiozaki et al., 1991). Our results suggest that the down-regulation of E-cadherin levels induced by TPA could be an important event in the process of scattering. However, at early times of treatment with TPA, scattered cells could be observed with detectable levels of membrane cadherin. It remains to be determined whether this cadherin is functionally active or not and if the interaction of this protein with some other component of the cell-cell adhesion system, like α-catenin (Nagafuchi et al., 1991), is disturbed by TPA. In any case, HT-29 M6 cells provide a system well suited for studying the loss of E-cadherin, an event presumably related to the increased tumorigenic properties of some malignant human cells.

Results from our laboratory have demonstrated that, in addition to its effect on cell scattering, TPA blocks the differentiation of HT-29 M6 cells (García de Herreros et al., 1993). This inhibition of cell differentiation might be a consequence of the alteration in cell-to-cell contacts caused by this phorbol ester, as correct cell-cell interactions are essential for the process of differentiation (Sztul et al., 1987; Pignatelli et al., 1992). In the HT-29 M6 cells, decreased cell-to-cell contacts are observed after TPA addition not only to preconfluent cells but also to confluent, differentiated cells (data not shown). It is, thus, possible that the tumour-promoting properties of TPA in colon cells are related to its ability to promote cell colony scattering, a process already related to increased invasiveness of epithelial cells (Weidner et al., 1990). Several authors have suggested a role for other activators of PK-C, commonly present in the bowel contents (faecal diglycerides, bile acids) in the development and/or progression of colon cancer (Friedman et al., 1984, 1989; Craven et al., 1987). Experimental studies have provided evidence that a high fat diet enhances colon cancer by acting at the stage of tumour promotion (Weinstein, 1991). Although the precise mechanism is not known, it has been suugested that this type of diet would increase the level of active diglycerides in the intestine (Morotomi et al., 1990). Interestingly, the stimulus to colonic epithelial proliferation induced by a high fat diet is prevented by a high Ca^{2+} intake (Lipkin and Newmark, 1985; Lipkin, 1988). It is possible that high Ca^{2+} intake would affect the action of this and other tumour promoters through its ability to interfere with the maintenance of normal epithelial cell contacts, as shown here for the HT-29 M6 cells.

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