The protein kinase C activator TPA modulates cellular levels and distribution of E-cadherin in HT-29 human intestinal epithelial cells

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Abstract The importance of E-cadherin protein in the establishment and maintenance of homotypic contacts in epithelial cells has already been determined. We report here that the association of E-cadherin to cytoskeleton, required for the functionality of this protein, increases progressively after seeding; in HT-29 M6 cells 4–5 days were required for detecting most of E-cadherin in the Triton X-100-insoluble (cytoskeleton-associated) fraction. The phorbol ester TPA differently affected E-cadherin levels in HT-29 M6 cells; at day 2–3, when most E-cadherin was found not-associated to the cytoskeleton, very important decreases (90%) in the total levels of this protein were detected as soon as 6 h after the addition of this compound. However, on later days (day 5), the predominant effect by 6 h was a translocation from the Triton-insoluble to the soluble fraction. The E-cadherin-associated proteins α-catenin and β-catenin were not significantly affected by treatment with TPA.

Key words: Phorbol ester; Protein kinase C; E-cadherin; Catenins; HT-29 cell

1. Introduction

Cadherins are a family of transmembrane proteins implicated in calcium-dependent cell–cell adhesion. The activity of these proteins has been reported to be necessary for the correct development of the embryo, as well as the morphogenesis of several tissues [1,2]. One of the members of this family, E-cadherin is a protein that plays a main role in the establishment of cell–cell adhesion in epithelial cells [3,4]. Downregulation of E-cadherin in transformed cell lines has been associated with de-differentiation and acquisition of the ability to invade, suggesting a possible role of this protein as a tumor supressor [5]. The activity of E-cadherin requires interactions at both sides of the membrane. On the extracellular side, different domains have been implicated in Ca^{2+} binding and homophilic interactions [2]. On the cytoplasmic side, it has been shown that E-cadherin binds several proteins: α-catenin, β-catenin, plakoglobin and p120, involved in linking E-cadherin to the cytoskeleton and modulating its activity [6,7]. From these proteins, interaction with α-catenin has been shown to be sufficient to promote association of E-cadherin to the cytoskeleton and to induce polarized cell–cell association in several lines of dispersed cells [8,9].

The phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) a potent and specific activator of protein kinase C (PK-C) [10] promotes a remarkable change in the phenotype of HT-29 M6 intestinal cells. TPA induces the scattering of HT-29 M6 colonies; it decreases cell–cell aggregation and enhances the cellular attachment to matrix substrates (collagen, laminin) [11]. The reported decrease in cell-to-cell adhesion was accompanied by a fast change in the cellular localization of E-cadherin and a slower downregulation of this protein, as it was evidenced by immunofluorescence [11]. In this article we have studied with more detail the effects of TPA over the distribution and levels of E-cadherin in HT-29 M6 by cell fractionation and immunoblotting.

2. Materials and methods

2.1. Cell culture

The HT-29 M6 cell line, a subpopulation of the HT-29 cells, isolated by selection with 10^{-6} methotrexate [12,13], was supplied by Dr. Alain Zweibaum (INSERM, Villejuif, France). Cells were seeded at 2×10^4 cells/cm^2 and 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; culture media was changed every other day to avoid nutrient depletion. Cells were used between passages 40 and 60.

2.2. Cell extraction and immunoblotting

Soluble and cytoskeletal fractions were prepared essentially as described by Nelson and coworkers [14]. Cells were rinsed in TBS plus 1 mM $CaCl_2$ and homogenized in CSK buffer (50 mM NaCl, 10 mM PIPES, pH 6.8, 3 mM $MgCl_2$, 0.5% Triton X-100, 300 mM sucrose, 1 mM phenylmethyl sulphonyl fluoride, 10 μg/ml leupeptin, 1 mM orthovanadate, 20 μM phenylarsine oxide) for 10 min at 4°C with gentle rocking. After centrifugation in a microfuge for 10 min at 4°C supernatant constituted the Triton-soluble fraction. Pellet was triturated in SDS buffer (20 mM Tris, pH 7.5, 5 mM EDTA, 2.5 mM EGTA, 1% SDS) and boiled at 100°C for 10 min. After centrifugation for 10 min in a microfuge, supernatant constituted the Triton-insoluble fraction. This fraction usually contained 5-fold less protein than the Triton-soluble fraction. Cell fractions were separated by SDS-PAGE, transferred electrophoretically to nitrocellulose and incubated for 1 h with mAbs anti-E-cadherin HECD-1, a kind gift of Dr. A. Cano Universidad Autónoma de Madrid), anti-α-catenin or anti-β-catenin (both from Transduction Labs, Lexington, KY) at a concentration of 1 μg/ml. After washing, membranes were incubated with peroxidase-conjugated goat anti-mouse antibodies (Dakopatts, Copenhagen, Denmark), and reacting antigens were visualized using ECL detection reagents (Amersham).

3. Results

3.1. Association to cytoskeleton of E-cadherin depends on the day of culture

HT-29 M6 cells grow in compact colonies that after confluence originate a cell monolayer with well-defined tight contacts. E-cadherin-mediated cell adhesion constitutes the first step in order to produce these structures. It has been reported that functionality of E-cadherin requires its association to the cytoskeleton [15]; therefore soluble and cytoskeletal fractions were prepared following the method of Nelson and coworkers

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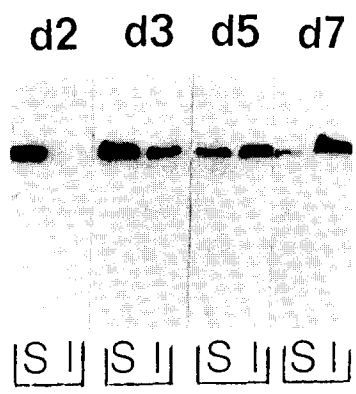

Fig. 1. Association to cytoskeleton of E-cadherin in HT-29 M6 progressively increases after seeding. Cells were seeded as described in section 2 and cultured in DME medium supplemented with FBS (10%) for the indicated days (2–7). Cell extracts were prepared and E-cadherin levels were determined by Western blot with mAb HECD-1. 35 μg of the Triton-soluble (S) and 7 μg of the Triton-insoluble (I) fractions were analyzed in this study. Only a major band of 120 kDa, corresponding to E-cadherin, was detected with this antibody.

[14] from HT-29 M6 cells at different times after seeding. This method, used succesfully in MDCK cells [14,16], is based in the high resistance to solubilization in nonionic detergents that present cytoskeleton-associated proteins. As shown in Fig. 1, at day 2 after seeding most of E-cadherin was detected in the Triton-soluble fraction, reflecting the fact of a very low association to the cytoskeleton (lower than 10–15% in three different experiments). The proportion of E-cadherin present in the Triton-insoluble fraction increased slowly after seeding, reaching a value of 80–90% at day 7 (four different experiments). Our experiments indicate that in HT-29 M6 cells 4–5 days are required for E-cadherin to be able to associate to the cytoskeleton. No significant changes in the total cellular amount of E-cadherin were detected at the different times studied.

3.2. TPA affects E-cadherin distribution and cellular levels

We have previously reported that short treatments (2–3 h) with the phorbol ester TPA induce phenotypic changes in HT-29 M6 cultures, characterized by the scattering of the cell colonies and the acquisition of a fibroblast-like morphology [11]. In this study, we observed a change in the distribution of E-cadherin by immunofluorescence, with a loss of reactivity in the cellular membrane. Levels and distribution of cadherin after addition of TPA were studied in greater detail and representative experiments are shown in Fig. 2. A kinetic study with cells at different days after seeding reflected a combined effect of the phorbol ester both on total levels and distribution of this protein. At day 2, when more than 90% of E-cadherin was found not-associated to the cytoskeleton, a rapid downregulation of this molecule was observed (85% by 6 h). Similar results

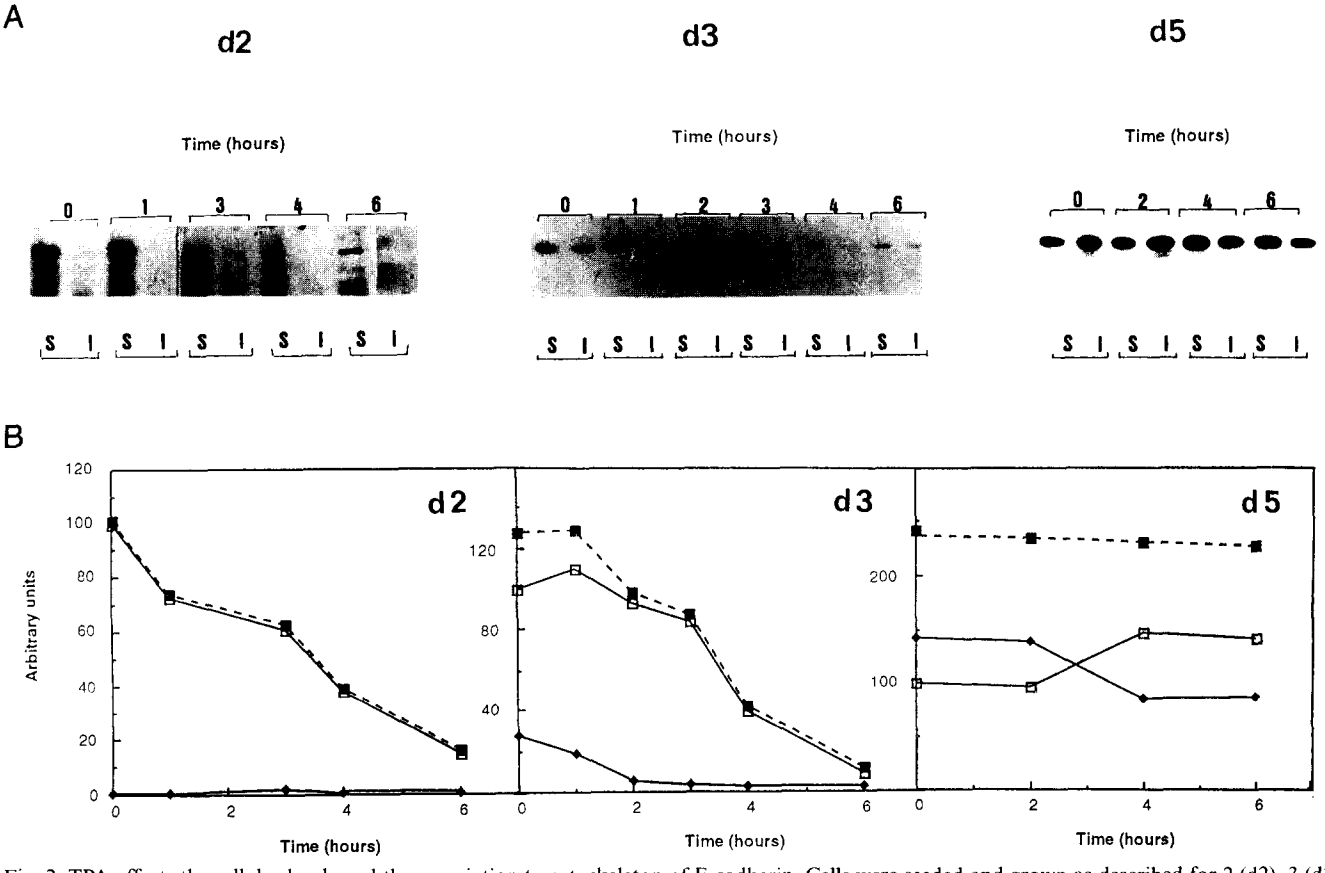

Fig. 2. TPA affects the cellular levels and the association to cytoskeleton of E-cadherin. Cells were seeded and grown as described for 2 (d2), 3 (d3) or 5 (d5) days; TPA (200 nM) was added and extracts were prepared at the indicated times after the addition of this phorbol ester. (A) E-Cadherin was analyzed by Western blot as described in the Triton-soluble (S) and insoluble (I) fractions. (B) Autoradiograms were scanned and E-cadherin levels were represented at different times after addition of TPA. □ = Triton-soluble fraction; ◆ = Triton-insoluble fraction; ■ = total cellular levels. An arbitrary value of 100 was assigned to the value of E-cadherin present in the Triton-soluble fraction before the addition of TPA.

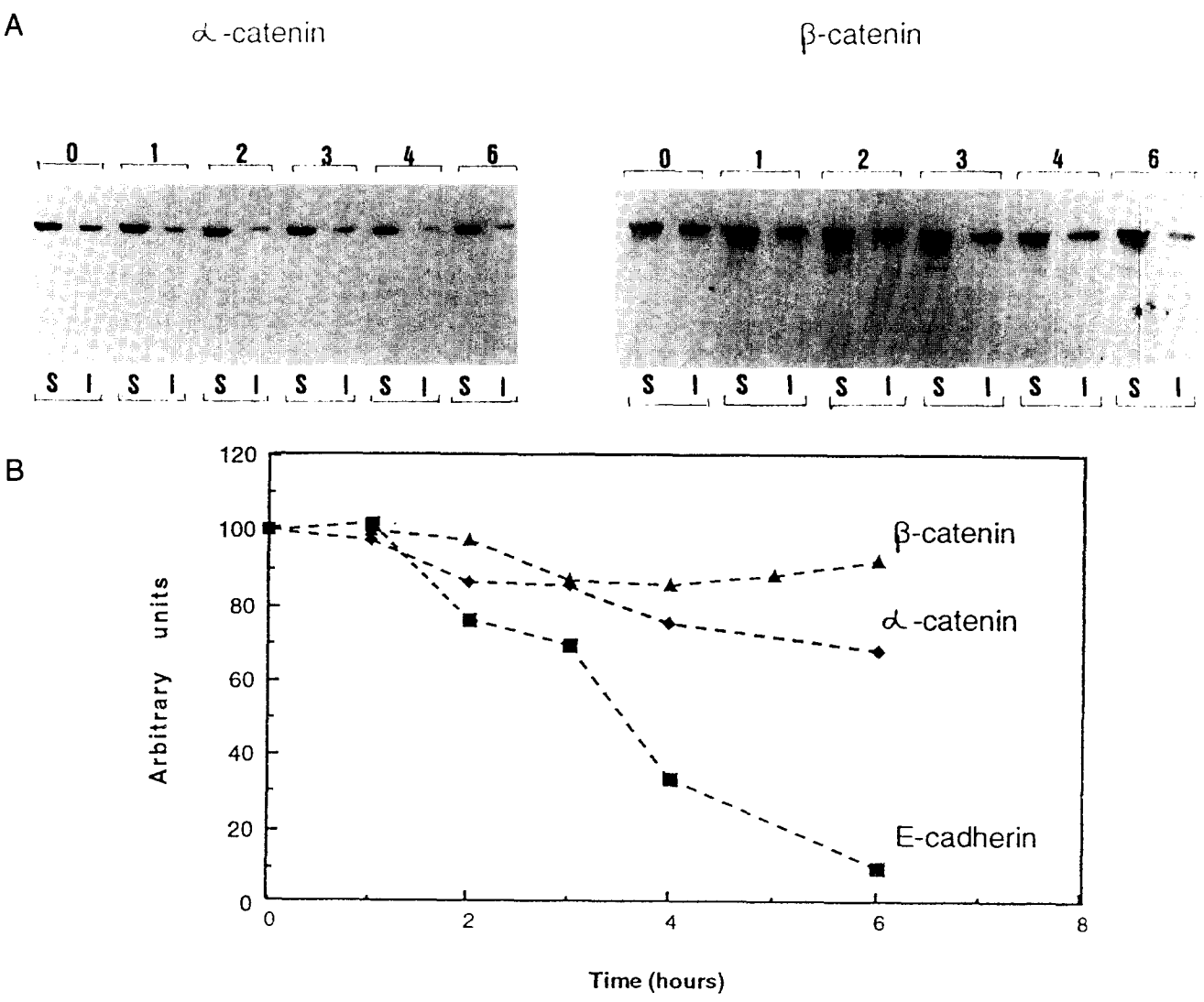

Fig. 3. TPA does not modify the cellular levels or the distribution of α and β-catenins. (A) Same extracts obtained from cells at day 3 after seeding were analyzed by Western blot using mAbs against α- or β-catenin. Only two bands of molecular weights 105 kDa (corresponding to α-catenin) and 95 kDa (corresponding to β-catenin) were detected with these antibodies. (B) Autoradiograms were scanned and total cellular levels of E-cadherin (taken from Fig. 2) (■), α-catenin (◆), and β-catenin (▲) were represented at different times after addition of TPA. A value of 100 was arbitrarily taken as the initial amount of these three proteins.

were obtained at day 3 with a slight difference; the disappearance of insoluble E-cadherin was observed before any decrease of the soluble protein, suggesting a possible translocation of E-cadherin from one fraction to the other. This translocation was clearly observed at day 5, where 60% of the protein was detected in the insoluble fraction; after 4 h of incubation the amount of insoluble E-cadherin had been decreased by 37%. In this case no significant decreases in the total amount of E-cadherin were detected by 6 h; after 15 h fell by 30–50% (three experiments) (not shown).

3.3. *Downregulation of E-cadherin by TPA is not accompanied by changes in the levels or distribution of α- or β-catenin*

Catenins are proteins that bind to E-cadherin and are needed for its activity [6]. Binding to α-catenin has been demonstrated to be sufficient to link E-cadherin to the cytoskeleton [8,9]; the role of β-catenin has not been established yet although it has been recently proposed that its binding could reduce E-cadherin activity [17]. Using mAbs specific for these proteins we determined if the changes induced by TPA in the levels of E-cadherin were accompanied by alterations of these proteins. Fig. 3 shows that the levels of α- or β-catenin did not change significantly, either in the Triton-soluble or insoluble fraction, after addition of TPA, contrarily to E-cadherin. The distribution of these two proteins was not affected either by this treatment; the proportion of α- or β-catenin in the cytoskeletal fraction (32 and 40%, respectively at day 3) remained unaltered after 6 hours of incubation with TPA.

4. Discussion

In this article we demonstrate that TPA modifies the E-cadherin levels in HT-2 M6 cells. The effect of this compound is dependent on the initial localization of E-cadherin; when the protein is soluble (early cultures) the phorbol ester causes a rapid depletion (80–90% at 6 h); however, when it is associated

to the cytoskeleton (late cultures) the most evident effect is the translocation to the soluble fraction and, only later, does a partial downregulation occur. In addition to these kinetic differences, the loss in E-cadherin was more complete in early than in late cultures of HT-29 M6 cells.

Very few examples of downmodulation of E-cadherin have been reported so far. It has been shown that E-cadherin is regulated by the ERBB2 proto-oncogene [18], an homologous of EGF receptor kinase. Overexpression of ERBB2 results in the transcriptional inhibition of the E-cadherin gene; inversely, addition to cells overexpressing ERBB2 of an antibody that blocks ERBB2 phosphorylation and signal transduction through the GRB2/Sem5 protein produces an increase in E-cadherin transcription [18]. These evidences point to a signal transduction pathway involving tyrosine kinases as an important element in the control of the levels of E-cadherin. We show in this article that the cellular content of this protein can also be controlled by another pathway initiated by activation of PK-C. It is likely that both pathways cross-talk, since we (A.S. and A.G.H., unpublished observations) and others [19] have shown stimulation of the tyrosine kinase c-src by PK-C activators in different HT-29 subpopulations. Additional evidence from our lab, showing increased levels of E-cadherin in cells treated with the tyrosine kinase inhibitor herbimycin (A.S. and A.G.H., unpublished observations), further support an involvement of these protein kinases in the control of E-cadherin levels in HT-29 cells.

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