Int. J. Cancer: **107,** 715–720 (2003)

Publication of the International Union Against Cancer

CYCLIC AMP INHIBITS TGFβ1-INDUCED CELL-SCATTERING AND INVASIVENESS IN MURINE-TRANSFORMED KERATINOCYTES

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Mouse-transformed keratinocytes cultured in the presence of transforming growth factor β1 (TGF-β1) acquire an array of morphologic and functional properties that give rise to a migratory phenotype that expresses mesenchymal molecular markers. This cellular conversion involves activation of the Ras-ERK pathway, enhancement of urokinase (uPA) and matrix metalloproteinase-9 (MMP-9) expression and induction of invasiveness. In our present work, we demonstrate that cAMP and forskolin are able to prevent the expression of these mesenchymal properties, probably due to blockade of the Ras-ERK pathway. Our results also show that cAMP and forskolin are able to abolish the TGF-β1-induced reorganization of the actin cytoskeleton that is characteristic of the mesenchymal phenotype and also inhibits the disruption of the E-cadherin cell to cell interactions. The latter responses seem to depend on the activity of protein kinase A, as demonstrated by the activation of the Ras-ERK pathway by specific protein kinase A inhibitors.

Key words: *cAMP; urokinase; matrix metalloproteinases; E-cadherin; epithelial-mesenchymal transdifferentiation*

Previous reports have shown that in transformed keratinocytes transforming growth factor β1 (TGF-β1) induces an epithelial-mesenchymal transition (EMT) associated with the development of highly invasive and metastatic spindle cell carcinoma.[1] We also demonstrated that this conversion proceeds through the activation of the Ras-ERK pathway and involves enhancement of uPA and MMP-9 expression and induction of invasiveness.[2,3]

The EMT is characterized by a set of transient phenotypic changes often associated with the acquisition of migratory properties by cancer cells and provides a means for cancer propagation through the organism.[4] The most conspicuous cellular EMT events can be grouped into the following categories: *(i)* cell scattering, which involves disruption of cell-cell association and further acquisition of cell motility; *(ii)* the production of tumoral matrix-degrading proteases; and *(iii)* remodeling of the actin cytoskeleton that provides the molecular framework for cellular motility.[5] Cell scattering is the only feature always present in EMT[6] and involves E-cadherin (E-cad) down-modulation. E-cad, a homotypic cell-to-cell interaction molecule that is expressed in epithelial cells, can act as a tumor suppressor that contributes to preserving cellular architecture and polarization by its association with F-actin through α and β catenin.[7–9]

It has been proposed that the second messenger cAMP can serve as an anticancer agent by acting as an inhibitor of the G1/S transition in the cell cycle or by inducing cell cycle-specific apoptosis.[10–12] Moreover, recent data provide evidence that the modulation of intracellular cAMP levels can also regulate some cellular properties involved in cell motility.[13,14] At the signaling level, it has been demonstrated that an active cross-talk between the cAMP and the Ras-ERK signaling pathways allows cAMP to inhibit or activate ERK depending on the cell type and the cellular context.[15] cAMP-activated protein kinase A (PKA) blocks Ras-dependent activation of ERK by 2 main mechanisms: *(i)* blocking Raf-1 activation by direct Ser phosphorylation (for example, phosphorylation of Ser 43 inhibits the ability of Raf-1 to bind GTP-loaded Ras)[16] and *(ii)* activating the Rap1 GTPase,[17] an inhibitor of ERK in rat fibroblasts and a potential mediator of the inhibition of Ras-dependent signaling to ERKs by cAMP.

In our present work, we demonstrate for the first time that TGF-β1-mediated activation of Ras-ERK in transformed keratinocytes is blocked by pretreatment with cAMP and forskolin. Our results show the TGF-β1-dependent acquisition of migratory-invasive capabilities, including growth factor-induced expression of uPA and MMP-9 and the delocalization of E-cad, were blocked by either cAMP or forskolin. The reversion of the malignant phenotype by cAMP opens a wide range of possibilities for the regulation of the invasive properties of cancer cells.

MATERIAL AND METHODS

Cell cultures and treatment conditions

The PDV cell line used in our study has been described elsewhere.[1] Cells were cultured in Ham's F-12 medium supplemented with amino acids and vitamins (Gibco, Rockville MD), 10% fetal bovine serum (FBS; Gibco) and 80 μg/ml Gentamycin. Cells were maintained at 37°C in a 5% CO_2 humidified atmosphere. For TGF-β1 treatment, PDV cells were incubated with human recombinant TGF-β1 (Calbiochem-Novabiochem, La Jolla, CA) at a final concentration of 10 ng/ml for the indicated times. MAPK inhibitor PD98059, PKA inhibitor H89 and 14-22 peptide (PKAI 14-22) were from Calbiochem and were added 30 min before the addition of TGF-β1 or dbcAMP. In the experiments designed to evaluate the effect of dbcAMP and forskolin (Sigma, St. Louis MO), these drugs were added to the culture medium 30 min before TGF-β1 supplementation or stimulation at concentrations of 1 mM and 10 μM, respectively.

Cell fluorescence analysis

Indirect immunofluorescence assays. PDV cells were grown on glass coverslips, fixed with 4% p-formaldehyde in PBS and stained with the anti E-cadherin rat monoclonal antibody ECCD-2,[18] at a dilution of 1:100. The secondary antibody was FITC-labeled anti-rat IgG (Sigma).

Fluorescent actin staining. Cells were grown and fixed in a manner similar to that described above and permeabilized with 0.1% Triton X-100 in PBS for 2 min. F-actin was visualized using Phalloidin-Alexa Fluor (Molecular Probes, Eugene, OR) with a microscope equipped for epi-fluorescence.

Western blot and activation of ERK1,2 and Smad2

Semi-confluent cultures (3×10^5 cells) of PDV cells, seeded in 6-well plates, were pretreated with cAMP or forskolin and stimulated with TGF-β1 at indicated times prior to a 4 hr incubation in serum-free Ham's F-12 medium. Thereafter, cells were lysed with 300 μl of lysis buffer (100 mM phosphate buffer, pH 7.2, 1% NP-40, 150 mM NaCl, 5 mM EDTA, 10 mM β-glycerophosphate,

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Received 12 February 2003; Revised 7 May, 25 June 2003; Accepted 4 July 2003

DOI 10.1002/ijc.11457

1 mM sodium orthovanadate, 5 mM NaF, 10 μg/ml leupeptin, 1 μg/ml pepstatin and 1 mM PMSF). After boiling, lysates were separated by SDS-PAGE on a 10% polyacrylamide gel. Gels were blotted onto PVDF membranes (BioRad, Hercules, CA). ERK 1,2 and Smad 2 activation was assessed as previously described[3] using an antibody that recognized the phosphorylated form of ERK1,2 (Santa Cruz Biotechnology, Santa Cruz, CA) or Smad2 (Upstate, Lake Placid, NY) . Duplicate membranes were probed with antibody to the unphosphorylated forms of ERK1,2 or Smad2 (Santa Cruz) to verify equal loading. When required, PKA inhibitors were added at 10 μM 30 min before the addition of dbcAMP.

Zimogram for gelatinase and u-PA

MMP and uPA activities were assayed in aliquots of serum-free media conditioned by 10^5 PDV cells cultured for 24 hr in the presence or absence of TGF-β1 and/or dbcAMP and forskolin and evaluated by SDS-PAGE zymograms containing 1 mg of gelatin per ml of polyacrylamide gel, as previously reported.[3] uPA activity was assessed in the same samples of conditioned media by a reverse diffusion assay.[19]

Invasion and migration assays

The invasive capacity of PDV cells was evaluated using Transwell chambers (Costar, Cambridge MA) with 8.0 μm-pore polycarbonate filters coated with 30 μg of Matrigel in the upper side of the filter (Collaborative Research, Bedford, MA). Cells resuspended in serum-free medium were seeded on the upper compartment of the well chamber and incubated for 72 hr in the presence or in the absence of 10 ng/ml TGF-β1 on both sides of the filter. After incubation, the number of cells present in each compartment was determined with MTT (Sigma), as described.[20] Invasive capacity was expressed as the percentage of cells in the lower side of the filter relative to total number of cells. Cellular motility was assessed by an *in vitro* "wound" assay as previously described.[20] Briefly, confluent cell monolayers were gently scratched with a plastic pipette tip to produce the "wound". Then, cultures were allowed to grow for 24 hr in complete culture medium in the presence or absence of TGF-β1 and/or dbcAMP or forskolin.

RESULTS

cAMP and forskolin block TGF-β-stimulated morphologic transdifferentiation in transformed keratinocytes

Treatment of PDV cells with TGF-β1 induces morphologic changes associated with the acquisition of a spindle-like morphology.[1] To assess whether cAMP was able to inhibit the TGF-β1-induced phenotypic transformation, we analyzed the organization of the actin cytoskeleton in TGF-β1-treated and control cells that had been preincubated with cAMP or forskolin. Figure 1 shows that TGF-β1-treated control cells exhibited a Phalloidin-staining actin organization with prominent ruffles of polymerized actin emanating from well-established focal adhesions. Also, some lamellipodia formations were observed. Pretreatment of cells with cAMP or forskolin induced a shift toward an epithelial architecture with a strong cortical actin staining that, surprisingly, caused the disappearance of the majority of the stress fibres present in untreated control cells.

cAMP and forskolin block TGF-β-stimulated delocalization of E-cadherin in transformed keratinocytes

TGF-β is one of the most prominent growth factors that causes cell scattering in epithelial cells. In the majority of the cases, this phenomenon is part of a reversible transition to a mesenchymal phenotype.[21] In PDV-transformed keratinocytes, we found that TGF-β1 induces cellular dispersion characterized by the loss of intercellular E-cad-dependent cell-cell contacts. In control untreated cells, E-cad appeared at the cell surface as a continuous line at the boundaries between neighboring cells. After TGF-β1 treatment, immunostaining decreased markedly and displayed a more diffuse pattern. When these cells were preincubated either with dbcAMP or with forskolin previous to TGF-β1 treatment, the integrity of cell-cell contacts was preserved presumably by antagonizing the TGF-β1-dependent scattering effect (Fig. 2*a*). Interestingly, loss of adherent junctions occurs without a significant change in the expression level of E-cad, as revealed by the immunoblot assay (Fig. 2*b*).

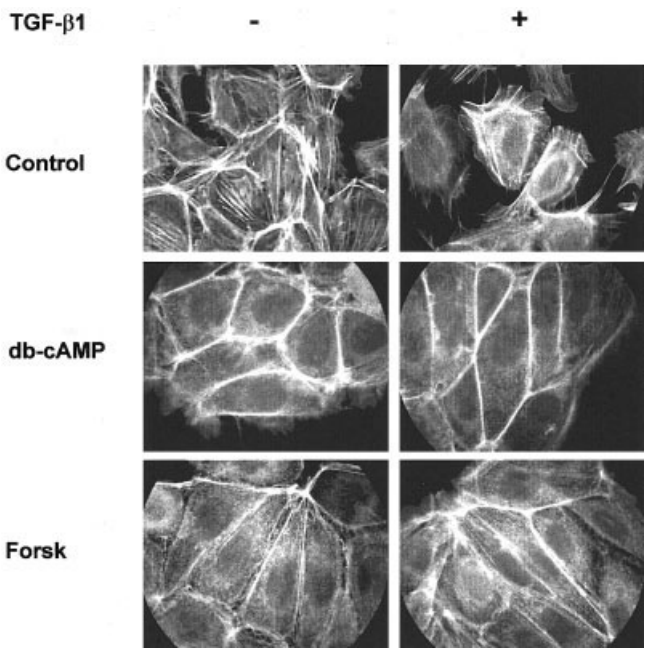

FIGURE 1 – cAMP and forskolin abolish the TGF-β1-induced morphologic changes in actin cytoskeleton in PDV cells. Cells were stimulated for 30 min with 1 mM dbcAMP (middle row), 10 μM forskolin (bottom row) or vehicle (top row). Cells were further incubated (+, right column) or not (−, left column) for 24 hr with 10 ng/ml of TGF-β1. Once fixed and permeabilized, actin cytoskeleton was stained using Phalloidin-Alexa Fluor.

cAMP and forskolin attenuates the TGF-β1-dependent stimuli on uPA and MMP-9 production

Production of extracellular matrix proteases facilitates cellular movement by degrading ECM proteins and constitutes a critical event for the expression of the malignant phenotype.[22] We have previously demonstrated that TGF-β1-stimulated invasiveness of transformed epithelial cells is concomitant to upregulation of uPA and MMP-9.[3,19] To test whether cAMP was able to counteract TGF-β1-dependent stimulation of protease secretion, we incubated PDV cells with dbcAMP or forskolin before the 24 hr treatment with TGF-β1. As shown in Figure 3, preincubation with any of these agents abrogated the TGF-β1-dependent production of both uPA and MMP-9, as measured by casein and gelatine zymograms, respectively.

TGF-β1-stimulated migratory and invasive properties of PDV cells are blocked by cAMP and forskolin

In previous reports we have demonstrated that TGF-β1-dependent expression of uPA and MMP-9 is required to promote an invasive and migratory cell behavior.[3,19] To test if these stimuli were sensitive to cAMP, we preincubated control and TGF-β1-treated PDV cells with dbcAMP or forskolin, and the migratory and invasive properties of the cells were evaluated by the "wound" and Transwell chambers assays. As Figure 4*a* shows, the TGF-β1-dependent enhancement of migration, expressed as the total closure of cellular monolayer "wound" and the factor-stimulated invasive capacity (Fig. 4*b*) of PDV cells were totally abolished by dbcAMP and forskolin.

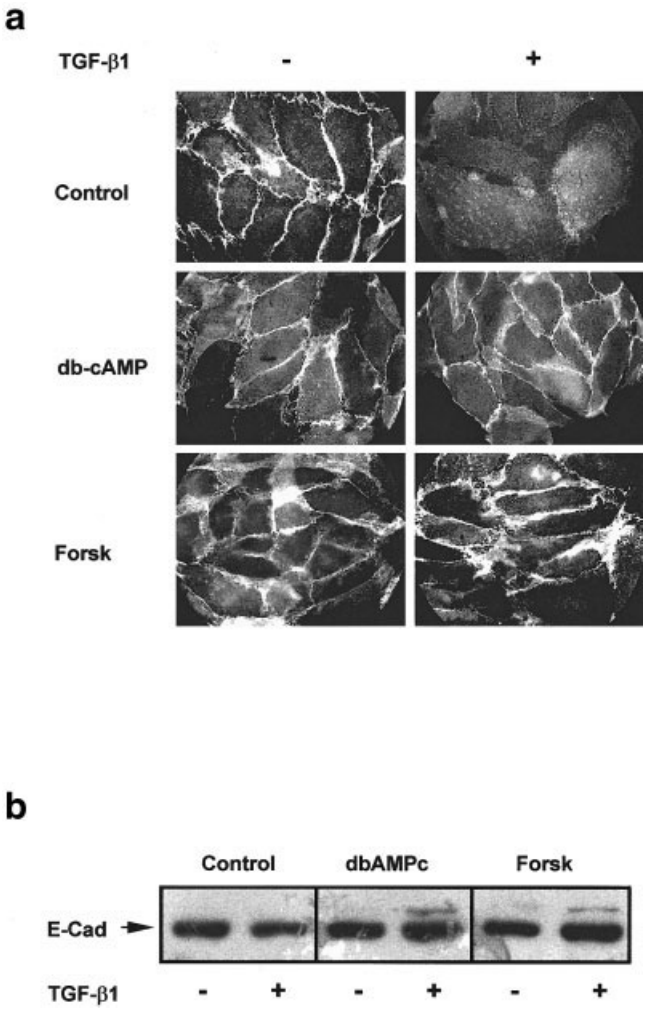

FIGURE 2 – cAMP and forskolin block TGF-β-stimulated delocalization of E-cadherin in PDV cells. *(a)* The integrity of the E-cadherin-dependent cell-cell interaction was evaluated by immunofluorescence to E-cad in cultured cells treated in the same conditions as in Figure 1. *(b)* Western blot of total expression of E-cadherin in cells shown in *(a)*.

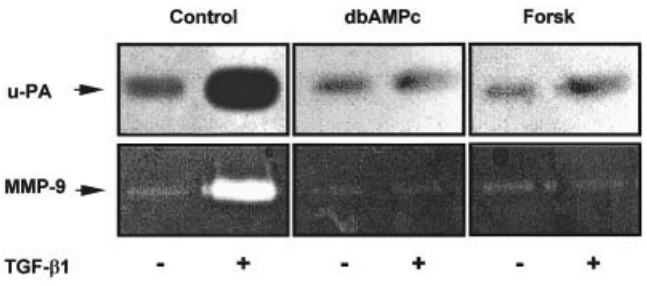

FIGURE 3 – cAMP and forskolin inhibit the TGF-β-stimulated expression of uPA and MMP-9 in PDV cells. Serum-starved PDV cells were stimulated for 30 min with 1 mM dbcAMP, 10 μM forskolin or vehicle and further incubated or not for 24 hr with 10 ng/ml TGF-β1. Media conditioned by these cells were used to determine u-PA activity and MMP-9 activity according to that described in Material and Methods.

TGF-β1-dependent ERK, but not Smad-2 activation, was inhibited by cAMP and mediated by PKA activity

It has been previously established that the Ras-ERK pathway is involved in the TGF-β1-dependent EMT in epithelial cells.[3,23] To further understand the effect of cAMP on TGF-β1 signaling, we investigated the role of cAMP on the activation of its 2 main signaling routes, Ras-ERK and Smad2. As depicted in Figure 5, TGF-β1 treatment of PDV cells provokes a rapid activation of both ERK1,2 (Fig. 5*a*) and Smad2 pathways (Fig. 5*c*). However, when these cells were preincubated with dbcAMP, only the phosphorylation of ERKs is completely abolished. In contrast, the activation of Smad2 by TGF-β1 was unaffected by pretreatment with dbcAMP (Fig. 5*c*). To test if PKA mediates the inhibitory effect of cAMP, prior to dbcAMP preincubation cells were treated with 2 well-known PKA inhibitors, H-89 and the myristoylated form of the inhibitory peptide contained in thermostable PKA inhibitor (PKAI 14-22).[24] As Figure 5*b* shows, both inhibitors relieved the inhibitory effect of dbcAMP in the presence or absence of TGF-β1, and the cells behaved as without dbcAMP.

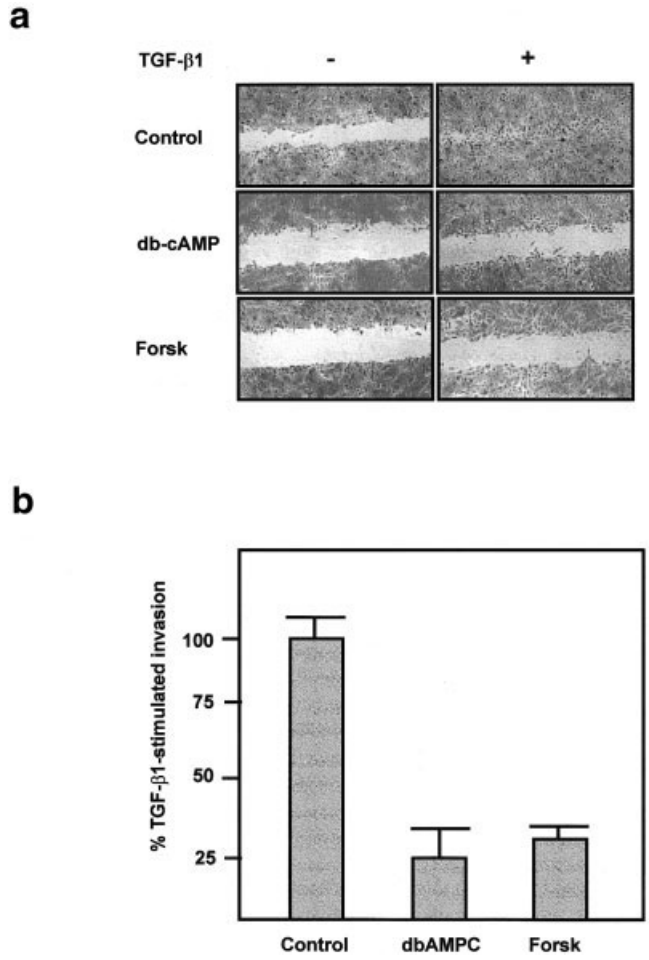

FIGURE 4 – Effect of cAMP and forskolin on TGF-β-stimulated cellular motility and invasiveness. *(a)* Wound healing assay of cells pretreated with 1 mM dbcAMP, 0.1 mM forskolin or vehicle and further incubated (+) or not (−) for 24 hr with 10 ng/ml TGF-β1. Afterwards, cells were fixed with ice-cold methanol and stained with crystal violet. *(b)* Inhibition of the TGF-β-stimulated Matrigel invasion by 1 mM dbcAMP, 10 μM forskolin assayed as described in Material and Methods.

MEK inhibitor PD 098059, cAMP and forskolin antagonize the effect of TGF-β1 on fibronectin production and modulation of E-cad

In epidermal carcinogenesis, the development of spindle carcinoma is associated with a high level of fibronectin (Fn) expression and the organization of this protein in an extracellular matrix.[25,26]

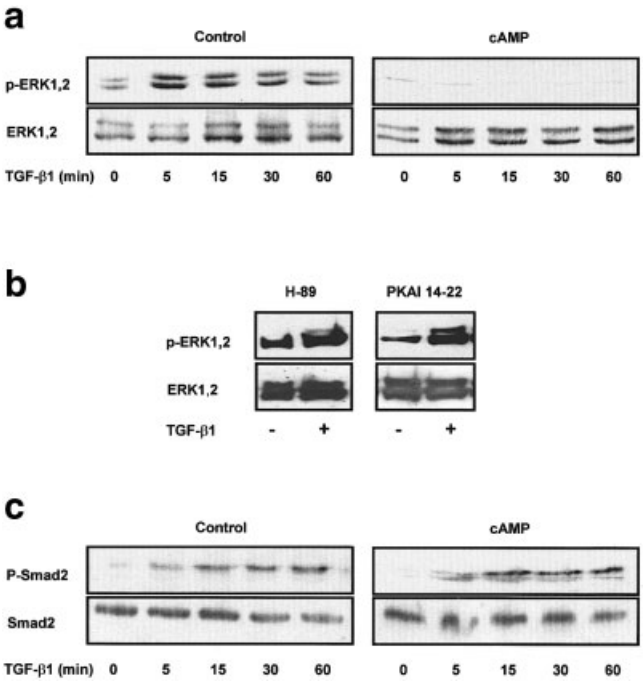

FIGURE 5 – TGF-β1-dependent ERK but not Smad-2 activation was inhibited by cAMP and mediated by PKA. *(a)* Serum-starved PDV cells pre-incubated (cAMP) or not with dbcAMP were stimulated with 10 ng/ml of TGF-β1 for the times indicated. The levels of activated ERK were analyzed by Western blot as described in Material and Methods. *(b)* PDV cells previously incubated for 30 min with PKA inhibitors H-89 and PKAI 14-22 (10 μM) followed by 30 min treatment with dbcAMP were treated or not with TGF-β1 for 15 min and activated ERK was determined. *(c)* PDV cells treated as in *(a)* were analyzed for Smad-2 activation as described in Material and Methods.

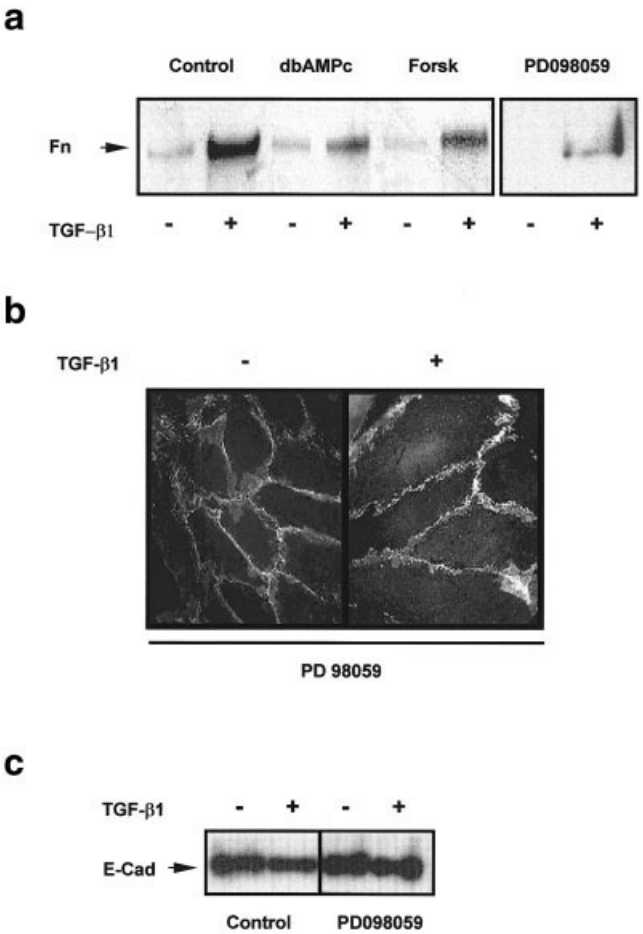

FIGURE 6 – MEK inhibitor PD 098059, cAMP and forskolin have similar effects on TGF-β1-stimulated production of Fibronectin and modulation of E-cad. *(a)* PDV cells were pre-incubated with cAMP, forskolin or PD 098059 for 30 min and further incubated with 10 ng/ml of TGF-β1 for 24 hr. After this, cells were lysed and analyzed by Western blot for Fibronectin (Fn). *(b)* PDV cells were pre-incubated with PD 098059 for 30 min and then treated (+) or not (−) with TGF-β1 for 24 hr. Cells were analyzed by immunofluorescence to E-cad according that described in Material and Methods. *(c)* Western blot for E-cad in control and PD 098059-treated PDV cells.

TGF-β1-treated PDV expressed high levels of Fn, which was reduced when the cells were pretreated with dbcAMP or forskolin (Fig. 6*a*). Similar results were obtained when cells were treated with the MEK inhibitor PD 098059, revealing the importance of the Ras-MAPK pathway in TGF-β1-induced Fn production. To test if the action of this MEK inhibitor also affected cell-cell interactions, we evaluated its role on the integrity of E-cad-dependent cell-cell association in TGF-β1-treated and untreated cells. As shown in Figure 6*b*, E-cad-dependent cell-to-cell interactions and E-cad protein expression were unaffected by TGF-β1 when these cells were pretreated with the MEK1,2 inhibitor PD 098059 (Fig. 6*b,c*).

DISCUSSION

Results presented here demonstrate that cAMP inhibits the TGF-β1-induced conversion of PDV cells into a motile spindle phenotype. These cells respond to TGF-β1 inducing cell scattering and increasing their migration and invasiveness, concomitant to stimulation of uPA and MMP-9 expression.[2,3,19,27] As we have previously shown, these cellular events occur as a consequence of the stimulation of the Ras-MAPK pathway.[2]

Despite that the Smad 2/3 pathway has been proposed as the canonical route for TGF-β1 signal transduction,[28] it is now well accepted that the Ras-MAPK pathway cannot only mediate some of the effects of TGF-β1,[29] but also is involved in the induction of the expression of this growth factor.[30] The existence of a cross-talk mechanism that modulates the activation of each signaling pathway by TGF-β1 seems to depend on the physiologic status of the cell and is a matter of active research.[29] In different cell types, it has been demonstrated that the ability of TGF-β1 to promote tumor cell invasiveness depends on the constitutive activation of MAPK by mutated Ras.[23,31] It has been recently shown that a hyperactive Raf/MAPK pathway is required for TGF-β1-induced EMT, tumorigenesis and metastasis in mouse mammary epithelial cells. TGF-β1 alone, without enhanced Ras signaling, induces reversible cellular changes characterized by cell scattering and morphologic conversion to a more mesenchymal phenotype but not an overt EMT.[32] PDV cells contain a mutated H-Ras allele and express relatively low levels of activated Ras protein.[33] However, treatment of these cells with TGF-β1 provokes a rapid activation of the Ras MAPK signaling, as evidenced by an enhancement of Ras-GTP levels and phosphorylation of ERK1,2.[34] On the other hand, previous data of our group demonstrated that PDV cells transfected with a dominant negative Ras N17 mutant gene were unable to respond to TGF-β1 neither by activating ERK1,2 nor by stimulating the production of MMP-9. By contrast, nontumorigenic MCA3D cells transfected with an active form of Ras acquired the capacity to express MMP-9 associated with an increased MMP-9 promoter activity.[3] Nevertheless, in spite of the requirement of a mutated Ras for TGF-β1 activity, some tumor cells are able to overcome this restriction and respond to this growth factor by activating alternative signaling routes. Thus, the TGF-β1-in-

duced EMT in NmuMG breast epithelial cells depends largely upon the integrity of Smad 2,3 signaling and is insensitive to MEK1 inhibitor PD 098059.[35] Recent data about the relevance of Smad route in TGF-β1-mediated motility add some controversial elements for discussion. In one study, it was demonstrated that the abrogation of autocrine TGF-β1 signaling in the highly invasive mammary cell line MDA-MB-231 resulted in an impairment of basal cell migration. Since motility could not be restored by reconstitution of the Smad pathway, these results suggest that Smad signaling alone is not sufficient for autocrine TGF-β1-mediated motility and that this property is more directly associated with the phosphorylation of AKT and ERK.[36] In another work, it was proposed that activation of Smad2 alone stimulates migration of murine keratinocytes, but the expression of overt EMT was obtained after H-ras activation. These authors propose that Ras and Smad cooperate to promote metastasis and that H-ras induces nuclear accumulation of phosphorylated Smad2 and upregulation of Smad2-mediated transcription.[37]

Our results emphasize that in PDV cells the inhibition of Ras-MAPK pathway by cAMP prevents the expression of malignant traits induced by TGF-β1. These results are in agreement with previous data showing that the elevation of intracellular cAMP antagonizes EGF-stimulated responses including migration and MMP-9 induction.[38] Interestingly, in this work, authors showed that cAMP did not inhibit growth factor-dependent activation of ERK1,2, but instead disrupted the EGF-dependent stimulation of JNK and p38 pathways. A hallmark of cAMP activity is its ability to exert either a positive or a negative regulation on the ERK cascade depending largely upon the cellular context and tissue specificity.[39] Thus, it has been proposed that in mouse fibroblasts cAMP counteracts growth factor-induced Ras/ERK signaling, keeping it within a physiologic range according to cellular functions and requirements.[40] On the other hand, in PC12 cells an elevated level of intracellular cAMP increases cell survival, stimulates neurite outgrowth and activates ERK1,2.[41] Recent data suggest that this can be explained by the cAMP-dependent activation of the GTPase Rap1, which can activate or inhibit ERK signaling in a cell-specific manner.[15] In fact, in Raf-1-expressing cells, PKA-activated GTP-loaded Rap1 antagonizes Ras activation of Raf-1 and ERKs as it binds to and sequesters Raf-1 away from Ras.[42] On the other hand, in B-Raf-expressing cells, GTP-loaded Rap1 activates B-Raf and the MAP kinase cascade.[43] PDV cells are Raf-1-expressing cells (data not shown) and thereby our observation of cAMP inhibition of the TGF-β1 stimulus on the ERK pathway could be explained by the inhibitory mechanism mentioned above. On the other hand, results shown in Figure 5*b* demonstrate that the inhibitory effects of cAMP are very likely mediated by the activation of PKA as indicated by the increase on phosphorylated ERK1,2 in the presence of PKA inhibitors. In addition, this result could be explained through the reported activation of the MAP kinase phosphatases (MAPKPs) by PKA.[44] Our observation of an enhancement of ERK phosphorylation under the stimulus of orthovanadate, which cannot be neutralized by cAMP (data not shown), supports this proposal. Thus, we can speculate that in PDV cells, cAMP-activated PKA can block the ERK pathway by acting at 2 levels: modulating Raf-1 activity or activating MAPKPs. In a different system, it has been recently demonstrated that the fibrotic response to TGF-β1, assessed as the induction of connective tissue growth factor (CTGF) expression, was inhibited by Iloprost, a prostacyclin derivative that blocks the Ras-ERK pathway through the activation of PKA.[45] These results permit us to propose that the blockade of TGF-β1 signaling by cAMP could correspond to a general mechanism available not only in the tumorigenic phenomenon but in other cellular responses.

Our data sustain the idea that TGF-β1, a growth factor that signals through the formation of heteromeric complexes between type I and type II Serine/Threonine kinase receptors, induces the expression of the malignant phenotype by activating the Ras-MAPK pathway, a prototype of the signaling route associated to tyrosine kinase receptors. Recent data reveal that TGF-β1 and EGF, growth factors known to exert antagonistic effects on cell cycle control and apoptosis in epithelial cells, can act synergistically by stimulating EMT through a MEK-dependent mechanism in primary cultured pig thyrocytes.[46] These data support the relevance of Ras-MAPK pathway in the acquisition of malignant traits in culture cells, however, do not allow ruling out the possibility that other signaling pathways known to transduce TGF-β1 are also operative. It has been reported that PI3K plays a central role in the TGF-β1-induced EMT.[47] However, we have observed that wortmannin and LY294002 decrease TGF-β1-induced cell migration but do not affect delocalization of E-cad (data not shown).

The similarity of the results using cAMP or the MEK1 inhibitor PD 098059 in Ras-ERK activation or in the expression of cellular malignant traits, such as invasiveness, uPA and MMP expression and cell dispersion,[2,3] reinforces the hypothesis that the MAPK signaling route represents a nodal point of control in the expression of malignancy. Our data allow us to suggest that cAMP or analogs may be useful as antineoplastic agents. However, at present, we lack experimental data to support the existence of an external factor that could inhibit the acquisition of malignant properties by increasing the intracellular concentration of cAMP.

ACKNOWLEDGEMENTS

We thank Drs. M. Rosemblatt and C. Rojas for their comments and critical reading of the manuscript. This work was supported by the Fondo Nacional de Ciencia y Tecnología, Fondecyt 3000045 (to J.F.S.) and 1010703 (to J.M.).

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