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Phorbol ester phorbol-12-myristate-13-acetate promotes anchorage-independent growth and survival of melanomas through MEK-independent activation of ERK1/2

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Abstract

The phorbol ester, phorbol-12-myristate-13-acetate (PMA), an activator of PKCs, is known to stimulate the in vitro growth of monolayer cultures of normal human melanocytes whereas it inhibits the growth of most malignant melanoma cell lines. We examined the effect of PMA on proliferation and survival of melanoma cells grown as multicellular aggregates in suspension (spheroids), and aimed to elucidate downstream targets of PKC signaling. In contrast to monolayer cultures, PMA increased cell proliferation as well as protected melanoma cells from suspension-mediated apoptosis (anoikis). Supporting the importance of PKC in anchorage-independent growth, treatment of anoikis-resistant melanoma cell lines with antisense oligonucleotides against PKC-α, or the PKC inhibitor Gö6976, strongly induced anoikis. PMA induced activation of ERK1/2, but this effect was not prevented by the MEK inhibitors PD98059 or by U0126. Whereas PD98059 treatment alone led to marked activation of the pro-apoptotic Bim and Bad proteins and significantly increased anoikis, these effects were clearly reversed by PMA. In conclusion, our results indicate that the protective effect of PMA on anchorage-independent survival of melanoma cells at least partly is mediated by MEK-independent activation of ERK1/2 and inactivation of downstream pro-apoptotic effector proteins.

Keywords: Bim; Bad; Anoikis; Cell cycle; MAPK

Most normal cells are dependent on adhesion to extracellular matrix (ECM) for continued growth and survival. Upon detachment from the substratum, anchorage-dependent cells either become arrested in the G1 phase of the cell cycle or they trigger a death program referred to as anoikis. Resistance to anoikis is likely to play an important role in the three-dimensional growth of solid tumors allowing detached tumor cells to survive as single cells or small aggregates in the bloodstream, thereby giving rise to more aggressive and metastasizing tumors. In vitro, loss of anchorage-dependent growth is the property that best correlates with tumorigenicity in vivo [1,2].

A number of studies have implicated members of the Bcl-2 family to play important roles in anoikis [2]. In normal epithelial cells, anoikis can be abrogated by overexpression of the anti-apoptotic protein Bcl-2. Furthermore, depending on the cell type, anoikis has been accompanied by changes in pro-apoptotic Bad, Bax or Bak and anti-apoptotic Mcl-1 protein levels or phosphorylation/activation status [3–5]. Recently, the pro-apoptotic protein Bim was shown to be a critical mediator of anoikis in epithelial cells [6] and phosphorylation of Bim by ERK1/2 has been shown to diminish its pro-apoptotic function and promote its degradation [7].

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The PKC family plays key regulatory roles in a multitude of cellular processes like cell growth, differentiation, apoptosis, malignant transformation, and metastasis (reviewed in [8]), and represents one of the most important mechanisms for signal transduction induced by extracellular stimuli, such as growth factors, hormones, and neurotransmitters [9]. To date, 12 PKC isoenzymes have been identified, and based on their biochemical properties, cofactor requirements, and sequence homology, they have been divided into three subfamilies. The classical PKCs (α, βI, βII, and γ) are Ca^{2+}- and diacylglycerol (DAG) dependent whereas the novel PKCs (δ, ε, η, θ, ν, and μ) are Ca^{2+}-independent but respond to DAG. The atypical PKCs (ζ, λ) are insensitive to both Ca^{2+} and DAG [10,11].

Several lines of evidence suggest a role for PKC in the progression of cells through the cell cycle. In the G1 phase, PKC affects the expression of cyclin D, E, and A proteins. Furthermore, PKC overexpression or activation has been shown to induce expression of the cdk-inhibitors $p21^{WAF1/CIP1}$ and $p27^{Kip1}$ whereas PKC-mediated aberrations in cdc-2 activity are most likely responsible for abrogations in the G2/M transition [12,13].

Activation of PKC has been demonstrated to affect central signaling pathways such as the MAPK/ERK1/2 and the PI3-K pathways. In this regard, Wolch et al. [14] demonstrated phosphorylation and activation of RAF-1 by PKC-α. Moreover, induction of $p21^{WAF1/CIP1}$ by the PKC activator phorbol-12-myristate-13-acetate (PMA) depends on RAF-1 and the MAPK/ERK1/2 pathway [15]. In prostate cancer cells, PKC was shown to induce apoptosis through activation of p38/MAPK and inhibition of the PI3-K pathways [16].

PMA activates classical as well as novel PKC isoforms. Upon activation, PKC is translocated from the cytosol to the membrane followed by its degradation [17]. Whereas normal melanocytes are dependent on PMA to grow in vitro as monolayer cultures, most malignant melanoma cell lines are growth arrested in the G1 and/or G2/M phases of the cell cycle under the same conditions [18–20]. However, the role of PKCs in progression of malignant melanoma is poorly understood, although it has been anticipated that PKC-α, due to the enhanced level/activation status observed concomitantly with increased aggressiveness, may be of particular importance [21–24]. Moreover, Dennis et al. [25] observed that transfection of human melanoma cell lines with antisense oligonucleotides directed against PKC-α suppressed metastasis formation in nude mice.

In the present study, we report that PMA increases proliferation as well as promotes anchorage-independent survival of melanoma cells cultivated as multicellular aggregates in suspension (spheroids). Furthermore, our results suggest that the anti-apoptotic effect of PMA is mediated at least partly through PKC and MEK-independent activation of ERK1/2 and inactivation of Bim and Bad pro-apoptotic proteins.

Materials and methods

Cell cultures and conditions. All human melanoma cell lines used in this study were routinely cultured in RPMI 1640 medium (BioWhittaker Europe, Verviers, Belgium) supplemented with 5% fetal bovine serum (FBS) (Biochrom KG, Berlin, Germany). The cell lines of the WM series were kindly provided by Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA) and have been described in detail elsewhere [26]. The MeWo cell line was derived from a lymph node metastasis [27]. PMA was from Sigma–Aldrich (St. Louis, MO), whereas the signaling inhibitors PD98059 and U0126 (inhibitors of MEK) and LY294002 (inhibitor of PI3-K) were from Cell Signaling (Beverly, MA) and Gö6976 (inhibitor of classical PKC) was obtained from Calbiochem (La Jolla, CA). For treatment of asynchronously growing monolayer cultures with PMA, cells were plated at 70% confluence overnight in RPMI medium containing 5% FCS. The medium was thereafter replaced with fresh complete medium containing 50 ng/ml PMA dissolved in DMSO. PD98059 (25 μM), LY294002 (25 μM), and Gö6976 (500 nM) were added to the cultures 45 min prior to addition of PMA. Multicellular aggregates (spheroids) were prepared as previously described [28]. Briefly, Seaplaque agarose (BioWhittaker Molecular Application, Rockland, ME) was diluted to 1% with serum-free medium and used to coat 24-well plates. Tumor cells (2×10^5 in 1 ml complete medium) were then plated on top of the solidified agarose. For treatment of spheroid cultures, PMA was added when plating in suspension, whereas the inhibitors in combination experiments were added 45 min prior to plating as spheroids and addition of PMA.

Transfection with antisense PKC-α. Anoikis-resistant human melanoma cell lines were plated overnight at a concentration of 2×10^5 cells per well in 6-well plates. Thereafter, the cells were transfected overnight with 0.5 μM phosphorothioate oligodeoxynucleotides using lipofectin as described by the manufacturer (Gibco-BRL, Gaithersburg, MD). The following oligodeoxynucleotides were used: 5′-GTTCTCGCTGGTGAGTTTCA-3′ (specifically targeting the 3′-untranslated region of human PKC-α). As a control a scrambled version of the same oligodeoxynucleotide (5′-GGTTTTAC-CATCGGTTC TGG-3′) was used [25,29]. Following transfection, the medium was replaced by RPMI 1640 medium supplemented with 5% FCS and the cells were left to recover for 6 h before plating as spheroids for an additional 48 h.

Flow cytometric analysis. Cells were harvested for flow cytometric analysis by trypsinization and washed in cold PBS before fixation in 70% ethanol for at least 1 h at 4 °C. The cells were thereafter washed twice with PBS and resuspended in a solution of 2 μg/ml Hoechst in PBS. Data acquisition and analysis was done on a Becton–Dickinson FACStar$^+$ using ModFit software.

Apoptosis and proliferation assays. The rate of apoptotic cell death was estimated using The Cell Death ELISA Plus Kit provided by Roche Diagnostics (Mannheim, Germany), which quantitates nucleosomes released into the cytoplasm of dying cells. Briefly, cells were collected by trypsinization, lysed, and incubated with a mixture of biotinylated antihistone antibody and peroxidase-conjugated anti-DNA antibody, both of which bind to histone DNA complexes and initiate a color reaction in the presence of the substrate 2,2′-azino-di(3-ethylbenzthiazolinesulfonate) (ABTS). The OD reading at 405 nm was corrected according to negative control and expressed as fold increase in apoptosis as compared to the level in untreated cells.

Proliferation rate was examined using the CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI), which quantitates the formation of formazan from the tetrazolium

compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium. The amount of formed formazan was measured by OD reading at 490 nm. Alternatively, cells were harvested as above, and viable and dead cells were counted using trypan blue exclusion (Gibco-BRL, Gaithersburg, MD).

Antibodies. Antibody to the retinoblastoma protein (pRb) was obtained from PharMingen (San Diego, CA, USA) and antibodies to $p21^{WAF1/CIP1}$, cyclin A, cyclin E, cdk4, Mcl-1, cdk2, Bcl-X_L, and total ERK2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cyclin D3 and Bcl-2 antibodies were from Dako (Glostrup, Denmark), cyclin D1 and α-tubulin were from Oncogene Research Products (Boston, MA), and $p27^{Kip1}$ and PKC antibodies were purchased from Transduction Laboratories (Lexington, KY) whereas antibody against Bax was from Neomarkers (Fremont, CA). Antibodies against active phosphorylated ERK1/2 and Bim were from Promega (Madison, WI) and Chemicon Int. (Temecula, CA), respectively. Antibodies against activated (phosphorylated) as well as total Bad and Akt proteins were from Cell Signaling (Beverly, MA).

Immunoblotting. Cells were lysed in ice-cold NP-40 lysis buffer (1% NP-40, 10% glycerol, 20 mM Tris–HCl, pH 7.5, 137 mM NaCl, 100 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.02 mg/ml each of aprotinin, leupeptin, and pepstatin, and 10 μl/ml phosphatase inhibitor cocktail I). All protease inhibitors were from Sigma–Aldrich (Sigma–Aldrich, St. Louis, MO). Lysates were sonicated and clarified by centrifugation. Protein quantitation was done by Bradford analysis and 25 μg protein/lane was resolved by SDS polyacrylamide gel electrophoresis. Transfer and hybridization were as described [30]. To ensure even loading, filters were stained with naphthol-blue black (Sigma–Aldrich) and hybridized with α-tubulin.

Results

PMA promotes cell proliferation and survival of human malignant melanoma spheroids

Several studies have demonstrated that most in vitro monolayer cell cultures of malignant melanomas are growth inhibited following treatment with the PKC activator PMA [18–20]. However, treatment of melanomas with antisense PKC-α oligonucleotides has demonstrated decreased tumor growth in vivo [25]. In order to reveal the mechanism behind these seemingly contradictory results, we chose to cultivate five human melanoma cell lines as multicellular aggregates in suspension (spheroids), the growth condition that mimics the in vivo growth conditions the best, and analyze the effect of PMA on proliferation and survival. As shown in Fig. 1A, PMA clearly increased cell proliferation in two and marginally in one of the five spheroid cultures. Of particular interest is the marked increase in proliferation of WM35 cells (265% of control). This cell line is poorly tumorigenic [31] and will normally die by apoptosis (anoikis) when cultivated in suspension. Furthermore, when WM35 spheroids were assayed for apoptotic cell death using the Cell Death ELISA Plus Assay we observed that PMA-treated spheroids demonstrated significantly lower degree of apoptosis compared to untreated cultures (Fig. 1B). Similar results were obtained by estimating cell death by trypan blue exclusion (data not shown). Only marginal effects on spheroid survival were observed for the more anoikis-resistant cell lines (WM1341B, WM902B, WM239, and WM45.1) (data not shown).

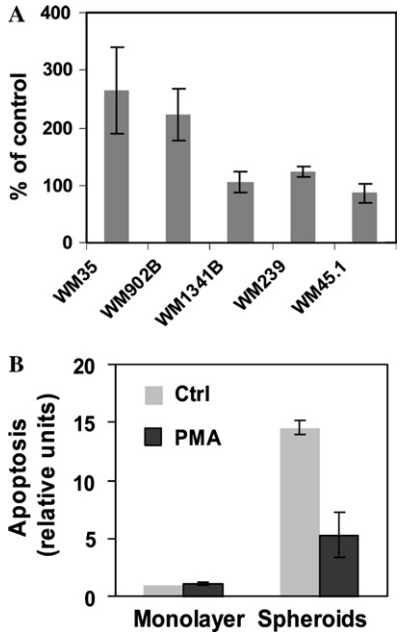

Fig. 1. PMA promotes cell proliferation and survival of melanoma cells grown as spheroids. (A) Spheroid cultures of human melanoma cell lines were treated with 50 ng/ml PMA for 3 days and analyzed for proliferation rate using the MTS assay (Promega). Results are presented as the percentage of untreated controls. (B) Monolayer and spheroid cultures of the WM35 cell line were treated with PMA as in (A) and analyzed for apoptosis using the Cell Death ELISA Plus Kit (Roche). Degree of apoptosis is expressed as fold increase as compared to the level in untreated monolayer cells. Data are means of at least three independent experiments ± SD.

Effect of PMA on markers of cell cycle progression

In order to examine the effect of PMA on cell cycle progression in more detail, the WM35 cell line was chosen as a model. The cells were first cultivated as spheroids for different time periods without or with 50 ng/ml PMA and analyzed for effects on cell cycle distribution by flow cytometry. As shown in Table 1, both

Table 1
Cell cycle distribution of WM35 spheroids treated with 50 ng/ml PMA

Hours with PMA	G0/G1		S		G2/M	
	Control	PMA	Control	PMA	Control	PMA
0[a]	46[b]		36		18	
6	75	71	16	15	9	14
12	71	73	15	16	14	11
24	63	59	22	25	15	16
48	84	68	8	17	8	15
72	86	75	8	12	6	13

[a] Exponentially growing monolayer cells.
[b] Percentage of cells.

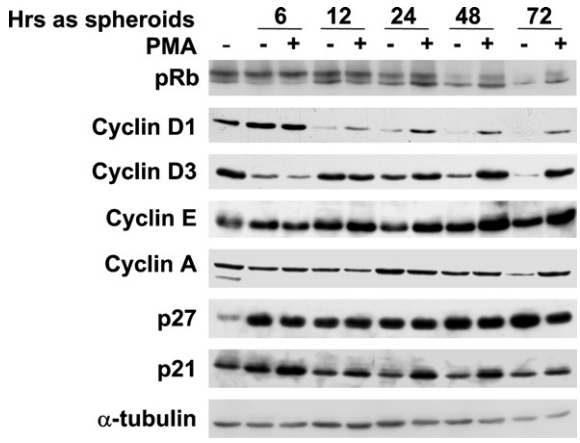

Fig. 2. Effect of PMA on protein levels of G1 phase regulators. WM35 cells cultured as spheroids without or in the presence of 50 ng/ml PMA were harvested after different time periods and analyzed by Western blotting for expression of G1 to S phase cell cycle regulators. Results from exponentially growing monolayer cultures are shown in lane 1. The filters were hybridized with an antibody against α-tubulin as a loading control. The results shown are representative examples of at least three individual experiments.

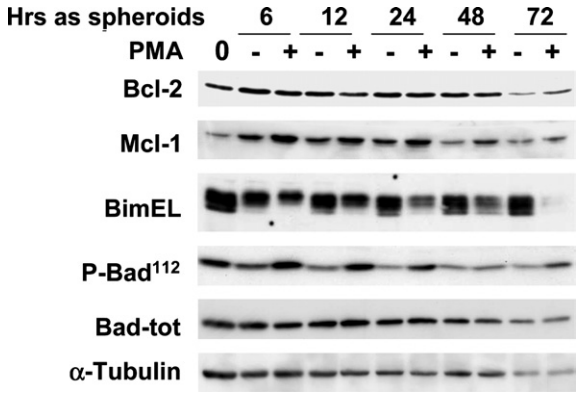

Fig. 3. Effect of PMA on regulators of apoptosis. WM35 cells treated as in Fig. 2 and examined by Western blot analysis for expression of anti-apoptotic Bcl-2 and Mcl-1, and pro-apoptotic Bim proteins, as well as phosphorylation status of Ser^{112} of pro-apoptotic Bad. The filter was rehybridized with α-tubulin as loading control. Similar results were obtained in two additional experiments.

untreated and treated spheroids gradually accumulated in the G1 phase, but in the presence of PMA this response was delayed. Consistently, altered expression of G1 phase regulators indicated continued cell cycle progression following treatment with PMA (Fig. 2). Whereas unphosphorylated pRb protein accumulated in control spheroids, pRb remained in the hyperphosphorylated state for a longer time period in the treated cells. Furthermore, PMA led to a rise in cyclin E protein level and prevented downregulation of cyclin A, D1, and D3 proteins. The level of the cdk inhibitor $p27^{Kip1}$ was relatively low in monolayer WM35 cells, but increased rapidly in both untreated and PMA-treated spheroids. An additional slight increase in $p27^{Kip1}$ levels was observed after 72 h in control spheroids but not following treatment with PMA. After a transient induction of $p21^{WAF1/CIP1}$ protein in both control and PMA-treated spheroids after 6 h, $p21^{WAF1/CIP1}$ fell in untreated, but less so in treated cells to a level comparable to what was observed in monolayer control cells (Fig. 2).

Effect of PMA on regulators of apoptosis

Our results so far have demonstrated that PMA treatment rescues melanoma cells from suspension-mediated cell death (anoikis) (Fig. 1B). Recently, the pro-apoptotic protein Bim was demonstrated to play a key role in anoikis [6]. In agreement with this, we found that treatment of WM35 spheroid cultures with PMA (50 ng/ml) as above led to increased phosphorylation, as shown by the slower migration rate, and subsequently to diminished protein level of the extra-long isoform of Bim (BimEL) that was not seen in control spheroids. The pro-apoptotic Bad protein was phosphorylated at Ser^{112} by PMA, suggesting its inactivation [32]. Moreover, PMA treatment resulted in rapid, but transient, accumulation of Mcl-1, an anti-apoptotic protein of the Bcl-2 family, further supporting the apoptosis-protective effect of PMA. Finally, PMA caused a slight, but marked, delay in the downregulation of Bcl-2 after 72 h (Fig. 3), whereas no differences in the levels of Bax and Bcl-X_L proteins were revealed between control and PMA-treated spheroids (data not shown).

Induction of anoikis by antisense PKC-α

In order to investigate whether the observed anoikis resistance was achieved through the effect of PMA on PKC-α, the isoform that in several studies (reviewed in [21]) has been shown to play an important role in the tumorigenesis of melanoma, the anoikis-resistant WM1341B cell line was transfected with an antisense oligonucleotide directed against PKC-α. The day after transfection the cells were plated for an additional 2 days as spheroids before being analyzed for degree of apoptosis. As shown in Fig. 4A, antisense PKC-α led to a close to threefold increase in anoikis as compared to the level in WM1341B spheroids transfected with lipofectin alone or a scrambled version of the antisense oligonucleotide. Specific downregulation of PKC-α protein by the antisense oligonucleotide was confirmed by Western blot analysis of PKC isoforms expressed by WM1341B cells (Fig. 4B). Of the classical PKCs, both WM35 and WM1341B cells express only PKC-α (data not shown). To further confirm the impact of PKC-α on anchorage-independent survival, WM35 and WM1341B spheroids were treated for 3 days with Gö6976 (500 nM), a specific inhibitor of classical PKCs. As shown for WM35 cells in Fig. 4C, Gö6976 increased

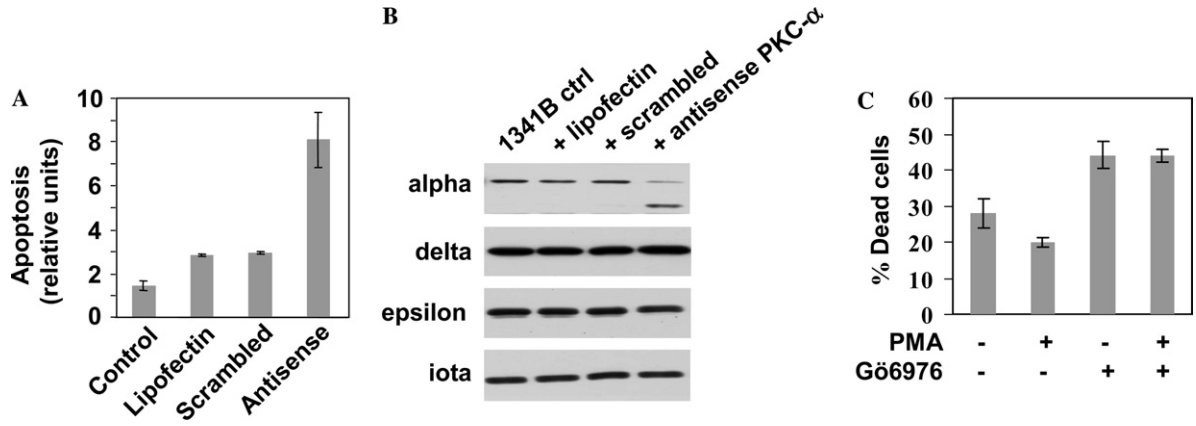

Fig. 4. Induction of anoikis by antisense PKC-α. (A) Anoikis-resistant WM1341B cells were transfected with antisense oligonucleotides directed against PKC-α. As controls the cells were transfected with a scrambled version of the antisense oligonucleotide or with lipofectin alone. Following transfection the cells were plated as spheroids for 48 h and analyzed for degree of apoptosis using the Cell Death ELISA Plus Kit. (B) Western blot analysis demonstrating downregulation of PKC-α by antisense PKC-α. To ensure specific downregulation of PKC-α, the filter was rehybridized with antibodies detecting the other PKC isoforms expressed by WM1341B. (C) WM35 spheroids were treated with the PKC inhibitor Gö6976 (500 nM) alone or in combination with PMA (50 ng/ml) as described in Materials and methods. Percentage of viable and dead cells was estimated by cell count and trypan blue exclusion. Data are means of at least three independent experiments ± SD.

anoikis, even in the presence of PMA. Together, these results suggest that PMA mediates a positive effect on anchorage-independent growth and survival through PKC-α activation rather than downregulation of the protein.

PMA activates the MAPK/(ERK1/2) signaling pathway downstream of MEK

Several studies have reported activation of the MAPK/ERK1/2 signaling pathway by PMA and PKC [33–35]. Evidence has also been provided demonstrating the involvement of MAPK/ERK1/2 in anoikis. To define whether this pathway is activated by PMA in melanomas, WM35 spheroid cultures were treated with 50 ng/ml PMA for 3 days and analyzed by Western blot analysis for protein expression of activated ERK1/2. As shown in Fig. 5A, PMA treatment resulted in activation of ERK1/2. When the cell cultures were treated with the MEK inhibitor PD98059 (25 μM) for 45 min, a nearly complete inhibition in ERK1/2 phosphorylation was seen. Surprisingly, however, PMA clearly reversed the effect of PD98059, even in the continuous presence of the inhibitor. This latter effect was visible as soon as 30 min after addition of PMA (data not shown).

To further ensure that the observed effect of PMA on ERK1/2 was mediated by PKC, WM35 cells were incubated with PD98059 in combination with Gö6976 (500 nM) for 45 min prior to addition of PMA. As expected, due to the numerous signaling events with potential capability to activate ERK1/2, Gö6976 alone had no effect on ERK1/2 phosphorylation. Notably, however, Gö6976 at least partially prevented PMA-mediated reactivation of ERK1/2 in the presence of PD98059 (Fig. 5A).

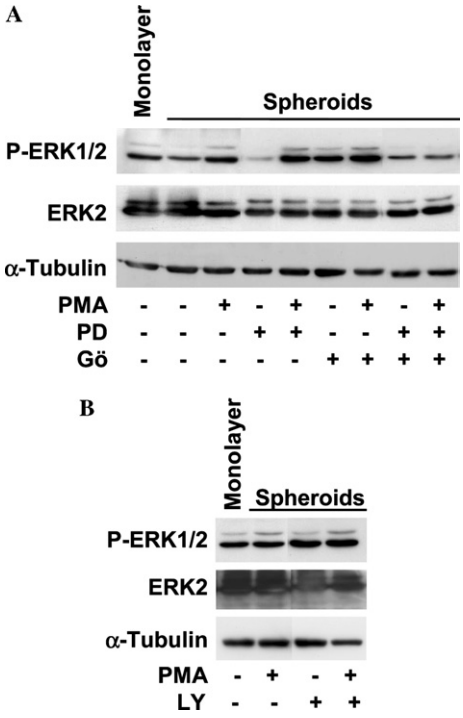

Fig. 5. Effect of PMA, PD98059, LY294002, and Gö6976 on the activation status of ERK1/2. In all combination experiments, the inhibitors were added 45 min prior to PMA and were present for the whole treatment period. (A) Spheroid cultures of WM35 cells were treated for 3 days with PMA (50 ng/ml), PD98059 (25 μM) or Gö6976 (500 ng/ml)or in combinations as indicated and analyzed by Western blotting for the expression of activated ERK1/2 and total ERK2, and for expression of α-tubulin as a loading control. (B) WM35 cells were treated with PMA and LY294002 (25 μM), and analyzed for ERK1/2 activation as in (A). In the total ERK2 controls, some crossreactivity with ERK1 is seen. Similar results were obtained in two other individual experiments.

PMA reverses anoikis induced by ERK1/2 inhibition: inactivation of Bim and Bad proteins

We next wished to examine whether the observed effect on ERK1/2 activity could be responsible for the PMA-mediated effects on anchorage-independent survival. To do so, WM35 cells were treated with PD98059 or PD98059 in combination with PMA as above and analyzed for degree of cell death by cell counting and trypan blue exclusion. As shown in Fig. 6A, PD98059 alone clearly increased anoikis. However, PMA reversed PD98059-mediated anoikis to a level comparable to PMA alone treated spheroids. To examine whether this latter effect could be due to increased phosphorylation and inactivation of the pro-apoptotic Bim and Bad^{112} proteins, protein lysates from treated and untreated spheroids were examined by Western blot analysis. As shown in Fig. 6B, PD98059 mediated dephosphorylation of Bad and Bim, whereas this effect was clearly reversed by PMA, suggesting that part of the anoikis suppressive effect of PMA is mediated through activation of ERK1/2 and inactivation of pro-apoptotic Bim and Bad proteins.

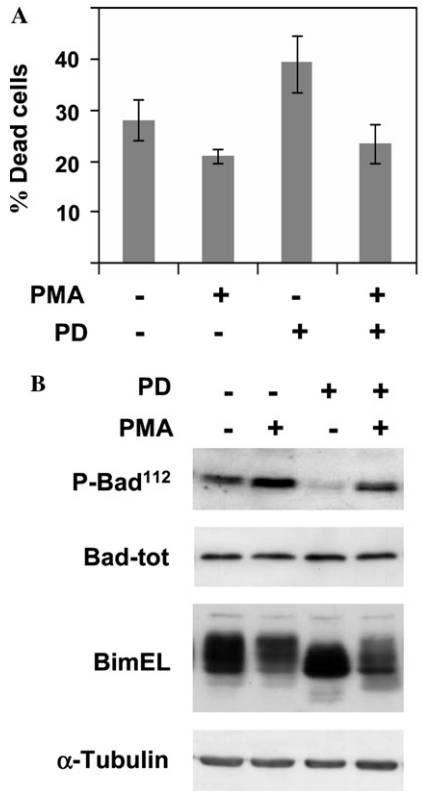

Fig. 6. Effect of PMA on PD98059 induced apoptosis. (A) WM35 spheroids were treated for 3 days with PMA (50 ng/ml) and/or PD98059 (25 μM) as indicated and the percentage of viable and dead cells was estimated by cell count and trypan blue exclusion. Data are means of at least three independent experiments ± SD. (B) Western blot analysis of total protein lysates from the cells in (A), demonstrating reversal of PD98059-mediated effects on Bad and Bim by PMA. The filter was rehybridized with antibodies against total Bad and α-tubulin as loading controls. Two additional experiments gave similar results.

Several studies have demonstrated a crosstalk between the MAPK/ERK1/2 and the PI3-K signaling pathways. Moreover, the PI3-K pathway has been widely associated with survival mechanisms including the ability to survive anchorage-independently [36–39]. In order to reveal whether PKC could activate ERK1/2 through PI3-K, WM35 spheroids were treated for 3 days with the PI3-K inhibitor LY294002 (25 μM) in combination with PMA. As demonstrated in Fig. 5B, inhibition of PI3-K did not prevent PMA-mediated ERK1/2 phosphorylation.

Discussion

Numerous studies have documented the importance of PKC activation in regulating cell growth, differentiation, and survival (reviewed in [8]). Whereas normal melanocytes are dependent on the PKC activator PMA to proliferate in vitro, most malignant melanoma cell lines are growth inhibited by PMA [18–20]. Intriguingly, on the other hand, treatment of human melanoma xenografts with antisense oligonucleotides directed against the PKC-α isoform has been shown to decrease tumor formation and metastasis [25]. However, the mechanisms by which PKC fulfills these effects on melanoma cell growth are still controversial. In the present study, we explored the effect of PMA, a potent activator of classical and novel PKC isoforms, on growth and survival of human melanoma cells grown as multicellular aggregates (spheroids). Furthermore, we have aimed in revealing downstream targets of PKC activation. Surprisingly, and in contrast to what has been reported for monolayer cultures [19,20], we observed that PMA increased proliferation when melanoma cells were cultivated as spheroids. Although the expression of G1 cell cycle proteins (cyclins D1, D3, E, and A) in the treated cells in general indicated higher proliferation rate, we observed that the level of the cdk inhibitor p21$^{WAF1/CIP1}$ protein was increased in PMA-treated spheroids as compared to controls. Our results support, however, findings in glioma cells, suggesting that PMA promotes proliferation through upregulation of p21$^{WAF1/CIP1}$, thereby facilitating formation of active cyclin–CDK complexes [40,41]. Furthermore, Mueller et al. [41] reported that loss of p21$^{WAF1/CIP1}$ impaired the ability of HCT colon carcinoma cells to grow anchorage-independently. In contrast, Detjen et al. [17] showed that PMA treatment caused p21$^{WAF1/CIP1}$-mediated G1 arrest in pancreatic cancer cells. Altogether, these results support the notion that PMA may promote different effects depending on the growth conditions and the cell type studied.

In addition to promoting anchorage-independent proliferation, we also observed that PMA was a strong inhibitor of suspension-meditated cell death (anoikis), a finding that may explain why antisense oligonucleotide inhibition of PKC-α suppresses melanoma growth and metastasis in animal models [25]. Our findings are in agreement with those of Ways et al. [42] who reported induction of anchorage-independent growth of MCF7 cells treated with PMA, but do not fully support the study by Bretland et al. [43] who demonstrated that PKC protects HaCaT keratinocytes against anoikis by inducing cell cycle arrest. In contrast to these former studies, in gastric cancer cells, addition of PMA led to anoikis, but had little impact on survival of monolayer cultures [44].

To further support the role of PKC, and in particular of PKC-α, in mediating the observed effects of PMA, we found that transfection of an anoikis-resistant melanoma cell line with an antisense PKC-α oligonucleotide, shown to be specific for PKC-α [29], reversed the anoikis-resistant phenotype. Similar results were also obtained using the PKC inhibitor Gö6976. Altogether, these results do not support the hypothesis that downregulation of PKC upon prolonged PMA treatment rather than PKC activation may be responsible for the observed effects of PMA [45,46]. Our antisense and inhibitor results furthermore contrast a recent study [47] in which a low level of PKC-α was shown to stimulate melanoma cell proliferation.

Apoptosis/anoikis is a complex process likely to involve a number of events that combined eventually decide on life or death of a cell. Numerous studies have demonstrated the importance of members of the Bcl-2 family as regulators of anoikis. For instance, cells overexpressing the anti-apoptotic proteins Bcl-2 or Bcl-X_L are highly resistant to anoikis and recently it was demonstrated that the BH3-only pro-apoptotic protein Bim is an important mediator of anoikis in epithelial cells [6]. Moreover, PMA has been shown to phosphorylate Bad at Ser^{112}, thereby favoring cell survival [32,48]. In agreement with these studies, we found that PMA delayed downregulation of Bcl-2 protein observed after anchorage deprivation of anoikis-sensitive melanoma cells. Furthermore, addition of PMA to spheroid cultures led to a marked mobility shift and clearly reduced the protein level of the BimEL isoform as well as increased the phosphorylation status of Bad^{112}. In contrast to results obtained by Meinhardt et al. [49] demonstrating PMA-mediated induction of anti-apoptotic protein Mcl-1 in adherent leukemia cells but not in cells in suspension, we found that PMA induced Mcl-1 expression in melanoma spheroid cultures.

The MAPK/ERK1/2 signaling pathway has been suggested to play an important role as a downstream target of PKC [33–35]. In the present study, we confirm this observation. Thus, Western blot analysis of protein lysates from spheroid cultures revealed that PMA treatment increased the phosphorylation status of ERK1/2. Interestingly, pretreatment with the MEK inhibitors PD98059 or U0126 did not prevent ERK1/2 activation by PMA, suggesting that PKC either directly or through activation of other signaling pathways may phosphorylate ERK1/2 independently of MEK1/2. Our results are in agreement with those of Grammer and Blenis [50] and Bapat et al. [51] who reported that PKC plays a major role in MEK-independent MAPK activation by platelet-derived growth factor signaling and peroxynitrite, respectively. In the former [50] study, it was suggested that PKC may act downstream of MEK by inhibiting an ERK phosphatase. Although we have not examined our cells for ERK phosphatase activity, our other results do not support such a hypothesis. Thus, PMA was found to reverse PD98059-mediated downregulation of activated, phosphorylated ERK1/2, even in the continuous presence of the inhibitor. Our results are, however, in stark contrast to others, demonstrating PMA-mediated ERK1/2 activation at an early stage upstream of MEK [37,52].

In the present study, we demonstrated that the MAPK/ERK1/2 pathway plays an important role in anchorage-independent survival of melanoma cells. Interestingly, we found that PMA reversed PD98059-induced anoikis, further indicating that the PMA-mediated effect on anchorage-independent growth is dependent on activation of PKC and the MAPK/ERK1/2 pathway. On the other hand, we observed a slight increase in the level of activated ERK1/2 also when anoikis-sensitive cells were grown as spheroids for 3 days. This seemingly contradictory finding is supported by the hypothesis put forward by Grammer and Blenis [50], stating that, depending on the cell type, the type and timing of stimuli, the MAPK pathway may be activated by MEK-dependent or -independent mechanisms and produce different end results.

Recently, it was demonstrated that PMA increases phosphorylation of BimEL protein through activation of ERK1/2, thereby leading to degradation of BimEL [7]. Likewise, phosphorylation and inactivation of Bad by ERK1/2 has been described [53]. Consistent with these findings, we observed a marked dephosphorylation of BimEL and Bad proteins after treatment of spheroids with the MEK inhibitor PD98059. Furthermore, we found that PMA, at least partly, reversed this effect of PD98059, a finding strengthening the hypothesis of MEK-independent regulation of ERK1/2 and the involvement of Bim and Bad in anchorage-independent survival. In support of Bad being of importance for melanoma cell survival, Eisenmann et al. [54] recently showed that the MAPK/ERK1/2 pathway mediates melanoma-specific survival signaling by differentially regulating RSK-mediated phosphorylation of Bad.

In addition to MAPK/ERK1/2, the PI3-K/Akt pathway has been found to act as downstream targets of PKCs [16,55]. Moreover, evidence has been provided showing a profound crosstalk between these two central signaling pathways [37,56], a finding that spurred us to examine whether PKC through PI-3-K/Akt could mediate phosphorylation of ERK1/2. However, treatment of our cells with the PI3-K inhibitor LY294002 did not abrogate PMA-mediated activation of ERK1/2 ruling out the possibility that PI3-K is the major target of PKC signaling in melanomas.

In summary, we show that anchorage-independent growth and survival of melanoma cells strongly depend on PMA-mediated PKC activation and the MAPK/ERK1/2 pathway leading to inactivation/degradation of the pro-apoptotic proteins Bim and Bad. Of particular interest are our findings demonstrating MEK-independent PKC activation of ERK1/2. Altogether, our results indicate that targeting PKC activity in combination with the MAPK/ERK1/2 pathway may prove to be an important therapeutic strategy in the treatment of melanomas.

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