SHORT REPORT

Protein kinase C α promotes apoptotic cell death in gastric cancer cells depending upon loss of anchorage

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Disruption of interactions between epithelial cells and extracellular matrix proteins leads to apoptosis of the cells, a phenomenon termed anoikis. Anoikis seems to play important roles in control of cellular positioning and inhibition of inappropriate cell growth. Here we found that a protein kinase C (PKC) activator phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA) promoted cell death in human gastric cancer cell lines MKN45 and MKN74 only when they lost anchorage. Loss of anchorage slightly increased enzymatic activity of PKC α , and an addition of TPA promoted cell death with further increase of PKC α activity, but not PKC β in MKN45 cells, implicating an involvement of PKC α in anoikis. Furthermore, vaccinia virus-mediated overexpression of PKC α strongly increased CPP32 activity in the detached MKN45 and MKN74 cells, and augmented anoikis, however it had little effect on viability and CPP32 activity in the attached cells. Taken together, PKC α promotes apoptotic cell death in gastric cancer cells depending upon loss of anchorage, thereby may be a modulator of anoikis.

Keywords: anoikis; apoptosis; protein kinase C α ; gastric adenocarcinoma

Most types of normal cells require attachment to extracellular matrix (ECM) proteins to grow or even survive. When anchorage-dependent cells are detached from their substrate by incubation with RGD peptides, they are arrested in the G1 phase and eventually undergo apoptosis (Guadagno *et al.*, 1993; Meredith *et al.*, 1993). This type of apoptosis is named anoikis (Frisch and Francis, 1994). Anoikis seems to be responsible for removal of differentiated epithelial cells, which lose contact with their underlying matrix and are no longer necessary or are dangerous to the host. In this context, anoikis may block growth of detached cells at inappropriate locations. This implies that a disturbance of anoikis may lead to abnormal cell growth and thus is likely to be associated with tumor progression in a variety of malignancies originating from epithelial cells. In agreement with this idea, dependence on anchorage is reduced in transformed cells (Folkman and Moscona, 1978), and inhibition of apoptosis by overexpression of Bcl-2 or BAG-1

promotes metastasis of malignant melanomas and abdominal dissemination of gastric cancer cells (Takaoka *et al.*, 1997; Yawata *et al.*, 1998). Thus, augmentation of sensitivity to anoikis is likely to become a good technique to prevent cancer-cell metastasis or dissemination. For this purpose, it is crucial to explore a signaling responsible for enhancement of anoikis.

Anoikis can be abrogated by attachment to integrin, and thus the signals generated by integrin ligation seem to somehow prevent anoikis (Meredith *et al.*, 1993). However, relatively little is known about how the signals are transduced by integrins into host cells. In fibroblasts, the integrin-mediated signals cause alternations of intracellular pH and Ca²⁺ levels, and activation of focal adhesion kinase (FAK) and Src family protein tyrosine kinases (Schwartz, 1993; Juliano and Varner, 1993; Cobb *et al.*, 1994), followed by transient activation of MAP kinase family members ERK1 and ERK2 (Chen *et al.*, 1994). In epithelial cells, however, the same signals do not activate ERK1 and ERK2 significantly (Frisch *et al.*, 1996a), strongly suggesting that integrin-mediated intracellular signaling may alter in different cell types. Conversely, disruption of interaction between epithelial cells and ECM proteins leads to activation of other MAP kinase family members JNK and p38 (Frisch *et al.*, 1996b; Derijard *et al.*, 1994), though there is a report showing a lack of correlation between activation of JNK and induction of anoikis (Khwaja and Downward, 1997). These controversial data imply that several kinases and other signaling molecules may regulate anoikis either positively or negatively. In the present study, we demonstrate that loss of anchorage preferentially increased PKC α activity and overexpression of PKC α promoted anoikis in human gastric cancer cell lines MKN45 and MKN74, but it had little effect on their viability in the attached cells. This implies that PKC α may be a modulator of anoikis.

TPA promotes anoikis of MKN45 cells

MKN45 cells were established from a poorly differentiated type of human gastric adenocarcinoma (Asao *et al.*, 1994), but are anchorage-dependent. To confirm their anchorage dependence, MKN45 cells were counted and plated onto 100-mm petri-dishes that had been coated with polyHEME. They could no longer attach to the plate and gradually reduced viability after 24 h culture in suspension. At 3 days of culture in suspension, more than 50% of MKN45

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cells lost their viability (cell survival was $44.9 \pm 2.6\%$) as shown in Figure 1a, indicating that MKN45 cells are anchorage-dependent. We next analysed the effect of TPA on anoikis of MKN45 cells. TPA induces growth, differentiation or apoptosis, depending upon the cell type and culture conditions (Clemens *et al.*, 1992). These divergent effects of TPA led us to consider whether TPA affects the anoikis of MKN45 cells. When MKN45 cells were placed in suspension, approximately 16% of the cells lost viability at 2 days of culture in suspension. An addition of 10 nM TPA to the culture medium greatly reduced their cell viability (cell survival was $43.4 \pm 3.1\%$) as shown in Figure 1b and led to a substantial increase of DNA fragmentation (Figure 1c). In sharp contrast, the apoptotic effect of TPA was not seen in the attached MKN45 cell (cell survival was $96.0 \pm 2.0\%$ at day 2). Thus, TPA augmented loss of viability of MKN45 cells depending upon suspension culture. We next explored possible ability of integrin-mediated signals to suppress the effect of TPA on apoptosis in the detached MKN45 cells. The anti-integrin $\beta 1$ murine monoclonal antibody (Ab) K20 stimulates tyrosine phosphorylation of proteins of 115 to 130 kDa and mimics the attachment of the cells via $\beta 1$ integrin to fibronectin (Kornberg *et al.*, 1991) when the antibodies were cross-linked on the surface of the cells by addition of anti-mouse immunoglobulin Ab (α -M). When MKN45 cells grew in suspension with TPA plus K20 for 24 h, loss of their viability was clearly suppressed (Figure 1d) and reached a level (cell survival was $81.5 \pm 1.8\%$) similar to those observed in the cells cultured without TPA

(Figure 1a and b). However, the addition of α -M alone did not induce the effect. These data confirmed that apoptotic effect of TPA depended upon loss of anchorage, excluding a possibility that the effect is due to nonspecific toxic effect of TPA.

Preferential activation of PKC α during augmentation of anoikis

TPA selectively activates both conventional and novel PKCs, but not atypical PKCs (Nishizuka, 1988; Ways *et al.*, 1992; Hug and Saue, 1993). We thus analysed whether the TPA-mediated enhancement of anoikis required activation of conventional PKCs. We first utilized several PKC inhibitors Gö 6976 and myr- ϕ PKC, which selectively suppress activity of conventional PKCs (Martiny-Baron *et al.*, 1993; Eicholtz *et al.*, 1993). The PKC inhibitors clearly suppressed the effect of TPA on anoikis in a dose-dependent manner (Figure 2). Thus, conventional PKCs may be responsible for the effect of TPA on anoikis. Among several conventional PKC isoforms, PKC α and β are expressed in epithelial cells (Verstovsek *et al.*, 1998) and thus we examined their PKC activity followed by either suspension culture and/or TPA treatment in MKN45 cells. TPA increased enzymatic activity of both PKC isoforms by a 1.4-fold increase (P value is <0.02) in the attached cells (Figure 3a). Interestingly, suspension culture alone slightly increased enzymatic activity of PKC α (Figure 3a). This implies that PKC α is likely to be persistently activated by suspension

Figure 1 Effect of TPA on anoikis in MKN45 cells. (a) Loss of anchorage reduced cell viability in MKN45 cells. Human gastric cancer MKN45 cells, provided from Japanese Cancer Research Resources Bank (Tokyo, Japan), were cultured for the indicated days at 5×10^5 cells/ml onto petri dishes that had been coated with polyHEME (Sigma, St Louis, MO, USA). Cell survival was assessed by trypan blue exclusion assay. (b) Augmentation of anoikis by TPA treatment. MKN45 were cultured at 5×10^5 cells/ml onto uncoated petri-dishes (-) or polyHEME coated dishes (+) for the indicated days with (+) or without (-) 10 nM TPA (Sigma). Cell survival was assessed by trypan blue exclusion assay. (c) Enhancement of DNA fragmentation by TPA in the MKN45 cells in suspension. The MKN45 cells were seeded at 4×10^6 cells per 100-mm plate into petri dishes coated with polyHEME for 40 h with (+) or without (-) 10 nM TPA. The low molecular weight genomic DNA was extracted with 0.5% Triton X-100, 10 mM EDTA, and 10 mM Tris, pH 7.4, treated with 400 μ g/ml of RNase A and Proteinase K for 1 h at 37°C, ethanol precipitated and analysed on 1.8% agarose gels. The gels were stained with 1 μ g/ml of ethidium bromide. (d) Inhibition of apoptotic effect of TPA by integrin-mediated signals. MKN45 cells were cultured in suspension with 10 nM TPA for 24 h with (+) or without (-) addition of anti-integrin $\beta 1$ murine monoclonal Ab K-20 (10 μ g/ml; Coulter, Cedex 9, France) and/or polyclonal rabbit anti-mouse Ab (α -M; 20 mg/ml; Jackson Laboratories, Bar Harbor, ME, USA). The rates of cell survival were assessed by the ability to exclude trypan blue. All data represent the mean $\pm$ standard deviation of three independent experiments in a, b and d

culture and this activation might involve in signaling for anoikis. Moreover, in the detached MKN45 cells, 10 nM TPA further increased PKC α activity (a 2.2-

Figure 2 Effect of PKC inhibitors on TPA-mediated augmentation of anoikis in MKN45 cells. **(a)** Inhibition of TPA-mediated augmentation of anoikis by Gö 6976 (Calbiochem, La Jolla, CA, USA). **(b)** Inhibition of TPA-mediated augmentation of anoikis by a pseudosubstrate myr- ψ PKC (Promega, Madison, WI, USA). Cells were cultured at 5×10^5 cells/ml onto the poly-HEME coated petri dishes with 10 nM TPA plus Gö 6976 (nM) or myr- ψ PKC (μ M) at the indicated concentrations for 24 h. The rates of cell survival were assessed by the ability to exclude trypan blue. All data represent the mean $\pm$ standard deviation of three independent experiments

fold) compared with that in the untreated cells (Figure 3a). By contrast, TPA stimulated PKC β to the same extent in the attached and detached cells. Thus, PKC α was predominantly activated by TPA when the cells lost anchorage.

To preliminary explore a possible mechanism for preferential activation of PKC α in the TPA-treated detached cells, we compared protein levels of PKC α , β , ϵ and δ in the detached MKN45 cells with those in the attached cells after 18 h of culture with or without 10 nM TPA. PKC's α and β are conventional, while PKC's ϵ and δ are novel PKCs (Dekker and Parker, 1994). These PKCs are known to be expressed in epithelial cells (Verstovsek *et al.*, 1998). In the detached MKN45 cells, TPA increased expression levels of PKC α , but not those of the other isoforms examined. In contrast, TPA did not affect their protein levels in the attached MKN45 cells (Figure 3b). From these data, the elevated PKC α protein levels may partly explain preferential increase of PKC α activity by TPA in the detached MKN45 cells.

Overexpression of PKC α augmented anoikis in MKN45 cells

To confirm whether activation of PKC α alone promotes anoikis, we established a vaccinia virus recombinant expressing the PKC α gene. MKN45 cells

Figure 3 Activation of PKC isoforms by TPA in the MKN45 cells. **(a)** MKN45 cells cultured either in suspension (+) or onto uncoated dishes (-) in the presence (+) or absence (-) of 10 nM TPA. The cells were lysed in 100 μ l of RIPA buffer containing 100 mM NaCl, 2 mM EDTA, 10 mM sodium orthovanadate, 1 mM PMSF, 1% NP-40 and 50 mM Tris [pH 7.2]. Lysates (200 μ g/assay) were immunoprecipitated with either anti-PKC α or anti-PKC β Ab (Transduction Laboratories, Lexington, KY, USA), and thereafter subjected to PKC enzyme assays. The PKC activities were assayed by measuring the incorporation of ^{32} P into a PKC specific substrate peptide from [γ - ^{32} P]ATP using Protein kinase C enzyme assay system (Amersham, Buckinghamshire, UK) as previously described (House *et al.*, 1987). All data represent the mean $\pm$ standard deviation of three independent experiments. **(b)** Expression of α , β , ϵ and δ PKC isoforms in MKN45 cells. Total cell lysates (80 μ g/lane) from the detached (suspension) and the attached (adhesion) MKN45 cells cultured with (+) or without (-) 10 nM TPA were subjected to SDS-PAGE and immunoblotted with 0.25 μ g/ml of anti-PKC α , anti-PKC β , and anti-PKC ϵ (Transduction Laboratories) or 0.1 μ g/ml of anti-PKC δ Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h. The blots were developed by a standard enhanced chemiluminescence (ECL) method (Amersham)

infected with the PKC α -recombinant vaccinia virus highly expressed PKC α protein in a virus-dose-dependent manner though the control MKN45 cells infected with the vaccinia virus-influenza HA recombinant expressed much less (Figure 4a). Overexpression of PKC α led to an enhancement of PKC α activity up to approximately 1.7-fold increase compared with the parental and control infected MKN45 cells (Figure 4b). When MKN45 cells were infected with either vaccinia virus-PKC α or vaccinia virus-influenza HA recombinant and cultured for 36 h in the regular tissue culture dishes, their viability was not significantly affected (Figure 4c). However, by suspension culture after 12 h-incubation with these recombinants, MKN45 cells overexpressing PKC α began to lose their viability, and reached survival rates of $42.4 \pm 3.5\%$ of control cells at 24 h-suspension culture, whereas the rate of survival was $88.7 \pm 3.8\%$ in case of the MKN45 cells infected with the vaccinia virus-influenza HA (Figure 5a). Representative data of trypan blue exclusion analysis revealed an extensive loss of viability in the detached MKN45 cells infected with vaccinia virus-PKC α (Figure 5b). Significant loss of viability in the PKC α -overexpressing MKN45 cells led to substantial increase of DNA fragmentation, but the control detached MKN45 cells revealed much less DNA fragmentation (Figure 5c). In addition, overexpression of PKC α significantly increased CPP32 activity (2.7-fold increase) in the detached MKN45 cells (Figure 5d). The control detached cells also revealed an increase of CPP32 activity due to loss of anchorage but the increase was far less. These data indicate that overexpression of PKC α alone clearly augmented anoikis in MKN45 cells.

Overexpression of PKC α augmented anoikis in MKN74 cells

To investigate PKC α -mediated augmentation of anoikis is restricted to MKN45 cells, we also examined the effect of overexpression of PKC α on anoikis of MKN74 cells which are also anchorage-dependent. MKN74 cells infected with the recombinant PKC α -vaccinia virus highly expressed PKC α protein (Figure 6a). When MKN74 cells were cultured for 24 h in suspension, the rate of cell survival was $70.3 \pm 2.0\%$, while overexpression of PKC α significantly reduced the survival rates ($40.7 \pm 2.2\%$). However, overexpression of PKC α did not affect their viability when they attached to plates (Figure 6b). In addition, overexpression of PKC α significantly increased CPP32 activity (3.4-fold increase) in the detached MKN74 cells (Figure 6c). However, the same treatment did not increase CPP32 activity significantly when they attached to plates. These data indicate that overexpression of PKC α alone also augmented anoikis in MKN74 cells, did not affect cell viability in the attached MKN74 cells.

In this report, we have shown that activation of PKC α caused augmentation of anoikis in gastric cancer cells. It is notable that overexpression of PKC α increased apoptotic death of MKN45 and MKN74 cells only when they grew in suspension, but had little effect on the attached cells. This indicates that PKC α -mediated enhancement of apoptotic death depends upon loss of anchorage. It has been described that integrin-mediated signals suppress anoikis by induction of Bcl-2 expression (Zhang *et al.*, 1995) and inactivation of ICE activity (Boudreau *et al.*, 1995). So far,

Figure 4 Overexpression of PKC α in MKN45 cells infected with vaccinia virus-PKC α recombinant. (a) Overexpression of PKC α protein in MKN45 cells. MKN45 cells were infected with the control vaccinia virus-influenza HA (Influenza HA) or the indicated multiplicity of infections (m.o.i.) of the vaccinia virus-PKC α (PKC α) recombinant. Vaccinia virus-PKC α recombinants were made by inserting a full-length cDNA of human PKC α into the *Sall* and *StuI* digest of pSC11SS, a derivative of the transfer vector pSC11 (Chakrabarti *et al.*, 1985). Recombinant vaccinia viruses were produced by standard homologous recombination technique and plaque purified three times. Total cell lysates (80 μ g/lane) were immunoblotted with anti-PKC α Ab using a standard ECL method. The filter was reprobed with anti-actin AC-40 Ab (Sigma) to verify loading of equal amounts of protein. As a control, we also used a recombinant vaccinia virus expressing the influenza virus hemagglutinin (HA) (Smith *et al.*, 1983). No detectable PKC α in the parent and control cells are due to short exposure for its detection. (b) Increase of PKC activity in MKN45 cells infected with the vaccinia virus-PKC α recombinants. The PKC activity was measured as described for Figure 3. (c) Cell viability of the attached MKN45 cells infected with the vaccinia virus recombinants. After infection with the vaccinia virus-influenza HA (Influenza HA; m.o.i. = 1.0) or vaccinia virus-PKC α (PKC α) recombinant at the indicated m.o.i., the cells were incubated onto uncoated petri dishes for 36 h and their cell viability was assessed by trypan blue exclusion assay. The results shown are the mean $\pm$ standard deviation of three independent experiments

Figure 5 Overexpression of PKC α augmented anoikis of MKN45 cells. (a) Increase of cell death in the detached MKN45 cells by overexpression of PKC α . After infection with either vaccinia virus-influenza HA (triangle; m.o.i. = 1.0) or vaccinia virus-PKC α (open circle, m.o.i. = 0.3; shaded circle, m.o.i. = 1.0; closed circle, m.o.i. = 3.0) or without infection (square), MKN45 cells were incubated for 12 h. They were then cultured onto the polyHEME coated plates for the indicated hours. Cell survival was assessed by trypan blue exclusion assay. (b) Phase contrast photomicrographs of the representative MKN45 cells infected with either vaccinia virus-influenza HA or vaccinia virus-PKC α (m.o.i. = 1.0). Following the inoculation of a recombinant virus, cells were incubated for 12 h, then re-seeded onto the polyHEME coated plates for 18 h, and stained with trypan blue. (c) Enhancement of DNA fragmentation by overexpression of PKC α in the detached MKN45 cells. Following a 12 h culture after the indicated recombinant viral infection (m.o.i. = 1.0), the MKN45 cells were cultured in suspension for 24 h. DNAs were isolated and loaded onto 1.8% agarose gel. (d) Overexpression of PKC α predominantly increased CPP32 activity in the detached MKN45 cells. MKN45 cells were infected with the recombinant vaccinia viruses, incubated for 12 h and transferred into suspension culture for 10 h. CPP32 protease activity (Nicholson *et al.*, 1995) was measured using ApoAlert CPP32 Assay Kits (Clontech, CA, USA). In brief, 5×10^6 cells infected with recombinant vaccinia viruses (m.o.i. = 1.0) were lysed with NP40 lysis buffer and these lysates were incubated with CPP32 substrate DEVD-pNA. Cleavage of the substrate was determined in the spectrophotometer at 400 nm by comparing the results with the level of uninfected cells. The samples were also incubated with a CPP32 inhibitor DEVD-fmk before addition of the substrate. There was no significant increase of CPP32 activity in the attached MKN45 cells infected with both recombinant vaccinia viruses (data not shown). All data represent the mean $\pm$ standard deviation of three independent experiments

Figure 6 Augmentation of anoikis in MKN74 cells by PKC α . (a) Overexpression of PKC α protein in MKN74 cells. MKN74 cells were infected with the control vaccinia virus-influenza HA (Influenza HA) or the vaccinia virus-PKC α (PKC α) recombinant (m.o.i. = 1.0). The total cell lysates (80 μ g/lane) of infected MKN74 cells were immunoblotted with anti-PKC α antibody using a standard ECL method. The filter was re-probed with anti-actin AC-40 Ab to verify loading of equal amounts of protein. (b) Increase of cell death in the detached MKN74 cells by overexpression of PKC α . After infection with either vaccinia virus-influenza HA or vaccinia virus-PKC α (m.o.i. = 1.0), MKN74 cells were incubated for 12 h, and thereafter transferred into suspension culture (+) or uncoated dishes (-) for 24 h. Cell survival was assessed by trypan blue exclusion assay. (c) Overexpression of PKC α predominantly increased CPP32 activity in the detached MKN74 cells. MKN74 cells were infected with the recombinant vaccinia viruses, incubated for 12 h and transferred into suspension culture (+) or uncoated dishes (-) for 8 h. CPP32 protease activity was measured as described in Figure 5. All data represent the mean $\pm$ standard deviation of three independent experiments

there has been few information concerning a direct linkage of PKC α -mediated signalings and these two events. Here we showed that suspension culture slightly increased PKC α , and that overexpression of PKC α increased CPP32 activity specifically in the detached human gastric cancer cells. These data raise a possibility that there is an integrin-mediated pathway to inhibit signalings from PKC α to activation of caspase cascade.

The existence of at least 12 members of the PKC gene family suggest that each isoform may have an individual function concerning different substrates and subcellular localization. In our studies, suspension culture slightly increased PKC α in human gastric cancer MKN45 cells. Together with our data using a biochemical reagent TPA and its selective inhibitors Gö 6976 and myr- ϕ PKC, we conclude that PKC α may be a crucial PKC isoform to confer augmentation of anoikis in gastric cancer cells. Similarly, in colon cancer cell line Caco-2, PKC α , but not other PKC isoforms, inhibits cell growth and induces differentiation (Abraham *et al.*, 1998), and conversely, down-regulation of PKC α enhances cell growth and induces the transformed phenotype of Caco-2 cells (Scaglione-Sewell *et al.*, 1998). Though these data are not directly linked to anoikis, persistent activation of PKC α may contribute to apoptotic cell death in a variety of epithelial cells. However, we cannot exclude a

possibility that other PKC isoforms may also augment anoikis.

We directly demonstrated that an overexpression of PKC α alone augmented anoikis in MKN45 and MKN74 cells using recombinant vaccinia viruses. To exclude a possible involvement of vaccinia virus itself on anoikis, we also studied the effects of gene transfer-mediated overexpression of PKC α on anoikis in COS7 cells and revealed that overexpression of PKC α also promoted anoikis in COS7 cells (data not shown). These data exclude an involvement of vaccinia virus itself in anoikis.

Our data enlighten a novel function of PKC α as a modulator of anoikis. It remains to be clarified how PKC α enhances anoikis and how the effect is inhibited by integrin-mediated signal(s). Understanding of these molecular mechanisms may help for introduction of anoikis in anchorage-dependent adenocarcinoma cells and provide useful information for anti-cancer therapy.

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