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The Overexpression of PKCδ Is Involved in Vascular Endothelial Growth Factor-Resistant Apoptosis in Cultured Primary Sinusoidal Endothelial Cells

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We have previously reported that primary sinusoidal endothelial cells from the rat liver are highly dependent on VEGF for cell proliferation in *in vitro* culture. However, even in the presence of VEGF, essentially all the SE cells could not survive longer than 7 days, leading to growth factor-resistant cell death. The death had characteristics typical of apopotosis, such as DNA fragmentation, staining with TUNEL reagent and nuclear condensation. We found that the cell death was blocked by the treatment of TPA in a dose-dependent manner and was preceded by a remarkable increase in PKCδ at a protein level. Furthermore, PKCδ-specific inhibitor, Rottrelin, significantly suppressed this VEGF-resistant apoptosis of cultured SE cells, whereas conventional PKC-specific inhibitor, Gö6976 could not. TPA was found to downregulate the overexpression of PKCδ. Thus, we suggest that the VEGF-resistant apoptosis is a new type of endothelial cell death and that PKCδ is an essential mediator for this process.

Key Words: **apoptosis; vascular endothelial cells; PKCδ.**

Apoptosis is a process by which cells activate their own death or cell suicide (1). In previous studies, it is considered to be essential for many physiological processes, including establishment of immune tolerance, morphogenesis, remodeling of tissues and so on (1). Cells undergoing apoptosis show a unique morphology in the form of shrunken cytoplasm with blebbing membranes, the formation of an apoptotic body and the condensation of nucleus (2). Apoptosis is also associated with the activation of a particular type of DNase, CAD, that degrades chromosomal DNA into small fragments (3). In the *in vitro* systems, apoptosis can be induced by various extrinsic and intrinsic factors such as activation of cell-surface receptors, withdrawal of growth factors, radiation, oxidative stress, hormonal stimulation and cell cycle perturbation (4). Although the morphological and biochemical features of apoptosis have been well demonstrated, its molecular mechanisms are not yet fully understood.

In vascular system, endothelial cells lining inside the blood vessels as well as smooth muscle cells are known to be essential components for the establishment of blood vessels. Proliferation, differentiation and cell death of endothelial cells are observed through the embryogenesis, particularly in the remodeling of vascular network (5). Furthermore, similar dynamic changes of the endothelial cells are also found in the process of a variety of pathological angiogenesis such as diabetic retinopathy and solid tumor growth *in vivo* (6, 7). Therefore, further analysis on the dynamics of the endothelial cells are important for a better understanding of the molecular basis of physiological and pathological blood vessel formation and remodeling.

Among the angiogenic factors so far described, vascular endothelial growth factor (VEGF) is considered to be a specific and fundamental regulator of the endothelial cell growth and differentiation (8, 9). We have previously shown that the primary sinusoidal endothelial cells obtained from rat liver are strictly dependent on VEGF as a natural ligand for their growth (10), and without VEGF these cells rapidly shrink and die similar to the cases of hematopoietic cells starved with growth-promoting cytokines such as IL-3 (11).

Interestingly, however, we also found that after 2 to 3 cycles of cell division, these primary endothelial cells start to die even in the presence of freshly supplemented VEGF. This process appears not to be a senescence of cells, since the senescence is usually thought to take place after 50 to 60 cycle cell division. Thus, we attempted to clarify this VEGF-resistant, yet unclassified endothelial cell death, which might reflect a process of vascular remodeling in certain conditions *in vivo.*

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In this study we show that this new type of endothelial cell death in the presence of VEGF is due to an apoptotic process and that an abnormal upregulation of PKCδ within the cell may be involved in this phenomenon.

MATERIALS AND METHODS

Cell culture. Recombinant human VEGF (165 amino-acid form) was purified by heparin column chromatography from the conditioned medium of Sf-9 insect cells expressing VEGF. Sinusoidal endothelial (SE) cells were isolated from the rat liver according to the procedure previously described (9), and grown in EGM-UV medium (Kurabo, Osaka) supplemented with VEGF (10 ng/ml).

Assay of cell growth. SE cells were grown in 24-well collagen-coated culture plates. They were unstimulated, or stimulated with 10 ng/ml VEGF or with 200 nM 12-O-tetradecanoyl phorbol 13-acetate (TPA; Sigma Chemical Co., St. Louis, MO) for 10 days. The medium was changed every 2 days. The number of cells in 1.0 × 1.5 mm rectangles were counted every 24 h. Data represented the average of triplicate samples. To analyse cell survival effects of TPA, SE cells were cultured in EGM-UV medium supplemented with 10 ng/ml VEGF. From the 3rd day on, various concentrations of TPA were added to the medium as indicated in the Fig. 3. On the 7th day, living cells were counted. Data represented the average of triplicate samples. Alternatively, for inhibition assay of apoptosis, the cells were incubated with various chemicals, such as 0.5 μM Gö6976 or 0.3 μM Rottlerin (Calbiochem Novabiochem, Nottingham, UK) or the vehicle on 4th day.

Assessment of apoptosis. For analysis of DNA fragmentation, low-molecular-mass DNAs were isolated from SE cells as described elsewhere (2). The samples were subjected to gel electrophoresis on a 2% agarose gel in Tris-acetate buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.0). The gel was stained with ethidium bromide and photographed on a UV transilluminator. Alternatively, SE cells were analyzed for the TUNEL staining using the In Situ Cell Death Detection Kit; fluorescein (Boehringer Mannheim Biochemicals. Indianapolis, IN). To assess nuclear morphology, SE cells were fixed with 4% paraformaldehyde in PBS at room temperature and then stained with 10 μM Hoechst 33258 (Wako, Tokyo) in PBS for 15 min. The stained cells were then observed under a fluorescence microscope.

Immunoblotting. SE cells were grown in EGM-UV medium containing VEGF or TPA. The cell lysates were prepared every day and Western blotting performed according to the previously described procedure (12). Isoform (α, β and δ)-specific anti-PKC, anti-Fas and anti-Fas ligand were purchased from Transduction Laboratories, Inc. (Lexington, KY). Anti-α-tubulin antibody (Sigma) was used as a control to verify that an equivalent amount of protein was loaded in each lane. Rabbit polyclonal antiserum against KDR/Flk-1 was prepared as described previously (12).

RESULTS

Cultured SE Cells in the Presence of VEGF Spontaneously Induce Cell Death

We have previously reported that primary sinusoidal endothelial (SE) cells from the rat liver are highly dependent on VEGF for cell proliferation in *in vitro* culture (10). As shown in Figs. 1A and 1B, VEGF stimulated proliferation of SE cells for up to 7 days. However, even in the presence of VEGF, essentially all the SE cells could not survive longer, leading to spontaneous induction of cell death. On the other hand, TPA, an activator of protein kinase C (PKC), also stimulated proliferation of SE cells for the first 4 days as recently reported (12). However, thereafter, TPA could not stimulate the cell proliferation any more, and only maintained the cell viability. Thus, we suggest that TPA exerts bidirectional growth regulatory effects on SE cells, growth stimulation of SE cells for the first 4 days and maintenance of the cell survival for the latter periods.

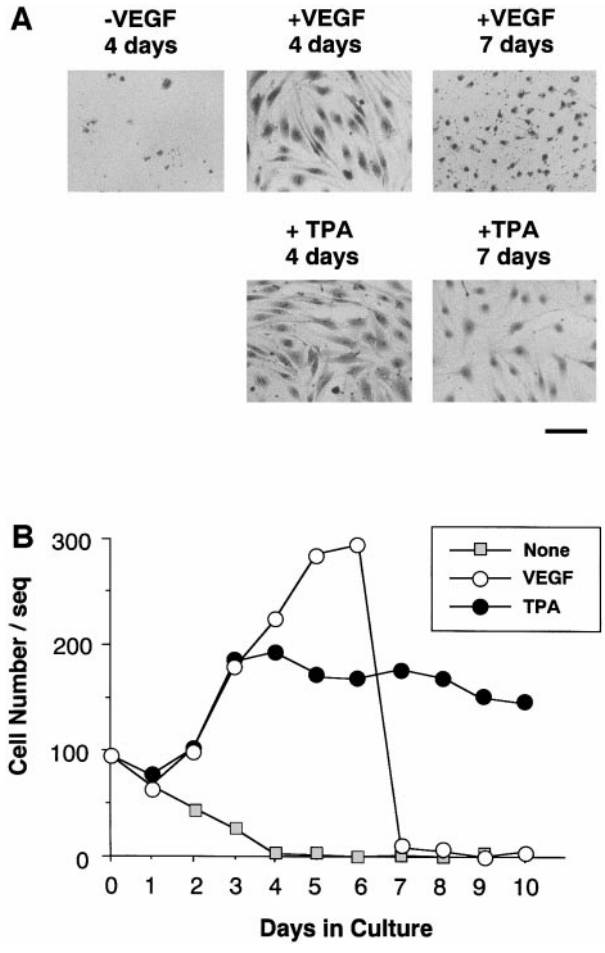

FIG. 1. Effects of VEGF or TPA on the proliferation of SE cells. (A) Morphological appearance of SE cells stimulated with VEGF or TPA. SE cells were cultured in EGM-UV medium on a collagen-coated plate. Cells were stimulated with 10 ng/ml VEGF or 200 nM TPA. Cells were fixed on day 4 or 7 with 4% paraformaldehyde and stained with crystal violet. (B) Growth curve of SE cells in response to VEGF or TPA. SE cells in 1.0 × 1.5 mm rectangles at three wells were counted every 24 h and the average number plotted. Bar, 50 μm.

SE cells undergoing death showed reduction in cell volume, shrunken cytoplasm and condensation of chromatin, which represent similar morphological features to apoptosis (Figs. 1A and 2B) (2). In contrast, morphological characterization of TPA-treated cells was almost the same as that of VEGF-treated growing cells

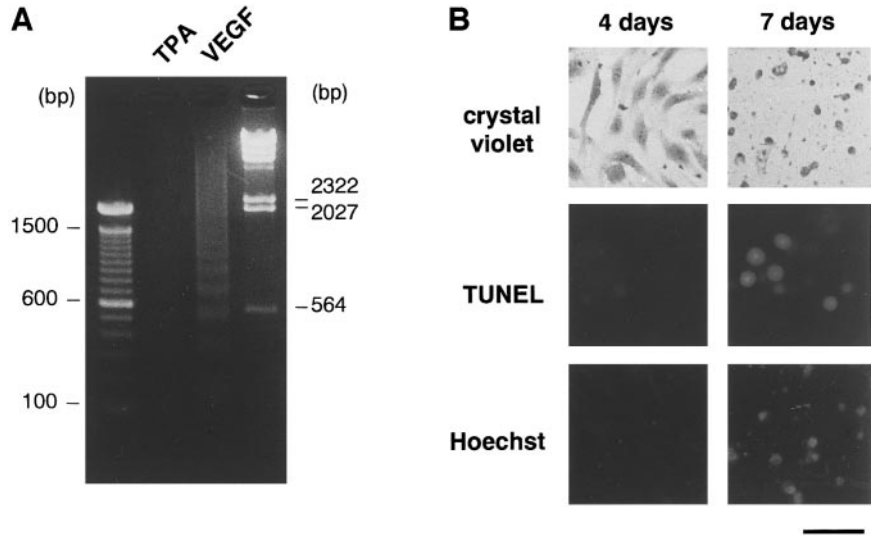

FIG. 2. (A) DNA degradation of SE cells. SE cells were cultured in the presence of 10 ng/ml VEGF or 200 nM TPA. When most SE cells were dead (on the 7th day), they were lysed and cellular DNA was isolated as indicated in Materials and Methods. Isolated cellular DNAs or DNA markers (shown in the right and left lanes) were subjected to gel electrophoresis on a 2% agarose gel. The gel was stained with ethidium bromide and photographed on a UV transilluminator. (B) Morphological features of endothelial cell death. SE cells from either the 4th-day culture or 7th-day culture were fixed with 4% paraformaldehyde and then stained with crystal violet (upper panels), TUNEL reagent (middle panels) and Hoechst 33258 (lower panels) as described in Materials and Methods. Bar, 50 μm.

on day 4. This SE cell death in the presence of VEGF is unique among cultured primary cells, since growth factors usually stimulate cell prolifetration and survival.

SE Cells Undergo Death by Apoptosis

It has recently been reported that apoptosis was characterized by the activation of endogenous deoxyribonuclease CAD and subsequent DNA degradation (3). Thus, to examine whether the mode of SE cell death in the presence of VEGF is related to apoptosis or not with respect to DNA structure, we assessed DNA fragmentation on agarose gels as a biochemical marker of apoptosis. As shown in Fig. 2, DNA isolated from SE cells undergoing cell death exhibited considerable degradation and ladder formation. In contrast, cells treated with TPA, which escaped from cell death, did not show any sign of DNA degradation. Morphological examination of cells stained with Hoechst dye 33258 revealed that a large number of nuclei from the 7th day culture show condensed chromatin, as compared to those from the 4th day control culture. The pattern of DNA fragmentation and these morphological features of the nuclei support the notion that the VEGF-resistant endothelial cell death involves an apoptotic process.

TPA Rescues the Death of SE Cells in a Dose-Dependent Manner

Based on the findings that SE cells did not undergo apoptosis in the presence of TPA (Fig. 1), we hypothesized that TPA treatment rescues the VEGF-resistant apoptosis in the presence of VEGF. To examine the effect of TPA on the survival of SE cells with VEGF, we treated SE cells with various concentrations of TPA from the 3rd day to 7th day. When SE cells exert apoptotic cell death even in the presence of VEGF at about 7 days, TPA suppressed the cell death in a dose-dependent manner (Fig. 3).

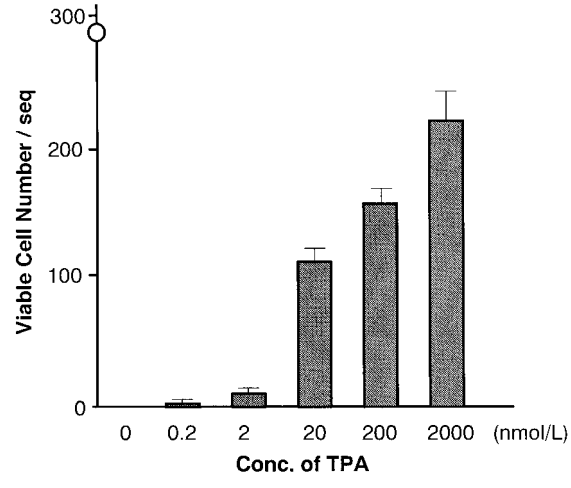

FIG. 3. Dose-dependent survival effects of TPA in SE cells. SE cells were cultured in EGM-UV medium supplemented with 10 ng/ml VEGF. From the 3rd day onward, various concentrations of TPA were added to the medium as indicated. On the 7th day, living cells were counted. Data represent the average of triplicate samples. Open circle represents the cell number on the 5th day when most of the SE cells are proliferating.

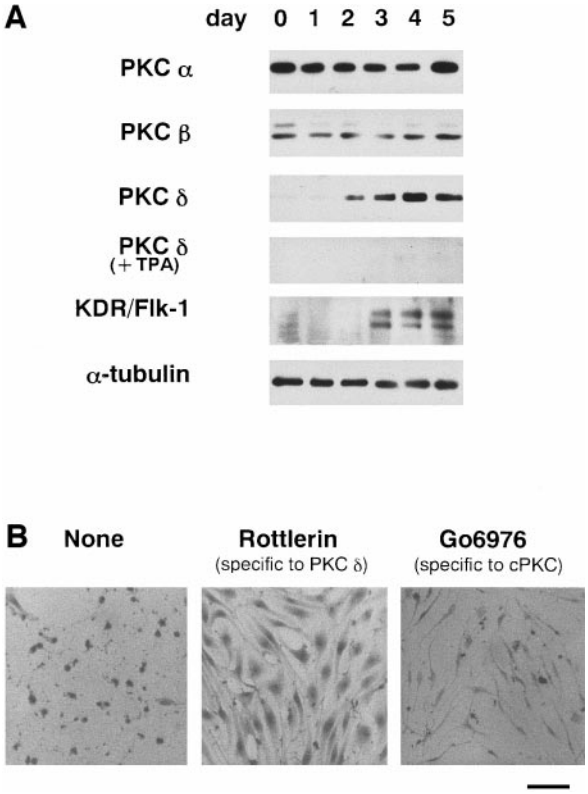

FIG. 4. Involvement of PKCδ upregulation in VEGF-resistant apoptosis. (A) Overexpression of PKCδ in SE cells. SE cells were cultured in the presence of 10 ng/ml VEGF for 5 days. Alternatively, SE cells were cultured with 200 nM TPA instead of VEGF. Cell lysates were prepared (see Materials and Methods) and subsequently analyzed by Western blotting using the appropriate antibodies as indicated. (B) PKCδ-specific inhibitor delayed the time of apoptosis. SE cells were treated with vehicle alone, Rottlerin (an inhibitor specific for PKCδ) or Gö6976 (an inhibitor specific for cPKC) in culture. When cells treated with vehicle alone underwent apoptosis (at about 7 days), all the cell samples were fixed with 4% paraformaldehyde and stained with crystal violet. Bar, 50 μm.

The Apoptosis Was Preceded by an Increase in PKCδ

Since one of the major targets of TPA is known to be PKC, initially we examined the time course of the increase or decrease of each PKC isozyme to know the involvement of PKC on apoptosis. As shown in Fig. 4A, PKCα, and PKCβ activated by VEGF stimulation, were almost unchanged at protein levels. Surprisingly, only the level of PKCδ were significantly increased up to 5 fold at 4th or 5th day, just before undergoing apoptosis. In five independent experiments, VEGF consistently caused a marked increase in PKCδ, preceding the apoptosis. In contrast, in cells treated with TPA which was escaped from cell death, PKCδ was undetectable possibly due to the downregulation of PKCs. Thus, we suggest that anti-apoptotic effects of TPA were at least in part due to downregulating PKCδ.

Another important system inducing apoptosis, Fas-Fas ligand system (13) does not appear to be involved in this apoptotic process, since the level of Fas was at undetectable level in SE cells. In contrast, the expression of Fas ligand was not affected during the observation period (data not shown). Moreover, one of the VEGF receptors, Flk-1/KDR, which is the major mediator of VEGF signals for cell proliferation, was still significantly expressed on day 5 (Fig. 4A). Therefore, SE cell death in the presence of VEGF is not considered to be due to the downregulation of Flk-1/KDR.

PKCδ-Specific Inhibitor Delayed the VEGF-Resistant Apoptosis

We next examined whether the increase in PKCδ is responsible for apoptosis in SE cells. The treatment of SE cells with Rottlerin, a PKCδ- specific inhibitor, significantly delayed the onset of apoptosis. In contrast, Gö6976, another inhibitor specific for conventional PKC, namely PKCα, β and γ, had no inhibitory effects on apoptosis, but rather accelerated the apoptosis of SE cells (Fig. 4B). These results strongly suggest that the abnormal increase in the activity of PKCδ via its overexpression in the protein levels triggers the apoptotic cell death of primary endothelial cells in culture.

DISCUSSION

In this study we found that, in addition to the growth factor-deficient cell death (10), rat liver primary sinusoidal endothelial cells undergo growth factor-resistant apoptotic cell death even in the presence of VEGF. Furthermore, this cell death was associated with overexpression of PKCδ, and a PKCδ-specific inhibitor was able to suppress the cell death. These results strongly suggest an involvement of abnormally expressed PKCδ as a crucial mediator for this process. A mechanism of senescence in this cell death appears unlikely, since this process started only after a few cell divisions were stimulated with VEGF.

One of the major questions on this apoptotic process is that why a typical growth factor VEGF specific to endothelial cells does not transduce an efficient survival signal. One possible explanation is that the VEGF receptors, Flt-1 and KDR/Flk-1 tyrosine kinases, particularly the major signal transducer KDR/Flk-1, do not generate a strong cell survival signal. PI3 kinase and Akt, a serine/threonine kinase pathway has been shown to be one of the important pathways for anti-apoptosis in a variety of cells (14). We have recently shown that KDR/Flk-1 has a strong tyrosine kinase activity and generate a cell proliferation signal from VEGF via PLC-γ-PKC-MAP kinase pathway, but only poorly activate the PI3 kinase pathway in SE cells (12). Therefore, the

growth-promoting signal from VEGF receptor appears to be not tightly linked to the cell survival signal in these cells. Recently, Fujio and Walsh have reported that endothelial cell survival induced by VEGF is PI3 kinase-Akt dependent in human umbilical vein endothelial (HUVE) cells (15). Since we found that the activation of PI3 kinase via VEGF receptor is very weak compared with a typical tyrosine kinase receptor such as PDGF receptor, we suggest that only a small amount of signal from VEGF receptor is transduced for the activation of PI3 kinase.

Our results strongly suggest that PKCδ, but not a conventional PKC isoform, PKC-α, -β or -γ, is the crucial mediator for the induction of VEGF-resistant apoptosis in SE cells. PKCδ has already been shown to induce growth arrest (16–18) or apoptosis (19, 20) of cultured cells in a mitochondria-dependent manner. Furthermore, caspase-3, a key protease in the apoptosis-inducing cascade, was able to cleave the PKCδ to generate a constitutively active catalytic fragment of this PKC isoform (21). Since the PKCδ-induced apoptosis was shown to be blocked by caspase-3-specific inhibitor, a positive feedback mechanism between the activation of PKCδ and caspase(s) may be involved in the molecular basis of VEGF-resistant SE cell apoptosis.

Another important question based on our results is why only the PKCδ among the PKC isoforms was upregulated in the presence of VEGF. Although transcriptional regulation of PKCδ gene has not yet been extensively examined, a transcriptional activation or a post-transcriptional mechanism including stabilization of protein or both may be involved in VEGF-induced PKCδ upregulation.

In addition, it is quite interesting to see how many types of vascular endothelial cells in the body carry a VEGF-resistant cell death mechanism shown here. It seems important to identify in which types of vascular endothelial cells and what kind of blood vessels under physiological and pathological conditions the VEGF-resistant and PKCδ-dependent cell death is utilized for the vascular remodeling *in vivo*.

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