Protein Kinase C Lies on the Signaling Pathway for Vascular Endothelial Growth Factor-mediated Tumor Development and Angiogenesis

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ABSTRACT

The growth of any solid tumor depends on angiogenesis. Among the known angiogenic factors, vascular endothelial growth factor (VEGF) has been shown to play a pivotal role in tumor angiogenesis. However, to date, the signal transduction pathway initiated by VEGF is still not fully understood. It has been suggested that protein kinase C (PKC) plays an important role in the VEGF-induced signal transduction pathway in vivo, although the role of PKC in tumor angiogenesis in vitro still remains to be elucidated. By delivering the VEGF gene within the self-contained tetacycline-regulated retroviral vector (Retro-Tet) into hepatocellular carcinoma (HCC) cells, we manipulated VEGF expression by providing tetacycline in the drinking water to assess the tumor kinetics mediated exclusively by VEGF. In this study, we combined this Retro-tet system and LY333531, an inhibitor of the PKC-β isoform, to elucidate the role of PKC-β in tumor development and angiogenesis. Using a syngeneic xenograft model, tumor augmentation induced by VEGF overexpression in HCC was markedly suppressed by oral administration of the PKC-β inhibitor, with an accompanying reduction of neovascularization and p44/42 mitogen-activated protein kinase activation. This inhibitory effect was achieved even after the tumor was fully established. Immunohistochemical analysis revealed that apoptosis increased markedly in the tumor upon PKC-β inhibitor treatment, whereas tumor cell proliferation itself did not change. Furthermore, with orthotopical transplantation, PKC-β inhibition suppressed HCC tumor development in the liver. These results suggest that PKC-β lies on the signal transduction pathway by which VEGF augments development and angiogenesis not only at the initial stage but also after the tumor is fully established.

INTRODUCTION

The growth of a solid tumor is now widely recognized to depend on the process of neovascularization. Without angiogenesis, tumors cease to grow beyond even a few millimeters in volume (1–5). It has been shown that tumor vascular density is an independent prognostic marker in several types of human tumors and is correlated with a poor prognosis (6–14). To date, many positive and negative angiogenic factors have been identified (1, 3, 15). Among the positive factors, VEGF,2 originally identified as a vascular permeability factor, is the most intriguing factor with regard to tumor angiogenesis. VEGF is a specific mitogen for vascular ECs in vitro and can be an angiogenic factor for neovascularization in vivo (1–5). In contrast to basic fibroblast growth factor, which is also a representative positive angiogenic factor, VEGF has a typical signal peptide composed of 26 amino acids. Accordingly, it has been shown that VEGF is secreted abundantly in several human tumors and animal experimental models. An increase in VEGF expression in human surgical specimens has been shown to be correlated with aggressive behavior and prognosis (5–14). In animal experimental models, overexpression of VEGF enhanced tumor growth, angiogenesis, and dissemination, whereas suppression of VEGF inhibited tumor growth in several tumor cell types (16–22). VEGF has been shown to act mainly through two distinct type-III tyrosine-kinase receptors, flt-1 and Flk-1/KDR, which are specifically expressed in the vascular ECs. Recent studies revealed that VEGF and its receptor interaction play an important role in a number of pathological events such as tumor angiogenesis, and these two receptors have different roles and signaling pathways (2, 23–25). Flk-1/KDR appears to play a predominant role in angiogenesis process both in vitro and in vivo (23, 26–30). However, the biological process that underlines these signaling pathways remains largely unknown.

The Tet system is a novel tetracycline-regulated gene expression system (31). This system allows manipulation of the interest gene in an “on and off” manner in vivo, which the conventional expression system can’t do for their constitutive gene expression level either overexpression or suppression. In this study, we used a retrovirus-mediated modified vector in which the two components of the Tet system have been organized within the same vector (32, 33). With this Retro-Tet System, it is possible to observe tumor kinetics that can be achieved only by VEGF gene expression (21, 34). PKC is composed of a family of serine-threonine kinases. It has been postulated that activation of PKC is an important intracellular signaling pathway for cellular growth (35, 36). Several reports have shown that PKC plays an important role in the VEGF-initiated angiogenesis process (37–41). In KDR-transfected NIH3T3 cells and ECs, MAP kinase was activated upon VEGF stimulation. This MAP kinase activation was mediated mainly by the PKC pathway, especially the β isoform (42). Other reports showed that the PKC-β inhibitor strongly suppressed VEGF-dependent EC growth in vitro (37). In a rat diabetic model, VEGF-induced retinal permeability was suppressed by the PKC-β inhibitor (39, 40). However, the role of PKC in tumor angiogenesis has not yet been clarified.

In this study, in both a xenograft model and an orthotopical experimental model, we examined the role of PKC, especially the β isoform, in tumor development and angiogenesis at different stages, which was due exclusively to VEGF gene expression by means of a combination of the PKC-β inhibitor and the Tet system. We report here for the first time that PKC, especially the β isoform, is a major regulator of VEGF-mediated tumor development and angiogenesis.

MATERIALS AND METHODS

Construction of the Retroviral Vector. The tetracycline aspect of the retroviral vector is conferred by the response and regulator units of the previously described Tet-inducible promoter system (31). A complete description of the construction of this Retro-Tet system vector has been reported previously (32, 33). The parent PBSTR-1 vector was generously provided by Dr. S. A. Reeves (Massachusetts General Hospital, Boston, MA). Human VEGF cDNA was cloned into the multicloning site of the PBSTR-1 vector at a BamHI site, forming the Tet-VEGF vector.

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2 The abbreviations used are: VEGF, vascular endothelial growth factor; EC, endothelial cell; PKC, protein kinase C; HCC, hepatocellular carcinoma; MAP, mitogen-activated protein; FCNA, proliferative cell nuclear antigen; RT-PCR, reverse transcription-PCR; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

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Cell Culture and Retroviral Production. BALB/c mice-derived BNL. 1. 7R. 1 HCC (BNL-HCC) and BOSC 23 retrovirus packaging cells were purchased from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and American Type Culture Collection (Manassas, VA), respectively. Cells were grown in a supplier-recommended medium. Stable Tet-VEGF-expressing clone (Tet-VEGF-HCC) was generated as described previously (34). Briefly, after the transduction of Tet-VEGF into BNL-HCC cells, followed by 14 days of selection with puromycin (1.5 μg/ml; Sigma), surviving single cell colonies were picked up and expanded. The VEGF expression level in the condition media for each colony was measured by an ELISA (R&D Systems). For further study, we used the colony that showed the highest and lowest VEGF clone in the absence or presence of tetracycline (tet: 1 μg/ml; Sigma), respectively. Tet-lacZ vector was also transduced into BNL-HCC cells and used as control cells (Tet-lacZ-HCC).

Growth of VEGF-transduced HCC Cells in Vivo. In the xenograft model, 1 x 10^6 Tet-VEGF or lacZ-HCC cells were syngenuously inoculated into the flank of BALB/c mice. PKC-β inhibitor (LY333531) was administered orally to mice by gavage on the same day as transplantation. For the dose-ranging study, mice were given 10, 50, and 100 mg/kg PKC-β inhibitor, respectively. The tumor was measured twice a week using calipers, and the tumor volume was calculated as described previously (21). The next experiment was conducted to examine the effect of the PKC-β inhibitor on the fully established tumor. In this experiment, PKC-β inhibitor administration started on day 18 (tumor volume was 438 mm^3). To suppress VEGF expression in vivo, the mice were given tet-containing drinking water (1 mg/ml). For the orthotopical transplantation experiment, 1 x 10^6 Tet-VEGF or lacZ-HCC cells were injected directly into the liver, and mice were given 10 mg/kg of PKC-β inhibitor. Fourteen days after injection, mice were sacrificed and examined for HCC tumor development in the liver, macroscopically and microscopically.

Semi-quantitative RNA Expression of CD31. To evaluate the expression of platelet/EC adhesion molecule (PECAM/CD31) RNA, which is widely used as a neovascularization marker, we used semi-quantitative RT-PCR analysis. The tumor pool was prepared from three of Tet-VEGF, lacZ-transduced mice and snap-frozen in the liquid nitrogen immediately. The mRNA was extracted from the tumor pools, and RT-PCR was performed as described previously (34). PCR products of CD31 were examined by 1.2% agarose gel electrophoresis and stained by ethidium bromide. Densitometric analysis was performed by measuring the absorbance with a Fuji BAS 2000 image analyzer. The level of gene expression was calculated after normalization with the glyceraldehyde-3-phosphate dehydrogenase internal control.

Assay of MAP Kinase Activity. The tumor pool was prepared in the same manner as described for the CD31 expression evaluation. MAP kinase activity was examined with some modification, as described previously (41, 42). Equal concentrations of protein from concentrated tumor lysate were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane, and Western blot analysis was done using a phospho-specific p44/42 MAP kinase antibody (New England BioLabs) diluted 1:1000. Blots were developed using an amplified alkaline phosphates immune-blot assay kit (Bio-Rad).

Immunohistochemistry. Immunohistochemistry of CD31 was analyzed as described previously (43). Frozen sections were allowed to thaw at room temperature for 30 min and then washed once in PBS. Nonspecific binding was conducted by incubation with 0.1% BSA in PBS for 15 min and with normal rabbit serum for 30 min. Endogenous biotin was blocked with 0.1% avidin for 15 min, followed by biotin for 15 min. The section was washed once with PBS after each 15-min incubation period, then reacted with a primary rat anti-mouse CD31 antibody (PharMingen, San Diego, CA; 1:100) for 1 h. After washing twice with PBS, the sections were incubated with a second biotin-labeled rabbit antirat IgG (Novoceastra). Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in PBS for 30 min, followed by a 5-min washing with PBS. Sections were then incubated with conjugated streptavidin for 30 min, rinsed again in PBS, and incubated with diaminobenzidine for 3 min.

In vivo tumor cell proliferation was evaluated by quantitation of cells positive for PCNA and mitotic activity in tissue sections. Apoptosis was detected with the DNA fragmentation products that were stained by in situ 3′ end labeling (TUNEL). Immunohistochemistry for PCNA and TUNEL was performed as described previously (43, 44). Paraffin-embedded sections were used for the PCNA and TUNEL assay.

Statistical analysis. The statistical significance was analyzed by Tukey’s multiple comparison test.

RESULTS

Regulation of VEGF-mediated Tumor Growth Kinetics by Tetracycline. In vitro VEGF expression in Tet-VEGF-HCC was tightly regulated by tetracycline (1 μg/ml), as described previously (34). In vivo, Tet-VEGF-HCC cells showed a marked increase in tumor development compared to the control group (lacZ-HCC). When VEGF expression was inhibited by the addition of tetracycline (1 mg/ml) to the drinking water, tumor growth was significantly decreased, almost to the same level as the lacZ-HCC cells (Fig. 1). This indicates that the tumor growth kinetics of this study should be attributed exclusively to VEGF expression. Detectable endogenous VEGF was not seen in the wild-type BNL-HCC cells (data not shown).

Effect of PKC-β Inhibition on VEGF-mediated Tumor Development. First, we performed the syngeneic xenograft experiment to examine the role of PKC-β in VEGF-induced tumor development. To maintain VEGF overexpression, Tet-VEGF-HCC-bearing mice (the Tet-VEGF group) were given tetracycline-free normal water throughout the experiment. Three different concentrations of PKC-β inhibitor were given orally on a daily basis to the Tet-VEGF group at doses of 100, 50, and 10 mg/kg. As shown in Fig. 2A, even 10 mg/kg PKC-β inhibitor significantly inhibited tumor growth compared to the non-treated Tet-VEGF group (P < 0.001). Next we examined the effect of the PKC-β inhibitor on tumor development in fully established tumors. Because 10 mg/kg PKC-β showed a sufficient inhibitory effect, a 10 mg/kg dose was used for additional experiments. All mice of the Tet-VEGF group were allowed to grow in the absence of tetracycline (switch “on”) until day 18 (tumor volume was 438 mm^3). Next, half of the mice were given the PKC-β inhibitor starting on day 18. The tumor growth rate of the PKC-β inhibitor-treated mice was markedly reduced to almost the same growth rate observed in mice receiving the PKC-β inhibitor from day 0 (Fig. 2B). These results indicated that tumor augmentation induced exclusively by VEGF was significantly reduced by LY333531 at a dose of 10 mg/kg. Furthermore, this inhibitory effect appeared even after the tumor was established. In this xenograft experimental model, PKC activity in the tumor was significantly inhibited by the 10-mg/kg dose of the PKC-β inhibitor (data not shown).

Because an orthotopical transplantation experiment has been shown to be much closer to the real biological situation (45, 46), we also examined the effect of PKC-β inhibitor on HCC development in the liver. Table 1 indicates that the PKC-β inhibitor significantly reduced the tumor growth rate of the PKC-β inhibitor-treated mice (P < 0.001).
the number of tumors in the liver ($P < 0.001$). In addition, most mice of Tet-VEGF group had a large tumor (over 5 mm in diameter; seven of seven mice; 100%), whereas such a large tumor was seldom found in the PKC-β-inhibitor-treated mice (one of seven mice: 14.2%; Table 1). Neither tumor invasion into the other organ nor metastasis was observed at the sacrifice period (day 14). Systemic administration of PKC-β-inhibitor had no obvious effect on the state of health of the mice in both the xenograft and orthotopical experiments (data not shown).

Tumor Neovascularization. To determine whether the inhibitory effect of the PKC-β inhibitor on tumor development was accompanied by the suppression of tumor angiogenesis, we examined CD31 expression in the tumor. First we performed an immunohistochemical analysis for CD31. As shown in Fig. 3, CD31-positive vessels were significantly decreased in PKC-β inhibitor-treated mice. However, it seemed difficult to obtain reliable vascular counts because there were only small slit-positive vessels in PKC-β inhibitor-treated mice. Accordingly, we next performed the semiquantitative RT-PCR analysis for $CD31$ gene expression. CD31 RNA expression in PKC-β inhibitor-treated mice was five times lower than that in Tet-VEGF mice without PKC-β inhibitor treatment (Fig. 4 and Table 2).

MAP Kinase Activity in Situ. The p44/42 MAP kinases are shown to be associated with cell proliferation induced by a number of mitogens, including VEGF and basic fibroblast growth factor (42, 47, 4415).

![Fig. 2. A, the growth effect of PKC-β inhibition on Tet-VEGF-HCC tumor development. To stimulate VEGF overexpression, mice were given tetracycline-free normal drinking water throughout the experiment. The PKC-β inhibitor was administered orally each day at doses of 100, 50, and 10 mg/kg from the beginning of the experiment. Each point represents the mean ± SD ($n = 6$). * represents a statistically significant difference compared to the Tet-VEGF-HCC ($P < 0.001$). B, the effect of PKC-β inhibition in established tumors. Mice were given PKC-β inhibitor (10 mg/kg) from day 0 or from day 18 (tumor volume was 438 mm$^3$). Arrows indicate the time points of administration of PKC-β inhibitor. The tumor growth rate of PKC-β inhibitor-treated mice from day 18 was markedly reduced to almost the same level as in mice which received PKC-β inhibitor from day 0. Each point represents the mean ± SD ($n = 6$). * and ** represent a statistically significant difference compared to the Tet-VEGF-HCC group ($P < 0.001$ and $P < 0.005$, respectively).

![Fig. 3. Effect of PKC-β inhibition on platelet/EC adhesion molecule (PECAM/CD31) expression in Tet-VEGF-HCC tumors. Untreated Tet-VEGF-HCC tumors (A) and Tet-VEGF-HCC tumors from animals receiving 10 mg/kg PKC-β inhibitor (B) are shown. Tumor vascularization visualized by immunostaining of CD31 vascular endothelial adhesion protein. CD31-positive vessels significantly decreased by PKC-β inhibition (original magnification, ×200).

![Fig. 4. CD31 mRNA expression in tumors measured by RT-PCR. In mice treated with the PKC-β inhibitor, CD31 RNA expression was significantly lower than in Tet-VEGF-HCC mice that did not receive the PKC-β inhibitor. Lane 1, Tet-VEGF-HCC (VEGF overexpression). Lanes 2 and 3, Tet-VEGF-HCC mice treated with the PKC-β inhibitor at daily doses of 10 and 100 mg/kg, respectively.]
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Table 2 Effect of PKC-β inhibitor on CD31 gene expression in animals with Tet-VEGF-HCC tumors

<table>
<thead>
<tr>
<th>VEGF</th>
<th>VEGF + PKC-β inhibitor (100 mg/kg)</th>
<th>VEGF + PKC-β inhibitor (10 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD31 gene expression</td>
<td>4.826a</td>
<td>0.948</td>
</tr>
</tbody>
</table>

a Gene expression presented after normalization with the glyceraldehyde-3-phosphate dehydrogenase internal control.

48). In KDR-transfected NIH3T3 cells and ECs, the p44/42 MAP kinase was activated upon VEGF stimulation, and signals from VEGF receptors to the MAP kinase were mediated mainly by PKC, especially the β isoform-dependent pathway (41, 42). To assess the role of PKC-β on the activation of MAP kinase in VEGF-mediated tumor development, we examined the MAP kinase with the p44/42 MAP kinase antibody, which recognized only the catalytically active, phosphorylated active species. Fig. 5 revealed that the phosphorylated activation of p44/42 MAP kinase was significantly suppressed upon administration of the PKC-β inhibitor at a dose of 10 mg/kg.

Apoptosis and Tumor Cell Proliferation in vivo. To examine whether the tumor inhibition by the PKC-β inhibitor was reflected by tumor cell proliferation and apoptosis, we performed an immunohistochemical analysis of PCNA and TUNEL. We also counted the mitotic index with routine H&E-stained tumor sections. As shown in Table 3, PCNA-positive cells and the mitotic index did not show a remarkable difference between PKC-β inhibitor-treated mice and nontreated mice. In vitro, even 500 nM PKC-β inhibitor, which corresponds to the expected blood concentrations of LY335351 in mice treated with a dose of 100 mg/kg in vivo, did not affect the proliferation of Tet-VEGF-HCC cells (data not shown). Our in vivo result was consistent with this in vitro data. On the contrary, the number of TUNEL-positive cells was significantly increased by treatment with 10 mg/kg PKC-β inhibitor (P < 0.001; Table 3). Morphological examination of routine H&E-stained sections showed an increase of inflammatory cells consisting mainly of macrophages and extensive necrosis in the PKC-β inhibitor-treated mice (data not shown).

DISCUSSION

The present study revealed that PKC-β played an important role in VEGF-mediated tumor development not only at the initial stage of tumor development, but also after the tumor was fully established. The role of VEGF as a major angiogenic factor is well established from a number of reports, including those regarding HCCs (1–5). In most of these studies used the conventional gene modification system, such as cellular differentiation, gene regulation, and proliferation (35, 36). It has been shown that activation of PKC is important for EC growth during angiogenesis (51–54). Selective inhibition of the PKC-β isoform strongly suppressed VEGF-dependent EC growth in a concentration-dependent manner. In vitro, VEGF appears to mediate its mitogenic effects through activation of the PKC pathway, predominantly involving PKC-β isoform activation in ECs (37). In vivo, it has also been shown that oral administration of the PKC-β inhibitor normalized many of the early retinal and renal hemodynamic abnormalities in rat models of diabetes (39, 40). However, the role of PKC in tumor development and angiogenesis has not yet been clarified. In this study, using a combination of the Retro-Tet system and the PKC-β inhibitor, we elucidated the role of PKC-β in VEGF-mediated tumor development and angiogenesis. We found that PKC-β inhib-

![MAPK](image)

Fig. 5. Effect of PKC-β inhibition on p44/42 MAP kinase activity. Tumors were resected from three mice and pooled. The tumor lysate was concentrated and Western blotted as described in “Materials and Methods.” Activation of p44/42 MAP kinase, as assessed by its phosphorylation status, was significantly suppressed with the administration of the PKC-β inhibitor at a daily dose of 10 mg/kg. Lane 1, treatment of Tet-VEGF-HCC-bearing animals with the PKC-β inhibitor. Lane 2, untreated animals with Tet-VEGF-HCC tumors.

Table 3 Effect of PKC-β inhibitor on PCNA, mitotic activity and apoptosis in Tet-VEGF-HCC tumors

<table>
<thead>
<tr>
<th>VEGF</th>
<th>PCNA-positive cells</th>
<th>Mitotic index</th>
<th>TUNEL-positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>32.43 ± 2.47a</td>
<td>1.45 ± 0.17</td>
<td>0.72 ± 0.21</td>
</tr>
<tr>
<td>VEGF + PKC-β inhibitor</td>
<td>30.86 ± 3.02</td>
<td>1.39 ± 0.15</td>
<td>2.38 ± 0.53b</td>
</tr>
</tbody>
</table>

a Mean ± SD (n = 30).
b Statistically significant difference as compared with the Tet-VEGF-HCC (VEGF) group (P < 0.001).
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PKC-b produced the size and number of HCC tumors. In summary, from the in vitro way. Ras activation was not required for VEGF signaling toward cell proliferation induced by a number of mitogens, including VEGF (42, 47, 56). The p44/42 MAP kinase pathway consists of a protein kinase cascade linking growth and differentiation signals with transcription in the nucleus. These MAP kinases are known to be associated with cell proliferation induced by a number of mitogens, including VEGF (42, 47, 56). The p44/42 MAP kinase was activated upon VEGF stimulation in ECs, and signals from VEGF receptors to MAP kinase were mediated mainly by PKC, especially the β isoform-dependent pathway. Ras activation was not required for VEGF signaling toward cell proliferation (41). Consistent with these in vitro data, we found that PKC-β inhibition significantly suppressed p44/42 MAP kinase phosphorylation in tumors. Furthermore, this PKC-β inhibitor affected neither the in vitro nor in vivo proliferation of Tet-VEGF-HCC cells. Accordingly, these findings suggest that this PKC-β inhibitor may inhibit p44/42 MAP kinase of ECs in the tumor rather than the tumor cell itself. Additional studies are required to identify the mechanism and the type of cells responsible for this MAP kinase inhibition.

Some reports, on the contrary, showed that VEGF utilized a different signaling pathway than PKC. Human umbilical vein endothelial cell survival depended on the activation of the phosophatidylinositol 3'-kinase/Akt signaling pathway (57). Phosphatidylinositol 3'-kinase/Akt signal was also utilized in Ha-ras-transfected NIH3T3 cells in a Ras-dependent manner (58). It may be possible that VEGF utilizes the cell type-specific signaling cascade to selectively elicit specific cellular responses. VEGF has been shown to act as a survival factor, preventing the apoptotic death of microvascular ECs (24, 59). In glioma cells, VEGF inhibition led to the detachment of ECs from the walls of preformed vessels and to their subsequent death by apoptosis (44). Angiostatin, a potent endogenous angiogenic inhibitor, has been shown to induce apoptosis of tumor cells, whereas tumor cell proliferation itself was not changed in vivo (60). In this experiment, PKC-β inhibition also significantly increased the number of apoptotic cells. On the contrary, tumor cell proliferation and mitotic index were not changed. Taken together, these data suggest that PKC-β inhibition reduced the tumor growth by decreasing VEGF-dependent MAP kinase activation, which in turn may stimulate the induction of apoptosis.

Orthotopic transplantation has been reported to be a preferential site for transplanted tumor cell growth, enhanced tumor growth, and development of metastatic ability in several types of tumors (45, 46). Accordingly, we examined the effect of PKC-β inhibition on HCC development of an orthotopic model in the liver. Similar to the xenograft experimental model, PKC-β inhibition significantly reduced the size and number of HCC tumors. In summary, from the present study, we suggest for the first time that PKC-β lies on the signaling pathway in VEGF-mediated tumor development and angiogenesis not only at the initial stage, but also after the tumor has become established in murine HCC cells.

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3 C. Garner, unpublished observations.
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