Role of protein kinase Cμ isoform expression in dimethylhydrazine-induced vascular endothelial proliferation

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Received April 3, 2012; Accepted May 23, 2012

DOI: 10.3892/mmr.2012.932

Abstract. 1,2-Dimethylthdrazine (DMH) has been known to induce vascular neoplasms, such as malignant endothelioma, in animal experiments through the induction of abnormal proliferation of human umbilical vein endothelial cells (HUVECs). We studied the effect of protein kinase C (PKC) isoforms on DMH-induced abnormal proliferation of vascular endothelium to identify the isoforms with higher relevance. The study was conducted with pure culture HUVECs in a control group and a $1x10^{-9}$ M DMH-treated group. The mRNA and protein expression of PKC isoforms in DMH-treated HUVECs was evaluated by reverse transcription-polymerase chain reaction and western blotting. DMH-induced PKC production was detected by a PKC activity assay. To investigate the role of the PKC isoforms in DMH-induced abnormal HUVEC proliferation, we modulated PKCμ expression in DMH-treated HUVECs using small interfering RNA. We determined the expression of 11 PKC isoforms by PCR analysis in both the control and DMH-treated groups, and the results were statistically analyzed to detect any differences. According to the results, both groups expressed 6 out of 11 isoforms. Expression of PKCμ was significant in the DMH-treated group, and downregulation of PKCμ inhibited DMH-induced abnormal HUVEC proliferation. The PKCμ isoform is believed to be important in the abnormal growth of vascular endothelial cells induced by DMH, and this was confirmed by an objective siRNA experiment, which showed a clear decrease in PKCμ expression. Therefore, it is believed that PKCμ is a key factor in the abnormal proliferation of vascular endothelial cells, and these results can be used as fundamental research data for abnormal vessel development or the embryologic mechanisms of vessel development.

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Key words: human umbilical vein endothelial cells, 1,2-dimethylthdrazine, protein kinase Cμ

Introduction

Angiogenesis is required for many physiological processes, including embryogenesis and wound healing, and neoangiogenesis is central to the development of various disorders, particularly the rapid growth and metastasis of solid tumours (1-5). For this reason, it is important to study angiogenesis and neoangiogenesis, as such information may ultimately provide treatments for these disorders. Hemangioma is a benign vascular lesion and is the most common tumor of infancy. However, the pathogenesis of hemangiomas is not well understood (6).

1,2-Dimethylhydrazine (DMH) is a strong colon carcinogen that induces colorectal tumors in experimental animals (7,8) and induces intestinal tumors in rats when given orally (9). DMH was shown to cause proliferation of human umbilical vein endothelial cells (HUVECs), and the sequential expression of growth factors was demonstrated (10,11). In addition, we found that protein kinase C (PKC) is highly associated with the abnormal growth of HUVECs induced by DMH (12,13).

PKC is a well-known regulator of cell proliferation. In particular, PKC can mediate G1-phase progression of the cell cycle (14-16). Some studies have shown that overexpression or inhibition of some PKC isoforms can enhance or reduce tumor growth, respectively, *in vivo* (17,18). In some human tumor tissues, PKC activity is higher than in normal tissues, and elevated PKC activity was found to be associated with increased metastatic or invasive potential in some human carcinoma cells, including human bladder carcinoma cells (19,20). 11 PKC isoforms have been identified in mammalian cells, and these isoforms have distinct cellular localizations and functions (21).

In the current study, we examined the PKC isoforms present in HUVECs and investigated the role of these PKC isoforms in the abnormal proliferation of HUVECs induced by DMH.

Materials and methods

Reagents. DMH was obtained from Sigma Aldrich Chemical Co. (Milwaukee, WI, USA). Calphostin C was purchased from BIOMOL (Plymouth Meeting, PA, USA).

Cell culture. Vascular endothelial cells, specifically HUVECs, were obtained from Modern Tissue Technologies, Inc., and

in these experiments, the cells were sub-cultured fewer than 8 times. The HUVECs were maintained at 37˚C in 5% CO_2 in the control medium, endothelial cell basal medium [EBM with 0.1% human epidermal growth factor (hEGF), 0.1% hydrocortisone, 0.1% GA-1000, 0.4% bovine brain extract (BBE), and 2% FBS].

MTT assay (proliferation assay). Proliferation was determined by the indirect colorimetric MTT assay. The MTT assay is metabolized by NAD-dependent dehydrogenase to form a colored reaction product, and the amount of dye formed is directly correlated with the number of cells. To determine the number of cells, HUVECs were seeded into a 24-well plate at $1x10^4$ cells/well, cultured for 48 h in EBM complete medium (with 0.1% hEGF, 0.1% hydrocortisone, 0.1% GA-1000, 0.4% BBE, and 2% FBS), serum-starved for 24 h, and then treated with or without various reagents for the indicated times. For the MTT assay, the stock solution (5 mg/ml MTT) was added to each well of the 24-well plates, which were seeded with HUVECs, and then the plates were incubated at 37˚C for 2 h. The formazan granules generated by the live cells were dissolved in 100% dimethyl sulfoxide (DMSO), and the absorbance at 570 nm was monitored using a Power-Wavex microplate spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT, USA).

Reverse-Transcription-Polymerase Chain Reaction (RT-PCR) analysis. Total RNA was isolated from HUVECs using an RNeasy mini kit (Qiagen, Valencia, CA, USA). Total RNA was reverse-transcribed with AMV reverse transcriptase (Promega, Madison, WI, USA). PCR amplification was performed using the primer sets shown in Table I, as well as 5'-GACTATGACTTAGTTGCGTTA-3' and 5'-GCCTTCATACATCTCAAGTTG-3' for β-actin. All of the primer sequences were generated using GenBank sequences. PCR amplifications were performed in duplicate. β-actin was used as control to assess PCR efficiency. PCR products were electrophoresed on 2% agarose gel and visualized by ethidium bromide staining.

Real-Time PCR. Total RNA was extracted, and the expression of β-actin and PCK mRNA were evaluated by real-time RT-PCR. The primer sequences used in the experiment were as shown in Table I, and 5'-GACTATGACTTAGTTGCGTTA-3' and 5'-GCCTTCATACATCTCAAGTTG-3' for β-actin. Real-time quantitation was based on the LightCycler assay, using a fluorogenic SYBR Green I reaction mixture for the PCR and the LightCycler Instrument (Roche). The amplification program consisted of 1 cycle of 95˚C with a 60-s hold ('hot start'), followed by 45 cycled of 95˚C with 0-s hold, specified annealing temperature with a 5-s hold, 72˚C with 12-s hold, and specified acquisition temperature with 2-s hold. All experiments were conducted 3 times, and included both negative and positive controls. β-actin mRNA was amplified as an internal control. LightCycler software version 3.3 (Roche Diagnostics) was used to analyze the PCR kinetics and calculate quantitative data. A standard curve generated in a separate run was loaded into the runs of each sample (without standard curves). Each run included 1 sample of known concentration, which was in the range covered by the standard curve, thus allowing for estimation of the exact copy number by the second derivative maximum method. For each sample, the copy number of target gene mRNA was divided by that of β-actin mRNA to normalize for target gene mRNA expression and avoid sample-to-sample differences in RNA quantity.

Table I. Specific primer sequences of PKC isoform.

Name		Sequence
PKCα	Sense	5'- cgg aag ccc cac ctt ctg c
	Antisense	5'-ctt tgt tgc cag cag ggc
PKCβI	Sense	5'-cgt gat gaa tgt tcc cag c
	Antisense	5'-cgc agt tct tca ttg gc
PKCβII	Sense	5'-cgc tga caa ggg tcc agc
	Antisense	5'-cca atc cca aat ctc tac
PKCγ	Sense	5'-gca gcc cca cct tct gcg
	Antisense	5'-gcc ccc atg aag tcg ttg cg
PKCδ	Sense	5'-gca gat gca ct gca ccg
	Antisense	5'-gcc cac gac tgt gaa cg
PKCε	Sense	5'-gac ag aac tat ctt gag
	Antisense	5'-agt tgt cct gta gga aag
PKCζ	Sense	5'-gcg ctt taa cag gag agc gt
	Antisense	5'-gct tct ctg tct gta ccc ag
PKCη	Sense	5'-gct gct gcg cac gac cgg cg
	Antisense	5'-gcc acg ttc gct tgc cat cg
PKCθ	Sense	5'-gca ggc aaa ggt cca cca cg
	Antisense	5'-gcc acc tta atc atg gcc ag
PKCι	Sense	5'-cgg gtg aac gcc tac tac c
	Antisense	5'-cgc ctg ttg aaa cgc ttg gc
PKCμ	Sense	5'-gct gtg ggg gct ggt acg
	Antisense	5'-gca tct cgc cac tgt cg

Small interfering RNA (siRNA) transfection. Small interfering RNA (siRNA) duplex oligo (on-TARGET plus SMART pool Dharmacon, Lafayette, CO, USA) targeting PKCμ mRNA or non-targeting duplex oligo (on-TARGET plus siCONTROL, Dharmacon) as a negative control was transfected using DharmaFECT Transfection Reagent (Dharmacon, CO, USA).

Western blot analysis. Confluent serum-starved HUVECs were treated with the appropriate conditions, washed with ice-cold PBS, and then lysed in total cell lysis buffer (210 mM mannitol, 70 mM sucrose, 5 mM Tris, pH 7.5, and 1 mM EDTA). Protein content was determined with a protein assay kit (Bio-Rad Laboratories, Richmond, CA, USA). Proteins were loaded on a 10% SDS-polyacrylamide gel, electrotransferred to nitrocellulose membranes (Hybond-ECL; Amersham Pharmacia Biotech, Piscataway, NJ, USA), and then probed with monoclonal antibodies (GAPDH Cell Signaling Technology, Beverly, MA, USA; PKCμ Abcam, Cambridge, UK). Immunoreactive bands were detected using anti-mouse and anti-rabbit peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech) and visualized by enhanced chemiluminescence (ECL detection kit; Amersham Pharmacia Biotech).

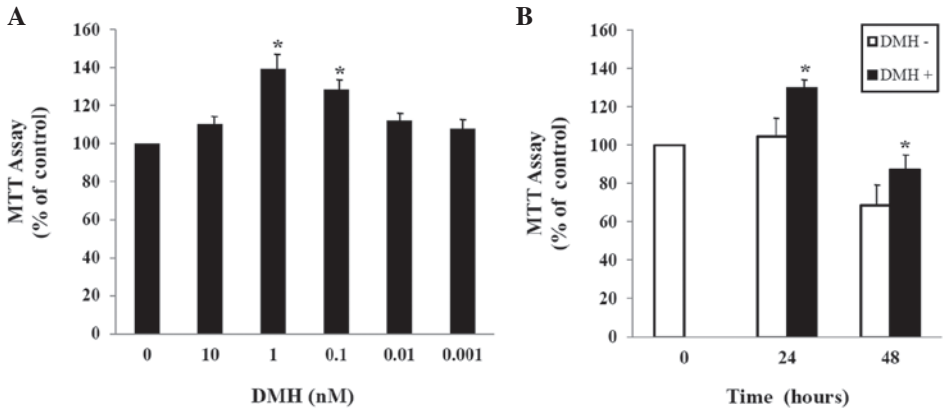

Figure 1. Effect of DMH on the proliferation of HUVECs. (A) HUVECs were seeded into 24-well culture plates at a density of $1x10^4$ cells/well, cultured for 48 h in growth medium, serum-starved for 24 h, and then treated with the indicated concentrations of DMH for 48 h. The number of cells was determined using the 3-(4,5-dimethyl-2-thiozol)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay as described in Materials and methods. Results are expressed as percentages of the vehicle-treated controls. (B) Serum-starved HUVECs were treated with vehicle (DMH-) or $1x10^{-9}$ M DMH (DMH+) for the indicated times. The number of cells was determined by the MTT assay, and the results shown are the percentages of the vehicle-treated controls (0 h). The data shown are the mean ± SEM of 4 different experiments. *p<0.05 compared to the control (DMH-).

PKC activity assay. Separated proteins were analyzed using the Protein Kinase C assay system according to the manufacturer's protocol (#V5330 Promega, Madison, USA). To compare the reaction to the PKC negative control, PKC Activator 5x buffer, Pep Tag PKC Reaction 5x buffer, Pep Tag C1 Peptide, and Peptide Protection solution were added. In the PKC positive control, these same 4 solutions, which were added to the negative control, were also added. In addition, purified PKC was also added. The third sample, the PKC standard, also had the same 4 solutions with the same amount of protein. All 3 samples were reacted at 30˚C for 30 min, and then incubated at 95˚C for 10 min to stop the reaction. Then, 80% glycerol was added to the reactions, which were electrophoresed in a 0.8% agarose gel containing 50 mM Tris-HCl at 100 V for 15 min. Phosphorylated PKC moved to the + charge while non-phosphorylated PKC moved to the – charge therefore, PKC was separated into 2 bands. The gel containing the phosphorylated protein was cut out and placed in 1.5 ml tube. Then, gel-solubilization solution was added and the tube was heated to 95˚C until the gel melted completely. This solution was transferred to another 1.5 ml tube containing gel-solubilization solution and glacial acetic acid. The solution was then transferred to a 96-well plate, which was placed in a scanning multi well spectrophotometer (ELISA reader Molecular Devices, Menlo Park, CA, USA) to measure the OD at 570 nm.

Statistical analysis. All results are presented as the means ± standard error of the mean. Comparisons between groups were analyzed using the t-test (two-sided) or analysis of variance. Post hoc range tests and pairwise multiple comparisons were conducted using the t-test (two-sided) with Bonferroni adjustments. P values of <0.05 were considered statistically significant.

Results

DMH induces abnormal proliferation of HUVECs. To explore the effect of DMH on the growth of HUVECs, HUVECs were exposed to the indicated concentration of DMH for 48 h, and proliferation was determined using the MTT assay. As shown in Fig. 1A, treatment of HUVECs with DMH for 48 h increased cell proliferation dose-dependently, with maximal stimulation at 1 nM. The DMH-induced abnormal proliferation was observed 24 h after exposure at $1x10^{-9}$ M DMH (Fig. 1B).

DMH-induced abnormal proliferation of HUVECs is mediated by activation of PKC. We previously reported that DMH treatment of HUVECs increased expression of PKC (12). In this study, we confirmed the effect of Calphostin C, an inhibitor of PKC, on the proliferation of DMH-treated HUVECs. As shown in Fig. 2A, treatment of HUVECs with $1x10^{-9}$ M DMH for 24 h induced PKC activation, while co-treatment with $5x10^{-9}$ M Calphostin C prevented DMH-induced PKC activation. Consistent with the result shown in the PKC activity assay, DMH-induced abnormal proliferation of HUVECs was completely inhibited by treatment with Calphostin C. The PKC inhibitor blocked the DMH-induced increases in both HUVEC proliferation and PKC activity (Fig. 2B).

DMH increases the expression of PKCμ in HUVECs. We showed that PKC activation affects the DMH-induced abnormal proliferation of HUVECs. Therefore, we next determined whether DMH regulates the expression of various PKC isoforms. We examined the mRNA levels of the various PKC isoforms by RT-PCR and real-time PCR. According to the results of both assays, 6 out of 11 isoforms were expressed. Statistical assessment showed higher PKCα, PKCμ, and PKCι expression in the DMH-treated group. As shown in Fig. 3A and B, exposure of the cells to DMH significantly increased the level of PKCμ mRNA. The increased PKCμ at the protein level was confirmed by western blot using a specific antibody. RT-PCR and western blot analysis showed that DMH stimulated the expression of PKCμ (Fig. 3C).

PKCμ is a key factor in DMH-induced abnormal proliferation of HUVECs. To determine the role of PKCμ in the DMH-induced abnormal HUVECs proliferation, we examined the effect of PKCμ down-regulation by interference technique using siRNA

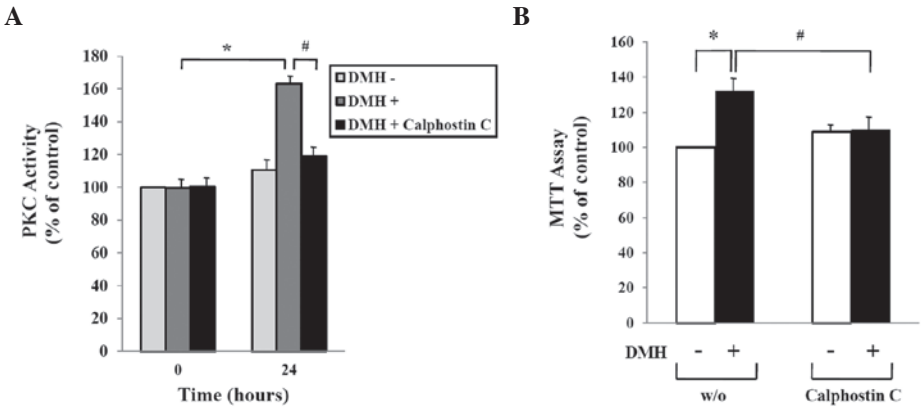

Figure 2. Role of PKC activation in DMH-induced abnormal proliferation of HUVECs. (A) HUVECs were seeded into 6-well culture plates at a density of $1x10^5$ cells/well, cultured for 48 h in growth medium, serum-starved for 24 h, and then treated with vehicle, $1x10^{-9}$ M DMH, or $5x10^{-9}$ M Calphostin C, a DMH inhibitor for 24 h as indicated. PKC activity in the supernatant was measured by ELISA as described in Materials and methods. (B) Serum-starved HUVECs were treated with vehicle, $1x10^{-9}$ M DMH, and $5x10^{-9}$ M Calphostin C, a DMH inhibitor, for 24 h as indicated. Cell proliferation was determined by the MTT assay and expressed as a percentage of the control (mock-incubated HUVECs). The data shown represent the mean ± SEM of 4 different experiments. $^*p<0.05$ compared to the control; $^\#p<0.05$ compared to DMH only-treated HUVECs for 24 h.

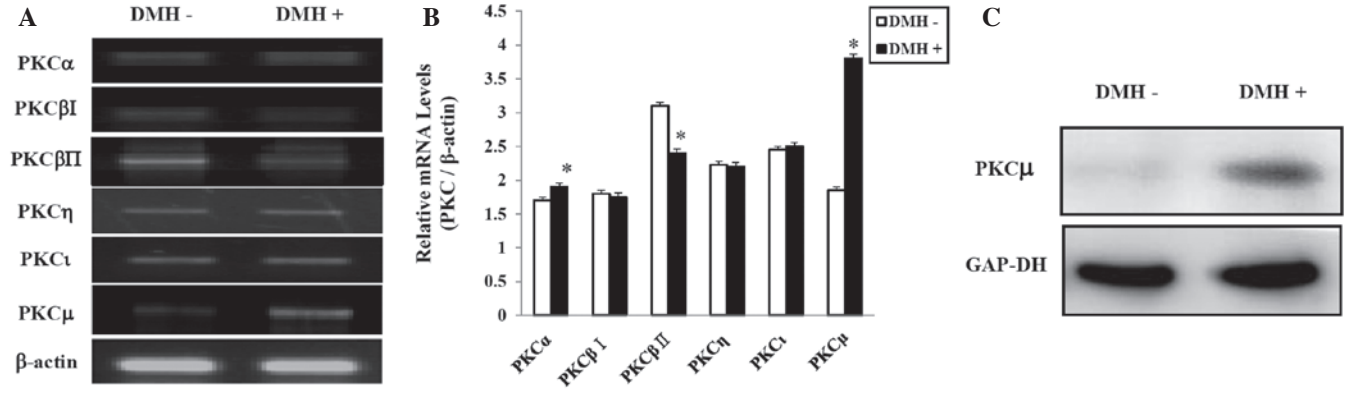

Figure 3. Expression of PKC isoforms in DMH-induced abnormally proliferating HUVECs. (A,B) Total mRNA was obtained from HUVECs treated with $1x10^{-9}$ M DMH for 24 h, and the expression of PKC isoforms in DMH-treated HUVECs was determined by (A) RT-PCR and (B) real-time PCR. (C) Serum-starved HUVECs were treated with $1x10^{-9}$ M DMH for 24 h. The expression levels of PKCμ and GAPDH were determined by western blotting with anti-PKCμ and anti-GAPDH antibodies, respectively. The data shown represent the mean ± SEM of 3 different experiments. $^*p<0.05$ compared to the control (DMH-).

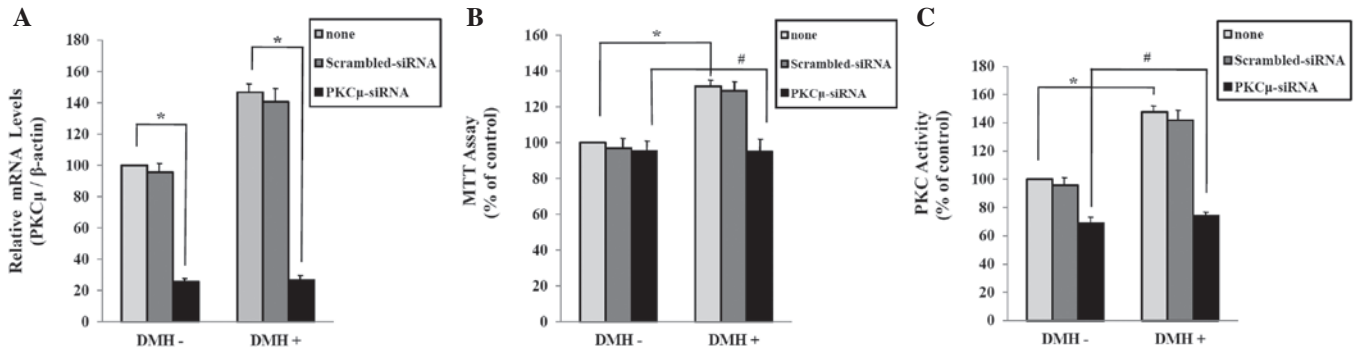

Figure 4. Role of PKCμ activation in DMH-induced abnormal proliferation of HUVECs. (A) Down-regulation of PKCμ expression by PKCμ small interfering RNA (siRNA) transfection. HUVECs were transfected with PKCμ siRNA or non-target siRNA (Scrambled-siRNA), serum starved for 24 h, and then incubated with $1x10^{-9}$ M DMH for 24 h. PKCμ mRNA levels were determined by real-time PCR analysis at 24 h after DMH treatment. The data shown represent the mean ± SEM of the ratio of the corresponding samples to the β-actin signal (n=4). (B) The effect of PKCμ down-regulation on DMH-induced abnormal HUVEC proliferation. HUVECs transfected with siRNA were serum starved for 24 h, and then treated with DMH for 24 h. Cell proliferation was determined by the MTT assay and expressed as a percentage of the control. (C) The effect of PKCμ on DMH-induced PKC activation. HUVECs were transfected with PKCμ siRNA or non-target siRNA (Scrambled-siRNA), serum-starved for 24 h in serum free medium, and then incubated with $1x10^{-9}$ M DMH for 24 h. PKC activity in the supernatant was measured by ELISA as described in Materials and methods. The data shown represent the mean ± SEM of 3 different experiments. $^*p<0.05$ compared to control cells (DMH-).

transfection. Real-time PCR analysis showed that the introduction of PKCμ siRNA oligonucleotides effectively inhibited the expression of PKCμ in HUVECs, and that PKCμ siRNA effectively inhibited DMH-induced expression of PKCμ (Fig. 4A). In the MTT assay, cell proliferation was increased in DMH-treated HUVECs. In contrast, down-regulation of PKCμ expression by the transfection of PKCμ siRNA decreased DMH-induced abnormal HUVECs proliferation (Fig. 4B). Next, we confirmed that whether PKCμ regulates DMH-induced PKC activation in HUVECs. The PKC activity assay showed that siRNA-induced down-regulation of PKCμ decreased the DMH-induced PKC activation in HUVECs (Fig. 4C).

Discussion

It has proceed the study that angiogenesis is critical to several physiological and disease processes, and understanding the mechanism underlying new blood vessel formation is required to develop treatments for blood vessel abnormalities, such as hemangioma, in the field of plastic surgery (22,23). For the present study, we thought that the best way to study abnormalities in blood vessel proliferation, such as hemangiomas, was to evaluate the mechanism of blood vessel formation. First, abnormal proliferation of HUVECs was induced *in vitro* by DMH, which is known to induce hemangiomas *in vivo*. We determined the specific concentration needed to induce the maximum effect of abnormal HUVEC proliferation. We also completed the experiment model as expressed growth factor in this process was confirmed by RT-PCR (10).

The mechanisms underlying angiogenesis and revascularization of blood vessel formation include the vascular endothelial cells and related mediators, such as interaction growth factor with delivery substance, and several signal transduction pathways. In this complex process, there are several enzymes, such as protein tyrosine kinase (PTK), PKC, and oxidase (24-27), which are important mediator molecules of signal transduction pathways. Because in abnormal proliferation, the process of blood vessel formation is in similar pathway, to explore the signal transduction process of abnormal cell proliferation, we examined the distribution of the signal transduction channel through use of an inhibitor. We found that signal transduction of PKC is important. We also confirmed that PKC plays an important role in the process of DMH-induced abnormal HUVECs proliferation by directly showing the activity of PKC through an enzymological approach.

In mammal cells, there are 11 known PKC isoforms. They are divided into 3 groups based on their structure and function: classic PKCs or cPKCs (α, βI, βII, and γ), novel PKCs or nPKCs (δ, ε, η, θ, and μ), and atypical PKCs or a PKCs (ζ and ι) (21). PKCα, βI, βII, δ, ε, and ζ exist mostly in the brain, lung and spleen (28,29). PKCα and ε facilitate proliferation of NIH3T3 cells, and these factors have been reported to be very important in cornea endothelial cells (30,31). The ones identified in this study are primarily found in liver and kidney tissue (28,32). In liver tissue, mostly PKC β and βII are present, but PKC α and ε are not. In contrast, PKC β and βII are not found in kidney, but PKC α, δ, ε, and ζ are. Based on this, the function of the isoforms is predicted to be different in each organ therefore, it is very important for us to predict their location or function so that they can be used in preliminary research of various diseases.

According to the results of PKC isoforms expression by RT-PCR during DMH-induced abnormal HUVECs growth, among the 11 PKC isoforms, 6 kinds of PKC which is PKCα, βI, βII, η, ι, and μ has been verified in the control and DMH-treatment groups. Among these 6 PKC isoforms, PKCμ had the highest expression in the DMH-treated group compared to the control. Therefore, it is thought that PKCμ plays an important role in DMH-induced HUVECs overgrowth.

In this study, we found that PKCμ may play a major role in the abnormal proliferation of HUVECs induced by DMH treatment through siRNA experiment. The study of PKC isoforms will allow us to manipulate gene expression in signal transduction leading to cell proliferation and differentiation. In addition, it will lead to a better understanding of abnormal angiogenesis and embryologic mechanisms, so that it can be used as preliminary study.

Acknowledgements

This study was supported by a Medical Research Institute Grant (2007-31) Pusan National University.

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