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Tyrosine phosphatase εM stimulates migration and survival of porcine aortic endothelial cells by activating c-Src

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Abstract

The cell growth, survival, and migration of vascular endothelial cells (ECs) are positively regulated by several protein tyrosine kinase receptors. Therefore, protein tyrosine phosphatases (PTPs) must also be important for these processes. The present study found that transmembranal PTPεM, but not cytoplasmic PTPεC, is expressed in porcine ECs and in rat smooth muscle cells, both of which were prepared from the aorta. The overexpression of wild-type PTPεM promoted cell survival and migration in porcine aortic ECs even in medium without and with 1% serum, respectively. A catalytically inactive, substrate-trapping mutant of PTPεM, respectively, did not affect and conversely suppressed cell survival and migration. Interestingly, the forced expression of wild-type PTPεC reduced cell viability in contrast to PTPεM in ECs lacking endogenous PTPεC, indicating the biological significance of selective expression of PTPε isoforms in the vasculature. PTPεM activated c-Src kinase probably by directly dephosphorylating phospho-Tyr527, a negative regulatory site of c-Src. The increases in cell survival and migration induced by overexpressed PTPεM were suppressed by the c-Src inhibitor SU6656. Considering the behaviors of vascular ECs in the pathogenesis of atherosclerosis, these data suggest that PTPεM negatively regulates the development of this disease by activating c-Src.

Keywords: Protein tyrosine phosphatase; PTPε; Protein tyrosine kinase; Src; Vascular smooth muscle cells; Endothelial cells; Atherosclerosis

The proliferation, migration, and survival of vascular smooth muscle cells (VSMCs) and endothelial cells (ECs) are closely related to the regulation of pathogenesis of the vascular disease atherosclerosis [1–4]. Accumulating evidence shows that multiple growth factors including platelet-derived growth factor, vascular endothelial growth factor, and hepatocyte growth factor (HGF) mediate these cellular events in the vasculature [1–4]. These facts indicate that protein tyrosine kinases (PTKs) play crucial roles in the pathogenesis of atherosclerosis because receptors for these growth factors possess intrinsic PTK activities. However, little is known about the potential roles of specific protein tyrosine phosphatases (PTPs) in this pathogenic process [5–7] although the regulation of protein phosphotyrosine (pTyr) levels is controlled by the opposite activities of PTKs and PTPs [8,9].

We have previously found the expression of six PTPs in rat cultured VSMCs and have suggested that among them, low molecular weight-PTP is a negative regulator for VSMC growth and migration [7]. The aims of this study were to identify several of PTPs expressed in vascular ECs and examine the functions of PTPs with respect to survival, growth, and migration of these cells. Especially, we focused on PTPε among the identified PTPs because of the following reasons. First, several

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reports have suggested the involvement of PTPε in the regulation of cell growth [10–13], survival [10], and reorganization of the cytoskeleton that is closely related to cell migration [13–15]. Second, the detection of receptor-type PTPεM (PTPεM), but not cytoplasmic PTPε (PTPεC) in both cultured VSMCs and ECs in the present study, prompted us to investigate the biological significance of the selective expression of these isoforms, which are transcribed from a single gene by alternative promoter usage [16–19].

Materials and methods

Materials. Antibodies specific for phospho-extracellular-regulated protein kinase (ERK) 1/2, phospho-p38, phospho-Akt, pTyr416, and pTyr527 of c-Src were purchased from Cell Signaling Technology (Beverly, MA). Hepatocyte growth factor was obtained from R&D Systems (Minneapolis, MN) and SU6656 was from Calbiochem (La Jolla, CA). Anti-v-Src antibody was from Oncogene Science (Manhasset, NY). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin, and streptomycin were obtained from Sigma Chemical (St. Louis, MO).

Reverse transcription (RT)-PCR analysis. Total RNAs were reverse-transcribed using random primers. The resultant cDNA mixtures were amplified by PCR to selectively detect PTPs of interest. Denaturation, annealing, and polymerase reactions proceeded at 94 °C for 1 min, at temperatures described below for 1 min, and then at 72 °C for 1.5 min, respectively. After 35 cycles of amplification, the incubation was continued at 72 °C for another 8.5 min to complete polymerization. The oligonucleotide primers and annealing temperatures were: PTP1B (49 °C), 5′-ctgggcggctatttaccagg-3′ (sense) and 5′-ggtgtagtggaaatgcaggatctc-3′ (antisense); PTP-MEG2 (62 °C), 5′-tctgctggacagagagctgtgg-3′ (sense) and 5′-atgtaatctgtctgagtgtggc-3′ (antisense); PTPεC (40 °C), 5′-aacttttccaggctcacct-3′ (sense) and 5′-ttgaactcctctcggaaccg-3′ (antisense); PTPεM (62 °C), 5′-cttgcagcctacttcttca-3′ (sense) and 5′-ttgaactcctctcggaaccg-3′ (antisense); PTP20 (59 °C), 5′-actgggtggcgttctcagg-3′ (sense) and 5′-cgtggccctttaggtcttcc-3′ (antisense); FAP-1 (52 °C),5′-caggaattcgtgtacattgcc-3′ (sense) and 5′-tagcagaagacatactgaccc-3′ (antisense); TC-PTP (59 °C), 5′-atacctgtctcgttctgatgg-3′ (sense) and 5′-taggtgtctgtcaatcttggc-3′ (antisense); and SHP-2 (57 °C), 5′-caagtgcaacaattcaaaacc-3′ (sense) and 5′-ttctctctgtgtttccctgg-3′ (antisense).

Cloning of rat PTPεC and PTPεM cDNAs, and construction of recombinant adenoviruses. The cDNAs encoding hemagglutinin (HA)-tagged rat wild-type PTPεM (WT-PTPεM) and C (WT-PTPεC) were generated by standard PCR procedures. The catalytically inactive, substrate-trapping mutant of HA-tagged PTPεM (DA-PTPεM) was generated by replacing Asp302 with Ala in PTP domain I. These cDNAs tagged with HA at the C-terminus were subcloned into the expression vector pcDNA3 (Invitrogen). Recombinant adenoviruses were constructed according to the manufacturer's protocol (Takara, Tokyo, Japan). In brief, cDNAs encoding WT-PTPεM, DA-PTPεM, and WT-PTPεC were inserted into the cosmid shuttle vector pAxCAwt. After homologous recombination, the resultant recombinant adenoviruses with WT-PTPεM (Ad-WT-PTPεM), DA-PTPεM (Ad-DA-PTPεM), WT-PTPεC (Ad- WT-PTPεC), and without insert (Ad-Cont) were purified and amplified in 293 cells.

Cell culture, infection, and preparation of cell lysates. Porcine ECs were prepared from the thoracic aorta of pigs slaughtered at a local abattoir and maintained as described by Shasby and Peterson [20]. We used ECs between passages 5 and 10 for all experiments. Cells were infected with the recombinant viruses at moi of 50 for 1 h. The virus suspension was then removed and the cells were cultured in DMEM containing 1% FBS for the appropriate hours. Lysates for Western blotting and immunoprecipitation were prepared as described [7]. Rat VSMCs were prepared from the thoracic aorta of adult Sprague–Dawley rats as described previously [7].

Measurement of DNA synthesis. Infected ECs in 96-well plates (3×10^3 cells/well) cultured in medium containing 1% FBS for 16 h were further incubated in the presence or absence of HGF. After 20 h, bromodeoxyuridine (BrdU) was added to a final concentration of 10 μM, and then the cells were incubated for another 24 h. Thereafter, incorporated BrdU was measured using the cell proliferation ELISA system, Version 2 (Amersham–Pharmacia Biotech), according to the manufacturer's protocol.

Cell migration assay. Cell migration was assessed using a wound-healing assay. Briefly, infected ECs (3×10^5 cells/3.5 cm dish) in cultured in medium containing 1% FBS for 16 h were incubated with or without SU6656 (2 μM) for 30 min, and then wounded with a 200 μl pipette tip. After culture with or without HGF (20 ng/ml) for 8 h, migrated cells were counted under a microscope.

Assessment of cell viability. After culture under various conditions, detached cells were collected, and then adsorbed cells were harvested by trypsin/EDTA treatment. Thereafter, all cells including detached cells were counted by Trypan blue staining.

Detection of activation of ERK, p38, Akt, and c-Src. Cell lysates (30 μg of protein) were fractionated by SDS–PAGE on 10% polyacrylamide gels, and then proteins were transferred to PVDF membranes. Activated ERK1/2, p38, and Akt were detected using antibodies specific for their activated forms, and pTyr416 and pTyr527 of c-Src were also detected with antibodies that specifically recognized pTyr416 and pTyr527, respectively. These protein bands were visualized using an enhanced chemiluminescence kit (Santa Cruz). Other experimental conditions were described in [21].

Results

PTPεM, but not PTPεC, is expressed in ECs and VSMCs

To identify PTPs expressed in porcine vascular ECs, total RNAs obtained from these cells were analyzed by RT-PCR. We investigated the presence of the PTPε isoforms PTPεM and PTPεC as well as of six cytoplasmic PTPs whose expression had been examined in VSMCs in our previous report [7]. As a result, we found the presence of PTP1B, PTP-MEG2, PTPεM, TC-PTP, and SHP-2, but not the other three PTPs including PTPεC in ECs (Fig. 1A). Sequencing confirmed that each PCR product encoded the predicted PTP (data not shown). Comparison of the data between ECs and VSMCs [7] indicates that at least PTP-MEG2 among the eight PTPs is selectively expressed in ECs, but not in VSMCs. We cannot exclude the possibility that ECs also express the three PTPs, which were not identified, because porcine nucleotide sequences of these PTPs have not yet been determined. The expression of PTPε in vascular ECs is in agreement with the findings of Thompson et al. [6] that PTPε is present in human umbilical vein ECs although they did not identify the isoform. Similarly, the band of PTPεM, but not that of PTPεC, was detected in rat VSMCs (Fig. 1C). The absence of PTPεC expression in ECs was not due to the experimental conditions because the PTPεC band

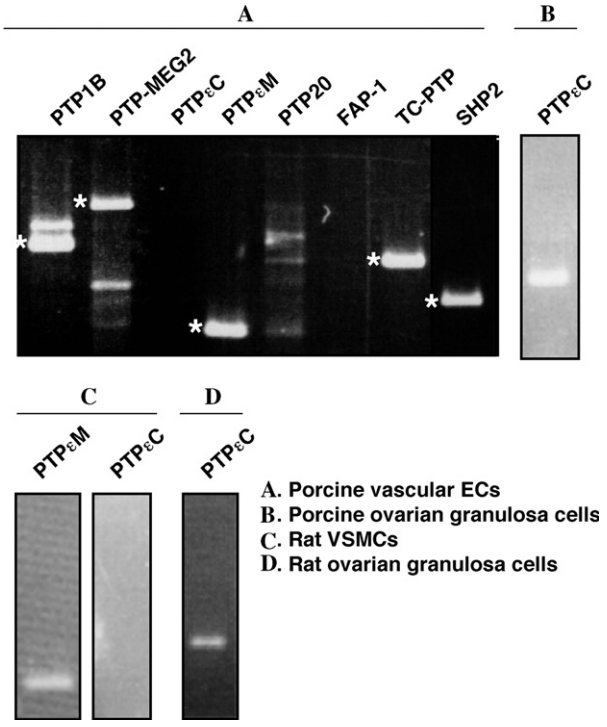

Fig. 1. Identification of PTPs expressed in ECs by RT-PCR. Total RNAs prepared from porcine ECs, rat VSMCs, and porcine and rat ovarian granulose cells were amplified by RT-PCR to detect indicated PTPs as described under Materials and methods. Each PCR product was resolved by electrophoresis on 2% agarose gels and stained with ethidium bromide.

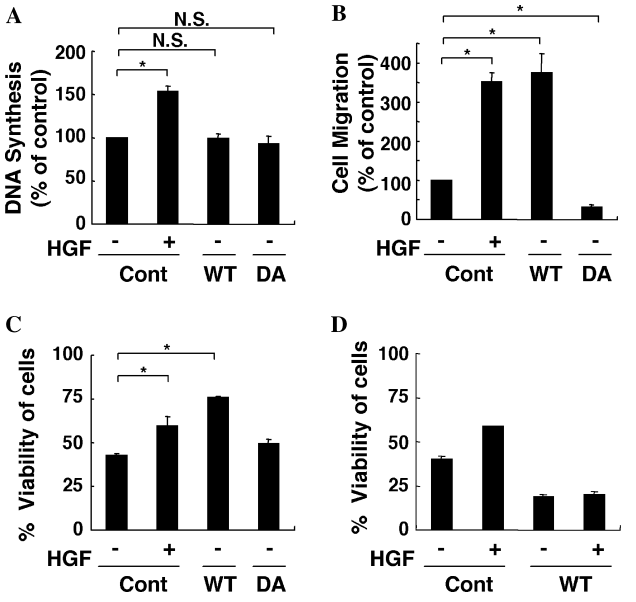

Fig. 2. Effect of overexpressed PTPεM on EC growth, migration, and survival. (A) DNA synthesis was assessed by incorporation of BrdU into cellular DNA. Cells infected with Ad-cont (Cont) were cultured with or without HGF (20 ng/ml) whereas Ad-WT-PTPεM (WT)- or Ad-DA-PTPεM (DA)-infected cells were cultured without HGF. After 20 h culture, BrdU was added and cells were incubated for another 24 h. (B) Cell migration was assessed using wound-healing assays. Infected cells were wounded with a 200 μl pipette tip. Thereafter, Ad-cont (Cont)-infected cells were cultured with or without HGF (20 ng/ml) whereas Ad-WT-PTPεM (WT)- or Ad-DA-PTPεM (DA)-infected cells were cultured without HGF. After 8 h culture, migrated cells were counted by microscope. (C) Cell viability was measured by Trypan blue staining. Cells infected with Ad-cont (Cont), Ad-WT-PTPεM (WT), or Ad-DA-PTPεM were incubated in medium without serum for 72 h, and then detached and adsorbed cells were counted. Cell viability is expressed as the percentage of surviving cells compared to the total number of cells. (D) Cell viability of Ad-cont (Cont)- or Ad-WT-PTPεC (WT)-infected cells was investigated as in (C). Data in (D) are expressed as means of two independent experiments, each determined in duplicate. All other data in (A–C) are means ± SE of three independent experiments, each performed in duplicate. $^{*}P < 0.05$. Other experimental conditions in (A–D) were described under Materials and methods.

was detected by RT-PCR using total RNAs prepared from porcine and rat ovarian granulose cells that express PTPεC (Figs. 1B and D) [21]. These data indicate that PTPεM, but not PTPεC, is expressed in vascular ECs and VSMCs, and suggest that the selective expression of PTPε isoforms is biologically significant.

PTPεM promotes EC survival and migration, but not growth

To investigate the biological functions of PTPεM in ECs, we expressed an HA-tagged wild-type (WT-PTPεM) or an HA-tagged catalytically inactive, substrate-trapping Asp302 to Ala mutant (DA-PTPεM) of PTPεM using adenovirus as a vector. More than 95% of ECs were transduced when infected at moi of 50 (data not shown). We examined the effect of the recombinant PTPεM on EC growth, migration, and survival (Fig. 2). Whereas HGF stimulated DNA synthesis to 154% of the control level, the overexpression of WT- or DA-PTPεM had no effect (Fig. 2A). On the other hand, the migration of cells infected with WT-PTPεM was increased to 375% compared with control cells, which was similar to the effect of stimulating the cells with HGF (Fig. 2B). In contrast, the DA mutant reduced cell migration to 32% of the control, indicating that the mutant acted as a dominant negative form. Cell survival was also increased by the expression of WT-PTPεM. That is, after 72 h culture without serum, only 43% of the control cells survived whereas cell viability was increased to 76% in the presence of overexpressed WT-PTPεM (Fig. 2C). The viability of cells expressing DA-PTPεM did not significantly differ from that of control cells. The overexpression of WT-PTPεM tended to increase the amount of cell migration and survival induced by HGF although the data for cell migration did not reach statistical significance (data not shown). These data obtained from the forced expression of DA-PTPεM and WT-PTPεM strongly demonstrate that PTPεM promotes the survival and migration of vascular ECs.

As shown in Fig. 1, PTPεC is not expressed in ECs. However, it is of interest to know whether this isoform

possesses the same function as PTPεM when expressed in ECs. We therefore investigated the effect of HA-tagged wild-type PTPεC on cell survival. Interestingly, overexpressed PTPεC obviously reduced cell viability in contrast to PTPεM (Fig. 2D), suggesting the biological significance of the selective expression of PTPεM in ECs.

Effect of PTPεM on several protein kinases involved in cell growth, survival, and migration

The protein kinases ERK, p38, Akt, and c-Src are critically involved in the regulation of the growth, survival, and migration of various types of cells. Thus, we investigated the effect of WT-PTPεM on the activities of these kinases with or without HGF stimulation in ECs (Fig. 3A). The activities were assessed by Western-blot analysis using antibodies that specifically recognized the activated forms of ERK, p38, and Akt, as well as the pTyr residues that are closely related to the kinase activity of c-Src. Stimulation of the cells with HGF enhanced the phosphorylation level of all kinases examined except for that of Tyr527 in c-Src (numbering as in chicken c-Src). Overexpressed PTPεM did not affect ERK, p38, and Akt, but increased the pTyr416 content of c-Src with a slight decrease in the pTyr527 level. To confirm the effect of PTPεM on c-Src, the phosphorylation levels of Tyr416 and Tyr527 in immunoprecipitated c-Src were examined (Fig. 3B). As expected, forced expression of the phosphatase increased the pTyr416 content, but obviously reduced the pTyr527 level. The two residues Tyr416 and Tyr527 of c-Src are positive and negative regulatory sites for the kinase activity, respectively. Together, these data suggest that PTPεM is a positive regulator of c-Src in vascular ECs, a notion that is consistent with the recently published data [15], and that c-Src activation may be crucial in the PTPεM-promoted survival and migration of ECs.

PTPεM probably directly associates with c-Src in ECs

Gil-Henn and Elson [15] have recently indicated that PTPεM and PTPεC can activate c-Src by directly dephosphorylating pTyr527 of the kinase in mouse embryonic fibroblasts and mammary tumor cells. We investigated whether c-Src is a substrate for PTPεM also in ECs. Figs. 4A and B show that c-Src activation depended on the phosphatase activity of PTPεM. That is, overexpressed WT-PTPεM, but not the catalytically inactive, substrate-trapping mutant DA-PTPεM significantly impaired the pTyr527 content with an obvious increase in the pTyr416 level. Moreover, DA-PTPεM effectively coimmunoprecipitated with c-Src whereas less WT-PTPεM associated with the kinase (Fig. 4C). This association profile is a key feature of the relationship

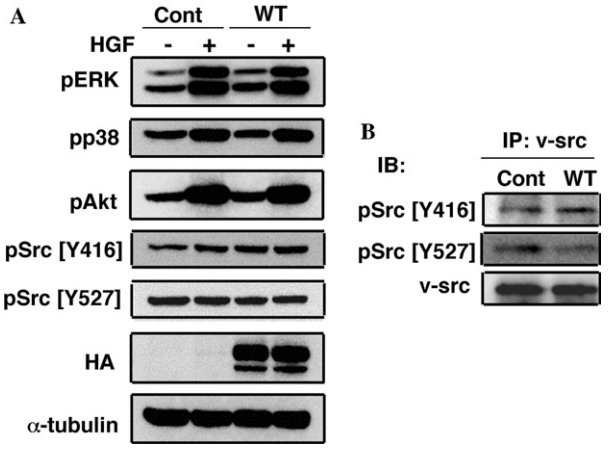

Fig. 3. Effect of overexpressed PTPεM on several protein kinases involved in cell growth, survival, and migration. (A) Ad-cont (Cont)- or Ad-WT-PTPεM (WT)-infected cells were incubated in medium containing 1% serum for 16 h, and then with HGF (20 ng/ml) or vehicle at 37 °C for 10 min. The activities of ERK, p38, and Akt, and the phosphorylation levels of pTyr416 and pTyr527 of c-Src were assessed as described under Materials and methods. Representative data are shown from three experiments that yielded similar results. (B) As in (A), infected cells were cultured in medium containing 1% serum for 16 h, lysates were prepared, and then c-Src was immunoprecipitated with anti-v-Src antibody followed by immunoblotting using antibodies specific for pTyr416 and pTyr527 of c-Src, respectively.

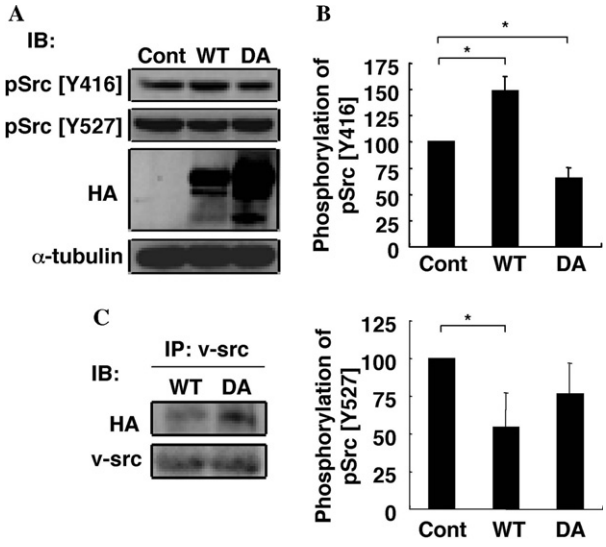

Fig. 4. Association of PTPεM with c-Src in ECs. (A) Ad-cont (Cont)-, Ad-WT-PTPεM (WT)-, or Ad-DA-PTPεM (DA)-infected cells were cultured in medium containing 1% serum for 16 h, then pTyr416 and pTyr527 of c-Src were detected as described under Materials and methods. (B) Band intensity of pTyr416 and pTyr527 of c-Src in panel A was quantified using a LAS-1000 imaging analyzer. Values of the band were normalized to the amount of α-tubulin and expressed as a percentage of the value of the control. Data are means ± SE of four independent experiments. $*P < 0.05$. (C) Cell lysates were prepared from cells treated as in (A), and immunoprecipitated with anti-v-Src antibody followed by immunoblotting using anti-HA antibody. The same experiment was repeated and results were similar.

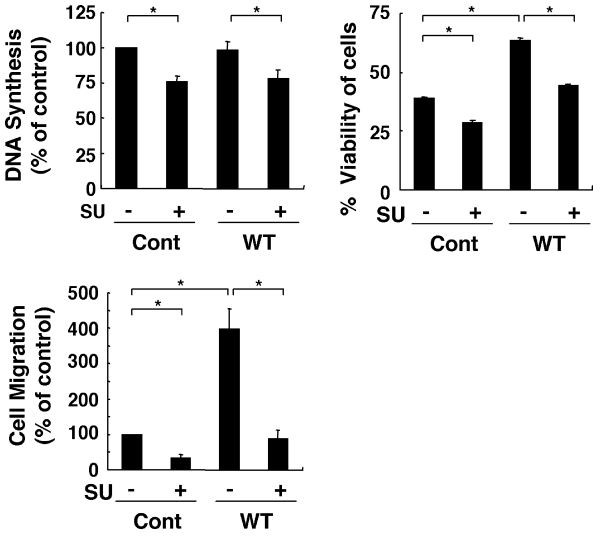

Fig. 5. Effect of SU6656 on EC growth, migration, and survival. Cells infected with Ad-cont (Cont) or Ad-WT-PTPεM (WT) were cultured in the presence or absence of 2 μM SU6656. Inhibitor was added 30 min before adding BrdU to measure DNA synthesis, before removing serum to determine cell survival, or before damaging cells to assess migration. Other experimental conditions were the same as those in Fig. 2. Data are means ± SE of three separate experiments, each performed in duplicate. $*P < 0.05$.

between PTP and its substrate. Accordingly, PTPεM activates c-Src probably by directly dephosphorylating pTyr527 in vascular ECs.

PTPεM promotes survival and migration of ECs through c-Src activation

We assessed the biological significance of c-Src activation in the process of PTPεM-promoted survival and migration using the c-Src inhibitor SU6656 (Fig. 5). All cell growth, migration, and survival were decreased by the addition of SU6656 in the absence of exogenously expressed WT-PTPεM although the decrease in cell migration was most obvious. The inhibitor also suppressed the increases in cell survival and migration induced by overexpressed PTPεM. Again, the effect of SU6656 was more remarkable on cell migration than on survival, which is in agreement with the PTPεM-induced increased levels in these cellular responses. These data demonstrate that PTPεM promotes EC survival and migration, but not growth, and that the dephosphorylation of pTyr527 of c-Src by the phosphatase with subsequent activation of the kinase crucially contributes to these PTPεM functions.

Discussion

The present study generated three key findings as follows. First, PTPεM promotes vascular EC survival and migration. Vascular endothelial injury by various factors, such as reactive oxygen species and increased blood pressure, is an early event in the pathogenesis of atherosclerosis. Molecules that can stimulate and mediate EC growth, survival, and migration are candidates for preventing the initiation and progression of atherosclerosis by repairing the injured endothelium. Therefore, our present findings demonstrate that PTPεM may be a negative regulator for the development of atherosclerosis. The fact that PTPεM exerted its functions even in medium without and with 1% serum in ECs suggests that PTPεM could crucially function under ischemic condition, in which the supply of growth factors and nutrients to ECs is limited. Second, PTPεM activates c-Src probably by directly dephosphorylating the negative regulatory site pTyr527, which critically contributes to PTPεM-promoted cell survival and migration in ECs. There is a report indicating that c-Src mediates the increased migration of bovine aortic ECs by the inhibition of the renin–angiotensin system [22]. The present findings are consistent with these data. Third, both ECs and VSMCs express PTPεM, but not PTPεC, and the action of artificially overexpressed PTPεC in ECs appears to be antagonistic to that of PTPεM. These results indicate that the accurate regulation of alternative promoter usage of the PTPε gene is essential for PTPε to accurately function in the vasculature. PTPεC as well as PTPεM decreased the Tyr527 content of c-Src with an increase in the pTyr416 level in ECs (data not shown), suggesting that PTPεC can suppress the signaling pathway(s) of cell survival by dephosphorylating its substrate(s) other than c-Src or by trapping the adapter protein Grb2 [23] under this artificial experimental condition.

In contrast to our data, one report indicated that overexpressed PTPεM inhibited the proliferation of human umbilical vein ECs [6]. Two reasons are mainly conceivable for explaining this discrepancy. One is the different vascular bed from which ECs were derived. We used porcine aortic ECs because arterial ECs are considered to be more suitable than venous ECs for studying cardiovascular diseases. In fact, arterial ECs differ from venous ECs in several aspects, such as the production of angiotensin-converting enzyme, ability to generate prostacyclin [24], and their response to cytokine stimulation [25]. The second explanation is the variability of ECs between species. Although this possibility cannot be ruled out, we think that the first explanation is more likely because it is well known that there are similarities between the human and porcine cardiovascular system [26].

The present study focused on PTPεM in ECs, but the functions of other PTPs also should be clarified for understanding the molecular mechanisms of pathogenesis of atherosclerosis. For example, in addition to PTPεM, we found that ECs express PTP1B and TC-PTP, both of which are also present in VSMCs [7].

These two PTPs negatively regulate insulin signaling pathway that is critically involved in the pathogenesis of diabetes. Because diabetes is a well-known risk factor for atherosclerosis, it is essential for elucidating the functions of PTP1B and TC-PTP in the vasculature.

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