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Endothelial Cell Protein C Receptor Opposes Mesothelioma Growth Driven by Tissue Factor

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Abstract

The pro-coagulant protein Tissue Factor (TF, F3) is a powerful growth promoter in many tumors but its mechanism of action is not well understood. More generally, it is unknown whether hemostatic factors expressed on tumor cells influence TF-mediated effects on cancer progression. In this study, we investigated the influence of TF, endothelial cell protein C receptor (EPCR, PROCR) and protease activated receptor-1 (PAR1, F2R) on the growth of malignant pleural mesothelioma (MPM), using human MPM cells that lack or express TF, EPCR or PAR1 and an orthotopic nude mouse model of MPM. Intrapleural administration of MPM cells expressing TF and PAR1 but lacking EPCR and PAR2 (F2RL1) generated large tumors in the pleural cavity. Suppression of TF or PAR1 expression in these cells markedly reduced tumor growth. In contrast, TF overexpression in non-aggressive MPM cells that expressed EPCR and PAR1 with minimal levels of TF did not increase their limited tumorigenicity. More importantly, ectopic expression of EPCR in aggressive MPM cells attenuated their growth potential, whereas EPCR silencing in non-aggressive MPM cells engineered to overexpress TF increased their tumorigenicity. Immunohistochemical analyses revealed that EPCR expression in tumor cells reduced tumor cell proliferation and enhanced apoptosis. Overall, our results enlighten the mechanism by which TF promotes tumor growth through PAR1, and they show how EPCR can attenuate the growth of TF-expressing tumor cells.

Keywords

endothelial cell protein C receptor; mesothelioma; protease-activated receptor; tissue factor; tumor growth

Introduction

It has been well recognized for many decades that tumors dramatically increase the risk for hemostatic abnormalities such as disseminated intravascular coagulation, pulmonary and venous thromboembolism (1-3). Prior studies have also established that hemostatic factors play a major role in cancer biology, particularly in tumor dissemination and metastasis (4-6). Tumor cell-associated tissue factor (TF) is known to contribute to tumor growth and

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progression either directly by TF-FVIIa or TF cytoplasmic tail-mediated cell signaling (7-9), or indirectly through generation of thrombin that leads to activation of platelets, fibrin deposition, and activation of PAR1-mediated cell signaling (10-13).

Recent studies showed promotion of tumor growth by TF, independent of its role in coagulation (14-17). Selective inhibition of TF-FVIIa signaling using a specific monoclonal antibody that blocks TF signaling, but not TF-mediated coagulation, was shown to reduce breast tumor growth (14). Blockade of PAR2 but not PAR1 cleavage by specific antibodies attenuated tumor growth. Consistent with the hypothesis that PAR2-mediated signaling contributes to tumor growth in breast cancer, mice lacking PAR2 but not PAR1, exhibited reduced tumor growth in a model of spontaneous mammary tumors (18). Our recent studies on progression of malignant pleural mesothelioma (MPM) showed that MPM cells that express TF generated large tumors within the pleural cavity, and inhibition of tumor cell TF by overexpression of TFPI in tumor cells blocked tumor growth and invasion (19). It is unknown at present whether TF-FVIIa-PAR2-mediated cell signaling contributes to growth of MPM as observed in breast cancer (14, 18).

Recent studies from our laboratory and others have established that FVIIa, the protein that initiates the coagulation upon binding to TF, also binds to EPCR (20-22). FVIIa binding to EPCR on the endothelium was shown to activate PAR1-mediated cell signaling providing barrier protective effect (23). Disse et al. reported that EPCR promoted PAR1 and PAR2 cleavage by FXa in the ternary complex of TF-FVIIa-FXa (24). Studies in cell model systems implicated that EPCR may promote tumor metastasis as EPCR-APC-mediated PAR1 signaling was shown to promote cancer cell migration, invasion, and angiogenesis (25, 26). *In vivo* studies gave conflicting data as EPCR-APC signaling decreased lung metastasis in melanoma model by preventing tumor cell migration through enhancement of endothelial barrier function (27, 28), whereas EPCR over expression increased metastasis in lung adenocarcinoma by promoting tumor cell survival (29). To date, there is no information on whether EPCR directly influences tumor growth.

In the present study, we show that MPM cells that express TF and PAR1 but not PAR2 generate large tumors in the thoracic cavity. Suppression of either TF or PAR1 reduces tumor growth in this model. However, overexpression of TF in less aggressive MPM cells that lack TF but express PAR1 failed to induce an aggressive phenotype. Interestingly, we found no EPCR expression in aggressive MPM cells whereas abundant EPCR expression was found in non-aggressive MPM cells. Introduction of EPCR expression to aggressive MPM cells by EPCR knock-in completely attenuated their tumorigenicity whereas the knock-down of EPCR expression in non-aggressive MPM cells engineered to overexpress TF markedly increased their tumorigenicity. The present study is the first to report that EPCR suppresses TF-driven tumor growth of mesothelioma.

Materials and Methods, (for detailed methods see Supplemental Material)

Cell lines

REN cells were from S. Albelda, University of Pennsylvania, MS-1 cells were from S-M. Hsu, The University of Texas Health Science Center at Houston, and M9K cells were from B. Gerwin, NIH. All three MPM cell types were obtained from the above investigators before 2008. Characterization of these cells when they were first used in our tumorigenesis model showed an epitheloid phenotype in culture and retained classical MPM markers, confirming their MPM origin (29, 30).

Generation of stable transfectants of MPM cells expressing/lacking TF, EPCR or PAR1

TF or PAR1 expression in REN MPM cells was selectively knocked-down by specific shRNA constructs cloned into pSilencer 2.1 U6-Puro expression vector. For generation of EPCR expressing REN cells, REN MPM cells were transfected with pZeoSV plasmid containing human EPCR cDNA (20). MS-1 and M9K MPM cells were stably transfected with pcDNA 3.1 containing TF cDNA. To suppress EPCR expression in MS-1 and M9K cells, native MS-1 and M9K cells or MS-1 and M9K cells engineered to overexpress TF were stably transfected with EPCR-specific shRNA constructs.

Tissue factor activity

The procoagulant activity of TF on intact cell surface of wild-type and stable transfectants was measured in a factor $\times$ activation assay (31).

Measurement of cytosolic Ca^{2+} release

Fluorescence microscopy was used for measurement of cytosolic Ca^{2+} release as described earlier (32).

Orthotopic murine model of thoracic human MPM

One hundred μl of MPM cell suspension containing 1×10^6 cells were injected into the pleural cavity of nude mice as described earlier (30) with a few minor modifications. Mice were sacrificed between 28 and 30 days following tumor cell implantation, and tumor growth was evaluated as described earlier (30).

Histology and immunohistochemistry

Tissues were processed for thin sectioning using standard procedures. Rehydrated tissue sections were processed for hematoxylin-eosin (H&E), elastin, collagen staining, or immunostaining for TF, EPCR, Ki67 or TUNEL staining.

Statistical analysis

Nonparametric statistical tests, Kruskal-Wallis or Mann-Whitney test, were used for determination of statistical significance.

Results

Status of TF, EPCR, PAR1, PAR2, TM and TFPI expression levels in MPM cells

TF expression was markedly higher in REN cells compared to MS-1 and M9K cells (Fig. 1A and B). TF expression was barely detectable in MS-1 and M9K cells. REN cells express very little EPCR whereas both MS-1 and M9K cells abundantly express EPCR, at levels found in endothelial cells (Fig. 1C). As reported earlier (19), REN cells lack TFPI expression whereas both MS-1 and M9K cells express TFPI (Fig. 1D). TM expression was not detectable in REN cells and barely detectable in MS-1 cells, but abundant in M9K cells (Fig. 1E). Western blot analysis revealed that all three MPM cell types express PAR1 whereas PAR2 expression was undetectable (Fig. 1F). Consistent with the antigen data, a PAR1 but not PAR2 agonist peptide induced intracellular Ca^{2+} release in REN cells (Fig. 1G). A similar pattern of Ca^{2+} release was observed in MS-1 and M9K cells, i.e., respond to PAR1, but not to PAR2 agonist peptide (data not shown).

REN MPM cells generate large invasive tumors and knock-down of TF reduces tumorigenicity of REN cells

As reported previously (30), implantation of REN cells into the thoracic cavity of nude mice resulted in multiple large tumors (> 2 mm) within the thoracic cavity (Fig. 2A). The number of large tumors in each mouse varied from 6 to 18. Some of the tumors were approximately the size of the heart (Fig. 2B). All tumors were limited to the thoracic cavity. These tumors were highly invasive and often penetrated deep into intercostal tissues on which they were attached (Fig. 2C and 2D). There was no evidence for metastasis as we found no tumors in distant organs such as liver. A variable number of very small tumors (< 2 mm) found in the thoracic cavity may reflect dispersed initial seeding of tumor cells rather than pleural metastases. In contrast to REN, intrapleural administration of MS-1, and M9K cells produced relatively few tumors; some of the mice developed no tumors at all. Most of the tumors that were generated from these cells barely reached the 2 mm size. None of the tumors grew as large as those found in mice injected with REN cells. Further, MS-1 and M9K cell-derived tumors were loosely attached to the tissue and remained non-invasive. Intrathoracic lavage of these animals showed small granular white spheroids. We found no significant differences in cell growth potential among REN, MS-1 and M9K cells *in vitro* either under basal conditions (in the absence of serum) or in the presence of serum. In all three cell types, the cell proliferation rate in the presence of 1% serum was about 2.5 to 3.5-fold higher over the cells cultured in the absence of serum (data not shown).

To investigate the role of TF on aggressiveness exhibited by REN tumors, we knocked-down TF expression in REN cells by stably transfecting with TF-specific shRNA (REN(-TF)). As a control, REN cells were stably transfected with a non-effective scrambled shRNA in pSilencer vector (REN(p)). As shown in Fig. 3A and 3B, TF antigen expression and cell surface TF activity was reduced by about 70% in REN(-TF) cells compared to that measured in naïve REN or REN(p) cells. Mice injected with naïve REN and REN(p) cells developed a similar number of large tumors (Fig. 3C), and no significant differences were found between them in their tumor growth or burden (Fig. 3D and Fig. 3E). In contrast, mice injected with REN(-TF) cells developed fewer numbers of large tumors (Fig. 3C). Although a few of the tumors in mice injected with REN(-TF) grew as large as those found in mice injected with naïve REN or REN(p) cells, most of the tumors were smaller. The total tumor volume and burden was significantly lower in mice injected with REN(-TF) cells in comparison to mice injected with naïve REN or REN(p) cells (Fig. 3D and 3E). In contrast to tumors originating from naïve REN and REN(p) cells, tumors that originated from REN(-TF) cells were less invasive (data not shown). Immunohistochemical analysis of tissue sections using anti-TF antibodies showed abundant expression of TF in tumors that developed in mice injected with naïve REN or REN(p) cells. In contrast, little expression of TF was found in tumors from mice injected with REN(-TF) (Fig. 3F). *In vitro* cell proliferation studies showed that FVIIa increased the cell proliferation of REN cells modestly, but consistently, and TF knock-down abrogated responsiveness to FVIIa (Supplementary Fig. 1). Immunohistochemistry analysis of tissue sections of tumors derived from mice injected with REN or REN(-TF) cells revealed a marked reduction in number of proliferating tumor cells (Supplementary Fig. 2) with a concomitant increase in number of apoptotic tumor cells (Supplementary Fig. 3) in tumors derived from REN(-TF) MPM cells.

To examine the involvement of PAR1 in TF-driven tumor growth of mesothelioma, PAR1 expression in REN cells was knocked-down by PAR1-specific shRNA (REN(-PAR1)). PAR1 knock-down reduced the PAR1 expression by more than 80% (Fig. 4A, 4B and 4C). Knocking-down PAR1 in REN cells resulted in more than 50% reduction in the number of tumors formed in the thoracic cavity of nude mice compared to nude mice injected with naïve REN and REN(p) cells (REN, 16.0 ± 3.5 ; REN(p), 12.9 ± 1.8 ; REN(-PAR1), 6.3 ± 0.8). As shown in Fig. 4D, 4E and 4F, inhibition of PAR1 expression in REN cells markedly

attenuated tumor growth and burden. In addition, PAR1 knock-down also eliminated the invasiveness of REN tumors. *In vitro* cell proliferation studies showed that PAR1 knock-down diminished a modest increase of thrombin-induced cell proliferation (Supplementary Fig. 1). Analysis of tumor tissue sections for the expression of nuclear proliferation antigen Ki67 showed a marked reduction in the percentage of proliferating tumor cells in tumors originating from PAR1 knocked-down REN MPM cells (Supplementary Fig. 2). TUNEL staining revealed a significant increase in the percentage of apoptotic tumor cells in REN(-PAR1) cell-derived tumors compared to REN(p)-cell derived tumors (Supplementary Fig. 3).

Over expression of TF in less aggressive MS-1 and M9K cells does not increase the tumorigenicity

To determine whether difference in TF expression alone is responsible for marked differences in the tumor growth between aggressive REN cells and less aggressive MS-1 or M9K cells, MS-1 and M9K cells were stably transfected to express human TF. MS-1 and M9K transfectants expressing similar levels of cell surface TF functional activity as to that found in REN cells (Fig. 5B) were selected for *in vivo* studies. Surprisingly, overexpression of TF in either MS-1 or M9K cells failed to alter their tumorigenicity. The number of tumors formed in the pleural cavity of nude mice injected with MS-1(+TF) or M9K(+TF) cells was comparable to those formed in the mice injected with naïve MS-1 and M9K cells, and these were markedly less than the number of tumors produced by injection of naïve REN cells (Fig. 5C). The tumors formed in nude mice injected with naïve or TF overexpressing MS-1 or M9K cells were small and barely reached the 2 mm size-threshold to be included in the total tumor count. Further, these small tumors, in contrast to the tumors formed in mice injected with REN cells, were loosely adhered to the pleura. No significant differences were observed in the tumor volume (Fig. 5D) or tumor burden (Fig. 5E) among mice injected with naïve and TF over expressing MS-1 or M9K cells, which were significantly lower ($P < 0.001$) compared to tumor volume and burden in mice injected with naïve REN cells. Immunohistochemical analysis of tumor sections revealed that MS-1 (Fig. 5F) and M9K cells (data not shown) stably transfected with TF continued to express TF in the tumor environment *in vivo*. Analysis of tumor tissue lysates for TF by Western blot analysis and the functional activity assay confirmed the expression of TF in small tumors generated by MS-1(+TF) and M9K(+TF) cells (data not shown). These data eliminate the possibility that loss of TF expression in tumor cells *in vivo* may be responsible for the inability of TF to promote tumor growth in these cell types.

EPCR regulates TF-driven tumor growth of MPM

Since REN cells do not express EPCR whereas MS-1 and M9K cells express abundant EPCR (Fig. 1C), we postulated that absence of EPCR in REN cells is responsible for their aggressive tumorigenicity. Therefore, we investigated the potential effect of tumor cell associated EPCR on suppression of MPM tumor growth. REN MPM cells were stably transfected to express EPCR (Fig. 6A). EPCR stable transfectants expressing levels of TF activity similar to that of naïve REN cells or REN cells stably transfected with a control vector were selected for intrapleural injection (Fig. 6B). Introduction of EPCR expression to REN cells markedly reduced the number of tumors formed in the thoracic cavity (Fig. 6C), and the few tumors that were formed remained very small (Fig. 6F). Thus, the total tumor volume and burden in mice injected with REN(+EPCR) cells was strikingly lower than that was observed with control REN(z) cells (Fig. 6D and 6E). Although *In vitro* studies showed no measurable differences in cell proliferation between REN and REN(+EPCR) in the presence or absence of various ligands (Supplementary Fig. 4), analysis of tumor tissue sections showed a dramatic decrease in the percentage of proliferating tumor cells in tumors derived from REN(+EPCR) cells compared to REN(z) cells (Fig. 6G). A higher percentage

of tumor cells were found to be apoptotic in tumors developed from REN(+EPCR) cells compared to tumors derived from REN(z) cells (Fig. 6H).

Consistent with our hypothesis that EPCR plays the critical role in suppressing tumor growth in MPM, knock-down of EPCR expression in TF overexpressing MS-1 and M9K cells (Fig. 7A-C) dramatically increased the tumorigenicity of these non-aggressive MPM cells (Fig. 7D-F). Mice injected with EPCR-knocked-down MS-1(+TF) and M9K(+TF) cells developed enormous tumor burden (Fig. 7F). These MPM cell types formed not only large nodular tumors but they grew on lung pleura and diaphragm forming large coalescing tumors that tightly attached the bottom of lungs to diaphragm and esophagus, creating a large thick mass containing tumor cells, organs, and extracellular matrix (Fig. 7G, H). In addition, small tumors on pericardial membrane and parietal pleura were clearly visible. Invasion of tumor cells into the intercostal space and along the ribs is also visible in all the mice injected with EPCR-knock-down MPM cells. The thoracic cavities of these mice were filled with bloody fluid (hemothorax). Finally, some of the mice injected with EPCR knock-down of MS-1(+TF) or M9K(+TF) cells lost about as much as 25% body weight in the last week. Analysis of TM and/or TFPI levels in M9K(+TF) and MS-1(+TF) cells and their corresponding EPCR knocked-downs showed no significant differences in their expression levels (Supplementary Fig. 5). In contrast to the increased tumor production obtained with EPCR knocked-down MS-1(+TF) and M9K(+TF) cells, EPCR knocked-down in parental MS-1 or M9K cells produced similar low tumorigenicity of parental cells (Fig. 7D-F). Overall, our data clearly illustrate that EPCR suppresses TF-driven tumor growth in MPM.

In vitro cell proliferation studies showed no significant differences in cell growth pattern among MS-1 and M9K cells expressing EPCR or EPCR knocked-downs (Supplementary Fig. 6A and 6B). However, evaluation of tumor tissues by immunohistochemistry for the presence of nuclear proliferation antigen Ki67 revealed that EPCR knocked-down of TF expressing MS-1 and M9K cells markedly increased the percentage of proliferating tumor cells in the tumor bed (Fig. 7I). TUNEL staining showed that EPCR knocked-down of TF expressing MS-1 and M9K cells significantly decreased tumor cell apoptosis (Fig. 7J).

DISCUSSION

The increased expression of TF in tumor cells has been shown to associate with various aspects of tumor progression, including tumor growth, angiogenesis, invasion, and metastasis (7-9). Tumor cell TF-induced activation of the coagulation pathway is believed to be primarily responsible for tumor cell dissemination and metastasis as down-stream coagulation activation results in thrombin-, platelet-, and fibrin-dependent pathways (6, 11, 12). In contrast, a direct tumor cell TF-mediated cell signaling was shown to be responsible for tumor growth (14). Although regulators of coagulation, such as EPCR and TM, were also shown to influence metastatic potential (27, 28, 33), their role in tumor growth was either unexplored or not completely understood (34). In the present study, we used MPM cells that express or lack TF, EPCR, PAR1 and PAR2, and a novel orthotopic murine model of MPM to investigate the influence of TF and EPCR on MPM tumor growth. The data provided herein demonstrate that TF promotes MPM tumor growth as observed with other cancers but TF-driven tumor growth in MPM does not require PAR2 but rather is dependent on PAR1. Interestingly, our data also reveal that TF expression in tumor cells in itself does not fully control tumor growth, as overexpression of TF in non-aggressive MPM cells expressing PAR1 failed to promote tumor growth. More importantly, our studies demonstrate for the first time that EPCR on tumor cells, at least in MPM cells, can suppress TF-driven tumor growth.

Recent studies have provided evidence that TF actively contributes to tumor growth through a non-hemostatic, TF-dependent signaling mechanism in melanoma (16) and breast cancer (14, 18). TF was shown to support tumor growth in breast cancer via TF-FVIIa-PAR2-mediated cell signaling, independent of PAR1 (14, 18). However, it remains unclear at present whether such a mechanism is required for tumor growth in other types of cancers. Our present observations that REN cells expressing TF and PAR1 but not PAR2 generate large primary tumors compared to MS-1 and M9K cells that express PAR1 but lack TF, and TF knock-down suppresses tumor growth of REN cells strongly suggest that TF drives tumor growth in MPM by a mechanism that does not involve PAR2. These data support the possibility that TF may promote tumor growth in different cancers by different mechanisms.

Similar to TF knock-down, PAR1 knock-down also markedly suppressed REN MPM tumor growth. The extent of tumor growth suppression in PAR1 knock-down appeared to be very similar to that observed with TF knock-down cells, which is consistent with the hypothesis that TF supports MPM tumor growth via a PAR1-dependent mechanism. However, it is unclear at present whether a direct TF-PAR1-mediated signaling or thrombin-induced PAR-1 mediated signaling is responsible for this tumor growth. Here, it is interesting to note that although overall reduction in tumor growth as measured in tumor volume and burden is similar in both TF- and PAR1 knock-downs, mice injected with PAR1 knock-down cells developed fewer tumors in total (average 10 tumors/mouse, including small tumors < 2mm size) compared to mice injected with TF knock-down cells (average 21 tumors/mouse). These data suggest, *albeit* indirectly, that TF-mediated PAR1 signaling may primarily drive tumor growth, whereas thrombin-mediated PAR1 signaling may contribute to tumor cell adhesion and initial seeding of tumor cells.

Earlier studies showed that TF expression levels determine the metastatic potential of tumor cells. Melanoma cells constitutively expressing high levels of TF were shown to be highly metastatic, whereas melanoma cells that lack TF failed to metastasize (35). Transfection of TF into non-metastatic melanoma cells positively transformed their metastatic potential (35). Similarly, transfection of CHO cells with TF enhanced tumor cell metastasis (36). A number of clinical studies found a correlation between TF expression and invasiveness/clinical stage of cancer (9). Based on these observations, we predicted that transfection of non-aggressive MS-1 or M9K cells with TF would increase their tumor growth potential. Therefore, it is quite surprising to find that transfection of TF into these cells to similar levels of TF found in REN cells failed to increase their tumor growth potential. Our earlier studies revealed that while REN cells lack TFPI, both MS-1 and M9K cells express abundant TFPI (37). However, it is unlikely that suppression of TF activity by TFPI on tumor cells is the reason for the failure of MS-1 or M9K cells expressing TF to generate large tumors since TF expression levels on the transfected cells far exceed TFPI levels as we selected stable transfectants based on TF functional activity levels. It is important to note here that TF functional activity in MS-1 or M9K cells stably transfected with TF was similar to that of REN cells. Moreover, we found similar TF activity levels in tumors excised from mice injected with MS-1 or M9K cells expressing TF and REN cells. Overall, our data indicate that although TF plays a crucial role in tumor growth in MPM, TF expression alone could not establish the tumor growth potential.

One additional difference between aggressive REN cells and non-aggressive MS-1/M9K cells is the expression of EPCR. Both MS-1 and M9K cells express EPCR levels similar to that found in endothelial cells whereas REN cells express no or very little EPCR. This raises an interesting possibility that EPCR acts as a tumor growth suppressor. Unlike numerous published studies correlating TF expression levels with tumor burden/metastasis and cancer survival, information on EPCR expression levels in cancer is very limited. Although EPCR expression was demonstrated in some cancer tissues (38-40), these studies were not

performed systematically as they were not related to tumor grade. It appears that expression of EPCR in tumor cells is a rare event (40) and more EPCR+ tumors could be found in stage pT1 than in pT2 (40). Although we are not aware of any studies in the literature that investigated the influence of EPCR on tumor growth, a number of studies reported that EPCR-APC signaling exert anti-apoptotic effects on a variety of cell types (41-46). Recently, Anton et al. (29) showed that APC/EPCR axis conferred a significant advantage to cell survival of lung adenocarcinoma cells, and this was responsible for robust prometastatic activity of lung adenocarcinoma. By extrapolation of these data, one would speculate that EPCR, as with TF, may promote tumor growth. However, the data presented in this study show clearly that EPCR, in fact, suppresses, tumor growth. This is an unexpected and novel finding, which runs contrary to the known functions of EPCR. At present, it is unknown whether tumor growth suppressive property of EPCR requires a specific pleural microenvironment or is specific to MPM cell types. Answering this requires extensive studies that involve deployment of other tumor growth models and tumor cell types, which are beyond the scope of the present work.

It is unknown, at present, whether the protective effect of EPCR on MPM tumor growth we observed is mediated by its anticoagulant function or cell signaling function. EPCR plays a key role in suppressing thrombin generation by promoting the activation of anticoagulant protein C to APC by thrombin-thrombomodulin complex. EPCR on tumor cells may simply inhibit the tumor growth by inhibiting thrombin generation, and thereby reducing thrombin-mediated PAR1-dependent tumor growth. However, it may be pertinent to note here that REN cells do not express TM, a cofactor needed for thrombin activation of EPCR bound protein C. Nonetheless, overexpression of EPCR in REN cells markedly reduced tumor growth. EPCR, in addition to supporting APC-mediated cell signaling, was shown to promote TF-FVIIa-FXa ternary complex signaling of PAR1 and PAR2 (24). It is unlikely that EPCR suppresses MPM tumor growth through its interaction with TF-FVIIa-FXa ternary signaling complex since this would presumably promote, and not suppress, tumor growth because upregulation of TF-dependent signaling in tumor cells may in general lead to activation of pro-angiogenic and pro-tumor growth pathways (7-9). Similar to TF, PAR1 signaling is also believed to contribute to cancer progression (13). However, at present, we can not exclude the possibility of PAR1 involvement in mediating the EPCR's protective effect as EPCR was shown to switch specificity of PAR1 signaling from damaging to protective signaling (47, 48). For example, it had been shown that cleavage of PAR1 by thrombin initiates proinflammatory responses in endothelial cells in the absence of EPCR ligation whereas when EPCR was ligated by protein C, the cleavage of PAR1 by thrombin initiates antiinflammatory response (48). Thus, it is conceivable that activation of PAR1 by thrombin or APC in tumor cells lacking EPCR could result in pro-tumor growth signaling whereas activation of PAR1 by thrombin in tumor cells expressing EPCR may initiate pro-apoptotic cell signaling.

At present, molecular mechanisms by which EPCR suppresses tumor growth are unclear. In our *in vitro* cell proliferation studies, we found no significant differences in cell proliferation between MPM cells lacking EPCR expression and MPM cells expressing EPCR in the absence or presence of FVIIa, APC, or thrombin. However, analysis of tumor cell proliferation in tumor tissues showed that EPCR expression in MPM cells suppresses tumor cell proliferation. Consistent with the finding that EPCR inhibits tumor cell proliferation *in vivo*, EPCR knocked-down markedly increased tumor cell proliferation in MS-1 and M9K cells overexpressing TF. In addition to inhibiting tumor cell proliferation *in vivo*, EPCR expression in tumor cells also promoted tumor cell apoptosis. These data were somewhat surprising because EPCR is known to primarily activate cytoprotective signaling pathways, including anti-apoptotic signaling pathways (46, 49). At present, it is unclear how EPCR suppresses tumor cell proliferation and promotes tumor cell apoptosis *in vivo*. A number of

studies have shown that EPCR-mediated signaling pathway inhibits NF- κ B-mediated proinflammatory pathways in monocytes and endothelial cells (42, 46, 48, 50). It is well-accepted now that tumor-associated inflammatory responses play a critical role in enhancing tumorigenesis and cancer progression (51-53). Thus, it is very tempting to speculate that EPCR's protective effect against tumor growth comes through its anti-inflammatory effect in tumor microenvironment. Notable differences in EPCR's effect on tumor cell proliferation in *in vitro* and *in vivo* indicate that host factors may significantly influence the mode of EPCR action.

Finally, our present data provide impetus for developing novel therapeutic strategies in treating cancer patients, particularly MPM patients. If EPCR is found to suppress MPM tumor growth through enhancement of APC generation, then administration of exogenous APC may restrict the progression of this tumor. If EPCR-mediated cell signaling in tumor cells is found to be responsible for curtailing tumorigenicity, then transduction of EPCR in tumor cells by gene therapy may become a viable option. MPM is an attractive target for gene therapy because of the paucity of conventional therapies, accessibility of the tumors in the pleural space for delivery of experimental therapeutics, and previous experience derived from gene therapy trials in MPM patients (54, 55).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Varied expression of TF, EPCR, TFPI, TM, PAR1 and PAR2 in human REN, MS-1 and M9K cells. (A) TF immunoblot; (B) Cell surface TF activity levels as measured in factor × activation assay (significance levels between the groups were determined by Kruskal-Wallis test and Dunn's post test was used to calculate statistical significant differences between REN and MS-1 or M9K; *** $P < 0.001$); (C-F) Immunoblots for EPCR (C), TFPI (D), TM (E), or PAR1 and PAR2 (F). Cell extracts of MDA231 tumor cells, human mesothelial cells (HMC) or human umbilical vein endothelial cells (HUVEC) were included in blots as controls. (G) Intracellular Ca^{2+} release in REN cells in response to control vehicle, PAR1 agonist peptide (50 μM) or PAR2 agonist peptide (50 μM). Arrow mark indicates the time

when the control vehicle or PAR peptide agonist was added to the cells. Fl. on Y-axis indicates mean fluorescence.

A

MS-1

M9K

REN

B

C

D

Figure 2.

REN and not MS-1 or M9K MPM cells generate large and invasive intrathoracic tumors. (A) Nude mice injected with MS-1, M9K or REN cells (1×10^6 cells/mouse) were killed 4 weeks following tumor cell injection and tumors generated by MPM cells in the thoracic cavity were measured, counted and weighed ($n = 10$ to 13 mice/group, combined from two independent experiments). Significance levels between the groups were determined by Kruskal-Wallis test; *** $P < 0.0001$. Dunn's post test was used to calculate statistical significant differences between REN and MS-1 or M9K; *** $P < 0.001$. (B) A representative chest cavity photograph showing tumors generated by MS-1, M9K or REN cells. Arrows indicate large tumors. H, heart; L, lung. (C) H&E staining of REN tumor showing the

invasion of tumor cells into diaphragm. D, diaphragm; T, tumor cells. (D) REN tumor invading through lung tissue. T, tumor; L, lung. Right hand picture shows a corresponding to region outlined with the box. Tissue section was stained for collagen.

Figure 3.

Suppression of TF in REN cells attenuates tumor growth. Naïve REN cells were stably transfected with a control non-effective scrambled shRNA (REN(p)) or TF-specific shRNA (REN(-TF)). The stable transfectants were analyzed for TF expression by western blot analysis (A) or measuring cell surface TF activity (B). Mice were injected with REN, REN(p) or REN(-TF) cells, and after 4 weeks mice were sacrificed, and tumor number (C), volume (D) and burden (E) were calculated (n = 10 to 13 mice/group, data from two experiments performed using two independent REN(-TF) stable transfectants, which gave similar results, were pooled). Significance levels between the groups were determined by Kruskal-Wallis test (** $P < 0.05$) with Dunn's post test to compare statistical significant

differences between REN vs. REN(p) or REN(–TF). ns, not significant; ** $P < 0.01$; *** $P < 0.001$. (F) Tumors sections were processed for histology by H&E staining or immunostaining with control IgG or anti-human TF IgG.

Figure 4.

PAR1 knock-down in REN cells reduced tumor growth and burden of REN tumors. REN cells were stably transfected with control non-effective scrambled shRNA or PAR1-specific shRNA. The stable transfectants were analyzed for PAR1 expression by western blot analysis (A) or measuring intracellular Ca^{2+} release in response to PAR1 peptide agonist (B and C). Stable transfectants of REN cells expressing control shRNA (REN(p)) or PAR1-specific shRNA (REN(-PAR1)) were injected intrathoracically into nude mice (1×10^6 cells/mouse). At the end of 4 weeks, mice were sacrificed, and tumor volume (D) and burden (E) were measured ($n = 7$ to 9 mice/group, data from two independent experiments performed with a single REN(-PAR1) clone was combined). Statistical significance between

the two groups was determined by Mann-Whitney test; *** $P < 0.001$. (F) A representative photograph showing differences in tumor growth in mice injected with REN(p) or REN(-PAR1) cells. Arrows indicate tumors. H, heart; L, lung.

Figure 5.

Over expression of TF did not increase the tumorigenicity of non-aggressive MS-1 and M9K cells. TF levels in MS-1 or M9K cells, and their derivatives were analyzed by Western blot analysis (A) or measuring cell surface TF coagulant activity (B). Mice injected with naïve REN, MS-1 or M9K MPM cells, and MS-1 and M9K cells stably expressing TF were killed at the end of 30 days and tumor number (C), volume (D) and burden (E) were measured. Data shown in panels C, D, and E comes from two independent experiments performed with two independent TF overexpressing transfectants, which gave very similar results; $n = 11$ to 14 mice/group). Statistical significance level between the groups was determined by Kruskal-Wallis test ($***P < 0.001$) with Dunn's post-hoc test to compare statistical

significant differences between the two groups (ns, not significant). (F) Tumor tissue sections were stained with H&E or immunostained with anti-humanTF IgG. T denotes tumor.

Figure 6.

EPCR expression in tumor cells reduced tumorigenicity of aggressive REN MPM cells. REN cells were stably transfected with pZeoSV or pZeoSV+EPCR expression vector and analyzed for EPCR expression (A) or cell surface TF activity (B). REN cells expressing the control vector (REN(z)) or EPCR expression vector (REN(+EPCR)) were injected intrathoracically into nude mice (1×10^6 cells/mouse). At the end of 30 days, mice were sacrificed, and tumor number (C), volume (D) and burden (E) were measured. Data shown in the figure were the combined data from two independent experiments performed with three independent EPCR overexpressing clones, which gave very similar results; $n = 11$ to 17 mice/group). (F) A representative photograph showing differences in tumor growth in

mice injected with REN(z) or REN (+EPCR) cells. Arrows indicate tumors. H, heart; L, lung. Tissue sections of tumors derived from mice injected with REN(z) and REN(+EPCR) cells were immunostained for expression of nuclear proliferating antigen Ki67 (G) or TUNEL staining (H), and the percentage of tumor cells stained positive were counted. Bar represents 100 μ m. Statistical significance between the groups is determined by Mann-Whitney test; *** $P<0.001$.

Figure 7.

Suppression of EPCR expression increases tumorigenicity of non-aggressive MPM cells engineered to express TF. MS-1, M9K, MS-1(+TF) and M9K(+TF) cells were stably knocked-down by puromycin resistant EPCR-specific shRNA plasmid. The stable transfectants were analyzed for EPCR and TF expression by Western blot analysis (A and B) or cell surface TF activity (C). MS-1, M9K cells, or its derivatives were injected intrathoracically into nude mice (1×10^6 cells/mouse). At the end of 30 days, mice were sacrificed, and tumor number (D), volume (E) and burden (F) were measured ($n = 6$ to 9 mice/group). (G) A representative photograph of thoracic cavities that depict marked differences in tumor growth and invasiveness between mice injected with EPCR knocked-

down in tumor cells expressing TF and all other tumor cell types. Arrows indicate tumors. H, heart; L, lung; D, diaphragm. (H) Tumor invasion. Tumor developed in a mouse injected with M9K (+TF-EPCR) was sectioned and stained for collagen or elastin. T, tumor; L, Lung; A, airway; B, blood vessel (4×). (I) Percentage of proliferating tumor cells as determined by the expression of nuclear proliferating antigen Ki67. (J) Percentage of apoptotic tumor cells as determined by TUNEL staining. Statistical significance level between the groups was determined by Kruskal-Wallis test (** $P < 0.001$; * $P < 0.01$, * $P < 0.05$).