PROTEINKINASE C ISOFORMS AND DIFFERENTIATION OF ENDOTHELIAL CELLS INTO THE FENSTRATED PHENOTYPE

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In the last couple of years considerable progress has been made in the identification of endothelial cell growth factors, such as VEGF and angiopoietin, and their specific receptors, such as flt-1 and flk-1, which play a role in vasculo- and angiogenesis. Using gene targeting and gene transfer the role of these genes in the early differentiation of endothelial cells and the formation of blood vessels has been characterized (for review see Risau, FASEB J. 9 (10):926-33, 1995). In contrast, surprisingly little is known about the molecular mechanisms of the later differentiation of endothelial cells in specific vascular beds. Endothelial cells display a remarkable heterogeneity in different organs (Garlandi and Dejana, Arterioscler Thromb Vasc Biol 17:1193-1202, 1997). Even in the same organ, the endothelium of large and small vessels, veins and arteries exhibit significant differences. Presently, microvascular endothelial cells are grouped into different phenotypes on a morphological basis. Continuous, fenestrated and discontinuous endothelium has been distinguished (Bennet et al., Am J Physiol. 196:381-390, 1959) . These morphological differences correlate with differences in permeability and adhesion. Continuous endothelium is mostly present in the brain capillaries (Kniesel et al., Brain Res Dev Brain Res 96 (1-2):229-40, 1996). The heterogeneity of the endothelial cells is not understood at the molecular level. The principal problems are the lack of organ-specific markers and imperfect endothelial cell preparations for in vitro analysis. We wanted to investigate the molecular mechanisms which lead to the generation of fenestrated endothelial cells.

Fenestrated endothelia are found in endocrine organs, the gastrointestinal tract , kidney glomeruli and capillaries, and specific regions of the brain, such as the choroid plexus and the circumventricular organs. Fenestrated endothelium possesses fenestrae i. e. specialized plasma membrane microdomains of approx. 60 nm in diameter (Esser et al. J Cell Biol. 140 (4):947-

59, 1998). Interestingly, fenestrated endothelial cells are present in many tumors (Roberts and Palade, Cancer Res. 57 (4):765-72, 1997). Several compounds which induce endothelial fenestrations in vitro have been previously described. These include retinoic acid, TGF-ß, the tumor promoter PMA, and extracellular matrix proteins (Lombardi et al., J Cell Biol. 102 (5):1965-70, 1986). However, the relevance of these factors for in vivo differentiation of the endothelium is not known. Recently, the endothelial cell growth factor VEGF has been demonstrated to induce fenestrations in vitro (Esser et al. J Cell Biol. 140 (4):947-59, 1998). Since VEGF is also persistently expressed in areas with fenestrated endothelial cells e.g. glomeruli, choroid plexus, and the corresponding VEGF-receptors 1 and 2 have been described in these locations, it seems reasonable to assume that the VEGF/VEGF-R plays a role in the differentiation of the fenestrated endothelium (Simon et al. J Am Soc Nephrol. 9 (6):1032-44, 1998). The effect of VEGF is possibly mediated by protein kinase C since we have recently shown that PKC isoforms play an important role in the proliferative effects of VEGF (Wellner et al., Arterioscler Thromb Vasc Biol 1999, in press). The experiments using phorbol-ester to induce fenestrated endothelium also suggest that protein kinase C plays a role in the generation of the fenestrated endothelium.

We have therefore tested the hypothesis that the expression of the VEGF receptors 1 and 2 are up-regulated in fenestrated endothelium and that this effect is mediated by specific protein kinase C isoforms. Induction of endothelial cell differentiation was achieved by exposing cultured endothelial cells (human umbilical vein endothelial cells HUVEC) to phorbol ester PMA (100 nM) for 24, 48 and 72 hr. Occurence of the fenestrated phenotype was analysed using electron microscopy and counting of fenestrae per cell. Secondly, western blot analysis for the expression of a previously described protein ESM-1 which is only expressed in fenestrated endothelium was measured (Wellner et al. JASN 1999, in press). VEGF receptor expression was measured by quantitative RT-PCR using taqman technology (Perkin Elmer, CA, USA) and western blot analysis. In addition, protein expression was assessed by immunocytochemistry. For selective inhibition of protein kinase C isoforms antisense oligonucleotides (ODN) and their respective sense and scrambled controls were used as previously described (Haller et al. Circ Res 1998; 82: 157-165).

Exposure of cultured human umbilical endothelial cells to phorbol ester led to an increase of fenestrae from 0.01±0.003 to 0.8±0.004 fenestrae/cell and an increased expression of ESM-1 (18 fold). The ESM-1 expression was stimulated after 24 hr of phorbol ester and remained upregulated for at least 72 hr. Concomitantly with ESM-1 the expression of both VEGF receptors was increased. VEGFR-1 (flk-1) increased by 260 % ($n=4$, $p<0.05$) and VEGFR-2 (flt-1) increased by 480 % ($n=4$, $p<0.05$). Immunocytochemistry revealed that both proteins were increased at the cell membrane and in the perinuclear area. RT-PCR for VEGFR-1 showed an 18-fold increase in m-RNA levels, while VEGFR-2 was increased 100-fold. No increase in VEGF protein levels was observed. We then investigated the role of PKC isoforms α, ε, and ζ in the induction of the fenestrated phenotype. Endothelial cells were incubated with the respective antisense ODN for 24 hr. Antisense ODN led to a significant downregulation of the respective PKC isoforms as has been described previously (Haller et al. Kidney Int 1998; 53: 1550-1558). We then analyzed the effects of PKC isoform inhibition on the expression of VEGF receptors. Inhibition of PKC α and ζ but not of PKC ε led to a significant down-regulation of the phorbol ester induced expression of both the VEGF receptor-1 (140 % vs 260 % , $n=4$, $p<0.05$) and VEGF receptor-2 (170 % vs 480 % , $n=4$, $p<0.05$). Using immunocytochemistrty we observed a decrease of both membrane-associated and cytosolic VEGF receptor proteins.

These results suggest that specific PKC isoforms play an important role in the induction of fenestrated endothelium. Kaya et al. recently provided further evidence for a role of PKC in the formation of fenestrated endothelial cells. They infused PMA continuously by an osmotic pump into the cerebral cortex of rats and induced around 30 % fenestrated endothelial cells. Fenestra development depended on concentration and time. Fenestra formation was reversible after 1-2 months . These findings support a physiological role of PKC in endothelial cell differentiation.

A variety of reports over the last ten years have provided ample evidence that PKC plays an important role in cell differentiation. Early on studies using phorbol ester for PKC activation have shown that differentiation of hematopoietic cells is influenced by PKC (Kaya et al., Exp Neurol 142: 6-13, 1996). Similar studies have been carried out in adipocytes, neuronal cells, smooth muscle cells etc. In addition, it has been shown that PKC expression

correlates with the degreee of cell differentiation i. e. PKC activity is highest in differentiated tissue as compared to dedifferentiated or rapidly growing tissue (Haller et al. Circ Res 76: 21-29, 1995).
PKC consists of a family of 12 isoforms which are expressed on different genes and have different functions (Haller et al, Ann NY Acad Sci 1994; 733: 313-24). Recently the role of the different PKC isoforms in cell differentiation has been investigated. Since the PKC isoforms are differentially expressed in different tissues the role of the PKC isoform in the differentiation process seems to be dependent on the cell type. PKC delta has been implicated in the differentiation of HL-60 cells. In neuronal cells both PKC ε and ζ have been associated with the differentiation process. We and others have previously shown that PKC α induces differentiation in vascular smooth muscle cells (Haller et al. Circ Res 76: 21-29, 1995). An important role for PKC α in cell differentiation and growth arrest has also been described by others (Cho et al., J Cell Physiol 172: 306-13, 1997). However, important questions regarding the exact role of PKC in cell differentiation are yet unsolved. Many substrates of PKC isoforms during cell differentiation have not been identified so far. Furthermore, it is not clear whether the effect of PKC is direct or whether other differentiation factors such as TGF-ß or urokinase are induced by PKC and in turn influence cell differentiation.

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