The protein C system in inflammation and cancer: a critical role for thrombomodulin

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The protein C system comprises the cellular receptors thrombomodulin (TM) and endothelial protein C receptor (EPCR), and the soluble proteins C and S. Thrombin binds to TM and this complex jointly with protein C bound to EPCR generates activated protein C (APC). One of the main functions of APC in blood coagulation is to regulate the rate of thrombin generation, in complex with protein S, by inhibiting factors Va and VIIIa through limited proteolytic cleavage.[1] Defects in one of the factor V cleavage sites for APC, e.g. the factor V Leiden variant, renders this protein partially resistant to cleavage by APC and the clinical result is an increased risk of venous thrombosis. Similarly, heterozygous deficiencies in PC or PS cause a marked increase in the risk of venous thrombosis. Complete absence of these proteins is hardly compatible with life due to massive disseminated intravascular coagulation.

In contrast, the evidence that defects at the level of TM or EPCR relate to (venous) thrombosis is limited. In fact, most evidence for a causal role of TM as anticoagulant protein comes from animal studies showing that mice with a selective defect in the TM gene (TM Pro) or a deletion in the TM gene in an endothelial cell specific manner renders these mice susceptible to venous as well as arterial thrombosis.[2] One reason for the lack of clinical evidence for a contribution to thrombosis may be that intact TM and EPCR are quite essential to health, not only because a protective activity against thrombosis, but possibly also because of a number of functions not related to thrombosis, e.g. in protection against excess inflammation and other biological processes.

APC (and its zymogen PC) has gained interest as an anti-inflammatory molecule since it was shown to protect baboons against death from sepsis. Recombinant human APC reduced mortality due to sepsis and it is believed that the combined anticoagulant and anti-inflammatory properties of APC explain this clinical benefit.[3] APC has been shown to induce several cellular effects related to inhibition of neutrophil adhesion and migration in inflammatory tissues, inhibition of cytokine production etc., part of which may be due to inhibition of NFkappaB induced transcription of pro-inflammatory and apoptosis genes.[4,5] In contrast, pro-inflammatory effects of APC have also been observed in cultured endothelial cells and the dominant actions of APC may therefore be prominently dependent on time, place and concentrations.

In the remainder of this presentation however, we will focus on the eminent role that TM appears to play in the cross roads between coagulation and inflammation on the one hand, and as a regulator of cell proliferation on the other hand.

Thrombomodulin and inflammation

Thrombomodulin is a glycosilated 557 aminoacid transmembrane receptor molecule that is constitutively expressed by vascular endothelial cells and can be induced by other cells including smooth muscle cells, keratinocytes and a large number of malignant cells.

Its structure and functions have been recently characterized.[2] The extracellular chain contains 6 EGF domains, of which 1-6 stimulate fibroblast growth, 3-6 are needed for activation of thrombin activatable fibrinolytic inhibitor (TAFI), 4-6 are involved in the binding of thrombin and the activation of PC. The N terminal part of the extracellular domain contains a lectin-like domain that appears to express functional properties in relation to inflammation. First, deletion of the lectin-like domain generated mice that were more susceptible to sepsis due to an increased adhesion of neutrophils and enhanced activity of MAP kinase pathways, leading to greater mortality in a sepsis model.[6] Second, the same domain binds high-mobility group-B1 protein (HMBG), a pro-inflammatory protein released by necrotic cells and acting as a pro-inflammatory cyto-

kine.[7] The net result is that the lectin-like domain, also as a soluble molecule, carries strong anti-inflammatory properties, with potential therapeutic benefit. The lectin domain is indeed more important in this regard than the thrombin binding moiety, because the TM Pro mutant mouse does not show a markedly different response to LPS stimulation (Franco, Reitsma, ten Cate, unpublished; although it has been reported to have a shortened survival in a terminal LPS model,[8] and its inflammatory defense against localized pulmonary inflammation is also unaltered as compared to wildtype mice.[9] Thus, it remains to be demonstrated whether the anti-inflammatory effect of TM is in part related to the scavenging of thrombin, preventing it from activating protease activated receptor-1 (PAR-1). A distinct anti-inflammatory property may be that the thrombin-TM complex activates TAFI, which is a potent inhibitor of activated complement anaphylatoxins.[1]

Due to the combination of anticoagulant (by its cofactor role in generating APC) and anti-inflammatory effects, the normal expression of functional TM is of importance in the defense against sepsis and thrombosis. The expression of TM is controlled by several factors: negative effects are inflicted by pro-inflammatory molecules including Il-1, TNFα, LPS and phorbolesters, in *in vitro* experiments. Such negative regulatory effects may be responsible for the observed decreased immunohistochemical staining of TM (and EPCR) in the subdermal microvasculature in patients with meningococcal septicemia.[10] In addition, increased shedding of TM from the endothelial surface when clipped by proteolytic enzymes such as elastase may also contribute to decreased immunostaining of TM and increased plasma concentrations of soluble TM in patients with inflammation and atherosclerosis.[2]

The expression of TM can also be positively influenced by diverse agents like statins, pentoxifylline and retinoic acid (see further). Both negative and positive regulatory mechanisms have however not yet been confirmed *in vivo*.

TM and cancer

Since its discovery TM has been investigated in relation to several different tumors in numerous papers (partially reviewed in 2). The initial assumption that TM was a specific endothelial cell protein and marker made it an interesting molecule for characterizing vascularization in specific tumors. In addition, it was established in several studies that several human tumor cells as well as cell lines expressed TM in rather high concentrations. Remarkably, epithelial cells from oesophagus, lung or breast tumor origin that normally are negative for TM stained positive and more importantly, the concentration of TM was positively correlated with cell differentiation, reduced infiltration and metastasis and a good clinical prognosis in tumors from different origin. As such TM has emerged as a powerful marker of tumor malignancy, in which more TM heralds a better prognosis.[11-13]

The mechanisms behind these observations are still uncertain. Given its structural similarity with endosialine, a tumor endothelial cell protein linked to cell proliferation, it is tempting to speculate that TM negatively controls cell proliferation *in vivo*. This action may be independent of its thrombin mediated anticoagulant function, but appears to require both the transmembrane and cytosolic domains of TM. Also the lectin like domain may be involved in regulation of tumor growth.[2]

As specific protective properties of TM in relation to cancer the following interactions may be relevant:[11]

1. TM may, by reducing fibrin formation in the circulation, limit the attachment of tumor cells to the endothelium, which is partially fibrin dependent;
2. deletion of the TM gene leads to embryonic death related to retardation in development which may be due to loss of cell differentiation; in tumors loss of differentiation due to reduced TM may cause more aggressive tumor progression;
3. while thrombin stimulates tumor progression by several different routes, TM scavenges thrombin and diminishes this effect;
4. TM directly inhibits cell proliferation in malignant melanoma;
5. TM-thrombin complex inactivates receptor bound pro-uPA and activates TAFI: both anti-fibrinolytic actions may reduce tumor infiltration.

An opposite effect may be that TM mediates thrombin induced activation of progelatinase A (MMP-2) in an APC dependent way, which could cause extracellular matrix degradation and tumor progression.[14] Tumorogenesis may be favored by reduced expression of TM; such may occur due to prolonged inflammatory exposure, infection by HSV[15] and may be other viruses and increased methylation of the TM gene, as noted in gastric cancer cells.[16]

The aforementioned observations suggest that pharmacological stimulation of TM gene expression may be an interesting target for controlling cancers of different origin; retinoic acid is one such example.[17]

Functional analysis of anticoagulant activity in tissues as function of TM

Using functional assays for tissue factor/factor VIIa and the Calibrated Automated Thrombin (CAT) generation assay with a fluorogenic substrate we analyze various tissues for pro- and anticoagulant properties.

We are particularly interested in determining functional heterogeneity in various organs under conditions of systemic inflammation. Under physiological conditions several tissues including heart and lungs have detectable levels of tissue factor activity, but the most prominent activity is an inhibition of thrombin generation activity. The latter appears to be fully TM dependent, because homogenates of mice with a functional TM defect (TM Pro) have a completely restored thrombin generation pattern. Murine lungs show the most profound anticoagulant activity, while brain homogenates lack TM activity.

Currently, we are investigating the pro- and anticoagulant effects of mouse organs after challenge with LPS. These data may provide a functional analysis of heterogeneity as well as functional regulation under different pathophysiological conditions.

Conclusions

The protein C system is not only a crucial anticoagulant pathway in humans, but may also be a highly efficient system for controlling inflammation as well as cell proliferation and differentiation networks. The abundant expression of TM in various tumor cells suggests a specific role for this molecule in cancer. Application of soluble lectin domain peptides or agents that regulate TM gene expression may become therapeutic tools to limit inflammation and tumor related mortality.

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