Activation mechanisms in vascular disease

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Chapter 7

General Discussion
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In this Chapter, I will elaborate on a number of issues that are derived from the studies described in this thesis, which have not yet been extensively discussed previously.

Serial analysis of gene expression (SAGE)
The "surfacing" of most human genes through the Human Genome Project has resolved some of the blank spots in our human endothelial cell (EC) SAGE data (Chapter 3). Genes, that were linked to an expressed sequence tag (EST), are now annotated and many tags that did not match any data in the databases now belong to an EST or are also annotated. Of note, a number of tags still remain unknown. Interestingly, some of these tags are regulated in a striking manner. For example, the corresponding mRNA of tag #30 is down-regulated in ECs upon stimulation with an atherogenic stimulus (see Table 1 of Chapter 3). In the combined SAGE databases from the National Center for Biotechnology Information (NCBI), tag #30 is found in only 7 out of the 100 deposited other SAGE libraries. Significantly, one of the tag #30-containing libraries is derived from ECs. In this EC-library, the tag #30 transcript is 4-fold up-regulated upon stimulation with vascular endothelial growth factor (VEGF), an angiogenic stimulus. Because of the diverse regulatory mechanism of this gene, it is not merely a marker for endothelial activation. The identification of the gene and gene product, corresponding to tag #30, will conceivably constitute an interesting future research project.

Tag #3 (Table 2) serves as an example of an EST clone that is now linked to an annotated gene, denoted as cardiac ankyrin repeat protein (CARP). This tag was also found among the three most extensively up-regulated genes in the human smooth muscle cell (SMC) SAGE data (Chapter 2), upon stimulation with the same atherosclerotic stimulus. A possible function for CARP in the vessel wall is pursued in Chapter 4. A number of the differentially expressed genes in both the SMC and EC SAGE databases were identical. Among these genes were interleukin 8 (IL-8), monocyte chemotactic protein 1 (MCP-1), gro-α, CARP, activin βA and plasminogen activator inhibitor 1 (PAI-1). It should be noted that the first three proteins are chemotactic proteins of which IL-8 and gro-α are known to attract neutrophils, while MCP-1 attracts monocytes. Thus, although monocytes/macrophages are the main inflammatory cells present in atherosclerotic lesions, apparently the chemotactic requirements are also provided to recruit neutrophils to the vessel wall. CARP, activin βA and PAI-1 are all potential inhibitors of atherosclerosis (Chapter 4 and 5) 1. These proteins were found to be expressed when the cells were activated in vitro by a strong atherosclerotic stimulus, namely the conditioned medium of oxidized low-density lipoprotein (ox-LDL) -stimulated monocytes. Apparently, apart from becoming activated, these cells also produce inhibitors of activation, presumably as a feedback mechanism to control the activation process. We established a
relationship between activin A and CARP in inhibition of SMC proliferation (Chapter 4). Collectively, it is concluded that stimulation of SMCs by activin A induces CARP mRNA expression. Since both proteins inhibit SMC proliferation, CARP is considered a downstream transcription factor-related protein of activin A. PAI-1 would inhibit SMC proliferation through another pathway, notably as an inhibitor of the SMC mitogen thrombin. Evidence for this mechanism is reported in Chapter 5.

Now the human genome project has elucidated most of the putative human genes it is likely that in the near future other techniques, particularly DNA-microarray, will be the preferred technique to study gene expression patterns in diseases. Theoretically, a DNA microarray includes spots of single-stranded DNA corresponding to all putative human genes. The mRNA expression pattern of all these genes can be observed in a single hybridization experiment. When multiple experiments are performed, using various cell types or tissues derived from different models of disease, computational clustering analysis may reveal genes that are associated with a certain type of disease, manifest in a particular cell type or tissue. In the future, these gene expression profiles may possibly lead to specialized DNA-microarrays for various disorders.

**Thrombin-mediated intima formation**

PAI-1 is best known for its inhibitory role in fibrinolysis. In addition, we have shown that in the presence of cofactors heparin or vitronectin (VN), PAI-1 can inhibit thrombin. This observation suggests that PAI-1 would also play a role in coagulation. Still, the latter option is probably irrelevant, since thrombin is inhibited more efficiently by antithrombin III (ATIII), the native thrombin inhibitor in blood. It is thought that inhibition of thrombin by PAI-1/VN complexes might be relevant in the vessel wall, where ATIII is virtually absent. We have demonstrated previously that PAI-1, VN and prothrombin co-localize in the vessel wall in human atherosclerotic plaques and that active thrombin is present in these lesions. Thrombin is a known mitogen for SMCs and PAI-1 is a candidate inhibitor of thrombin-mediated proliferation in atherosclerosis. Abundant prothrombin and VN in the vessel wall are presumably derived from plasma. In Chapter 5, we show that PAI-1 and VN are involved in inhibition of SMC proliferation, since the PAI-1 and VN-deficient mice demonstrate increased intima formation. The fact that this excessive SMC proliferation is thrombin dependent is demonstrated by using a specific thrombin inhibitor, hirudin, to reduce intima formation in PAI-1-deficient mice. Since a number of studies have demonstrated that fibrin is associated with SMC proliferation, inhibition of thrombin could also influence intima formation via this pathway. In contrast to our results in Chapter 5, PAI-1 is implicated in increased intima formation in these models by its anti-fibrinolytic function. Even though we hardly observe thrombi (fibrin) in the ligated carotids after one week, the ultimate proof for a thrombin-PAI-1 interaction would be to detect cleaved PAI-1 in the intima. In addition, generation of cleaved PAI-1 should be prevented upon
administration of hirudin. The rationale for this line of research is based on the following observations. PAI-1 is a serine proteinase inhibitor (serpin) that inhibits the serine proteinase thrombin via the so-called "branched-suicide-substate" mechanism\(^6\). According to this scheme of interaction, two products are feasible. First, thrombin can cleave PAI-1 between the P1 and P1' in the reactive center loop, resulting in inactive PAI-1 while thrombin is recovered as an active mitogen. Second, PAI-1 can inhibit thrombin in the presence of cofactor VN and form a ternary complex, hereby inactivating thrombin. Both reactions take place and the ratio is approximately seven cleaved PAI-1 molecules for one inhibited thrombin molecule, respectively. Therefore, the presence of cleaved PAI-1 in the vessel wall would further indicate that thrombin and PAI-1 interact. Inhibition of PAI-1 cleavage by hirudin would assign thrombin as the responsible serine proteinase. Cleaved PAI-1 may be revealed by a combination of an immunohistochemical and morphometric approach, to quantify the cleaved-PAI-1-positive area. Antibodies should be generated against the "free" P1-P12 or P1'-P14' peptides (which do not bind intact PAI-1), using the same peptide-based immunization strategy as has been employed to obtain antibodies against murine TF. This approach will be pursued in future studies.

**Thrombin-mediated coagulation**

In sepsis, the bacterial toxin lipopolisaccharide (LPS) causes acute inflammation and eventually coagulation in the microvasculature of numerous organs. Tissue factor (TF) is the main initiator of thrombin-mediated coagulation in this disease. It is known that TF is induced by LPS in various cell types during sepsis. In addition, components of the fibrinolytic system are modulated during sepsis as well. Specifically, urokinase-type plasminogen activator (u-PA) synthesis is decreased in the kidney upon LPS activation in mice\(^16\), while PAI-1 protein is increased in ECs of different vascular beds including the kidney (data not shown).

A critical role is established for activated monocytes during sepsis. The involvement of TF-positive monocytes has long been described to contribute to coagulation in this disease. Yet, the monocytes are outnumbered by the granulocytes in some of the affected organs during the development of the inflammatory response in sepsis. In addition, the observations of TF-positive granulocytes would suggest that granulocytes could also play an essential role in coagulation in this disease. Several researchers have shown TF-positive granulocytes in various sepsis models\(^17-21\). However, controversial data has been reported on the issue whether granulocytes are capable to synthesize TF and attribute to the increased coagulation of the microvasculature. We have generated polyclonal antiseras directed against five different peptide sequences of the extracellular murine TF protein. One of these peptide-derived antiseras is applicable in immunohistochemical experiments. It revealed TF in normal tissues, such as brain, trachea and the kidney and after exposure to LPS in the spleen, lung and liver (Chapter 6). In our experiments, the increase in TF mRNA in the
interfollicular space of the spleen coincided with the increase in TF protein-positive granulocytes in the same area. Therefore, we suggest that TF synthesis might only take place in the spleen after LPS administration. The observation of TF-positive granulocytes in our murine sepsis model did not stand alone, since we later discovered that one of the remaining four peptide-derived antisera only recognized TF-positive granulocytes upon LPS stimulation (data not shown). This antiserum was directed against amino acid (aa) sequence V1QQFEDGRKLNV (aa 144-157) of the murine TF protein. No TF immunostaining was observed in the normal control tissues with this antiserum. This may indicate that TF has a different conformation when exposed on the surface of granulocytes as compared to other TF expressing cell types. Moreover, two of the antisera that did not show immunoreactivity in any of the tissues were directed against aa sequences (aa 56-74 and aa 188-204) that contain residues involved in binding and activation of coagulation factors VII or X, respectively. These sites are possibly not exposed in encrypted TF and occupied by the coagulation factors upon activation of the extrinsic coagulation cascade. The controversy on TF synthesis and/or exposure on the surface of granulocytes is mostly based on the fact that researchers have not been able to induce TF mRNA or protein in in vitro experiments with this cell type, while monocytes have been shown to be inducible. Possibly, the spleen is the only environment for granulocytes, which become activated, to produce TF, a setting that can not be easily mimicked in vitro. Along this line, it has been shown that induction of TF in monocytes can be mediated by contact with lymphocytes. A similar mechanism may possibly apply for granulocytes and would be interesting to pursue.
General Discussion

References


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