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Interaction of vascular endothelial cells with leukocytes, platelets and cancer cells in inflammation, thrombosis and cancer growth and metastasis

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Adhesion and migration of mammalian cells are of crucial importance in a number of biological events, such as fertilization, embryogenesis, pattern, tissue and organ formation, and in a variety of physiological and pathological processes, including lymphocyte trafficking, leukocyte recruitment, hemostasis, wound healing, tumor angiogenesis and cancer metastasis. All these cellular interactions are precisely regulated by temporal and spatial presentation of various cell adhesion molecules and chemotactical molecules displaying appropriate specificity and affinity for proper development and functioning of the organism.

My research is focused on the structures and functions of vascular endothelial cells. I am especially interested in the biological roles of endothelial cells in mediating adhesion and migration of leukocytes, platelets and cancer cells as well as their involvements in inflammation, thrombosis, cancer growth and metastasis. They include the following aspects:

LEUKOCYTE-ENDOTHELIAL CELL INTERACTION

The leukocyte-endothelial cell interactions are mediated by at least four families of cell adhesion molecules. They are selectins (CD62), selectin ligands, integrins and IgG superfamily of cell adhesion molecules. The selectin family of cell adhesion molecules interacts with their cognate ligands; and these interactions are generally believed to mediate initial attachment, rolling and weak adhesion of leukocytes on the activated endothelial cells. The integrin family of cell adhesion molecules interacts with the cell adhesion molecules of immunoglobulin superfamily; and these interactions are mainly responsible for firm adhesion and signal transduction, which can then trigger diapedesis of leukocytes for transendothelial migration. Eventually, the emigrated leukocytes are guided by increasing concentrations of various soluble chemoattractants to move to their destinations, such as the site of infection or tissue injury. It is generally believed that the selectin mediated cellular interactions are the first step in the multi-step paradigm of leukocyte recruitment.

P-selectin (CD62P) is a cell surface adhesion molecule synthesized and stored in the Weibel-Palade bodies of endothelial cells and the α-granules of platelets. Upon inflammatory and thrombogenic challenges, P-selectin is rapidly expressed on stimulated endothelial cells and activated platelets by exocytosis. The cell surface P-selectin interacts with specific glycoprotein ligands on human leukocytes, platelets and certain cancer cells and these cellular adhesive interactions are thought to play important roles in inflammation, thrombosis and metastasis.

During my postdoctoral training in Dr Rodger P McEVER's lab, we discovered that P-selectin was a cell adhesion molecule for human neutrophils (Geng *et al*, Nature 1990, 343: 757). We successfully generated monoclonal antibodies to P-selectin (G1 mAb) which binds to the lectin domain of P-selectin and neutralizes leukocyte adhesion mediated by P-selectin. This blocking antibody is now widely used by researchers work-

ing in this field as a bench-marker for abrogation of leukocyte adhesion mediated by P-selectin.

After identification of the biological function of P-selectin, I went to study the structural and functional relationship of this cell adhesion molecule. We discovered that P-selectin had high affinity binding sites for Ca^{2+} and that the binding of Ca^{2+} can induce a conformational change in the lectin domain of P-selectin, which is required for the functional activity of P-selectin (Geng, *et al*, J Biol Chem 1991; 266: 22313). We also found that three separate peptide sequences from the lectin domain of P-selectin may directly mediate leukocyte adhesion (Geng, *et al*, J Biol Chem 1992; 267: 27739).

Using P- and E-selectin affinity chromatography and monoclonal antibody approach, we showed that a sialoglycoprotein, called PSGL-1 (P-selectin glycoprotein ligand-1; CD162) functioned as an important human leukocyte ligand for P-selectin (Ma, *et al*, J Biol Chem 1994; 269: 27739) and E-selectin (Asa, *et al*, J Biol Chem 1995; 270: 11662). It is now generally recognized that PSGL-1 functions as a high affinity ligand for all three selectins.

In collaboration with Dr Åke P ELHAMMER, we successfully sequenced all identifiable *O*-linked carbohydrates released from PSGL-1 isolated from the human myelocytic cell line HL-60 (Aeed, *et al*, Glycoconjugates J 1998; 15: 975). The sequence consenses between the *O*-linked glycans on PSGL-1 and on proteins from porcine *zona pellucida* of oocytes further lead us to the discovery of the expression of PSGL-1 in *zona pellucida* of porcine oocytes and P-selectin on the acrosomal membrane of sperm cells. These findings implicate that these molecules may be involved in porcine sperm-egg binding during fertilization (Geng, *et al*, J Cell Biol 1997; 137: 743).

E-selectin (CD62E), a cell adhesion molecule for most leukocytes, is known to be expressed exclusively on the cytokine stimulated endothelial cells mainly by inductive activation of NF-κB. Using immunohistochemistry and *in situ* hybridization, we showed that B lymphocytes and plasma cells in the spleens and lymph nodes from nude mice (T lymphocytes deficient), but not from SCID mice (T and B lymphocytes deficient), expressed E-selectin prior to cytokine stimulation. The expression of E-selectin was also confirmed on human B lymphocytes isolated from peripheral bloods. The mouse J774A.1 monocytes could adhere to the marginal zones of mouse spleens in an E-selectin Ab inhibitable manner, suggesting the functional activity of the expressed E-selectin. In addition, ARH-77 cells, a cell line derived from human plasma cells, were found to express E-selectin mRNA and protein and to have a NF-κB activity for an E-selectin promoter. NF-κB antagonists, such as TPCK (tosylsulfonyl phenylalanyl chloromethyl ketone), dexamethasone and a IκBα mutant plasmid could inhibit both the NF-κB activity and the expression of E-selectin. Transfection with an E-selectin promoter-driven reporter gene construct further verified the E-selectin promoter activity in ARH-77 cells. Again, TPCK, dexamethasone and the IκBα mutant plasmid could neutralize this activity. These findings suggest that B lymphocytes and plasma cells can express E-selectin, which is functional for monocytic leukocytes, by a mechanism of constitutive activation of NF-κB (Liu *et al*, Biophys Biochem Res Commun 2001; 286: 281; Xia *et al*, Biochem Biophys Res Commun 2001; 289: 851).

NF-κB and AP-1 are ubiquitous transcription factors and pleiotropic regulators of many genes involved in inflammation and oncogenesis. Act1 (also called CIKS) is a recently identified molecule, which activates NF-κB and AP-1. We identified alternatively spliced Act1 that lacked the exon 2 encoding the first nine amino acids in the amino terminus of the protein. Compared to full-length Act1, this truncated Act1 appeared to be equally active, as demonstrated by the luciferase gene reporter assay using E-selectin (a marker for NF-κB activation) and AP-1 promoters and by the gel-shift assay using NF-κB and AP-1 binding oligonucleotides. We further demonstrated that only the spliced Act1 was detected in cDNA libraries derived from human fetal brain, liver, leukocytes and bone marrow. In contrast, both the spliced and full-length Act1 templates were detected in a variety of human cancer cell lines. The expression of both the spliced and full-length transcripts was detected at 4-h time point following treatment of endothelial cells with tumor necrosis factor-α, interleukin-1β or bacterial endotoxin. Notably, the dominant amounts of the spliced Act1 over the full-length Act1 were amplified from both the cancer cell mRNAs and the stimulated endothelial cell mRNAs. Considering the *act1* chromosome localization at the 6q21 subregion, our findings indicate that the newly identified alternatively spliced Act1 is a major transcript of the molecule and that Act1 may play important roles in oncogenesis (Xia, *et al*, Biochem Biophys Res Comm 2002; 296: 406).

Heparin, a highly sulfated proteoglycan, is known

to have strong anticoagulant and anti-inflammatory activities. We have generated a discrete set of the chemically modified heparin derivatives and found that an *N*-desulfated heparin had 188-fold (compared to heparin) and 32-fold (compared to low molecular weight heparin; LMWH) reductions of anticoagulant activities as determined by measurements of activated partial thromboplastin time. The *N*-desulfated heparin inhibited adhesion of human promyeloid HL-60 cells to the stimulated human umbilical vein endothelial cells (HUVECs) under a physiological shear stress. It also prevented the transmigration of human neutrophils through the monolayers of the stimulated HUVECs. Further, intravenous administration of this compound attenuated the peritoneal infiltration of neutrophils in a mouse model of acute peritonitis, and reduced tissue edema and leukocyte deposition in a rabbit ear model of ischemia and reperfusion injury. It is to our best knowledge that the *N*-desulfated heparin has the lowest anticoagulant activity among LMWH and chemically modified heparin derivatives, while preserving a potent anti-inflammatory action. These combined properties appear to suggest it as a safer choice for treatment of inflammation (Geng & Wang, PCT/CN01/00922; Wang, *et al*, Inflammation Res 2002; 51: 435; Zhou, *et al,* World J Gastroenterol 2002; 8: 897).

PLATELET-ENDOTHELIAL CELL INTERACTION

P-selectin is stored in the α-granules of platelets and the Weible-Palade bodies of endothelial cells. Upon thrombogenic and inflammatory challenges, it is rapidly expressed, by exocytosis, on the cell surface of the activated platelets and the stimulated endothelial cells. Thus, P-selectin has been considered as a key linker molecule between thrombosis and inflammation. Using P-selectin knockout mice, it has been clearly shown that platelet P-selectin play indispensable roles in hemostasis and thrombosis.

Currently, little is known for the molecular mechanisms governing the regulation of the rapid mobilization of P-selectin in these cells. We found that phenylarsine oxide (PAO) and diamide (both were inhibitors for protein tyrosine phosphatases), but not genistein (an inhibitor for protein tyrosine kinases), adenosine, wortmannin and LY294002 (all were inhibitors for phosphatidylinositol 3- and 4-kinases), could inhibit P-selectin exocytosis on activated platelets and could abolish the P-selectin mediated aggregation of activated platelets to neutrophils. However, PAO did not attenuate the P-selectin mediated adhesion of human promyeloid HL-60 cells on the stimulated endothelial cells under flow conditions. Further, PAO had no detectable effects on the exocytosis of P-selectin in the stimulated endothelial cells. These results indicate that protein tyrosine phosphatases are necessary for P-selectin exocytosis on the activated platelets, but not on the stimulated endothelial cells, and suggest that inhibitors for protein tyrosine phosphatases may be potentially valuable for treatment of platelet/leukocyte aggregation (Chen & Geng, Biochem Biophys Res Commun 2001; 286: 609).

Resting and activated platelets have been shown to roll on endothelial P-selectin, indicating that platelets express (a) ligand (s) for P-selectin. We show that P-selectin specifically precipitated one 28-kDa glycoprotein from the whole lysates of human platelets in a Ca^{2+}-dependent manner. Further, the purified 28-kDa molecule could inhibit the binding of P-selectin to human resting and activated platelets. In contrast, KPL1 (a leukocyte adhesion blocking monoclonal antibody to PSGL-1) did not neutralize the binding of P-selectin to human platelets, even though it abolished the binding of P-selectin to human promyeloid HL-60 cells. Our results thus indicate that this newly identified 28-kDa glycoprotein is an important platelet ligand for P-selectin (Li, *et al,* Thromb & Haemost 2002; 87: 706). Currently, we have obtained its molecular identity using mass spectroscopy and are working on its functional roles in the homotypic aggregations of platelets and heterotypic aggregation of platelets with leukocytes or endothelial cells.

CANCER CELL-ENDOTHELIAL CELL/PLATELET INTERACTIONS

P-selectin reacts with a variety of human cancers and human cancer derived cell lines and mediates the interactions of the stimulated endothelial cells and the activated platelets with cancer cells. Using P-selectin knockout mice, it has been demonstrated that P-selectin deficiency significantly attenuates the growth and metastasis of human colon carcinoma. In search for P-selectin ligands on human cancer cells, we were successful in isolation and characterization of the glycoprotein ligands from NKI-4 cells, a cell line derived from a human malignant melanoma (Kaytes & Geng, Biochemistry 1998, 37: 10514) and NCI-H345 cells, a cell

line derived from a human small cell lung cancer (Li, *et al*, Biochem Biophys Res Commun 2001; 288: 637). We are now working on the protein sequencing for molecular cloning and the preparation of adhesion blocking monoclonal antibodies to these molecules.

Structurally, the extracellular portion of P-selectin has an NH2-terminal domain of ~120 residues homologous to the Ca^{2+}-dependent, carbohydrate-recognition domain (C-type lectin). Functionally, the binding of P-selectin to leukocytes requires sialylated and fucosylated carbohydrate structures (a lectin-carbohydrate pair); the prototype of them is Siaa2-3Galb1-4 (Fuca 1-3) GlcNAc, called sialyl Lewisx (SLex). However, these conclusions are mainly reached by extensive studies of leukocytes and high endothelial venules of lymph nodes. The carbohydrate moieties of P-selectin ligand on human cancer cells remain undetermined. We recently show that heparan sulfate-like proteoglycans can mediate adhesion of malignant melanoma A375 cells, a cell line of human non-blood borne, epithelial-like cancer cells, to P-selectin under flow conditions. This finding indicates that heparan sulfate-like proteoglycans on cancer cells can function as cell surface ligands for P-selectin (Ma & Geng, J Immunol 2000; 165: 558).

The interaction of P-selectin with PSGL-1 is known to require tyrosine sulfation. However, it is unknown whether sulfation is necessary for P-selectin binding to somatic cancer cells. We showed that P-selectin mediated adhesion of Acc-M cells, a cell line derived from a human adenoid cystic carcinoma of salivary gland. These cells had a moderate expression of heparan sulfate-like proteoglycans, but had no detectable expressions of PSGL-1, CD24, Lewisx and sialyl Lewisx. Treatment with sodium chlorate (a sulfation biosynthesis inhibitor), but not β-*D*-xyloside (a proteoglycan biosynthesis inhibitor) or heparinases, reduced adhesion of these cells to P-selectin. Sodium chlorate also inhibited the P-selectin precipitation of the ~160-kDa, ~54-kDa and ~34-kDa molecules from the cell surface of Acc-M cells. Further, P-selectin could bind to human breast carcinoma ZR-75-30 cells in a sulfation dependent manner. Our results thus indicate that sulfation is essential for adhesion of non-blood borne, "epithelial-like" human cancer cells to P-selectin (Ma & Geng. J Immunol 2002; 168: 1690).

P-selectin (CD62P), expressed on stimulated endothelial cells and activated platelets, reacts with P-selectin glycoprotein ligand-1 (PSGL-1, CD162) for leukocyte rolling. It also binds to heparin and heparan sulfate proteoglycans (HSPGs), which attenuates P-selectin mediated adhesions of leukocytes and cancer cells. Here we report that P-selectin mediated adhesion, but not rolling, of the HSPGs bearing human malignant melanoma A375 cells under shear stress. To understand its underlying molecular mechanism, we measured the biophysical properties of this interaction. Heparin inhibited the adhesion of A375 cells to immobilized P-selectin under flow (IC_{50}=3 μmol/L heparin) and neutralized the binding of P-selectin to A375 cells (IC_{50}=4 μm heparin). Using surface plasmon resonance technique, we found that P-selectin bound to heparin with a dissociation constant (K_d) of (115±6) nmol/L. The measured off rate ($k$off) was $(3.15\pm0.34)\times10^{-3}\ s^{-1}$ and the calculated on rate ($k$on) was $(2.75\times10^{4})\ mol^{-1}\cdot L\cdot s^{-1}$. Taken together, our data suggest that the very slow $k$off and the reduced $k$on, but apparently not the K_d, are responsible for adhesion, but not rolling of A375 cells, to P-selectin under flow (Wang & Geng, Thromb Haemost 2003; 90: 309).

Slit is a secreted protein known to function through the Roundabout (Robo) receptor as a chemorepellent in axon guidance and neuronal migration, and as an inhibitor in leukocyte chemotaxis. We found Slit2 expression in a large number of solid tumors and Robo1 expression in vascular endothelial cells. Recombinant Slit2 protein attracted endothelial cells and promoted tube formation in a Robo1- and phosphatidylinositol kinase-dependent manner. Neutralization of Robo1 reduced the microvessel density and the tumor mass of human malignant melanoma A375 cells *in vivo*. These findings demonstrate the angiogenic function of Slit-Robo signaling, reveal a new mechanism in mediating the crosstalk between cancer cells and endothelial cells, and indicate the effectiveness of blocking this signaling pathway in treating cancers (Wang *et al*, Cancer Cell 4: 19, 2003).