CELL ADHESION AND HUMAN DISEASE
The Ciba Foundation is an international scientific and educational charity (Registered Charity No. 313574). It was established in 1947 by the Swiss chemical and pharmaceutical company of CIBA Limited—now Ciba-Geigy Limited. The Foundation operates independently in London under English trust law.

The Ciba Foundation exists to promote international cooperation in biological, medical and chemical research. It organizes about eight international multidisciplinary symposia each year on topics that seem ready for discussion by a small group of research workers. The papers and discussions are published in the Ciba Foundation symposium series. The Foundation also holds many shorter meetings (not published), organized by the Foundation itself or by outside scientific organizations. The staff always welcome suggestions for future meetings.

The Foundation's house at 41 Portland Place, London W1N 4BN, provides facilities for meetings of all kinds. Its Media Resource Service supplies information to journalists on all scientific and technological topics. The library, open five days a week to any graduate in science or medicine, also provides information on scientific meetings throughout the world and answers general enquiries on biomedical and chemical subjects. Scientists from any part of the world may stay in the house during working visits to London.
Contents

Symposium on Cell adhesion and human disease, held at the Ciba
Foundation, London 17-19 May 1994
Editors: Joan Marsh (Organizer) and Jamie A. Goode

R. O. Hynes  Chairman's introduction  1

D. D. Wagner  P-selectin knockout: a mouse model for various human
diseases  2
Discussion  10

L. Kwee, D. K. Burns, J. M. Rumberger, C. Norton, B. Wolitzky,
R. Terry, K. M. Lombard-Gillooly, D. J. Shuster, F. Kontgen,
C. Stewart, K. McIntyre, S. Baldwin and M. A. Labow  Creation and
characterization of E-selectin- and VCAM-1-deficient mice  17
Discussion  28

Z. M. Ruggeri  Von Willebrand's disease and the mechanisms of platelet
function  35
Discussion  45

adhesion deficiency (LAD) II  51
Discussion  58

and endothelial adhesion molecules in ischaemia/reperfusion injuries  63
Discussion  72

General Discussion  77

M. J. Elices  The integrin $\alpha_4\beta_1$ (VLA-4) as a therapeutic target  79
Discussion  85
J. N. W. N. Barker  Adhesion molecules in cutaneous inflammation  91
Discussion  101

J. R. Stanley  Defective cell–cell adhesion in the epidermis  107
Discussion  120

W. Birchmeier, J. Hülsken and J. Behrens  E-cadherin as an invasion suppressor  124
Discussion  136

Discussion  151

Discussion  170

General discussion II  174

M. J. Humphries, J. Sheridan, A. P. Mould and P. Newham  Mechanisms of VCAM-1 and fibronectin binding to integrin $\alpha_4\beta_1$; implications for integrin function and rational drug design  177
Discussion  191

General discussion III  195

R. Rothlein and J. R. Jaeger  Treatment of inflammatory diseases with a monoclonal antibody to intercellular adhesion molecule 1  200
Discussion  208

Discussion  222

Final discussion  227

Index of contributors  233

Subject index  235
Participants

J. N. W. N. Barker  St John's Institute of Dermatology, St Thomas's Hospital, Lambeth Palace Road, London SE1 7EW, UK

W. Birchmeier  Max-Delbrück-Centrum für Molekulare Medizin, Robert-Rössle-Strasse 10, D-13125 Berlin, Germany

M. J. Elices  Cytel Corporation, 3525 John Hopkins Court, San Diego, CA 92121, USA

B. Ernst  Zentrale Forschungslaboratorien, Ciba-Geigy Ag, Rosenthal R1060.3.34, CH-4002 Basle, Switzerland

A. Etzioni  Department of Pediatrics, Rambam Medical Center, B. Rappaport Medical School, IL-31096 Haifa, Israel

D. R. Garrod  CRC Epithelial Morphogenesis Research Group, School of Biological Sciences, University of Manchester, 3.239 Stopford Building, Oxford Road, Manchester M13 9PT, UK

D. O. Haskard  Department of Medicine (Rheumatology Unit), Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK

P. Herrlich  Kernforschungszentrum Karlsruhe GmbH, Institut für Genetik, PO Box 3640, D-76021 Karlsruhe, Germany

N. Hogg  Imperial Cancer Research Fund, PO Box No 123, Lincoln's Inn Fields, London WC2A 3PX, UK

M. J. Humphries  School of Biological Sciences, University of Manchester, 2.205 Stopford Building, Oxford Road, Manchester M13 9PT, UK
R. O. Hynes (Chairman)  Center for Cancer Research, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139-4307, USA

G. Koopman (Ciba Foundation Bursar)  Department of Pathology, Academic Medical Center, University of Amsterdam, Meibergdreef 9, NL-1105 AZ Amsterdam, The Netherlands

M. Labow  Department of Biotechnology, Hoffman La Roche, 340 Kingland Street, Nutley, NJ 07110-1199, USA

S. V. Ley  University Chemical Laboratory, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, UK

S. T. Pals  Department of Pathology, Academic Medical Center, University of Amsterdam, Meibergdreef 9, NL-1105 AZ Amsterdam, The Netherlands

R. B. Parekh  Oxford GlycoSystems Ltd, Unit 4, Hitching Court, Blacklands Way, Abingdon OX14 1RG, UK

J. S. Pober  Boyer Center for Molecular Medicine, Yale University School of Medicine, POB 9812, 295 Congress Avenue, New Haven, CT 06536-0812, USA

G. Riethmüller  Institut für Immunologie, Universität München, Goethestrasse 31, D-80336 München, Germany

R. Rothlein  Department of Immunology, Boehringer Ingelheim Pharmaceutical Inc, 900 Ridgebury Road, PO Box 368, Ridgefield, CT 06877, USA

Z. M. Ruggeri  Department of Molecular and Experimental Medicine, Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037, USA

F. Sánchez-Madrid  Seccion de Immunologia, Universidad Autónoma de Madrid, Hospital de la Princesa, Calle de Diego de Leon 62, E-28006 Madrid, Spain

S. Shaltiel  Department of Chemical Immunology, The Weizmann Institute of Science, PO Box 26, IL-76 100 Rehovot, Israel

A. Sonnenberg  Division of Cell Biology, Netherlands Cancer Institute, Plesmanlaan 121, NL-1066 CX Amsterdam, The Netherlands
J. R. Stanley  Dermatology Branch, National Cancer Institute, Building 10, Room 12N238, Bethesda, MD 20892, USA

P. Verrando  Unité INSERM 387 Adhésion Cellulaire, Hôpital de Sainte-Marguerite, 270 Boulevard de Sainte Marguerite, F-13277 Marseille Cedex 9, France

D. D. Wagner  Center for Blood Research, Harvard Medical School, 800 Huntington Avenue, Boston, MA 02115, USA

R. K. Winn  Department of Surgery, University of Washington School of Medicine, Harborview Medical Center, 325 Ninth Avenue, ZA-16, Seattle, WA 98104-2499, USA
Chairman’s introduction

Richard O. Hynes

Howard Hughes Medical Institute and Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139-4037, USA

Cell adhesion plays an important role in many disease states. These include cancer (invasion and metastasis), thrombosis, inflammatory diseases and problems arising from ischaemia/reperfusion injury (heart attacks, stroke, organ transplantation, frostbite). In all these situations, cells show alterations in their adhesive properties; basically, they stick where they should not or they stick too much.

In recent years, advances in our understanding of the molecular basis of cell adhesion have revealed the existence of families of cell surface receptors. Each cell adhesion event involves one, or more often several, adhesion receptors from the various families of molecules. Detailed analyses of these receptors have in many cases defined their binding sites. This presents the opportunity to block the binding sites using antibodies, peptides, carbohydrate groups or synthetic analogues of these reagents. In this way, it is hoped that undesired adhesive elements can be blocked, providing novel therapies for human diseases.

The challenge is to define with adequate specificity which of the many receptors are involved in a given disease and which present the best targets for therapeutic intervention. Potential anti-adhesive therapeutic drugs can enter human clinical trials only after adequate testing in animal systems. In this context, recent advances in methods for generating mice with alterations in specific genes allow the development of animal models of human genetic deficiencies and also model systems lacking one or more adhesion receptors. In this way, the roles of individual adhesion systems in specific diseases can be defined precisely, allowing better planning of therapeutic approaches.

Finally, there are some questions I think we should attempt to answer during this symposium (and this is obviously not an exhaustive list). First, which adhesion molecules should we try to block the function of in any given disease and how will we find out which are the best ones to target? Second, once we have identified them, which strategy should we choose for blocking the function of these molecules? Third, is there the possibility of gene therapy for some adhesion diseases? The final question is a serious issue, which I hope we can discuss: how does one go about blocking adhesion in a chronic fashion in the treatment of diseases such as rheumatoid arthritis, psoriasis and cancer?
P-selectin knockout: a mouse model for various human diseases

Denisa D. Wagner

The Center for Blood Research, Harvard Medical School, 800 Huntington Avenue, Boston, MA 02115, USA

Abstract. P-selectin is a transmembrane adhesion receptor specific to platelets and endothelial cells. It has an N-terminal lectin domain that recognizes specific carbohydrate moieties on monocytes, neutrophils and some other subsets of leukocytes. P-selectin is stored in granules and is expressed on the plasma membrane only after the cells are stimulated by vascular injury or during inflammation. Physiologically P-selectin is likely to be involved in the recruitment of leukocytes that promote wound healing and fight infection. There are many disorders in which the excessive recruitment of leukocytes is characteristic, including chronic inflammation, atherosclerosis, arthritis, diabetes, asthma and reperfusion injury. Because certain cancer cells also express the ligand for P-selectin it is possible that this receptor is involved in metastasis. To study the specific role of P-selectin in these pathological processes, we have prepared a mouse lacking P-selectin through gene targeting. Leukocyte interaction with the vessel wall is defective in these animals as leukocytes do not roll in the mesenteric venules and their extravasation at sites of inflammation and vessel injury is limited. We are testing these animals in models of the various diseases mentioned above in order to evaluate when the absence of P-selectin is beneficial.


The vessel wall is no longer considered to be only an inert barrier to blood. For example, the endothelial cells forming the inside wall of vessels can modify the composition of their plasma membranes in response to environmental cues. This can happen either slowly, by de novo synthesis of new membrane components, or rapidly, by the mobilization of preformed membrane components to the plasma membrane. The endothelial cells can also modify their secreted products depending on the circumstances, thus influencing the environment. The general research interest of my laboratory is the rapid response of endothelium to injury. We have found that endothelial cells store adhesive proteins in storage granules called Weibel–Palade bodies (Weibel & Palade 1964),
which are rapidly secreted at the time of a vascular injury. These proteins are von Willebrand factor, the soluble adhesion molecule for platelets (Wagner et al 1982) and P-selectin, the transmembrane receptor for leukocytes (Bonfanti et al 1989, McEver et al 1989). The granules exocytose their contents within minutes of endothelial stimulation with secretagogues such as histamine, thrombin, fibrin or complement components C5b–9 (Wagner 1993). Von Willebrand factor and P-selectin are also found together in platelet α-granules, from where they are released upon platelet activation. In this chapter I will discuss primarily P-selectin, as von Willebrand factor is covered elsewhere in this volume (Ruggeri 1995).

P-selectin is a member of the selectin family of adhesion receptors (Lasky & Rosen 1992, Bevilacqua & Nelson 1993). The name ‘selectin’, thought of by Bevilacqua (Bevilacqua et al 1991), indicates the function of the protein in selective interactions mediated by a lectin domain. Indeed, the Ca²⁺-dependent (C-type) lectin domains of these molecules bind to specific negatively charged carbohydrate structures presented by various mucins (Springer 1994). The N-terminal lectin domain is followed by an epidermal growth factor-like domain and several complement-binding protein-like repeats. The proteins contain a single transmembrane domain and a short cytoplasmic tail. L-selectin, which is present on leukocytes, is also called the homing receptor, as it mediates homing of lymphocytes to the peripheral lymph nodes (Gallatin et al 1983). L-selectin is also present on neutrophils where it contributes to neutrophil adhesion and extravasation at sites of inflammation. E-selectin is specific to endothelial cells, but in contrast to P-selectin, it is synthesized only after exposure of the endothelium to inflammatory cytokines (Bevilacqua et al 1987), at which time it becomes directly expressed on the plasma membrane (Fig. 1). After internalization, E-selectin is rapidly degraded in the lysosomes. P-selectin, which is also endocytosed after its expression on the surface, is directed to the Golgi region where it is incorporated into nascent Weibel–Palade bodies (Fig. 1, Subramaniam et al 1993). P-selectin can therefore be involved both in acute processes, when it becomes available immediately after stimulation, and in chronic phenomena, when it may cycle several times between the membrane and the storage granule under repeated stimulation. In addition, the synthesis of P-selectin is up-regulated when cells are treated with endotoxin or inflammatory cytokines (Sanders et al 1992). Since the two endothelial selectins mediate primarily adhesion to neutrophils and monocytes (Bevilacqua & Nelson 1993) and may be present simultaneously on activated endothelial cells, their functions could overlap. To determine the specific functions of P-selectin, in both normal physiology and pathological conditions, we prepared P-selectin-deficient animals in collaboration with Richard Hynes. This was accomplished (Mayadas et al 1993) through homologous recombination in embryonic stem cells (Capecchi 1989). The engineered animals, which do not contain any P-selectin in their platelets and endothelial cells, are grossly normal and fertile.
P-selectin

E-selectin

FIG. 1. The expression of P- and E-selectins on endothelial cells. P-selectin is stored in Weibel–Palade bodies and it becomes rapidly expressed on the plasma membrane upon degranulation. After endocytosis, the P-selectin returns to storage granules (Subramaniam et al 1993). In the presence of inflammatory cytokines endothelial cells begin to synthesize E-selectin. The expression may last many hours and eventually all the protein is degraded in lysosomes.

This shows that P-selectin is dispensible for normal embryonic development and angiogenesis. The disruption of the P-selectin gene did not affect the expression of E- and L-selectin, whose genes are located in close proximity to P-selectin (Watson et al 1990).

The total peripheral leukocyte and platelet counts in the P-selectin-deficient animals are similar to those of wild-type animals. In contrast, the basal neutrophil counts in the homozygous-deficient animals were two to three times higher than in the wild-type animals. Since the numbers of progenitors in the bone marrow were determined to be comparable, this difference is very likely due to a longer half-life of the neutrophils in the mutants. Indeed, by injecting radiolabelled human neutrophils into the tail vein of mutant and wild-type animals, Robert Johnson (unpublished results) showed that these cells survive longer in the bloodstream of the mutant mice.

Several lines of investigations have implicated the selectins in the first step in leukocyte extravasation, leukocyte rolling. First, P-selectin embedded in a lipid bilayer supports leukocyte rolling under physiological flow conditions in vitro (Lawrence & Springer 1991). Second, infusion of anti-L-selectin antibody significantly inhibits leukocyte rolling in vivo (von Andrian et al 1991, Ley et al 1991). To investigate the effect of the absence of P-selectin on leukocyte rolling, we performed intravital microscopy of the mouse mesentery. In this model (Atherton & Born 1973), the mesentery of anaesthetized animals is pulled out and spread onto a microscope stage, and the behaviour of leukocytes is directly observed and recorded. To increase rapidly the expression of P-selectin on the vessel wall, we treated the mesentery with the Ca\(^{2+}\) ionophore A23187, which we knew to be an excellent in vitro secretagogue for Weibel–Palade bodies.
FIG. 2. Schematic representation of the experimental design of an intravital microscopy study. Following the 10 min baseline period, 10 µM calcium ionophore A23187 was applied to the mesentery by superfusion, at the times indicated. In the wild-type animals the baseline rolling was 10.5 ± 2.5, first response to the ionophore was 24.9 ± 7.1 and second response 17.3 ± 3.3 (values represent mean number of rolling leukocytes passing through a perpendicular plane per min, ± SEM [n = 8 for wild-type, 5 for mutant]). In the P-selectin-deficient animals we did not observe any rolling leukocytes at baseline. First response was 0.10 ± 0.06 and second response 0.04 ± 0.04 (Mayadas et al 1993). (Sporn et al 1986). The complete experimental design is presented in Fig. 2. In the wild-type mice, the numbers of leukocytes observed rolling on the vessel wall more than doubled after the ionophore treatment, as if the vessel were stickier. In contrast, there were no rolling leukocytes in the P-selectin-deficient mesentery under the baseline conditions and only one leukocyte for every 10 min after the ionophore treatment (Mayadas et al 1993). It is clear from these results that P-selectin plays a crucial role in the initial interaction of the leukocytes with the vessel wall. The L-selectin of the leukocyte cannot alone support baseline rolling. We have performed intravital microscopy studies using animals with experimentally induced peritonitis. Our preliminary results indicate that when the mesentery is inflamed (i.e. several hours after injection of thioglycollate), leukocytes can roll in the absence of P-selectin (Robert Johnson, unpublished observations). Therefore, under inflammatory conditions, other adhesion molecules can mediate leukocyte rolling. Interestingly, in the P-selectin-deficient mice after thioglycollate injection, the leukocytes roll much more slowly than in wild-type animals. This is likely due to a different strength of adhesive interactions in this case from that generated by P-selectin and its ligand. In addition, in the wild-type animals many more leukocytes are in contact with the vessel wall, demonstrating the importance of P-selectin even under the inflammatory conditions.

The defect in the initial contact of the leukocyte with the vessel wall in the P-selectin-deficient animals is likely the cause of an observed delay in extravasation (Mayadas et al 1993). Injection of thioglycollate in the peritoneum of wild-type animals stimulates a rapid onset of neutrophil extravasation, whereas in the P-selectin-deficient animals there is a 2 h delay. After this time,
it is possible that a different molecule, perhaps E-selectin, may be expressed on the endothelium, thus allowing leukocyte extravasation. Currently, we are preparing mice deficient in both P- and E-selectin: it will be interesting to see whether the neutrophil extravasation to the peritoneum in response to thioglycollate will be ablated completely in these animals. We have also examined the effect of P-selectin deficiency on the recruitment of macrophages into chronically inflamed peritoneum. We have found that the numbers of macrophages recruited 48 h after a thioglycollate injection are significantly reduced in the absence of P-selectin (Robert Johnson, unpublished observation). This result further indicates that P-selectin can play a role in chronic inflammation.

Another chronic situation we have investigated is contact hypersensitivity. In the murine model, although the response is initiated by CD4\(^+\) lymphocytes, the neutrophilic response is more intense than in humans. To elicit the contact hypersensitivity response, we sensitized mice with oxazolone, followed by injection of \([^{125}\text{I}]\text{iododeoxyuridine}\), which labels monocytes and lymphocytes. Twenty-four hours post-challenge, the mutant mice had 50% lower counts in sensitized ears than the wild-type mice. In addition, histological sections of the ears showed much less neutrophil infiltration in the mutant mice (M. Subramaniam, unpublished observations). Lack of P-selectin therefore affects the recruitment of both mononuclear cells and neutrophils in this model.

Weibel-Palade bodies and \(\alpha\)-granules are massively exocytosed at the time of a vascular injury, when thrombin is generated (Fig. 3). The released von Willebrand factor is important for the formation of a platelet plug and the P-selectin probably participates in the recruitment of phagocytic cells to fight infection and to clear debris. To evaluate the actual role of P-selectin in the recruitment of phagocytes to the site of a wound, we have adapted the following wound healing model: full thickness skin excisional wounds (3–4 mm in diameter) were generated on the flanks of wild-type and P-selectin-deficient mice. Biopsies were taken from killed mice at intervals of hours or days following wounding. Staining of the 1 h wound sections showed neutrophils exiting along post-capillary venules and veins in the wild-type mice, but very few in the mutant mice. By 4 h, the numbers of extravasated neutrophils in the mutant mice approached those in the wild-type animal wounds (M. Subramaniam, unpublished observations). It will be interesting to see whether the P-selectin deficiency has an effect on wound closure.

To summarize, we have learned the following about the phenotype of the P-selectin-deficient mice: the mice have an elevated neutrophil count that is probably due to defective neutrophil clearance; their leukocytes do not roll in mesenteric venules in the absence of an inflammatory stimulus; their neutrophils show a 2 h lag in extravasation at sites of inflammation and at wound sites; macrophage recruitment to chronically inflamed peritoneum is reduced; and the mice have a significantly reduced contact hypersensitivity response.
P-selectin knockout mice

FIG. 3. Diagram of an injured venule. The degranulation of platelet α-granules and endothelial Weibel–Palade bodies mobilizes P-selectin to the cell surface where it serves as receptor for monocytes, neutrophils and other subsets of leukocytes. It mediates leukocyte rolling on endothelium at sites of inflammation and recruitment of phagocytes to a wound. (Reproduced with permission from Wagner 1993.)

Because of the striking phenotype of the P-selectin-deficient mice, these animals could provide an interesting model system with which to study the role of P-selectin in various diseases and pathological conditions. The most obvious condition in which P-selectin is likely to play a role is inflammation. P-selectin binds myeloid cells and, more recently, has been shown also to bind natural killer cells and subpopulations of memory T cells of both the CD4+ and CD8+ type (Moore & Thompson 1992, Damle et al 1992, de Bruijne-Admiraal et al 1992), and may therefore mediate migration of all these cell types to inflammatory sites. Some rather well known diseases are linked to inflammation. For example, in insulin-dependent diabetes mellitus, the defect in insulin secretion results from the destruction of pancreatic islets by a chronic, slowly evolving, inflammatory process called insulitis, which is thought to be of autoimmune origin. Histological studies show islet infiltration with macrophages and both CD4+ and CD8+ T cells (Rossini et al 1993). It would be interesting to know whether P-selectin is one of the adhesion molecules involved in this inflammatory process.

Atherosclerosis is another prominent disease in which P-selectin is very likely to play a role. In this vascular disorder, monocytes adhere to injured endothelium (that is likely to express P-selectin), extravasate and become engorged with lipid vesicles while remaining under the endothelium (Ross 1986). Some of the lipid may be obtained from phagocytosed platelets (Sevitt 1986), known to be present in advanced lesions, a process that could also be mediated by P-selectin. Mice fed a high-fat diet develop atherosclerotic lesions and therefore can be used as
animal models for atherosclerosis (Paigen et al. 1985). Because genetic manipulation of mice is possible, the role of an individual gene in the complex process of atherosclerosis can now be studied. This should be done for P-selectin.

Selectins were also shown to bind to carbohydrate on some cancer cells and therefore may be involved in the metastatic spread of cancer. E-selectin has been shown to bind to colon cancers (Rice & Bevilacqua 1989, Lauri et al. 1991) and we have shown that P-selectin mediates binding of platelets to neuroblastoma and small-cell lung cancer (Stone & Wagner 1993). Since P-selectin, and not E-selectin, is expressed on platelets in addition to endothelial cells, it may have a unique importance for the development of successful metastases. Experimental animal models of metastasis have convincingly demonstrated the involvement of platelets in this process (Gasic et al. 1968, 1973). With the existence of the P-selectin-deficient mouse one will actually be able to test the hypothesis that this selectin participates in the hematogenous spread of cancer cells.

These are just a few examples of diseases and conditions where P-selectin may be implicated. Understanding of the situations where adhesive interactions through P-selectin do play a role will undoubtedly lead to the development of new drugs that inhibit these interactions and therefore arrest or reverse the disease process. We hope that the development of the P-selectin-deficient animal model will bring us closer to this goal.

**Acknowledgements**

This work was supported by National Institutes of Health grants HL41002 and HL53756.

**References**


Bonfanti R, Furie BC, Furie B, Wagner DD 1989 PADGEM (GMP-140) is a component of Weibel-Palade bodies of human endothelial cells. Blood 73:1109–1112

Capecchi MR 1989 Altering the genome by homologous recombination. Science 244:1288–1292


DISCUSSION

Labow: In the thioglycollate model of inflammation, where are the neutrophils that invade the peritoneum coming from?

Wagner: The leukocytes are coming from the blood vessels that are in contact with the peritoneum, because they can sense the cytokines that are produced in a response to the thioglycollate, such as IL-8.

Ruggeri: I was intrigued by your comments about the interaction that you see in the P-selectin knockout between the neutrophils and the vessel wall during inflammation. You suggested that another adhesion mechanism is responsible for the ability of these cells still to interact and crawl on the surface. Why didn't you also see that in the wild-type? This would be a mechanism that should exist in the wild-type. Is it possible that it has been overlooked in the wild-type because only a minority of cells use this pathway? If there is such a pathway, why does it work only in a small number of cells?

Wagner: We may be looking here just at small subsets of leukocytes that can roll slowly, for example, on E-selectin. This may also be the case in wild-type mice, but it seems that the majority of the cells are rolling fast on P-selectin. Another possibility is that P-selectin, being a larger molecule than E-selectin, may be sticking out further into the bloodstream. Therefore, P-selectin may grab the leukocyte first and shift it to another P-selectin molecule—like playing volleyball. So the leukocyte would be flying from one P-selectin to another and E-selectin could never reach it.

Ruggeri: If it is true that there is a molecule that mediates a stronger adhesion than that mediated by P-selectin, at some point you should be able to differentiate the relative functional role of the different pathways by increasing the flow rate. On the arterial surface you don't see any rolling, so there's obviously a shear rate above which selectins cannot function. Consequently, if you set up a model where you can vary the flow under controlled conditions, you may be able to clarify what the different mechanisms could be.

Wagner: Again, you're absolutely right. In the arterioles the shear rate could be so high that although the selectins are expressed, they are no longer able to function properly. We have compared blood vessels of very similar location
and diameter in two sets of animals that were treated in the same way. We would expect the shear rates in the two to be similar. It would be interesting to vary shear rates, but at the moment we don’t know how to do it. Lawrence & Springer (1993) have immobilized P-selectin and E-selectin on plates and studied the leukocytes rolling under flow conditions in this system. They saw that the leukocytes rolled more slowly on E-selectin, which suggests that this may be the molecule expressed in our knockout mice under inflammatory conditions.

_**Ernst:** Under *in vitro* conditions, P-selectin shows up immediately after stimulation and is gone after 20–30 min, whereas E-selectin is expressed only after 2–4 h. What is the picture *in vivo*?

_Wagner:* Our studies with cultured endothelial cells have shown that P-selectin is not destroyed after surface expression, but it returns to the Weibel–Palade bodies. Therefore it could become available again. We hypothesize that this is how P-selectin could also be involved in chronic situations *in vivo*.

_Haskard:* You’re talking about E-selectin as being a possible molecule on which the leukocytes could roll in the absence of P-selectin, but what about L-selectin? Have you thought of doing experiments with anti-L-selectin antibodies to see if that abrogates the residual rolling?

_Wagner:* Others have done this with wild-type animals. Injection of anti-L-selectin antibodies inhibits leukocyte rolling, but not completely (Ley et al 1991, von Andrian et al 1991). Maybe the L-selectin on the leukocyte has to find a ligand—perhaps CD34—on the endothelium, and at the same time P-selectin has to find a carbohydrate on the leukocyte. It could be that for optimal rolling, independent interactions mediated through two selectins are needed.

_Hynes:* There’s not much of a depression in the ‘baseline’ leukocyte rolling in the L-selectin knockout that Tom Tedder has made (unpublished results).

_Wagner:* That is correct. P-selectin is clearly the most important selectin for rolling under these baseline conditions.

Some people have also proposed that P- and L-selectin may be ‘holding hands’, i.e. that P-selectin may be recognizing carbohydrate presented by L-selectin, because L-selectin contains sialyl Lewis X structures. But that is not always the case. For example, with Reina Mebius we have looked at adhesion of wild-type leukocytes to high endothelial venules in peripheral lymph nodes of P-selectin knockout mice, an interaction known absolutely to require L-selectin. But these knockout lymph nodes didn’t have P-selectin. So, if L- and P-selectin had to ‘hold hands’, then the leukocytes wouldn’t have been able to bind to the P-selectin-deficient lymph nodes, but they do bind just as well as to wild-type lymph nodes.

_Shaltiel:* Has anyone made an estimate of the number of points of contact during ‘good’ rolling? How would rolling be affected by changing the density of P-selectin expression on the cell? For instance, if you were to do an experiment in the presence of increasing levels of antibodies against P-selectin, how would this affect rolling?
Wagner: Tim Springer’s lab has some *in vitro* results (Lawrence & Springer 1993) showing that the density of a selectin affects the speed of leukocyte rolling: the velocity of rolling decreases with increase in selectin density. The speed of rolling is also dependent on shear stress. But you reach a plateau for the density of a particular selectin when more shear no longer increases velocity.

Shaltiel: So the level of selectin release would form a sort of steady-state—would this be a mechanism of regulating the process?

Wagner: Yes, you could regulate or fine-tune the rolling process by controlling the release of P-selectin from the Weibel–Palade bodies, because secretion from Weibel–Palade bodies (as we know from our own experience) is not an all-or-none phenomenon—you can have partial secretion of the storage granules. So you could up-regulate or down-regulate the rolling or change the speed of rolling in that way. In addition, you could control rolling by up-regulating *de novo* synthesis of selectins by inflammatory cytokines.

Birchmeier: You mentioned cancer cells: have you actually initiated studies to look at metastasis, for instance, by using wild-type cells in mutant mice?

Wagner: We are starting to do these studies.

Pober: Part of the general paradigm for the difference between P- and E-selectin is that P-selectin pre-exists in resting endothelial cells, but that’s clearly not always the case. You raise the issue of the role of P-selectin in atherosclerosis; it’s not so clear that large arteries have much P-selectin in their Weibel–Palade bodies. What is known about regulation of P-selectin expression? How do you set the baseline?

Wagner: No one has really studied P-selectin expression in different blood vessels. We have seen some P-selectin expression in arteries, others have also reported this (McEver et al 1989). There is always the possibility that P-selectin, like E-selectin, is up-regulated by *de novo* synthesis, because cytokines and TNF have been shown to up-regulate P-selectin (Sanders et al 1992, Weller et al 1992). It is also possible that different levels of stimulation are necessary to bring the P-selectin to the cell surface in an arteriole than are needed in a venule, because we never see leukocyte rolling in arterioles. But this might be because the slight inflammation we created in our experiments is not enough to cause secretion from the Weibel–Palade bodies in arterioles.

Ruggeri: This is an interesting question; it might be related to the fact that von Willebrand factor expression in arterial cells is very heterogeneous. It’s widely assumed that von Willebrand factor is a marker for endothelial cells. Although this is certainly true *in vitro* (every cultured endothelial cell makes von Willebrand factor), when you look *in vivo*, at the level of both the protein and mRNA, you see a tremendous heterogeneity. There are actually some endothelial structures and vessels that lack von Willebrand factor entirely, particularly in arteries. A careful study of the expression of von Willebrand factor would be extremely valuable.
**Hynes:** If you take Denisa Wagner's theory that P-selectin is recruiting monocytes in atherosclerosis, isn't it possible that the P-selectin is coming from the platelets, not necessarily just from the endothelial cells?

**Pober:** That depends on whether or not there are ligands on the vessel wall to interact with P-selectin. You could have a platelet bridge between a leukocyte and the endothelial cell surface, but I'm not aware that there's any clear evidence that endothelial cells, for example, have P-selectin ligands.

**Hynes:** I agree, but what if the platelets got stuck there by some other process? They could then express P-selectin on activation.

**Wagner:** For example, if they were bound to von Willebrand factor deposited there as a result of some injury.

**Pober:** The evidence from the lipid-fed primates in Russell Ross' serial morphological studies does not implicate platelets in the adhesion of monocytes to the foam cell lesions (reviewed in Ross 1993). The initial recruitment of monocytes appears to be platelet independent.

I think everyone has partially ignored the issue of whether or not the regulation of expression of P-selectin is controlled by the same mechanisms as E-selectin, and whether one could think about pharmacological targeting to inhibit synthesis by the same approaches that are being widely tested for inhibiting synthesis of E-selectin.

**Wagner:** This is regulation at the mRNA level, but for P-selectin, in addition, one could try to target the regulated secretory pathway. Inhibitors of regulated secretion exist, such as microtubule-depolymerizing agents, but one would have to use something more subtle to inhibit the secretion of Weibel-Palade bodies. An inhibitor of Weibel-Palade body secretion might also be a useful therapeutic agent for people with thrombotic problems, which may be a result of release of the large von Willebrand factor multimers stored in these granules.

**Sonnenberg:** Does anybody know what the function of P-selectin is on platelets?

**Wagner:** In collaboration with Bruce and Barbara Furie, we showed a while ago that P-selectin on platelets mediates adhesion to monocytes and neutrophils (Larsen et al 1989). This interaction has now been confirmed to be mediated solely by P-selectin because, for the platelets in P-selectin knockout mice, this leukocyte binding is totally ablated. But we don't yet know whether or not this is the only function of P-selectin on platelets. When the platelets have been isolated and washed, we don't see a defect in platelet aggregation in response to collagen, for example. But in vivo this aggregation doesn't happen with just platelets as there are always leukocytes present and the cells 'talk' to each other. We don't know whether a thrombus that is formed in vivo in the P-selectin-deficient mice is the same size and is held together with the same strength as one in the wild-type mice. We are now looking for a machine that would allow us to measure platelet aggregation in whole blood, and it is not easy to come by.
Ruggeri: Aggregation is the most unrealistic scenario for platelet function, because it involves platelets in suspension sticking to one another—that's not how things work in vivo. If you don't have a surface and if things don't start on a surface, you are looking at an artefact.

Hynes: It's nice to hear a platelet person say that!

Ruggeri: Studying aggregation is probably like studying transgenic mice: it tells you a lot but it doesn't tell you the full story and you always have to go back to real life.

Etzioni: In the wound healing experiment, you showed that after 4 h there's an almost normal number of neutrophils in the area, whereas in the contact hypersensitivity model, even after 8 h, there was a marked increase in the neutrophil count. Do you have an explanation for this?

Wagner: Perhaps the stimulus is different in the hypersensitivity reaction and the other adhesion molecules are expressed more slowly.

Etzioni: Do you think that there is no E-selectin expression and that it is just a P-selectin-dependent reaction?

Wagner: I don't know. We will have to get some E-selectin knockout mice and look at their contact hypersensitivity response.

Labow: Have you followed the time course of the expression of E- and P-selectin by immunohistochemistry in these mice?

Wagner: No. One reason this is difficult is that antibodies that recognize mouse antigens are scarce. We plan to use some of your reagents to look at E-selectin. We intend to make P- and E-selectin double knockouts, to see what will happen in the absence of both endothelial selectins. Selectins may not be the whole story—in some regions the blood flow is so slow that you may not need selectins at all and other adhesion molecules may mediate leukocyte rolling.

Hynes: It's becoming clear that every situation has to be looked at separately. We can't generalize that it's P-selectin first and then E-selectin (it often isn't) and we can't generalize about how long P-selectin stays around (sometimes it may be transient and at other times it clearly isn't). We're just going to have to look at each situation separately.

Barker: Concerning your allergic contact dermatitis model: the theory is that you apply your allergen and it's taken up by antigen-presenting cells in the epidermis (Langerhans' cells), which migrate to lymph nodes. It is known that Langerhans' cells express certain selectin ligands on their cell surface which are up-regulated, at least in human skin (Ross et al 1994). Are the P-selectin-deficient mice more difficult to sensitize? This deficiency may well inhibit Langerhans' cell trafficking.

Wagner: We really don't know—there may be a defect in the way these mice are sensitized as well.

Barker: You mentioned that the neutrophil response in the skin of these mice is intense after induction of contact allergy; in humans you don't see any neutrophils at all—it's a completely lymphocytic infiltrate.
Wagner: The response in mice is very different from humans in that it’s much more neutrophilic, although it is initiated by T lymphocytes.

Pober: There are new data from Phil Askenase (unpublished results) which indicate that in this kind of contact sensitivity model the magnitude of the response is dependent on platelets, which are presumably delivering serotonin and other vasoactive mediators to the reaction. The defect in P-selectin-deficient animals that you’re seeing, rather than being involved in the ultimate recruitment of the neutrophils into the lesion through P- versus E-selectin adhesion, may instead reflect a platelet defect in terms of delivering the vasoactive mediators. Also, it’s a fairly controversial question as to whether T cells utilize P-selectin for homing to inflammatory sites, and your methods are not directly measuring T cell infiltration per se. Nevertheless, I suspect you will see fewer T cells in the lesions, because T cell recruitment is often dependent upon the initial infiltration of other leukocytes. Although human reactions are almost entirely mononuclear at 24 h (when they are usually measured), at 4 h they are predominantly neutrophilic—the human neutrophil response subsides very quickly, whereas the murine one doesn’t.

Wagner: We are now quantitating the difference in the monocyte infiltration and also labelling the CD4+ T cells in the dermis, so we should be able to see the effect of P-selectin deficiency on the recruitment of the different classes of leukocytes.

Garrod: Presumably, you keep the P-selectin knockout mice in a controlled clean environment, but are there any differences in their survival or their responses to infection and injury compared with normal mice?

Wagner: They are not kept under any special conditions; we don’t keep them like immunodeficient mice, for instance. Sometimes we even keep them in the lab for several days and they do fine. They don’t seem to be especially susceptible to infection, but we haven’t yet done a controlled study with an infectious agent. I expect if we really challenged these mice, they would have problems.

Garrod: Presumably they get small injuries?

Wagner: They may bite each other and they rub their noses on the cages, but we haven’t noticed much of a difference between the P-selectin knockouts and wild-type mice. Their longevity and fertility are similar.

References