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Second International Meeting

THE PLATELET ADP RECEPTORS

Biochemistry, Physiology, Pharmacology and Clinical Aspects

Chairmen:

M. Cattaneo (Milan, Italy), C. Gachet (Strasbourg, France)

October 3-5, 2002

S. Margherita di Pula, Italy

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3. The Royal Marsden Hospital Bone-Marrow Transplantation Team. Failure of syngeneic bone-marrow graft without preconditioning in post-hepatitis marrow aplasia. Lancet 1977; 2:242-4.
4. Red cell aplasia (Editorial). Lancet 1982; 1:546-7.
5. Karlsson S, Humphries RK, Gluzman Y, Nienhuis AW. Transfer of genes into hemopoietic cells using recombinant DNA viruses [abstract]. Blood 1984; 64(Suppl 1):58a.

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7. Hillman RS, Finch CA. Red cell manual. 5th ed. Philadelphia: FA Davis, 1985.
8. Bottomley SS. Sideroblastic anaemia. In: Jacobs A, Worwood M, eds. Iron in biochemistry and medicine, II. London: Academic Press, 1980:363-92.
9. DuPont B. Bone marrow transplantation in severe combined immunodeficiency with an unrelated MLC compatible donor. In: White HJ, Smith R, eds. Proceedings of the third annual meeting of the International Society for Experimental Hematology. Houston: International Society for Experimental Hematology, 1974:44-6.
10. Bieber MM, Kaplan HS. T-cell inhibitor in the sera of untreated patients with Hodgkin's disease (Abstract). Paper presented at the International Conference on Malignant Lymphoma Current Status and Prospects, Lugano, 1981:15.
11. Worwood M. Serum ferritin. In: Cook JD, ed. Iron. New York: Churchill Livingstone, 1980:59-89. (Chanarin I, Beutler E, Brown EB, Jacobs A, eds. Methods in hematology; vol 1).
12. Ranofsky AL. Surgical operation in short-stay hospitals: United States-1975. Hyattsville, Maryland: National Center for Health Statistics, 1978; DHEW publication no. (PHS) 78-1785, (Vital and health statistics; series 13; no. 34).

Forthcoming[13] or electronic material[14]:

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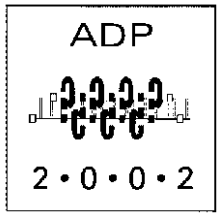

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table of contents

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table of contents

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Second International Meeting THE PLATELET ADP RECEPTORS
Biochemistry, Physiology, Pharmacology and Clinical Aspects

Santa Margherita di Pula (Sardinia, Italy), October 3-5, 2002

MAIN PROGRAM

SESSION 1

New P2Y receptors

BOEYNAEMS J-M,*§ ROBAYE B,* MARTEAU F,* GHANEM E,° BEAUWENS R,° SAVI P,◊ SUAREZ GONZALEZ N*

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While the identification of ligands of orphan receptors structurally related to known P2Y receptors (GPR87=GPR95, GPR91=P_{2U2}, GPR99= GPR80...) remains a priority but difficult task, we have made progress in characterizing the recently identified $P2Y_{13}$ receptor and in determining the role of the $P2Y_4$ receptor. The pharmacology of the human $P2Y_{13}$ receptor was characterized in detail following stable expression in CHO (cAMP inhibition), 1321N1 ([^{33}P]-2MeSADP binding) and $G_{\alpha 16}$-cotransfected 1321N1 (inositol phosphates) cells. The most salient differences from the $P2Y_{12}$ receptor were:

—ATP and 2MeSATP behaved as weak partial agonists;
—potency of 2MeSADP was greater than or equal to that of ADP depending on the expressing cell line and signaling pathway studied;
—AR-C67085X behaved as an antagonist with a μM potency;
—the active metabolite of clopidogrel was inactive.

Generation of knockout mice is currently underway. The functions of the $P2Y_4$ receptor remain poorly defined. Therefore $P2Y_4$-null mice were generated. Genotype frequencies were consistent with Mendelian X-linked transmission. $P2Y_4$-null mice were viable and displayed normal development, survival and reproduction. Chloride transport by the jejunal epithelium was assessed by the measurement of short circuit current in Ussing chambers, in the presence of phlorizin. The response to luminal UTP, which is maintained in $P2Y_2$-null mice, was abolished in $P2Y_4$-null mice. This is the first clear-cut demonstration of a role of the $P2Y_4$ receptor.

New P2Y receptors

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The G-protein-coupled (GPC) P2Y receptor family currently encompasses the $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptors,[1] the long-searched and recently identified platelet $P2Y_{12}$ receptor[2] and the recently cloned $P2Y_{13}$ receptor for ADP.[3] However, it is highly likely that the actual number of P2Y receptors largely exceeds the seven so-far cloned members of this family. This assumption is based on the following evidence: (i) pathophsyiological responses have been reported to be mediated by P2Y-like receptors characterized by response profiles that do not overlap with any of the already cloned members of the family,[4,5] and, (ii) based on sequence identity and on the presence of key aminoacid residues important for nucleotide binding in transmembrane domains 6 and 7, several orphan GPCRs sequences available in the public database may indeed represent novel P2Y receptor subtypes. One of these genes (KIAA0001; GPR105) has been recently identified as the uridine 5'-diphosphoglucose (UDPglucose) receptor and shows significant sequence identity with the cloned P2Y receptors.[6] KIAA0001 has a widespread human tissue distribution and shows 81% identity with the previously cloned VTR 15-20 rat receptor which has been reported to be regulated by immunologic challenge[7] and represents its rat ortholog.[8] The International Union of Pharmacology (IUPHAR) Subcommittee for P2Y receptor

nomenclature and classification is currently considering the inclusion of the UDPglucose receptor into the P2Y family as the $P2Y_{14}$ receptor.[9] Several other genes (including $P2Y_5$, $P2Y_9$, $P2Y_{10}$ and the orphan receptors GPR87, GPR91, GPR34 and H963) are also related with the cloned P2Y receptors and are currently being evaluated by several laboratories to assess if they indeed respond to nucleotides. Finally, it has been recently reported that, in human mast cells, the cysteinyl leukotriene (CysLT1) receptor unexpectidely serves as a dual-specific receptor for both the cysLTs and for the pyrimidine nucleotide UDP.[10] If confirmed by further studies, the existance of mixed receptors specifically responding to more than one endogenous ligand may open up completely new views in the pharmacological modulation of P2Y receptors, and, more in general, of GPCRs.

References

1. King BF, Burnstock G, Boyer JL, Boeynaems JM, Weisman GA, Kennedy C, et al. IUPHAR Compendium of Receptor Characterization and Classification. IUPHAR Media 2000; 307-20.
2. Hollopeter G, Jantzen HM, Vincent D, Li G, England L, Ramakrishnan V, et al. Identification of the platelet ADP receptor targeted by antithrombotic drugs. Nature 2001; 409:202-7.
3. Communi D, Gonzalez NS, Detheux M. Brèzillon S, Lannoy V. Parmentier M, Boeynaems JM. dentification of a novel human ADP receptor coupled to G(i). J Biol Chem 2001; 276:41479-85.
4. Brambilla R, Burnstock G, Bonazzi A, Ceruti S, Cattabeni F, Abbracchio MP. Cyclo-oxygenase-2 mediates P2Y receptor-induced reactive astrogliosis. Br J Pharmacol 1999; 126:563-67.
5. Windscheif U, Pfaff O, Ziganshin AU, Hoyle CHV, Baumert HG, Mutschler E, et al. Inhibitory action of PPADS on relaxant responses to adenine nucleotides or electrical field stimulation in guinea-pig taenia coli and rat duodenum. Br J Pharmacol 1995; 115:1509-17.
6. Chambers JK, MacDonald LE, Sarau HM, Ames RS, Freeman K, Foley JJ, et al. J Biol Chem 2000; 275:10767-771.
7. Charlton ME, Williams AS, Fogliano M, Sweetnam PM, Duman RS. The isolation and characterization of a novel G protein-coupled receptor regulated by immunologic challenge. Brain Res 1997; 764:141-8.
8. Freeman K, Tsui P, Moore D, Emson PC, Vawter L, Naheed S, t al. Cloning, pharmacology, and tissue distribution of G-protein-coupled receptor GPR105 (KIAA0001) rodent orthologs. Genomics 2001; 78:124-8.
9. Abbracchio MP, Barnard EA, Boeynaems JM, Boyer JL, Jacobson KA, Gachet C, et al. The UDPGlucose receptors renamed the P2Y14 receptor. Trends Pharmacol Sci 2002; (in press).
10. Mellor EA, Austen KF, Boyce JA. Cysteinyl leukotrienes and uridine diphosphate induce cytokine generation by human mast cells through an interleukin 4-regulated pathway that is inhibited by leukotriene receptor antagonists. J Exp Med 2002; 195:583-92.

New insights into P2X receptors

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Extracellular ATP stimulates a host of cellular responses by acting on two receptor subfamilies: P2Y and P2X.[1] The P2Y receptor (P2YR) sub-family comprises 8 subtypes ($P2Y_1$, $P2Y_2$, $P2Y_3$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and the UDP-glucose receptor), while the P2X receptor (P2XR) subfamily comprises 7 subtypes ($P2X_{1\text{-}7}$). P2YRs are seven membrane-spanning, G-protein-coupled (metabotropic) receptors mainly thought to mediate chemotactic, differentiative or proliferative responses, while P2XRs are ligand (ATP)-gated channels made by the assembly of tri/six-subunits of the same (homo-oligomers) or different (hetero-oligomers) P2X subtype. P2XRs are mainly thought to mediate short-term, fast cell-to-cell communication. Exceptions might be $P2X_7$ and $P2X_5$, which have also been proposed to mediate cell proliferation and/or differentiation.[2,3] Compelling biochemical data indicate that all P2X subunits assemble to form heteromeric receptors (trimers), an exception being P2X7 that is thought to form only homomeric receptors.[4] The main, if not exclusive signal transduction mechanism associated with P2XR activation is the flow of cations (Ca^{2+}, K^+ and Na^+) across the plasma membrane, although recent data suggest that, at least in the case of $P2X_7$, signaling could also be achieved by protein-protein interactions. It has long been thought that $P2X_7$, due to its extended cytoplasmic COOH tail, might interact with cytoplasmic or membrane protein, but this hypothesis has remained largely unproven. Two recent papers by Alan North *et al*. have described how that $P2X_7$ assembles in a signaling complex together with at least 13 cytoplasmic or plasma membrane proteins. Furthermore, a study by Paul Bertics *et al*.[7] unveiled the presence of a consensus sequence for LPS binding in the $P2X_7$ tail, thus lending further support to the suggestion that this receptor might be involved in LPS-dependent phagocyte activation. The function of P2XRs is well established only in the nervous system, where they mediate neuronal communication; however compelling evidence for $P2X_7$ and preliminary, but nonetheless intriguing, data for $P2X_1$ and $P2X_5$ suggest that these receptors might also have an important role outside the nervous system. While $P2X_7$

has almost always been considered a cytotoxic receptor, recently it has been proposed that it might have a hitherto unsuspected role in cell proliferation, and as such play a role in some hematopoietic tumors.[2,8] As regards $P2X_1$, this receptor has an established role in smooth muscle cell contraction, but it seems that it may also play an important role in platelet activation and the regulation of endothelial cell responses. $P2X_2/P2X_3$ heteromers are involved in sensory transduction and pain sensation. $P2X_4$ is expressed in both excitable and non-excitable cells, but its function is as yet ill defined. In cardiac muscle this receptor might mediate positive ionotropic responses. $P2X_5$ has been rather elusive until now, but very recent data suggest that it might have an important role in skeletal muscle differentiation.[3] $P2X_6$ is probably the P2XR with the most restricted distribution and the most elusive function. Preliminary data point to a possible role in cell adherence. In conclusion, ATP-gated ionotropic receptors are rapidly becoming a main focus of interest since they offer new insights into the molecular mechanisms involved in signal transduction and open intriguing perspectives for the development of new drugs.

References

1. Ralevic V, Burnstock G. Receptors for purines and pyrimidines. Pharmacol Rev 1998; 50:413-92.
2. Baricordi OR, Melchiorri L, Adinolfi E, Falzoni S, Chiozzi P, Buell G, et al. Increased proliferation rate of lymphoid cells transfected with the $P2X_7$ ATP receptor. J Biol Chem 1999; 274:33206-8.
3. Ryten M, Dunn PM, Neary JT, Burnstock G. ATP regulates the differentiation of mammalian skeletal muscle by activation of a $P2X_5$ receptor on satellite cells. J Cell Biol 2002; 158:345-55.
4. Kim M, Spelta V, Sim J, North RA, Surprenant A. Differential assembly of rat purinergic P2X7 receptor in immune cells of the brain and periphery. J Biol Chem 2001; 276:23262-7.
5. Kim M, Jiang LH, Wilson HL, North RA, Surprenant A. Proteomic and functional evidence for a P2X7 receptor signalling complex. EMBO J 2001; 20:6347-58.
6. Wilson HL, Wilson SA, Surprenant A, North RA. Epithelial membrane proteins induce membrane blebbing and interact with the $P2X_7$ receptor C terminus. J Biol Chem 2002; 277:34017-23.
7. Denlinger LC, Fisette PL, Sommer JA, Watters JJ, Prabhu U, Dubyak GR, et al. Cutting edge: the nucleotide receptor P2X7 contains multiple protein- and lipid-interaction motifs including a potential binding site for bacterial lipopolysaccharide. J Immunol 2001; 167:1871-6.
8. Adinolfi E, Melchiorri L, Falzoni S, Chiozzi P, Morelli A, Tieghi A, et al. $P2X_7$ receptor expression in evolutive and indolent forms of chronic B lymphocytic leukemia. Blood 2002; 99:706-8.

Interaction of ribose-modified nucleotides with P2Y receptors in platelets

Jacobson KA,* Xu B,* Cattaneo M,° Zighetti ML,° Stephens A,§ Kirschenheuter G,§ Greslin AF,§ Cheng X,§ Sennelo J,§ Kim SA,§ Kim HS,* Bischofberger N,§ Cook G§

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We have extensively explored the structure-activity relationships of nucleotide bisphosphate analogues as $P2Y_1$ receptor antagonists,[1,2] based on the initial discovery of receptor antagonism by A3P5P.[3] Modification of the ribose moiety of nucleotides and nucleosides has provided new insights into structural and conformational requirements for ligands at P2Y nucleotide receptors. The ability to constrain such analogs in a receptor-preferring conformation using the (N)-methanocarba ring system has aided the development of such antagonists (e.g. MRS 2279) as well as that of P2 receptor agonists.[1,2] In addition to introducing ribose ring constraints, we have released constraints of the ribose moiety in the form of acyclic analogs. A series of acyclic nucleotide derivatives (all bisphosphates, e.g. MRS 2298) were moderately potent $P2Y_1$ antagonists, demonstrating that the ribose moiety, or a cyclic substitute, was not required for antagonism of this receptor.[4] In fact, at least with respect to antagonists, the ribose appears to be nothing more than a spacer group, maintaining the preferred geometry of phosphate groups with respect to the adenine moiety. Using a high-resolution structure of rhodopsin as a template[5] and mutagenesis[6] we are examining detailed binding elements within purine receptors, with the long-range goal of identifying new leads in drug design. We have modeled the docking of nucleotide agonists and antagonists to $P2Y_1$ receptors[4,6] and have proposed a mode of overlay of multiple phosphate moieties. Activation by ADP of both $P2Y_1$ and $P2Y_{12}$ receptors in platelets contributes to platelet aggregation, and antagonists at these receptor subtypes have antithrombotic properties.[7-9] In an earlier publication we characterized the SAR as $P2Y_1$ receptor antagonists of acyclic analogs of adenine nucleotides, containing two phosphate groups on a symmetrically-branched aliphatic

chain, attached at the 9-position of adenine. In this study[10] we focused on anti-aggregatory effects of P2Y antagonists related to a 2-chloro-N6-methyladenine-9-(2-methylpropyl) scaffold, containing uncharged substitutions of the phosphate groups. For the known nucleotide (cyclic and acyclic) bisphosphate antagonists of $P2Y_1$ receptors, there was a significant correlation between inhibition of aggregation induced by 3.3 μM ADP in rat platelets and inhibition of $P2Y_1$ receptor-induced phospholipase C (PLC) activity previously determined in turkey erythrocytes.[4] Substitution of the phosphate groups with non-hydrolyzable phosphonate groups preserved platelet anti-aggregatory activity. Substitution of one of the phosphate groups with O-acyl greatly reduced the inhibitory potency, which tended to increase upon replacement of both phosphate moieties of the acyclic derivatives with uncharged (e.g. ester) groups. In the series of non-symmetrically-substituted monophosphates, the optimal antagonist potency occurred with the phenylcarbamate group (e.g. MRS 2401). Among symmetrical diester derivatives, the optimal antagonist potency occurred with the di(phenylacetyl) group (e.g. MRS 2412). MRS 2395, a dipivaloyl derivative, inhibited the ADP-induced aggregation of rat and human platelets, without affecting $P2Y_1$ receptor-induced PLC activity. MRS 2395 antagonized the ADP-induced inhibition of the cyclic AMP pathway in rat platelets. We propose that the uncharged derivatives are acting as antagonists of a parallel pro-aggregatory receptor present on platelets, i.e. the $P2Y_{12}$ receptor. Thus, different substitution of the same nucleoside scaffold can target either of two P2Y receptors in platelets.

References

1. Nandanan E, Jang SY, Moro S, Kim H, Siddiqui MA, Russ P, et al. Synthesis, biological activity, and molecular modeling of ribose-modified adenosine bisphosphate analogues as $P2Y_1$ receptor ligands. J Med Chem 2000; 43: 829-842.
2. Boyer J, Adams M, Ravi RG, Jacobson KA, Harden TK. 2-Chloro-N6-methyl-(N)-methanocarba-2'-deoxyadenosine-3,5-bisphosphate is a selective high affinity $P2Y_1$ receptor antagonist. Br J Pharmacol 2002; 135:2004-10.
3. Boyer JL, Romero-Avila T, Schachter JB, Harden TK. Identification of competitive antagonists of the P2Y1 receptor. Mol Pharmacol 1996; 50:1323-9.
4. Kim HS, Barak D, Harden TK, Boyer JL, Jacobson KA. Acyclic and cyclopropyl anlaogues of adenosine bisphosphate antagonists of the $P2Y_1$ receptor: structure activity relationships and receptor docking. J Med Chem 2001; 44:3092-108.
5. Palczewski K, Kumasaki T, Hori T, Behnke, CA, Motoshima H, Fox BA, et al. Crystal structure of rhodopsin: A G protein-coupled receptor. Science 2000; 289:739-45.
6. Moro S, Guo D, Camaioni E, Boyer JL, Harden TK, Jacobson KA. Human $P2Y_1$ receptor: Molecular modeling and site-directed mutagenesis as tools to identify agonist and antagonist recognition sites. J Med Chem 1998; 41: 1456-66.
7. Daniel JL, Dangelmaier C, Jin J, Ashby B, Smith JB, Kunapuli SP. Molecular basis for ADP-induced platelet activation. I. Evidence for three distinct ADP receptors on human platelets. J Biol Chem 1998; 273:2024-9.
8. Hechler B, Leon C, Vial C, Vigne P, Frelin C, Cazenave JP, et al. The $P2Y_1$ receptor is necessary for adenosine 5'-diphosphate-induced platelet aggregation. Blood 1998; 92:152-9.
9. Ingall AH, Dixon J, Bailey A, Coombs ME, Cox D, McInally JI, et al. Antagonists of the platelet P2T receptor: a novel approach to antithrombotic therapy. J Med Chem 1999; 42:213-20.
10. Xu B, Stephens A, Kirschenheuter G, Greslin A, Cheng X, Sennelo, et al. Acyclic analogs of adenosine bisphosphates as P2Y receptor antagonists: phosphate substitution leads to multiple pathways of inhibition of platelet aggregation. American Chemical Soc., 224th National Meeting, Boston, MA, USA, Aug. 20, 2002. Abstract MEDI 275.

Perspective in the use of P2 agonists / antagonists in clinical medicine

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During the past decade, the cloning and expression of seven P2X and eight P2Y receptors and the discovery of a myriad of effects triggered by extracellular nucleotides interacting with these receptors has provided a vast potential for development of novel therapeutic approaches for treatment of human diseases. One of the most advanced therapeutic areas targeting P2 receptor systems is antithrombosis where two approved drugs; Ticlopidine and Clopidogrel are available in the clinic. These products are pro-drugs that irreversibly inhibit one of the platelet ADP receptors, the $P2Y_{12}$ receptor. Intense research and development of reversible antagonists of platelet $P2Y_1$ and $P2Y_{12}$ receptors as antithrombotic agents is ongoing. Other therapeutic applications currently in clinical and advanced preclinical studies include treatment of dry eye disease ($P2Y_2$ agonist), cystic fibrosis ($P2Y_2$ agonist), allergic rhinitis ($P2Y_2$ agonist) chronic bronchitis ($P2Y_2$ agonist), pain (P2X2/3 antagonist), retinal detachment ($P2Y_2$ agonist) diabetes ($P2Y_2$ agonist), cancer, etc. Most of the inves-

tigational drugs being tested in clinical trials in humans are nucleotides or nucleotide analogs. Among the most important challenges facing the drug industry to aid in the understanding of the full therapeutic potential of P2 receptors are the discovery and development of receptor selective non-nucleotide pharmacophores for P2 receptors and the development of an integrated view of the complex release, metabolism and signaling properties of extracellular nucleotides.

MINISYMPOSIUM
Treatment and Prophylaxis of Bleeding Episodes in Patients with Defects of Platelet Function

Platelet transfusion

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Bleeding in a number of acute and chronic conditions that require medical and surgical treatment may be prevented and treated with platelet transfusion. The purpose of this intervention is to compensate insufficient production of platelets or correct defective platelet function. A detailed set of guidelines for the clinical use of platelet concentrates (PC) has been recently published by the *American Society of Clinical Oncology*.[1] These guidelines can be downloaded from the URL: *http://www.asco.com*.

PC may be obtained from whole blood collected into a multiple plastic bag set. The most popular procedures for the preparation of PC from whole blood donations are the platelet-rich plasma (PRP) and the buffy-coat (BC) methods. In the former, mostly used in the USA, whole blood is centrifuged at low speed, the PRP is transferred to a satellite bag, which is then centrifuged at high speed. The platelet button is left undisturbed in 50-70 mL plasma to favor disaggregation. Finally, platelets are resuspended and stored at 20-24°C under continuous gentle agitation for a maximum of 5 days. In the latter method, most popular in Europe, whole blood units are first centrifuged at high speed in order to concentrate most white cells and platelets into the BC layer formed at the interface between red cells and plasma. BC can be further processed into PC as individual units or as a pool of BC diluted in autologous plasma or in crystalloid media. Both options involve low speed centrifugation and transfer of the supernatant PC into an appropriate bag. Platelets may also be prepared by apheresis, the automated process of blood collection, on line centrifugation and return to the donor of the unwanted cells and plasma. PC may undergo leukoreduction to prevent significant side effects such as anti-HLA alloimmunization,[2,3] transmission of leukotropic viruses[4,5] or non-hemolytic febrile transfusion reactions.[2] To prevent transfusion-associated graft-versus-host disease in susceptible recipients, these products must be irradiated.[6] Good quality platelets obtained from whole blood and from apheresis show similar clinical effectiveness. The use of platelets to prevent hemorrhage is called the *prophylactic approach* while their use to *treat* the actual bleeding episodes is termed the *therapeutic approach*. The choice to transfuse platelets prophylactically stems from several studies showing a decrease in the incidence of hemorrhagic deaths in leukemic patients following this policy. The platelet count above which platelet transfusion is not necessary is termed the *platelet transfusion trigger*. Recently, in stable oncohematologic recipients the prophylactic trigger of platelet transfusion has been decreased from the traditional level of 20,000/μL to 10,000/μL.[7,8] Transfusion at higher levels may be necessary in patients with hemorrhage, fever, infection, or a large spleen, and in those receiving treatment with drugs affecting platelet function (the so called *detrimental factors*), or undergoing surgical treatment. The effectiveness of prophylactic platelet transfusion is determined by the evaluation of the corrected count increment (CCI), i.e. the post transfusion platelet count increment divided by the number of platelets transfused and multiplied by the patient's body surface area. The CCI can be determined 10-60 minutes and 20-24 hours after the transfusion. Platelet transfusion is considered effective when it is associated with a CCI ≥ 7,500/μL at 1 hour or 4,500/μL at 20-24 hours.

About 15% of chronic recipients of platelets become refractory to platelets from random donors, i.e. they present repeated CCI at 1 and 24 hours below the above values. This is mainly due to the development of antibodies against antigens of the human leukocyte antigen (HLA) system, which are present on the platelet membrane. Refractoriness may cause longer hospital stay and higher costs in addition to having clinical implications for the

patient's safety.[9] In fact, because of the lack of adequate post-transfusion platelet count increments, these patients tend to be repeatedly and ineffectively transfused. In our institution we have chosen to try to overcome refractoriness by random donor platelet cross matching after having experienced difficulties with a previous strategy based on the use of HLA typed platelets. This policy was associated with effective transfusions in approximately 50% of occasions. In disorders of platelet function such as Glanzmann's thrombasthenia, Bernard-Soulier, gray platelet and Scott syndromes and storage pool disease, other interventions, including the use of DDAVP, corticosteroids and antifibrinolytic agents, might be necessary.[10] Unfortunately, in these conditions specific triggers for platelet transfusion cannot be easily identified because platelet count may be normal. Therefore, in these cases careful observation of the clinical development of hemorrhage is of utmost importance and the ultimate target is to restore sufficient platelet function. The lack or incomplete expression of specific glycoproteins in some platelet function disorders has raised the concern that the administration of normal platelets could trigger the development of antibodies capable of decreasing the efficacy of future platelet transfusions. However, this seems quite uncommon and is not considered a reason to withhold platelet support. In conclusion, use of good quality products, regular monitoring og patients and close cooperation between the clinical staff and the transfusion service are the cornerstones of effective platelet transfusion.

References

1. Schiffer CA, Anderson KC, Bennett CL, Bernstein S, Elting LS, Goldsmith M, et al. Platelet transfusion for patients with cancer: clinical practice guidelines of the American Society of Clinical Oncology. J Clin Oncol 2001; 19:1519-38.
2. Lane TA. Leukocyte reduction of cellular blood components. Effectiveness, benefits, quality control, and costs. Arch Pathol Lab Med 1994; 118:392-404.
3. Heddle NM. The efficacy of leukodepletion to improve platelet transfusion response: a critical appraisal of clinical studies. Transfus Med Rev 1994; 8:15-28.
4. Bowden RA, Slichter SJ, Sayers M, Weisdorf D, Cays M, Schoch G, et al. A comparison of filtered leukocyte-reduced and cytomegalovirus (CMV) seronegative blood products for the prevention of transfusion-associated CMV infection after marrow transplant. Blood 1995; 86:3598-603.
5. Okochi K, Sato H. Transmission of adult T-cell leukemia virus (HTLV-1) through blood transfusion and its prevention. AIDS Res 1996; 2 Suppl 1:S157-S61.
6. Anderson KC, Goodnough LT, Sayers M, Pisciotto PT, Kurtz SR, Lane TA, et al. Variation in blood component irradiation practice: implications for prevention of transfusion-associated graft-versus-host disease. Blood 1991; 77:2096-102.
7. Rebulla P, Finazzi G, Marangoni F, Avvisati G, Gugliotta L, Tognoni G, et al. The threshold for prophylactic platelet transfusions in adults with acute myeloid leukemia. Gruppo Italiano Malattie Ematologiche Maligne dell'Adulto. N Engl J Med 1997; 337:1870-5.
8. Wandt H, Frank M, Ehninger G, Schneider C, Brack N, Daoud A, et al. Safety and cost effectiveness of a 10 × 10^9/L trigger for prophylactic platelet transfusions compared with the traditional 20 × 10^9/L trigger: a prospective comparative trial in 105 patients with acute myeloid leukemia. Blood 1998; 91:3601-6.
9. Meehan KR, Matias CO, Rathore SS, Sandler SG, Kallich J, LaBrecque J, et al. Platelet transfusions: utilization and associated costs in a tertiary care hospital. Am J Hematol 2000; 64:251-6.
10. Norfolk DR, Seghatchian J. Pharmacological therapies to minimise platelet transfusion. Transfus Sci 2000; 22: 149-53.

Recombinant activated factor VII for treatment and prophylaxis of bleeding in patients with platelet function defects

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Recombinant activated factor VII (rFVIIa) (Novoseven®, Novo Nordisk) is becoming an attractive alternative in the treatment of bleeding episodes and in the hemostatic coverage for invasive procedures in patients with functional platelet disorders. In this setting, the transfusion of platelet concentrates was the standard approach; however, repeated transfusions may produce anti-HLA or anti-glycoprotein IIb-IIIa alloimmunization, resulting in ineffectiveness future treatments, and also carry the risks related to blood products, including blood-borne virus infections. High-dose FVIIa is thought to be able to activate factors IX and X on the platelet surface also in the absence of tissue factor, thus enabling thrombin generation; thrombin is a strong signal for platelet activation and recruitment of other platelets. Therefore, enhanced thrombin generation and fibrin formation by high-dose FVIIa might provide an alternative mechanism for bypassing the functional defects in platelet disorders. Moreover, as shown in *in vitro* thrombocytopenia models, by increasing initial thrombin generation, fewer platelets are required to achieve effective hemostasis. Since the first report in 1996 of its use in a child with Glanzmann's thrombasthenia (GT) with severe nose bleeding, rFVIIa has been successfully administered to patients with

both congenital (GT, Bernard-Soulier syndrome, platelet-type von Willebrand's disease) and acquired (uremia, myelodysplastic syndromes) platelet disorders. However, in the reported cases, treatment protocols are quite heterogeneous, with respect to dosages, duration and modalities of administration (bolus or continuous infusion-CI), number and interval of bolus injections, associated treatments (antifibrinolytics, platelet transfusions), and parameters for monitoring treatment and evaluating efficacy. The relative rarity of these diseases hamper collection of sufficient data by a single Institution or country to define the optimal treatment modalities and assess its efficacy and safety; therefore, over the last years an *International Registry on rFVIIa and Congenital Platelet Disorders* has been established to collect clinical records on the use of rFVIIa in this setting. According to the last report of this Registry, data concerning 32 patients, most with GT, (aged <1 to 72 years, about half of them <15 years and with circulating platelet alloantibodies) and 57 bleeding episodes (2/3 of them nose or mouth bleedings) have been analyzed. In 95% of cases antifibrinolytics were associated and in 42% red cell transfusion were needed. After excluding 2 episodes, not evaluable because of concomitant platelet transfusion, successful rFVIIa use was recorded in 67% of cases, with a median number of doses of 3 (range 1-14), in all but 3 cases >85 μg/kg. Two recurrences within 48 hours after cessation of bleeding have been recorded, one of which stopping after additional rFVIIa injections. Of 15 failures (27.3%), 4 cases of gastrointestinal (GI) hemorrhage were included; these episodes were 44.4% of all GI bleedings reported (4/9), whereas the failure rate in non-GI-episodes was 22.9% (11/48). However, in most failures, doses <80 μg/kg or CI or a low number of doses (1-3) before alternative treatments, were employed. rFVIIa has been also used in 13 invasive procedures, in all cases with antifibrinolytics, either as bolus injections (75-120 μg/kg, 1-33 doses, median 5) or in CI (9-30 μg/kg/hr, 2.7-16 days), with success in 9 cases and failures in 2 (another 2 cases are not evaluable because of concomitant platelet transfusions). Only one severe adverse event was reported (a thromboembolism in an old woman who had undergone intestinal resection, 6 days after a prolonged CI with high rFVII dose). In conclusion, although the data are not sufficient for definitive recommendations, rFVIIa administered as bolus injections >85 μg/kg every 2 hours, together with antifibrinolytics, seems a safe and successful alternative to platelet transfusions in the treatment and prophylaxis of bleeding in these patients, especially in those with platelet alloantibodies or when local measures are ineffective. Our experience in a young woman with a severe bleeding tendency, who required surgery for an ovarian cyst, supports this approach.

Selected references

1. d'Oiron R, Menart C, Trzeciak MC, Nurden P, Fressinaud E, Dreyfus M, et al. Use of recombinant factor VIIa in 3 patients with inherited type I Glanzmann's thrombasthenia undergoing invasive procedures. Thromb Haemost 2000; 83:644-7.
2. Kjalke M, Johannesen M, Hedner U. Effect of recombinant factor VIIa (NovoSeven") on thrombocytopenia-like conditions in vitro. Semin Hematol 2001; 38 Suppl 12:15-20.
3. Monroe DM, Hoffman M, Allen GA, Roberts HR. The factor VII-platelet interplay: effectiveness of recombinant factor VIIa in the treatment of bleeding in severe thrombocytopathia. Semin Thromb Hemost 2000; 26:373-7.
4. Poon MC, Demers C, Jobin F, Wu JW. Recombinant factor VIIa is effective for bleeding and surgery in patients with Glanzmann thrombasthenia. Blood 1999; 94:3951-3.
5. Poon M-C, d'Oiron R. Recombinant activated factor VII (NovoSeven®) treatment of platelet-related bleeding disorders. Blood Coagul Fibrinolysis 2000; 11 Suppl 1:55-68.
6. Poon M-C, Katsarou O, Huth-Kuehne A, et al. Recombinant factor VIIa in congenital platelet bleeding disorders. Blood 2000; 96 Suppl 1:256a[abstract].
7. Poon M-C, d'Oiron R, Hann I, et al. Use of recombinant factor VIIa (Novoseven") in patients with Glanzmann's thrombasthenia. Semin Hematol 2001; 38 Suppl 12:21-5.
8. Tengborn L, Peturson B. A patient with Glanzmann's thrombasthenia and epistaxis successfully treated with recombinant factor VIIa. Thromb Haemost 1996; 75:981-2.

Desmopressin (DDAVP)

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Desmopressin (1-deamino-8-D-arginine vasopressin, DDAVP) is a synthetic analog of the antidiuretic hormone vasopressin. Like the natural antidiuretic hormone, desmopressin increases the plasma levels of factor VIII and von Willebrand factor (VWF), with the advantage, compared to vasopressin, that it produces little or no vasoconstriction, no increase in blood pressure, and no contraction of the uterus or gastrointestinal tract, so that it is well tolerated when administered to humans.[1] In 1977, desmopressin was used for the first time in patients with mild hemophilia A and von Willebrand's disease (VWD) for the prevention and treatment of bleeding.

The clinical indications for desmopressin quickly expanded beyond hemophilia and VWD. The compound was shown to be efficacious even in bleeding disorders not involving a deficiency or dysfunction of factor VIII or VWF, including congenital and acquired defects of platelet function and such frequent abnormalities of hemostasis as those associated with chronic kidney and liver diseases. Desmopressin has also been used prophylactically in patients undergoing surgical operations characterized by large blood loss and transfusion requirements. Some of these indications have been strengthened by the experience accumulated; others have not been supported by rigorous clinical trials or have been superceded by the advent of more efficacious treatments. *Desmopressin in the treatment of congenital disorders of platelet function*. Desmopressin shortens or normalizes the bleeding time of most patients with congenital defects of platelet function.[1] There is usually a good response in patients *with defects of the release reaction* and in those with *isolated and unexplained prolongations of the bleeding time*. Most patients with *storage pool deficiency* respond to desmopressin but a few do not, in particular those with severe deficiencies of platelet δ-granule content. Negative results have been reported in most patients with *Glanzmann's thrombasthenia*. The documented efficacy in patients with *Bernard-Soulier syndrome*, who lack the GPIb-IX-V complex, the platelet receptor for VWF that is essential for platelet adhesion to the vessel wall at high shear, supports the contention that desmopressin can shorten the prolonged bleeding time through mechanism(s) that are independent of released VWF (see later). Whether the effect on a laboratory test such as the bleeding time corresponds to a hemostatic effect is not well established. Although there are anecdotal reports of desmopressin successfully stopping or preventing bleeding in these patients, a clinical trial is necessary to determine the clinical efficacy of desmopressin. The mechanisms by which desmopressin induces shortening of the bleeding time in patients with normal factor VIII/VWF are unclear.

1) *Released VWF; a biologically plausible, but as yet unproven, mediator*. It is biologically plausible that the favorable effects of the compound may be mediated by increased platelet adhesion to the vessel wall[2] due not only to the rise of plasma VWF but also to the abluminal secretion of the protein toward the subendothelium[3] and to the fresh appearance in plasma of ultralarge VWF multimers.[4] These ultralarge VWF multimers are hemostatically very effective because they support platelet adhesion to the vascular subendothelium to a higher degree than other VWF multimers and induce platelet aggregation under conditions of high shear.[5] In fact, it has been shown that infusion of desmopressin improves the formation of platelet aggregates that form at the high shear rate levels that can be found in the microcirculation. This effect of desmopressin can be observed not only in patients with type 1 VWD, but also in patients with normal VWF but impairment of platelet aggregation at high shear due to congenital or drug-induced abnormalities of the secretory mechanisms or of the interaction of released ADP with its platelet receptors.[6-8] The improvement of platelet aggregation at high shear after desmopressin administration to these patients correlated with the shortening of the bleeding time and the increase in the plasma levels of VWF with ultralarge multimers, suggesting that these changes in VWF could indeed be responsible, at least partly, for the observed effects of desmopressin on primary hemostasis.

2) Mechanisms independent of released VWF: proven, but as yet uncharacterized. If the potentiation of platelet function that is mediated by desmopressin-induced release of VWF is biologically plausible, there is no direct evidence that it is responsible for the effects of the drug observed *in vivo*. In contrast, clear and direct evidence exists that other, as yet unknown, mechanisms are operating *in vivo*. In 1987, it was shown that desmopressin infusion in patients with type 3 VWD further shortened their prolonged bleeding times, which had been partially corrected by the administration of cryoprecipitate.[9] Since type 3 VWD patients lack VWF in tissue stores, the effect of desmopressin on their bleeding time was not associated with an increase in plasma VWF levels or the appearance of ultralarge VWF multimers. These results unequivocally indicated that the drug can affect primary hemostasis independently of released VWF. Subsequent studies in rabbits, who do not respond to desmopressin infusion with an increase in the plasma levels of factor VIII and VWF, gave further support to the concept that the drug can affect primary hemostasis independently of released VWF. In fact, in rabbits whose bleeding times had been prolonged by combined treatment with aspirin and the thrombolytic streptokinase, desmopressin infusion shortened the prolonged bleeding times without increasing the plasma levels of VWF.[10] Another, more indirect demonstration of a released VWF-independent mechanism comes from the finding that desmo-

pressin shortens the prolonged bleeding times of patients with Bernard-Soulier syndrome, who lack GPIb, the platelet receptor for VWF which is essential for platelet adhesion and activation at high shear. Effects of desmopressin on the platelet count or on agonist-induced platelet aggregation have been ruled out by many studies.[1] Several putative mechanisms or mediators have been proposed, but their role is uncertain.

Conclusions. Desmopressin is efficacious in mild hemophilia and type 1 VWD and usually permits the avoidance of factor concentrates, with significant reductions in costs and the risk of transmitting blood-borne viral diseases. Desmopressin has been used successfully to prevent or stop bleeding. In patients with defects of primary hemostasis not associated with abnormalities of VWF. However, there is still no well-designed clinical trial that truly shows efficacy of the compound in these conditions.

References

1. Mannucci PM. Desmopressin (DDAVP) in the treatment of bleeding disorders: the first 20 years. Blood 1997; 90: 2515-21.
2. Sakariassen KS, Cattaneo M, van den Berg A, Ruggeri ZM, Mannucci PM, Sixma JJ. DDAVP enhances platelet adherence and platelet aggregate growth on human artery subendothelium. Blood 1984; 64:229-36.
3. Takeuchi M, Naguza H, Kanedu T. DDAVP and epinephrine induce changes in the localization of von Willebrand factor antigen in endothelial cells of human oral mucosa. Blood 1981; 72:850.
4. Ruggeri ZM, Mannucci PM, Lombardi R, Federici AB, Zimmerman TS. Multimeric composition of factor VIII/von Willebrand factor following administration of DDAVP: Implications for pathophysiology and therapy of von Willebrand's disease subtypes. Blood 1982; 58:1272-8.
5. Moake JL, Turner NA, Stathopoulos NA, Nolasco LH, Hellum JD. Involvement of large plasma von Willebrand factor (vWF) multimers and unusually large VWF forms derived from endothelial cells in shear-stress induced platelet aggregation. J Clin Invest 1986; 78:1456-61.
6. Cattaneo M, Lombardi R, Bettega D, Lecchi A, Mannucci PM. Shear-induced platelet aggregation is potentiated by desmopressin and inhibited by ticlopidine. Arterioscler Thromb 1993; 13:393-7.
7. Cattaneo M, Zighetti ML, Lombardi R, Mannucci PM. Role of ADP in platelet aggregation at high shear: studies in a patient with congenital defect of platelet responses to ADP. Br J Haematol 1994; 88:826-9.
8. Cattaneo M, Pareti FI, Zighetti M, Lecchi A, Lombardi R, Mannucci PM. Platelet aggregation at high shear is impaired in patients with congenital defects of platelet secretion and is corrected by DDAVP: correlation with the bleeding time. J Lab Clin Med 1995; 125:540-7.
9. Cattaneo M, Moia M, Della Valle P, Castellana P, Mannucci PM. DDAVP shortens the prolonged bleeding times of patients with severe von Willebrand disease treated with cryoprecipitate. Evidence for a mechanism of action independent of released von Willebrand factor. Blood 1989; 74:1972-5.
10. Johnstone MT, Andrews T, Ware JA, Rudd MA, George D, Weinstein M, et al. Bleeding time prolongation with streptokinase and its reduction with 1-deamino-8-D-arginine vasopressin. Circulation 1990, 82:2142-51.

SESSION 2

The molecular basis of platelet secretion: the role of septins

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Studies are presented characterizing platelet CDCrel-1, a protein expressed to high levels by megakaryocytes and belonging to a conserved family of proteins, termed septins. Septin filaments were originally identified in yeast as being essential for budding. For years, the presence of septins in higher eukaryotic cells was dismissed since budding, or asymmetric cell division, is a process unique to yeast. However, recent studies have demonstrated that septins do exist in higher eukaryotes and, in those cases examined, are commonly associated with events in which dynamic membrane movement or cytoplasmic partitioning, such as in cytokinesis or vesicle trafficking, occurs. The physiological relevance of platelet CDCrel-1 will be presented, describing a variety of biochemical methods and characterization of platelet function in animals deficient in CDCrel-1. Biochemical characterization of platelet CDCrel-1 is presented including *in situ* localization, purification with a variety of associated proteins and the generation of targeted deletion in the mouse CDCrel-1 gene. Platelet function in CDCrel-1^{Null} platelets is presented. The immunopurification of CDCrel-1 revealed it to be part of a macromolecular complex containing a protein involved in platelet secretion, syntaxin 4. Moreover, CDCrel-1 was localized *in situ* to areas surrounding platelet storage granules. The relevance of CDCrel-1 in platelet secretion was established with the characterization of platelets from a CDCrel-1^{Null} mouse. As compared to platelets from wild-type littermates, CDCrel-1^{Null} platelets aggregate and release stored ^{14}C-serotonin in the presence of subthreshold levels of col-

lagen. These results provide new insights into the mechanisms regulating platelet secretion and identify platelet septins as a previously unrecognized protein family contributing to membrane trafficking within the megakaryocyte and platelet.

Oral Communications

IMMUNOLOCALIZATION OF $P2Y_1$ AND TPα RECEPTORS IN PLATELETS SHOWED LARGE INTERNAL POOLS

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$P2Y_1$ and TPα receptors on platelets belong to the G-protein-coupled seven transmembrane domain family. They transmit signals for platelet shape change, mobilization of calcium, and platelet aggregation. Aim of this study was to examine their distribution in platelets and to test the effect of platelet activation and desensitization on their partition between surface and internal membrane pools. Immunogold labeling on platelet sections with a monoclonal antibody to the amino-terminal domain of $P2Y_1$ and a polyclonal antibody to the C-terminal domain of TPα revealed that while present at the platelet surface, both receptors were abundantly represented in the internal compartment. Specifically, receptors were found in the membranes of α-granules and those of thin channels of the open-canalicular system. Activation of platelets by ADP and I-BOP (a TXA_2 analog) increased the labeling of $P2Y_1$ and TPα at the surface. However, as gold particle density was also increased intracellularly, activation may have resulted in increased antibody accessibility to the receptor. A return to a platelet discoid shape, and to basal values of labeling, characterized desensitization of each receptor. Platelets lacking the $P2Y_{12}$ ADP receptor normally expressed $P2Y_1$ and TPα, and changes in their distribution during platelet activation and after prolonged incubation with ADP were as for the control. The presence of so many receptors inside platelets suggests the potential for their activation by internally mobilized ADP or newly formed TXA_2 during thrombus formation and independently of the surface pool. Pharmacological antagonism of ADP or TXA_2 receptors in antithrombotic therapy may need to take this into account.

DIFFERENTIAL REGULATION AND RELOCALIZATION OF PLATELET P2 RECEPTORS AFTER LONG-TERM ACTIVATION

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Platelets activated by ADP become refractory to restimulation with ADP. This phenomenon called the *refractory state*, has been shown to be due to selective desensitization of the $P2Y_1$ receptor, while the $P2Y_{12}$ receptor remains functional.[1] The aim of the present study was to investigate the intracellular distribution of $P2Y_1$ and $P2Y_{12}$ receptors after ADP-induced desensitization. The fate of the ADP receptors was followed in 1321N1 cells expressing the $P2Y_1$ or $P2Y_{12}$ receptor coupled to green fluorescent protein (eGFP) and in platelets using selective anti-$P2Y_1$ or -$P2Y_{12}$ antibodies and electron microscopy, after treatment with ADPbS (1 mM) or 2MeSADP (1 mM). Both reagents induced desensitization of the calcium mobilization mediated by the $P2Y_1$-eGFP receptor, whereas the $P2Y_{12}$-eGFP receptor remained responsive to ADP as demonstrated by maintenance of the inhibition of cAMP production. Confocal fluorescence microscopy revealed that the $P2Y_1$-eGFP receptor underwent time-dependent and clathrin-dependent internalization after treatment with the ADP analogs, while the $P2Y_{12}$-eGFP receptor remained localised at the plasma membrane. In resting platelets, immunogold staining of the $P2Y_1$ and $P2Y_{12}$ receptors was mainly observed at the cell surface (58 and 65%, respectively) and weakly in the granules (14 and 22%, respectively). After treatment with ADPβS, the $P2Y_1$ receptor disappeared from the plasma membrane and its granule labeling increased strongly (46%), but the distribution of the $P2Y_{12}$ receptor remained unchanged. Similar results were obtained using 2MeSADP. In conclusion, these findings confirm previous observations that platelet refractoriness to ADP is entirely due to $P2Y_1$ desensitization and demonstrate that the native or recombinant $P2Y_1$ and $P2Y_{12}$ receptors are differentially regulated during ADP-induced activation, the $P2Y_1$ receptor being desensitized and internalized, whereas the $P2Y_{12}$ receptor is not.

1. Baurand A, Eckly A, Bari N, Leon C, Hechler B, Cazenave JP. Desensitization of the platelet aggregation response to ADP: differential down-regulation of the $P2Y_1$ and P2cyc receptors et al. Thromb Haemost 2000; 84:484-91.

RAC ACTIVATION DOWNSTREAM OF THE ADP RECEPTORS IN BLOOD PLATELETS

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The actin cytoskeleton reorganization plays a critical role in the regulation of platelet responses such as shape change, secretion and aggregation. The Rho GTPase family (Rho, Rac, Cdc42), highly expressed in platelets, is thought to play a major role in the control of these processes. Although ADP is a weak aggregating agent *per se*, it is a necessary co-activator, through its $P2Y_{12}$ receptor, for normal activation of platelets by other physiological agonists *in vivo* and *in vitro*. For instance, secreted ADP is an important co-factor of platelet activation by TRAP (PAR-1 thrombin receptor activating peptide) or through cross-linking of the FcγRIIa receptor *in vitro*. The activation of the Rho GTPase family downstream of the ADP receptors $P2Y_1$, $P2Y_{12}$ or $P2X_1$ has not been characterized yet. Aims of this study were: characterization of 1) ADP-mediated activation of Rac in platelets from knock-out mice lacking $P2Y_1$, $P2X_1$ or Gαq and 2) the impact of ADP as a co-activator of Rac by TRAP and FcγRIIa in human platelets. We used a pull-down assay with GST-PAK1 recombinant protein to visualize Rac-GTP, the activated form of Rac. The $P2Y_1$ receptor and its downstream effector Gq are essential for ADP-dependent Rac activation, whereas $P2Y_{12}$ and $P2X_1$ are not involved as shown with platelets from knock-out mice and in human platelets by using selective antagonists. Using the selective antagonist ARC69931MX, we showed that ADP, via $P2Y_{12}$, potentiates Rac activation in human platelets stimulated by TRAP or FcγRIIa clustering. Furthermore, Rac activation does not require GPIIb/IIIa engagement as evidenced using platelets from patients with Glanzmann's thrombasthenia or a GPIIb/IIIa blocking antibody in mouse platelets. The platelet P2 receptors play different roles in regulating Rac activation in blood platelets, the $P2Y_1$ receptor being necessary for ADP-induced Rac activation, the $P2Y_{12}$ receptor enhancing the response to other agonists and the $P2X_1$ having no detectable role.

CRUCIAL ROLE OF THE ADP RECEPTOR $P2Y_1$ IN PLATELET ADHESION AND SIGNALING UNDER HIGH FLOW

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ADP plays a crucial role in regulating platelet function through the activation of two major receptors, $P2Y_1$ and $P2Y_{12}$. $P2Y_1$ initiates platelet shape change and aggregation through the mobilization of internal calcium stores; $P2Y_{12}$, coupled to adenylyl cyclase inhibition, is essential for the full aggregation response to ADP and the stabilization of aggregates. We have recently identified the sequential cytoplasmic calcium signals in a two stage platelet activation process induced by the glycoprotein Ibα mechanoreceptor in a shear field: A Ca^{++} release from intracellular stores (type α/β peaks) that precedes stationary platelet adhesion, and type γ peaks that occur once adhesion is established. In the present study we investigated the role of $P2Y_1$ and $P2Y_{12}$ in platelet adhesion and signaling under flow using a parallel flow chamber. Fluo-3 AM loaded platelets reconstituted in whole blood were perfused over a VWF coated surface at wall shear rate of 3000 s^{-1} for 90 seconds. We first measured the surface distribution of single platelets and the formation of aggregates at different platelet counts, and found that a $P2Y_1$ antagonist (MRS2279) prevented the formation of platelet microaggregates, while a $P2Y_{12}$ antagonist (AR-C69931MX) had a slight inhibitory effect only on larger aggregates >200 μm^2. Analysis of the dynamics of individual platelets showed that the $P2Y_1$ antagonist increased the translocation velocity (5.5±0.7 μm/s compared to 1.9±0.3 of the control) and decreased the arrest time (1.2±0.8 s compared to 5.9±2.8 s of the control). In contrast, no significant effect on these parameters was observed using the P2Y12 antagonist. We then observed a distinct inhibition of $[Ca^{++}]_i$ elevation with the two ADP receptor antagonists. Blocking P2Y1 slightly inhibited the number of platelets showing α/β peaks (14.6±1.5% compared to 20.6±1.6% of the control, $p<0.01$) and the alfa peak Ca^{++} concentration (1,037±322 nM compared to 1,543±312 nM of the control, $p<0.05$), while it completely abolished γ peaks. The $P2Y_{12}$ antagonist had no inhibitory effect on either α–β or γ peaks. Our results demonstrate that $P2Y_1$ is the ADP receptor involved in the initial stages of platelet

adhesion and activation onto immobilized VWF. This receptor has a key role in the mechanism leading to the arrest of platelets on immobilized VWF under high shear stress, as it influences both the initial Ca^{++} release from intracellular stores (type α/β peaks) and the subsequent type γ peaks that depend on a transmembrane Ca^{++} flux and occur after stable platelet adhesion.

PRIMORDIAL ROLE OF THE $P2Y_{12}$ ADP RECEPTOR IN DIFFERENT PHASES OF PLATELET-COLLAGEN INTERACTIONS

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Adenosine diphosphate (ADP) plays a pivotal role in platelet aggregation induced by collagen, but the importance of the distinct ADP receptors expected to interact with released ADP during the process of platelet–collagen interactions, i.e. contact, spreading, and subsequent aggregation, remains to be better defined. In this work, the role of the two G-protein-coupled ADP receptors, $P2Y_1$ and $P2Y_{12}$, was studied during platelet deposition to immobilized fibrillar type I and type III collagen and to the KOGEOGPK peptide representing a platelet primary binding domain in type III collagen. Blocking $P2Y_{12}$ with the AR-C69931MX antagonist inhibited platelet deposition by about 75% for both types of collagen whereas blocking $P2Y_1$ with A3P5P or A2P5P inhibited platelet deposition maximally by 40-50%. Platelet deposition to the octapeptide was only inhibited by 26% in the presence of AR-C69931MX and not by A3P5P. In all cases, the platelet release of serotonin was only significantly decreased in the presence of AR-C69931MX. Whereas the blockage of P2Y1 only inhibited the recruitment of additional platelets onto the adherent platelet monolayer as shown by scanning electron microscopy, $P2Y_{12}$ played a crucial role both in platelet adhesion and recruitment. These results demonstrate the primordial role of $P2Y_{12}$ receptors during platelet deposition to fibrillar collagens. It is proposed that the binding of ADP, secreted from dense granules, to these $P2Y_{12}$ receptors during primary adhesion (contact) to collagen will potentiate dense granule secretion, platelet adhesion and recruitment of additional platelets onto the surface of adherent platelets.

SESSION 3

Ca^{2+}-influx via the platelet ion channel $P2X_1$ contributes to collagen-induced platelet activation

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Rapidly increasing knowledge on the mode of action of P2X ion channels in various tissues and organs has uncovered the critical role that these seven nucleotide-operated channels play in several physiological processes. Megakaryocytes and platelets express only one member of this family, $P2X_1$, the function of which is controversial in hemostasis. *Ex vivo*, platelet $P2X_1$ rapidly loses its function via nucleotide-induced desensitization, unless protected from inactivation by apyrase. Yet, because apyrase degrades ATP and ADP, this treatment in itself further complicates correct analysis of platelet function, in view of the critical role that ADP plays in platelet amplification reactions. In order to circumvent this dilemma involved in *in vitro* analysis of $P2X_1$, we have generated transgenic mice, overexpressing human $P2X_1$ in a platelet-specific fashion. When studied *ex vivo*, in the presence of physiological Ca^{2+}-levels and under apyrase protection, the combined analysis of human platelets and of these transgenic murine platelets has enabled us to conclude that $P2X_1$ plays a role in the control of selected pathways of platelet activation. Thus, its activation by specific ligands such as α,β-meATP triggers rapid Ca^{2+}-influx,[1] in its turn causing a rapid transient platelet shape change,[2] associated with some intracellular reorganization of the secretory dense granules. This Ca^{2+}-influx triggers the specific phosphorylation of at least two enzyme systems. The Ca^{2+}/calmodulin-dependent myosin light chain kinase is activated, resulting in myosin light chain phosphorylation within 2-5 seconds, thus constituting the basis for the rapid, reversible platelet shape change. At the same time, a protein kinase C is activated, triggering phosphorylation reactions via the ERK1/2 MAPK pathway. The stable ATP analog α,β-meATP, but not ADP, is capable of triggering ERK2 phosphorylation, in agreement with the concept that ADP and ATP activate differ-

ent receptors.[3,4] It, therefore, seems that P2Y signal transduction mediated via ADP does not include ERK2 phosphorylation. Although the selective stimulation of $P2X_1$ and the resulting ERK2 phosphorylation by themselves are insufficient to cause platelet secretion and platelet aggregation, the $P2X_1$ mediated Ca^{2+}-influx appeared to contribute to the collagen-induced platelet activation.[5] When platelets are exposed to low concentrations of collagen, ATP is rapidly released and activates the $P2X_1$ ion channel within 1 min. The resulting ERK2 phosphorylation then contributes to further dense granule release and amplifies ongoing platelet activation. Inhibition studies involving EGTA addition, prior $P2X_1$ desensitization with α,β-meATP or antagonism with ADP, as well as the use of specific inhibitors of ERK2 phosphorylation and of PKC activity, have confirmed a mechanism in which the $P2X_1$ mediated Ca^{2+}-influx is the exclusive trigger of the low-dose collagen-associated ERK2 phosphorylation.[6,7] $P2X_1$ contributes to the observed ERK2 phosphorylation via its activation of Ca^{2+}/calmodulin. Inhibition of this protein not only blocks the α,β-meATP mediated platelet shape change, but it also prevents the phosphorylation of ERK2. The *ex vivo* analysis of the collagen-induced aggregation of murine platelets overexpressing human $P2X_1$ has confirmed this concept: under conditions in which transgenic $P2X_1$ is active, enhanced platelet responses to collagen are observed, associated with increased ERK2 phosphorylation. Under non-protective conditions, i.e. when neither murine nor transgenic $P2X_1$ is preserved during platelet preparation, the response of the isolated platelets to collagen is not enhanced. The *ex vivo* perfusion of apyrase-protected murine blood over surfaces of collagen confirms the enhanced reactivity of transgenic over wild type platelets. When $P2X_1$ function is not preserved, no difference in aggregate formation occurs. These findings are compatible with a model in which functional $P2X_1$ is capable of positively modulating collagen-platelet interactions, also under conditions of flow. The induction of pulmonary embolism by i.v. injection of collagen is accompanied by an increased mortality in mice overexpressing $P2X_1$. Inhibition of ERK2 in wild type mice reduced the mortality provoked by a higher dose of collagen. These findings are compatible with a role for platelet $P2X_1$ in hemostasis and thrombosis. The correlation between the *ex vivo* analysis, always requiring the protection of $P2X_1$ with apyrase, and the *in vivo* data, obtained without such protective treatment, supports this conclusion.

References

1. Sage SO, Yamoah EH, Heemskerk JW. The roles of P(2X1)and P(2T AC)receptors in ADP-evoked calcium signalling in human platelets. Cell Calcium 2000; 28:119-26.
2. Rolf MG, Brearley CA, Mahaut-Smith MP. Platelet shape change evoked by selective activation of $P2X_1$ purinoceptors with α,β-methylene ATP. Thromb Haemost 2001; 85:303-8.
3. Mahaut-Smith MP, Ennion SJ, Rolf MG, Evans RJ. ADP is not an agonist at P2X(1) receptors: evidence for separate receptors stimulated by ATP and ADP on human platelets. Br J Pharmacol 2000; 131:108-14.
4. Oury C, Toth-Zsamboki E, Vermylen J, Hoylaerts MF. Does the P(2X1del) variant lacking 17 amino acids in its extracellular domain represent a relevant functional ion channel in platelets? Blood 2002; 99:2275-7.
5. Oury C, Toth-Zsamboki E, Thys C, Tytgat J, Vermylen J, Hoylaerts MF. The ATP-gated P2X1 ion channel acts as a positive regulator of platelet responses to collagen. Thromb Haemost 2001; 86:1264-71.
6. Oury C, Toth-Zsamboki E, Vermylen J, Hoylaerts MF. P2X1 mediated activation of ERK2 contributes to platelet secretion and aggregation induced by collagen. Blood (in press).
7. Toth-Zsamboki E, Oury C, Tytgat J, Vermylen J, Hoylaerts MF. The $P2Y_1$ receptor antagonist adenosine-2',5'-diphosphate non-selectively antagonizes the platelet $P2X_1$ ion channel. Thromb Haemost 2001; 86:1338-9.

New insights into the role of $P2X_1$ in platelet function

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Polymerase chain reaction amplification of human cDNA with oligonucleotides specific for the human $P2X_1$ subtype has shown the presence of $P2X_1$ transcripts in platelets and megakaryoblastic cell lines, and immunological and biochemical evidence confirmed that functional $P2X_1$ is expressed on platelets.[1-4] Since the selective $P2X_1$ agonist α,β-methylene-ATP initiates calcium influx into human platelets but not internal Ca^{2+} mobilization,[5] it has been proposed that $P2X_1$ could be responsible for the rapid influx across the plasma membrane of Ca^{2+} in platelets exposed to ADP. However, it has more recently been shown that this reported activation of the platelet ionotropic receptor by ADP is attributable to contamination of commercially

available ADP preparations with ATP, which is the physiological agonist of the $P2X_1$ receptor.[6] The role of $P2X_1$ in platelet activation is controversial. The failure of many studies to show that the non-hydrolyzable $P2X_1$ agonist α,β-methylene-ATP promotes platelet shape change or aggregation[7,8] is to be attributed to rapid desensitization of the receptor by adenine nucleotides released during the preparation of platelet suspensions, since a more recent study showed that the compound can induce platelet shape change in platelet preparations in which apyrase was added to prevent or rapidly reverse receptor desensitization.[9] Prevention of $P2X_1$ desensitization with apyrase improves the aggregation response of human platelets to threshold concentrations of collagen but has no effect on platelet aggregation induced by higher concentrations of collagen or ADP.[10] Therefore, the few available *in vitro* studies do suggest that $P2X_1$ may play a role in platelet function, but that this is probably of minor relevance. These *in vitro* findings contrast with the demonstration that a patient with a dominant negative mutation in the $P2X_1$ receptor gene had a severe bleeding disorder, suggesting that $P2X_1$ plays an important role in normal hemostasis.[11] The aforementioned *in vitro* studies were performed under experimental conditions in which platelets are stirred in an aggregometer and stimulated by soluble agonists. However, at sites of vascular injury *in vivo*, platelets interact with the thrombogenic surface of the exposed subendothelium under different flow conditions, which generate different levels of shear rate and shear stress. The highest levels of shear rate in the normal circulation (up to 5,000 s^{-1}) occur in small arterioles, while in pathological conditions, such as at the top of atherosclerotic plaques partially occluding coronary arteries, they can reach levels as high as 10,000 s^{-1}. Since it has been previously demonstrated that fluid shear stress activates Ca^{2+} influx into human endothelial cells via a receptor of the P2X family ($P2X_4$),[12] we tested the hypothesis that $P2X_1$ may play a relevant role in platelet function under high shear conditions. To this end, we studied platelet aggregation induced by high shear stress using a previously described cone-and-plate viscometer, as well as the development in real time of platelet thrombi on a collagen surface exposed to flowing blood without or with desensitization of the platelet $P2X_1$ receptor. We found that platelet aggregation induced by high shear stress (10^8 dynes/cm^2) was inhibited in samples in which $P2X_1$ had been desensitized. In addition, $P2X_1$ desensitization interfered with platelet thrombus formation on collagen type I fibrils exposed to high shear rates (2,000 s^{-1} and 6,000 s^{-1}). These results suggest that $P2X_1$ may play a role in platelet aggregation under flow conditions that exist in the arterial circulation.

References

1. Vial C, Hechler B, Leon C, Cazenave JP, Gachet C. Presence of $P2X_1$ purinoceptors in human platelets and megakaryoblastic cell lines. Thromb Haemost 1997; 78: 1500-4.
2. Clifford EE, Parker K, Humphreys BD, Kertesy SB, Dubyak GR. The $P2X_1$ receptor, an adenosine triphosphate-gated cation channel, is expressed in human platelets but not in human blood leukocytes. Blood 1998; 91:3172-81.
3. Scase TJ, Heath MF, Allen JM, Sage SO, Evans RJ. Identification of a $P2X_1$ purinoceptor expressed on human platelets. Biochem Biophys Res Commun 1998; 242:525-8.
4. Sun B, Li J, Okahara K, Kambayashi J. $P2X_1$ purinoceptor in human platelets. Molecular cloning and functional characterization after heterologous expression. J Biol Chem 1998; 273:11544-7.
5. MacKenzie AB, Mahaut-Smith MP, Sage SO. Activation of receptor-operated cation channels via $P2X_1$ not P2T purinoceptors in human platelets. J Biol Chem 1996; 271:2879-81.
6. Mahaut-Smith MP, Ennion SJ, Rolf MG, Evans RJ. ADP is not an agonist at $P2X_1$ receptors: evidence for separate receptors stimulated by ATP and ADP on human platelets. Br.J Pharmacol. 2000; 131:108-14.
7. Daniel JL, Dangelmaier C, Jin J, Ashby B, Smith JB, Kunapuli SP. Molecular basis for ADP-induced platelet activation. I. Evidence for three distinct ADP receptors on human platelets. J Biol Chem 1998; 273:2024-9.
8. Takano S, Kimura J, Matsuoka I, Ono T. No requirement of P2X1 purinoceptors for platelet aggregation. Eur J Pharmacol 1999; 372:305-9.
9. Rolf MG, Brearley CA, Mahaut-Smith MP. Platelet shape change evoked by selective activation of P2X1 purinoceptors with alpha,beta-methylene ATP. Thromb Haemost 2001; 85:303-8.
10. Oury C, Toth-Zsamboki E, Thys C, Tytgat J, Vermylen J, Hoylaerts MF. The ATP-gated P2X1 ion channel acts as a positive regulator of platelet responses to collagen. Thromb Haemost 2001; 86:1264-71.
11. Oury C, Toth-Zsamboki E, Van Geet C, Thys C, Wei L, Nilius B, et al. A natural dominant negative P2X1 receptor due to deletion of a single amino acid residue. J Biol Chem 2000; 275:22611-4.
12. Yamamoto K, Korenaga R, Kamiya A, Ando J. Fluid shear stress activates Ca(2+) influx into human endothelial cells via P2X4 purinoceptors. Circ Res 2000; 87:385-91.

New insights into the roles of $P2Y_1$ and $P2Y_{12}$ receptors in platelet function

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Since the identification of the $P2Y_1$ and $P2Y_{12}$ receptors responsible, respectively, for initiation and amplification of aggregation to ADP, their role in platelet function has been progressively elucidated. Transgenic mice overexpressing the $P2Y_1$ receptor selectively in the platelet lineage display platelet hyperreactivity, confirming this receptor's key role in hemostasis and thrombosis. Furthermore, ADP selectively promotes granule secretion of transgenic platelets, indicating that $P2Y_1$ receptor expression level is critical for this event. $P2Y_1$ also mediates ADP-induced p38 MAP kinase activation. It is rapidly desensitized and internalized after platelet exposure to ADP, while the $P2Y_{12}$ remains functional. Recent data indicate a role for $P2Y_1$ in the shape change induced by collagen. Concerning $P2Y_{12}$, it is essential for full aggregation not only to ADP but also to agonists acting on Gq, G12/13- or tyrosine kinase-coupled receptors, through a PI3-kinase pathway and potentiates platelet secretion. $P2Y_{12}$ also activates the small GTPase Rap1B. Furthermore, this receptor mediates partial aggregation without shape change in platelets from $P2Y_1$ knockout (KO) mice at high ADP concentrations. This receptor is also essential for normal thrombus build-up on a collagen surface in flowing blood. $P2Y_{12}$ KO mice display a phenotype identical to that resulting from clopidogrel intake and are completely insensitive to clopidogrel treatment, indicating that the $P2Y_{12}$ is a target of this drug, once again confirming its key role in thrombosis. The pharmacological profile of $P2Y_{12}$ has been reexamined using HPLC-purified nucleotides, indicating that ADP and its diphosphate analogs are agonists while the triphosphate analogs are antagonists. Finally, while both $P2Y_1$ and $P2Y_{12}$ are involved in thrombin generation *in vivo*, the $P2Y_{12}$ is selectively involved in thrombin generation *in vitro*. Altogether these new insights improve our understanding of the role of each ADP receptor in platelet function and are essential for the conception of new antithrombotic strategies.

Oral Communications

$P2X_1$-MEDIATED ACTIVATION OF Ca^{2+}-CALMODULIN LEADS TO MYOSIN LIGHT CHAIN AND ERK2 PHOSPHORYLATION IN HUMAN PLATELETS

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The ATP-gated P2X1 receptor is a non-selective cation channel, highly expressed in human platelets and megakaryocytes, but its significance has not yet been understood, in part due to its rapid desensitization in conventionally prepared platelet samples. Using transmission electron microscopy, we found that the $P2X_1$–mediated fast, reversible platelet shape change is accompanied by secretory granule centralization and pseudopodia formation. The specific, non-hydrolysable $P2X_1$ agonist, α,βme-ATP caused rapid, transient (2-5 s) and dose-dependent myosin light chain (MLC) phosphorylation in washed human platelets. The α,βme-ATP-induced MLC phosphorylation was abolished when extracellular Ca^{2+} or apyrase were omitted. In contrast, the IP_3-receptor inhibitor, 2-ABP (100 μM) did not affect this event. The non-specific P2 antagonist, suramin, dose-dependently inhibited the $P2X_1$-mediated MLC phosphorylation. Thus, the exclusive influx of Ca^{2+} ions via $P2X_1$ ionotropic activity suffices to trigger MLC phosphorylation. Since phosphorylation of MLC can be dichotomously regulated by the Ca^{2+}-calmodulin-dependent MLC kinase and/or by Rho-kinase, the effects of the Rho-kinase inhibitor, HA-1077 (20 μM) and of the calmodulin inhibitor, W-7, were tested. While HA-1077 had no significant effect, W-7 dose-dependently inhibited the $P2X_1$-induced shape change and MLC phosphorylation (IC_{50} ~25 μM). Thus, the $P2X_1$-mediated Ca^{2+} influx triggers MLC phosphorylation via activation of Ca^{2+}-calmodulin, a central player in cytoskeleton-related events such as platelet shape change and granule release. We have previously shown that the P2X1 receptor contributes to collagen-evoked platelet aggregation via the ERK2 pathway. Interestingly, W-7 was also able to inhibit the α,βme-ATP- and the collagen-induced ERK2 activation, indicating that Ca^{2+}-calmodulin is an upstream component of the ERK2 cascade in platelets. Recent studies reported the co-localization in

platelet membranes of calmodulin and the collagen receptor GPVI. In view of its contribution to collagen-induced platelet aggregation, we therefore postulate a role for the $P2X_1$-mediated Ca^{2+} influx in the activation of the calmodulin-dependent signal transduction triggered by collagen.

EFFECTS OF AMPLIFIED $P2X_1$ RECEPTOR CALCIUM INFLUX ON FUNCTIONAL RESPONSES IN HUMAN PLATELETS

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G-protein-coupled $P2Y_1$ and $P2Y_{12}$ receptors play key roles in platelet activation, however the importance of ionotropic $P2X_1$ receptors remains unclear. One of the key problems during studies of platelet $P2X_1$ receptors is the ease with which they desensitize *in vitro*. Therefore we have developed conditions that enhance $P2X_1$-evoked Ca^{2+} influx and investigated the effects on functional responses alone or in synergy with $P2Y_{12}$ receptors. $[Ca^{2+}]i$ was measured in fura-2-loaded platelets resuspended in artificial saline, with transmitted light at 578nm used to simultaneously monitor shape change and aggregation. $[Ca^{2+}]i$ increases evoked by the $P2X_1$ receptor agonist α,β-MeATP (10 μM) were slightly (1.3 fold), but not consistently increased by substitution of external Na^+ with the impermeant cation n-methyl-D-glucamine. In contrast, simple elevation of external Ca^{2+} in the range 1-10 μM enhanced peak $P2X_1$ $[Ca^{2+}]i$ responses by up to 3 fold, allowing the functional effects of this receptor to be studied for $[Ca^{2+}]i$ increases up to 800 nM. α,β-MeATP stimulated a shape change which saturated at peak $[Ca^{2+}]i$ of ≥ 400 nM, without evidence of aggregation. The maximal $P2X_1$-evoked transmission decrease was 82% of that obtained via $P2Y_1$ receptors (10 μM hexokinase-purified ADP). α,β-MeATP caused a disc to sphere transformation in virtually all (96%) platelets, but lacked the long processes produced by ADP. Following block of $P2Y_1$ receptors with A3P5PS (300 μM), co-stimulation with α,β-MeATP and ADP failed to induce aggregation despite the generation of peak $[Ca^{2+}]i$ responses similar to those stimulated via $P2Y_1$ receptors. Therefore, early transient Ca^{2+} influx via $P2X_1$ receptors can contribute to platelet activation by stimulating a significant morphological change, but does not readily synergize with $P2Y_{12}$ receptors to support aggregation. This also suggests that the $[Ca^{2+}]i$ increase responsible for the synergistic interaction between $P2Y_1$ and $P2Y_{12}$ receptors requires more than a single transient increase in $[Ca^{2+}]i$ as observed following a single application of the $P2X^1$ agonist α,βmeATP.

INCREASED PLATELET REACTIVITY TO COLLAGEN IN TRANSGENIC MICE OVEREXPRESSING THE $P2X_1$ ION CHANNEL

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The platelet ATP-gated $P2X_1$ ion channel mediates a rapid Ca^{2+} influx responsible for platelet shape change. $P2X_1$ also contributes, through ATP released from the dense granules, to collagen-induced platelet aggregation, a process involving the extracellular signal-regulated kinase 2 (ERK2) signaling pathway. To define the role of the $P2X_1$ ion channel further, we have generated transgenic mice overexpressing the human $P2X_1$ channel in the megakaryocytic cell lineage under the control of the murine GPIIb promoter. These mice displayed normal platelet count and morphology. In the presence of apyrase to minimize $P2X_1$ desensitization and of physiological Ca^{2+} concentrations, platelets from the transgenic mice showed increased α,β-meATP (1 μM)-evoked shape change. Most importantly, subthreshold concentrations of collagen, which only induced shape change of wild-type platelets, caused full aggregation of the platelets paralleled by maximal ERK2 phosphorylation. Perfusion of apyrase-treated whole blood from transgenic mice over a collagen surface at high shear rate (1000 s^{-1}) revealed greatly enhanced aggregate formation as well as exposure of negatively charged phospholipids in comparison to that occurring in wild-type controls. These platelet responses were inhibited by saratin, which blocks the collagen-vWF interaction, demonstrating the enhancement of an event initiated by collagen-bound vWF. Moreover, we measured the mortality induced by intravenous injection of a mixture of collagen (0.06 mg/kg body weight) and epinephrine (60 μg/kg). In these experiments, 80% of the transgenic mice died after 4 minutes, whereas the mortality rate was only 30% in wild-type mice. In wild-type mice, when thromboembolism was induced with collagen (0.125 mg/kg) plus epinephrine (60

μg/kg), blockade of the ERK2 pathway by preinjection of the MEK1/2 inhibitor U0126 (200 mg/kg, 1 min) significantly increased the number of survivors (from 40 to 90%) Hence, the platelet $P2X_1$ ion channel plays a role in hemostasis and thrombosis through its involvement in platelet responses to collagen via activation of ERK2. It may also enhance the response to surface-bound vWF at high shear.

A MINOR ROLE OF THE PLATELET $P2X_1$ RECEPTOR IN HEMOSTASIS. STUDIES IN $P2X_1$ KNOCK-OUT MICE

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The functional role of the platelet $P2X_1$ receptor has been difficult to assess due to the lack of selective antagonists and also the rapid desensitization of this receptor during preparation of platelets for *in vitro* studies. Therefore, we resorted to $P2X_1$ knock-out (KO) mice to investigate the role of this receptor in hemostasis and performed experiments in $P2X_1$ KO mice of mixed MF1-129Ola and pure MF1 genetic background. The bleeding time was mildly prolonged in both $P2X_1$ KO mouse strains as compared to in the respective wild-type (WT) strains. The selective $P2X_1$ receptor agonist α,βMeATP induced a transient calcium entry in WT platelets, whereas no calcium entry could be detected in $P2X_1$ KO cells. Platelet aggregation in response to ADP (100 μM), thrombin (0.1 U/mL) or the TXA_2 analog U46619 (2 μM) was similar in WT and $P2X_1$ KO mice, indicating that the $P2X_1$ receptor does not play a significant role when platelets are activated by these agonists. In contrast, P2X1 KO platelets derived from mice of either genetic background displayed a decreased response to a low concentration of collagen (1.25 mg/mL). This property was further characterized by using flowing whole blood to study platelet accumulation on a surface coated with type 1 collagen at wall shear rates corresponding to those in small arteries (1500 s^{-1}). Platelet deposition was monitored for 3 min under conditions preventing glycoprotein IIb-IIIa-mediated aggregation. Whereas accumulation of $P2X_1$ KO platelets from mice of the MF1-129Ola genetic background was greatly reduced (by 55%), accumulation of $P2X_1$ KO cells from mice of the MF1 genetic background was similar to that of WT platelets. Overall, these results point to a minor role of the platelet $P2X_1$ receptor in primary hemostasis. Whether or not the genetic background determines the contribution of this receptor to platelet interactions with collagen requires further investigations.

SESSION 4

Source and metabolism of nucleotides

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Extracellulary nucleotides, released from cells in order to act as signaling molecules in an autocrine or paracrine manner, include the mononucleotides ADP, ATP, UTP and GTP as well as the metabolically more stable dinucleotides. In this latter group, discovered recently, 5´,-5´-dinucleoside polyphosphates with Ap_nA, Ap_nG and Gp_nG (A: adenosine, G: guanosine, p: phosphate groups with n = 2 to 6) can be counted. Highly controlled and regulated release of intracellular nucleotides occurs in many types of cells under physiological conditions. Nucleotides are released from vascular and myocardial endothelial cells, red blood cells, platelets and from endocrine sources such as the adrenal chromaffin cells into the circulation. There are at least three possible mechanisms for the release of nucleotides. A major mechanism is the stimulus-dependent *exocytosis* of nucleotides-filled vesicles, especially in platelets, neurons and neuroendocrine cells, such as adrenal medullary chromaffin cells. The dense granules of mast cells, platelets, and chromaffin cells store nucleotides at very high concentrations (mM or higher). *Non-exocytotic* release of ATP likely occurs by conductive ATP transport through ATP release channels, by non-conductive passive transport mechanisms using the large gradient (cytosolic ATP concentrations 1–10 mM; extracellular concentrations about 10,000-fold lower). Non-conductive *bidirectional transporters* exist in the plasma membrane for nucleosides primarily in brain; therefore, it cannot be discounted that ATP-specific transporters may exist. Pathologically, ATP is released from cells in response to shear forces (e.g. endothelial cells), stretch, changes in osmolarity, oxidative stress and lipopolysaccharides.

In human blood plasma diadenosine polyphosphate concentrations in the order of magnitude of 10^{-6} mol/L to 10^{-5} mol/L were measured. ATP concentrations in the blood are significantly lower than 1 μmol/L. Once the nucleotides are released into the extracellular milieu they have several possible fates. They can mediate fast and slow responses via purinergic receptors. The concentration of ex-tracellular nucleotides necessary to activate the purinergic receptors is very low (0.1–10 μM). The cell needs to release only 0.1% or less of its intracellular ATP pool to trigger autocrine or paracrine ATP signaling. After release nucleotides may also diffuse away in static conditions or be swept away in a circulating environment. Nucleotides are rapidly cleared while passing through the vascular bed (half-time of nucleotides in perfused lung is 0.2 s). Therefore, ATP and its metabolites should be envisioned as local mediators or autocrine/paracrine factors that act within tissues or tissue microenvironments. As dinucleotides are more stable they may act as endocrine factors. ATP and dinucleotides can be enzymatically hydrolyzed by specific and unspecific ecto-enzymes as well as soluble nucleotide hydrolases. It is notable that soluble nucleotides may also be released by stimulus-dependent exocytosis, adding a further element in the control of the extracellulary nucleotide concentration. In summary, several factors must be considered that may influence the attainment of effective concentrations of nucleotides in the extracellulary space. These factors include, but are not limited to, the amount of the nucleotides released, their volume of distribution in the extracellular microenvi-ronment and the presence and activity of nucleotide hydrolyzing enzymes.

Role of nucleoside triphosphate diphosphohydrolases in hemostasis and thromboembolism

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Nucleoside triphosphate diphosphohydrolases (NTPDases) are a recently described family of ecto-nucleotidases that differentially hydrolyze γ and β phosphate residues of extracellular nucleotides.[1] It has been proposed that these biochemical properties, in tandem with 5'-nucleotidase activity, regulate extracellular nucleotide activation of purinergic/pyrimidinergic receptor (P2) mediated signaling responses (Zimmermann *et al.*).[2-4] CD39/NTPDase-1 is the dominant vascular endothelial ectonucleotidase and rapidly hydrolyzes both extracellular ATP and ADP to AMP.[5] Dualistic properties of NTPDase1 in the regulation of P2-receptor activation were demonstrated and confirmed by the generation of mutant mice with total deletion of *cd39* expression (*Enjyoji, Rosenberg et al.*).[2] As anticipated, these null mice exhibited a prothrombotic vascular phenotype, associated with accelerated infarction of transplanted organs[6] and developed profound vascular injury following ischemia-reperfusion insults to the hepatosplanchnic bed.[4] Somewhat paradoxically (but confirmed repeatedly by other laboratories; *Gachet, Takashima et al.*), mutant mice have also demonstrated perturbations in platelet activation, angiogenesis[7] and inflammatory responses.[8] Such alterations in hemostasis and these other effects have been considered to be secondary to selective and short-term $P2Y_1$-receptor desensitization.[2] It is of interest that newly originated transgenic mice, over-expressing CD39, have comparable changes in parameters of platelet activation and also exhibit prolonged bleeding times with resistance to systemic thromboembolism (*d'Apice et al., 2002*). Additionally, and in keeping with the assumption that high levels of CD39 expression abrogate P2-signaling by accelerated hydrolysis of extracellular nucleotides, administration of soluble NTPDases and/or induction of high level CD39 expression (viz. by adenoviral vectors) consistently result in decreased platelet microthrombi and substantial amelioration of vascular injury.[6,9,10] In contrast, CD39L1/NTPDase2, a preferential nucleoside triphosphatase, activates platelet aggregation by converting the competitive antagonist (ATP) of ADP-receptors to the specific agonist, ADP.[1] We have recently observed that NTPDase-2 is associated with the adventitia of muscularized vessels, microvascular pericytes and cell populations in the subendocardial space. Differential expression of NTPDases in the vasculature also suggests spatial regulation of nucleotide-mediated signaling.[11] Our data suggest a role for NTPDases in the differential regulation of P2-receptor activity and function in platelets. In this context, NTPDase-1 should abrogate platelet adhesion to intact endothelium while exposure of blood to cells expressing NTPDase-2 may promote platelet microthrombus formation, for example at sites of extravasation following vessel injury.[4,11]

References

1. Zimmermann H, et al. Proceedings of the Second International Workshop on Ecto-ATPases and Related Ectonucleotidases. Vanduffel and Lemmens, eds. Shaker Publishing, Maastricht, The Netherlands; 2000. p. 9-20.
2. Enjyoji K, Sevigny J, Lin Y, Frenette PS, Christie PD, Esch JS 2nd, et al. Targeted disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation. Nat Med 1999; 5:1010-7.
3. Zimmermann H. Nucleotides and cd39: principal modulatory players in hemostasis and thrombosis. Nat Med 1999; 5:987-8.
4. Robson SC, Enjyoji K, Goepfert C, Imai M, Kaczmarek E, Lin Y, et al. Modulation of extracellular nucleotide-mediated signaling by CD39/nucleoside triphosphate diphosphohydrolase-1. Drug Develop Res 2001; 53:193-207.
5. Kaczmarek E, Koziak K, Sevigny J, Siegel JB, Anrather J, Beaudoin AR, et al. Identification and characterization of CD39/vascular ATP diphosphohydrolase. J Biol Chem 1996; 271:33116-22.
6. Imai M, Takigami K, Guckelberger O, Enjyoji K, Smith RN, Lin Y, et al. Modulation of nucleoside [correction of nucleotide] triphosphate diphosphohydrolase-1 (NTPDase-1)cd39 in xenograft rejection. Mol Med 1999; 5: 743-52.
7. Goepfert C, Sundberg C, Sevigny J, Enjyoji K, Hoshi T, Csizmadia E, et al. Disordered cellular migration and angiogenesis in cd39-null mice. Circulation 2001; 104: 3109-15.
8. Mizumoto N, Kumamoto T, Robson SC, Sevigny J, Matsue H, Enjyoji K, et al. CD39 is the dominant Langerhans cell-associated ecto-NTPDase: modulatory roles in inflammation and immune responsiveness. Nat Med 2002; 8:358-65.
9. Imai M, Takigami K, Guckelberger O, Kaczmarek E, Csizmadia E, Bach FH, et al. Recombinant adenoviral mediated CD39 gene transfer prolongs cardiac xenograft survival. Transplantation 2000; 70:864-70.
10. Gangadharan SP, Imai M, Rhynhart KK, Sevigny J, Robson SC, Conte MS. Targeting platelet aggregation: CD39 gene transfer augments nucleoside triphosphate diphosphohydrolase activity in injured rabbit arteries. Surgery 2001; 130:296-303.
11. Sevigny J, Sundberg C, Braun N, Guckelberger O, Csizmadia E, Qawi I, et al. Differential catalytic properties and vascular topography of murine nucleoside triphosphate diphosphohydrolase 1 (NTPDase1) and NTPDase2 have implications for thromboregulation. Blood 2002; 99:2801-9.

Metabolic control of excessive extracellular nucleotide accumulation by CD39/ecto-nucleotidase-1: implications for ischemic vascular diseases

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Platelets are responsible for maintaining vascular integrity. In thrombocytopenic states, vascular permeability and fragility increase, presumably due to the absence of this platelet function. Chemical or physical injury to a blood vessel induces platelet activation and platelet recruitment. This is beneficial for the arrest of bleeding (hemostasis), but when an atherosclerotic plaque is ulcerated or fissured, it becomes an agonist for vascular occlusion (thrombosis). Experiments in the late 1980s cumulatively indicated that endothelial cell CD39 – an ecto-ADPase – (rather than prostacyclin or nitric oxide) reduced platelet reactivity to most agonists. As discussed herein, CD39 rapidly and preferentially metabolizes ATP and ADP released from activated platelets to AMP, thereby abolishing platelet aggregation and recruitment. Since ADP is the final common agonist for platelet recruitment and thrombus formation, this finding highlights the significance of CD39. A recombinant, soluble form of human CD39, solCD39, has enzymatic and biological properties identical to those of the full-length form of the molecule, and strongly inhibits human platelet aggregation induced by ADP, collagen, arachidonate, or TRAP (thrombin receptor agonist peptide). In a murine model of stroke, driven by excessive platelet recruitment, solCD39 reduced the sequelae of stroke, without an increase in intracerebral hemorrhage. CD39 null mice, generated by deletion of apyrase-conserved regions 2-4, exhibited a decrease in post-ischemic perfusion and an increase in cerebral infarct volume when compared to controls. Reconstitution of CD39 null mice with solCD39 reversed these changes. We hypothesize that solCD39 has potential as a novel therapeutic agent for thrombotic diatheses.

Oral Communications

EFFECTS OF INHIBITION OF $P2Y_1$ AND $P2Y_{12}$ ON COLLAGEN-RELATED PEPTIDE INDUCED FIBRINOGEN BINDING, WHOLE BLOOD CLOTTING, COAGULUM ELASTICITY AND FIBRINOLYSIS RESISTANCE

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Activation of platelets in whole blood has previously been shown to shorten the coagulation time. Since the release of endogenous ADP is supposed to be important for a full and sustained activation of platelets, we have begun to investigate how ADP receptor antagonists affect blood coagulation in freshly drawn blood samples. Free oscillation rheometry (FOR) enables investigation of clotting and clot properties of whole blood clots. We have used FOR to investigate the effect of the $P2Y_1$ antagonist MRS2179 and the $P2Y_{12}$ antagonist AR-C69931MX on whole blood coagulation, coagulum elasticity and tPA-induced fibrinolysis. The platelets were activated by the addition of a collagen related peptide (CRP). The inhibitors were also studied by flow cytometry measuring platelet fibrinogen binding after stimulation by ADP or CRP. Complete inhibition of fibrinogen binding in response to ADP was observed in blood with more than 0.01 μmol/L AR-C69931MX or 10 μmol/L MRS2179. AR-C69931MX inhibited fibrinogen binding in response to CRP with 70% in doses above 0.1 μmol/L, while MRS2179 maximally inhibited 40% of the binding in doses above 1 μmol/L. In the FOR experiments, AR-C69931MX, and probably MRS2179 prolonged the clotting time, but no large effects were seen on clot elasticity or clot fibrinolysis resistance. It is clear that a part of the activation measured as fibrinogen binding in response to CRP is due to platelet autoactivation by ADP, since both ADP receptor inhibitors also affected the CRP-induced fibrinogen binding, but to different degrees. The inhibitors also seem to prolong the clotting time of whole blood samples, but more experiments utilizing FOR are needed to reveal whether ADP receptor inhibition also affects the properties of the coagulum formed.

DIFFERENTIAL INVOLVEMENT OF THE $P2Y_1$ AND $P2Y_{12}$ RECEPTORS IN PLATELET-MEDIATED ASPECTS OF COAGULATION

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Previous *in vivo* studies have demonstrated a role for $P2Y_1$ and $P2Y_{12}$ receptors in the generation of thrombin.[1,2] The aim of the present work was to evaluate the respective roles of these platelet ADP receptors in thrombin generation *in vitro*. MRS2179 (100 μM), a selective $P2Y_1$ antagonist, and AR-C69931MX (10 μM), a selective $P2Y_{12}$ antagonist, were employed to discriminate between the receptors. Leukocyte tissue factor (TF) and platelet P-selectin exposure were measured in whole human blood by flow cytometry, while FITC-annexin V was used to detect exposure of phosphatidylserine (PS) at the surface of washed human platelets. Thrombin generation was triggered by addition of recombinant TF (Innovin®) to human platelet-rich plasma (PRP) and measured by the Thrombogram® technique.[3] TF and P-selectin exposure were increased following activation of blood with ADP (100 μM) or collagen (10 μg/mL). AR-C69931MX and MRS2179 inhibited ADP-induced TF exposure by 80 and 60%, respectively, and P-selectin exposure by 93 and 85%, respectively. Likewise, they decreased collagen-induced TF exposure by 50 and 20% and P-selectin exposure by 90 and 60%. AR-C69931MX delayed the onset of thrombin generation in PRP (33 vs 25 min) and reduced its maximum rate (134 vs 163 nM), whereas MRS2179 was ineffective. Similarly, AR-C69931MX decreased FITC-annexin V labeling of thrombin-activated platelets but MRS2179 was ineffective. This is in accordance with previous studies performed with platelets from $P2Y_1$ knock-out mice or using A3P5P as an antagonist of the $P2Y_1$ receptor.[4] In conclusion, the $P2Y_1$ and $P2Y_{12}$ receptors are both involved in platelet-induced exposure of leukocyte TF, while the $P2Y_{12}$ receptor also participates in PS exposure during platelet activation. Further studies will be required to assess the causal relationship between these *in vitro* observations and the *in vivo* effects of antiplatelet drugs targeting the P2 receptors.

1. Herault JP, Dol F, Gaich C, Bernat A, Herbert JM. Effect of clopidogrel on thrombin generation in platelet-rich plasma in the rat. Thromb Haemost 1999; 81:957-60.
2. Leon C, Freund M, Ravanat C, Baurand A, Cazenave JP, Gachet C. Key role of the P2Y(1) receptor in tissue factor-induced thrombin-dependent acute thromboem-

bolism: studies in P2Y(1)-knockout mice and mice treated with a P2Y(1) antagonist. Circulation 2001; 103:718-23.
3. Hemker HC, Giesen PL, Ramjee M, Wagenvoord R, Beguin S. The thrombogram: monitoring thrombin generation in platelet-rich plasma. Thromb Haemost 2000; 83:589-91.
4. Tiacci E, Luppi M, Barozzi P, Gurdo G, Tabilio A, Ballanti S, et al. Fatal herpesvirus-6 encephalitis in a recipient of a T-cell-depleted peripheral blood stem cell transplant from a 3-loci mismatched related donor. Haematologica 2000; 85:94-7.

PARTIAL $P2Y_{12}$ RECEPTOR BLOCKADE BY CLOPIDOGREL PRODUCES MODERATE INHIBITION OF PLATELET PROCOAGULANT ACTIVITY IN PATIENTS WITH ACUTE CORONARY SYNDROMES

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Platelets play a major role in catalyzing the generation of thrombin at the site of arterial thrombosis through agonist-induced procoagulant responses consisting of phosphatidylserine exposure (detectable by annexin V) and shedding of microparticles. Clopidogrel and AR-C69931MX are antithrombotic agents and antagonists of the $P2Y_{12}$ receptor. Previous studies of AR-C69931MX have demonstrated that the $P2Y_{12}$ receptor amplifies platelet procoagulant responses. We studied whether clopidogrel also affects PAR1-induced platelet procoagulant responses in patients with acute coronary syndromes and at the same time assessed the *in vitro* effects of AR-C69931MX both before clopidogrel (BC) and after clopidogrel (AC). Patients admitted with acute coronary syndromes were studied before and the day after clopidogrel 300mg loading dose then 75mg daily (n=12). All patients were already on aspirin and LMW heparin. PRP was prepared from hirudin-anticoagulated blood. Platelet aggregation (PA) was measured by turbidimetry in response to TRAP 20 μM. AR-C69931MX (400 nM) or saline control was added *in vitro* 2 minutes prior to addition of TRAP. After 4 minutes PA aliquots were taken and added to Hepes Tyrode's buffer containing annexin V-FITC, $CaCl_2$ and anti-CD42a-RPE. Annexin V binding and microparticle formation were assessed by flow cytometry. *Results.* Clopidogrel weakly inhibited TRAP-induced PA (4 min PA: BC 76±24%, AC 59±27%, p=0.004) compared to AR-C69931MX (18±22%, p<0.001). Clopidogrel inhibited TRAP-induced Annexin V binding moderately (%positive: BC 28±15, AC 17±12, p=0.011) compared to a greater effect of AR-C69931MX (BC %positive 6±6, p<0.01). TRAP-induced microparticle formation was inhibited by clopidogrel (%microparticles: BC 16±8, AC 7.7±4.3, p=0.001) and dramatically inhibited by AR-C69931MX (BC 1.8±0.6, p<0.01). Clopidogrel has weak effects on platelet aggregation and moderate effects on procoagulant responses induced by TRAP. More complete $P2Y_{12}$ receptor blockade with AR-C69931MX yields greater inhibitory effects on these parameters supporting the development of strategies that yield more effective $P2Y_{12}$ receptor blockade than the current regimen of clopidogrel.

INHIBITION OF PLATELET AGGREGATION AND THROMBOSIS BY NOVEL SELECTIVE $P2Y_{12}$ RECEPTOR ANTAGONISTS

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ADP-induced platelet aggregation requires the simultaneous activation of two nucleotide receptors: $P2Y_1$ and $P2Y_{12}$. We have developed a new class of potent and selective inhibitors of the human platelet $P2Y_{12}$ receptor. 2',3' phenylacetaldehyde acetal derivatives of nucleotides and dinucleotide polyphosphates inhibited in a concentration-dependent manner ADP-induced aggregation of human platelets with IC_{50} values as low as 50 nM. Increasing concentrations of these compounds produced parallel shifts to the right in the concentration-effect curves for ADP, consistent with competitive antagonism. The inhibitory effect of these compounds on platelet aggregation was completely reversed by removal of the antagonist prior to the addition of ADP, indicating that these compounds interact with the receptor in a reversible manner. *In vivo* studies of intravascular thromboembolism in mice induced by collagen plus adrenaline were also inhibited by intravenous administration of the antagonists, resulting in significant decreases in mortality rates. These compounds had no effect on $P2Y_1$ receptors, although they did, potently inhibit the Gi-coupled P2Y receptor expressed in C6 glioma cells, indicating that the inhibitory effect of platelet aggregation is mediated by antagonism of the $P2Y_{12}$ receptor.

Posters

ATP IS AN ANTAGONIST OF THE P2Y$_{12}$ RECEPTOR

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Since the cloning of the platelet P2Y$_{12}$ receptor, studies of its pharmacological profile have reported results which do not fit previous knowledge concerning the effects of triphosphate analogs. Thus, ATP is known to be an antagonist of ADP-induced platelet activation but at the recombinant receptor it is proposed to be an agonist. The aim of this study was, therefore, to re-examine the pharmacological profile of the P2Y$_{12}$ receptor. Human 1321N1 cells stably expressing the human P2Y$_{12}$ receptor and P2Y$_1$ knock-out (KO) mouse platelets were used to evaluate the agonistic or antagonistic properties of various adenine nucleotide analogs by determining their effects on forskolin- or PGE1-induced cAMP accumulation, respectively. ADP, 2MeSADP or ADPβS induced a concentration-dependant inhibition of cAMP accumulation in both P2Y$_1$ KO platelets and 1321N1-P2Y$_{12}$ cells with an identical rank order of potency: 2MeSADP > ADPβS≈ADP. ATP and its analogue 2ClATP behaved as full agonists at the P2Y$_{12}$ receptor in both platelets and 1321N1-P2Y$_{12}$ cells. However, their agonistic properties were totally abolished in the presence of the triphosphate nucleotide regenerating system creatine phosphate/creatine phosphokinase, indicating that this effect was due to breakdown of the nucleotides into their diphosphate analogs. Furthermore, ATP (50 μM) antagonized the inhibition of cAMP accumulation induced by ADP (0.5 μM) in P2Y$_1$ knockout mouse platelets, confirming that ATP is an antagonist of the platelet P2Y$_{12}$ receptor. In conclusion, triphosphate adenine nucleotides have no agonistic properties when care is taken to remove contaminating diphosphate nucleotides. ATP behaves as an antagonist of the platelet P2Y$_{12}$ receptor and hence the two platelet ADP receptors, P2Y$_1$ and P2Y$_{12}$, share a common pharmacological profile whereby ADP and its diphosphate analogs are agonists while the triphosphate analogs are antagonists.

RIBOSE AND NUCLEOBASE MODIFICATIONS TO NUCLEOTIDES THAT CONFER ANTAGONIST PROPERTIES AGAINST THE PLATELET P2Y$_{12}$ RECEPTOR

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ADP serves as an agonist for both P2Y$_1$ and P2Y$_{12}$ receptors in platelets, and activation of both is required to induce aggregation. As antagonism of either receptor results in inhibition of platelet aggregation, these proteins are attractive drug targets for the treatment of cardiovascular diseases. We have studied the SAR of modified nucleotides interacting with platelet ADP receptors, and have identified ribose and base substituents that work in concert to render the modified compounds potent and selective antagonists against the platelet P2Y$_{12}$ receptor. While ADP is a potent agonist of both P2Y$_1$ and P2Y$_{12}$, the analog 2',3'-phenylacetaldehyde acetal ADP (INS48800) produced a concentration-dependent inhibition of aggregation stimulated by ADP and 2MeSADP (IC$_{50}$ = 24.7±2.62 uM). Further modification of INS48800 with a 6-phenylaminocarbonyl (*urea*) moiety (INS49266) led to a dramatic increase in potency (IC$_{50}$ = 52.1±21.6 nM). ADP modified with the urea alone (INS49267) was an antagonist with potency (IC$_{50}$= 10.8±1.75 uM) comparable to that of INS48800. The monophosphate analog of INS49266 (INS50000) was also able to antagonize the action of ADP at P2Y$_{12}$, albeit with approximately 10-fold lower potency. A CDP analog of INS49266 (4-(p-fluoro)phenylaminocarbonyl, 2',3'-phenylacetaldehyde acetal CDP; INS49286) was likewise 10-fold less potent than INS49266. The inhibitory effect of these compounds was surmounted by increasing concentrations of the agonist 2MeSADP, suggesting a competitive mechanism.

A ROLE OF THE $P2Y_1$ RECEPTOR IN LOCALIZED ARTERIAL AND VENOUS THROMBOSIS IN RODENTS

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Studies in $P2Y_1$-deficient mice have revealed that the $P2Y_1$ receptor plays a key role in the general intravascular thromboembolism induced by infusion of collagen and adrenaline or injection of tissue factor. *Aim.* The aim of the present work was to use models of localized arterial and venous thrombosis to assess the contribution of the $P2Y_1$ receptor to these processes. Arterial thrombosis was induced in a mesenteric arteriole of a mouse by application of $FeCl_3$ and was quantified by attributing a score ranging from 0 (uninjured vessel) to 4 (occlusive thrombus). Venous thrombosis was studied in a Wessler model adapted to rats and the extent of thrombosis was evaluated by weighing the thrombi. $P2Y_1$-deficient mice displayed less arterial thrombosis than wild-type mice, thrombosis scores being significantly reduced from 3.1± 0.16 (n = 11) to 2.5±0.19 (n = 12, $p < 0.05$). Thrombosis was also inhibited by the $P2Y_1$ antagonist MRS2179, scores decreasing from 3.27±0.24 (n = 12) to 2.42 ±0.19 (n = 11, $p = 0.01$). Clopidogrel (50 mg/kg, iv), to date the reference antiplatelet drug targeting the $P2Y_{12}$ receptor, decreased arterial thrombosis to the same extent as $P2Y_1$ inhibition, from 3.1±0.25 (n = 11) to 2.1±0.2 (n = 17, $p < 0.01$). A combination of $P2Y_1$ deficiency and inhibition of $P2Y_{12}$ with clopidogrel produced additive effects (n = 10, $p < 0.01$). Venous thrombosis was slightly but significantly reduced in rats treated with MRS2179 since the average thrombus weight decreased from 14.85±1 mg (n = 13) to 9.24±1.1 mg (n = 15, $p < 0.01$). These results demonstrate a role of the $P2Y_1$ receptor in both arterial and venous thrombosis, further establishing this receptor as a potential target for antithrombotic drugs.

THE $P2Y_1$ RECEPTOR PLAYS A KEY ROLE IN THE PLATELET SHAPE CHANGE INDUCED BY COLLAGEN IN THE ABSENCE OF THROMBOXANE A_2 FORMATION

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Studies in $P2Y_1$ deficient mice have revealed that this receptor might be involved in collagen-induced platelet activation. The aim of the present study was to characterize the roles of the $P2Y_1$ and $P2Y_{12}$ receptors in collagen-induced platelet activation, using the $P2Y_1$ antagonist MRS2179 and the $P2Y_{12}$ antagonist AR-C69931MX, in association or not with aspirin. Inhibition of the $P2Y_1$ receptor alone (100 μM MRS2179) prolonged the lag phase preceding aggregation induced by collagen (2.5 to 100 μg/mL) without affecting the amplitude of aggregation (90%) or level of secretion (85%). At collagen concentrations below 2.5 μg/mL, the extent of aggregation and level of secretion were also affected. In contrast, inhibition of the $P2Y_{12}$ receptor (10 μM AR-C69931MX) or of TXA_2 production (1 mM aspirin) diminished the extent of both aggregation and secretion without affecting the lag phase. Combining aspirin with MRS2179 resulted in complete inhibition of platelet shape change and aggregation even at the highest concentration of collagen, with no more than 21% residual secretion, whereas combining aspirin with AR-C69931MX had only a modest additive effect on aggregation at collagen concentrations below 25 μg/mL. Electron microscopy showed that in the presence of MRS2179 and aspirin, isolated platelets and platelets in contact with collagen fibers conserved the typical morphology of discoid resting cells and did not aggregate. Conversely, in the presence of AR-C69931MX and aspirin, platelets were still able to change shape and aggregate. At the intracellular level, tyrosine phosphorylation of $PLC\gamma_2$ was delayed by MRS2179 during collagen-induced platelet activation but not by AR-C69931MX or aspirin alone. Altogether these results highlight a selective role of the $P2Y_1$ receptor in the early steps of signal transduction and in the shape change induced by collagen in the absence of TXA_2 formation.

ANTIPLATELET ACTIVITY OF GEMFIBROZIL CHIRAL ANALOGS

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Platelet aggregation has been assessed using a recently developed platelet function analyzer (PFA-100®, Dade Behring, Milan), a system which allows for rapid evaluation of platelet function on small

samples of anticoagulated human whole blood. The instrument is a controlled microprocessor in which the process of platelet adhesion and aggregation, following a vascular injury, is simulated *in vitro*. This method has been designed to provide an in vitro measure of primary platelet-related hemostasis simply, quickly, quantitatively and accurately in the routine screening of patients with potential hemorrhagic risk due to abnormal platelet plug formation. Herein we report the results obtained by testing chiral analogs of gemfibrozil, in order to evaluate their ability to inhibit human platelet aggregation. All tested compounds revealed a dose-dependent inhibitory activity toward human platelet aggregation. Moreover, a similar inhibitory effect is detectable in their precursor, the well-known gemfibrozil used as the basic reference compound. The inhibitory activity of these compounds is generally detectable at concentrations ranging from 1 to 5 mM. Considering the well-known activity of acetylsalicylic acid against platelet aggregation, we used acetylsalicylic acid as a clearly established reference compound and confirmed that it exerts a good anti-aggregating activity. The findings allow us to surmise that all tested compounds and gemfibrozil act at the platelet level with a mechanism different to that of acetylsalicylic acid, even if with a different potency. In conclusion, the simplicity of the PFA-100® system facilitates preliminary screening tests in order to find new anti-aggregating compounds. The use of this method allows us to demonstrate that the synthesized gemfibrozil analogs inhibit human platelet aggregation. Our study is still continuining as we are setting up an alternative system based on the use of properly collected and anticoagulated bull blood. This new method, used to study the previously described compounds and a new series of gemfibrozil analogs, offers the advantage of predicting the ability of these drugs to modulate the fibrinolytic system using more easily available standardized blood.

THE FREQUENCY OF PLATELET NON-RESPONSIVENESS TO ACETYLSALICYLIC ACID AMONG PATIENTS HANDICAPPED BY VASCULAR THROMBOEMBOLIC DISEASES

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A comparison was made regarding the frequency of non-responsiveness to acetylsalicylic acid (ASA) between 92 cardiology outpatinets of a district hospital and 97 heavily handicapped patients of the Raliway Hospital. The main differences were in the populations of patients: the duration of the treatment with ASA, the numbers of the combined atherothrombotic events, the rate of totally and 67% handicapped patients. There were no significant differences in gender and age. The non-responsiveness to ASA was measured and determined by platelet aggregometry using the Born method. Dose-response curves were plotted with the various concentrations of the following inducers: ADP, epinephrine, arachidonic acid and collagen. Patients were considered as non-responders if the aggregation of the platelets of the ASA-treated patients was not inhibited. Compliance was also taken into consideration. *Results*. The frequency of ASA-non-responders was 28.86% among the non-handicapped cardiology patients of the district hospital, whereas it was 45.8% among the handicapped patients. The latter had been taking the drug for a long period (average 5.33 years) without the pharmacodynamic effect of ASA having been checked, whereas the patients of the other hospital were treated for a shorter period (1.5 years) and the platelet aggregometry was performed in the 1st year of the treatment. The clinical significance of these data and the importance of the pharmacodynamic control of ASA are emphasized along with the appropriate choice of another platelet inhibitor for patients with cardio-, and/or cerebrovascular ischemic events.

SESSION 5

Congenital defects of the platelet P2 receptors – clinical aspects

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Platelets possess at least two P2 receptors for adenosine diphosphate (ADP). The $P2Y_1$ receptor, which is coupled to Gq and phospholypase C-β, is responsible for mobilization of ionized calcium from internal stores and mediates the ADP-induced platelet shape change and initial wave of rapidly reversible aggregation. The other receptor, $P2Y_{12}$, is negatively coupled to adenylyl cyclase through Gi and mediates a progressive and sustained ADP-induced aggregation not preceded by shape change. In addition, this receptor plays an important role in the potentiation of platelet secretion induced by several platelet agonists. The combined action of $P2Y_1$ and $P2Y_{12}$ is necessary for the full platelet aggregation response to ADP. Only patients with congenital defects of the platelet $P2Y_{12}$ receptors have been described so far.

Severe defects of $P2Y_{12}$

The first patient (VR) with congenital severe defect of $P2Y_{12}$ was described in 1992 by Cattaneo *et al*.[1] He is a man from Southern Italy, aged 49 years at the time of diagnosis, who had a lifelong history of excessive bleeding, prolonged bleeding time and abnormalities of platelet aggregation that are similar to those observed in patients with defects of platelet secretion (reversible aggregation in response to weak agonists and impaired aggregation in response to low concentrations of collagen or thrombin), except that the aggregation response to ADP was severely impaired. Other abnormalities of platelet function found in this patient were in common with those induced by thienopyridine compounds including: i) no inhibition by ADP of PGE_1-stimulated platelet adenylate cyclase, but normal inhibition by epinephrine; ii) normal shape change and normal (or mildly reduced) mobilization of cytoplasmic ionized calcium induced by ADP; iii) presence of about 30% of the normal number of platelet binding sites for [^{33}P]2MeSADP(2) or [^{3}H]ADP. Three additional patients, one male (ML)[3] and two sisters (IG and MG)[4] with very similar characteristics were described in 1995 and 2000. Similar to VR, these patients displayed a severely decreased number of binding sites for radiolabeled 2MeS-ADP on their platelets.[3,4] The molecular defects that are responsible for the abnormal phenotypes of these patients with severe defect of P2Y12 have been characterized.[3,5,6]

Congenital dysfunction of $P2Y_{12}$

More recently, a new patient with a congenital bleeding disorder associated with abnormal $P2Y_{12}$-mediated platelet responses to ADP has been characterized.[7] Platelets from this patient underwent normal shape change but reduced and reversible aggregation in response to 4 μmol/L ADP, similar to normal platelets with blocked $P2Y_{12}$ receptor. The response to 20 μmol/L ADP, albeit still decreased, was more pronounced and was decreased by a $P2Y_{12}$ antagonist, indicating residual receptor function. ADP failed to lower the adenylyl cyclase activity stimulated by prostaglandin E_1 in the patient's platelets, even though the number and affinity of [^{33}P]-2MeSADP binding sites was normal. Analysis of the patient $P2Y_{12}$ gene revealed, in one allele, a G to A transition changing the codon for Arg^{256} in the sixth transmembrane domain (TM6) to Gln and, in the other, a C to T transition changing the codon for Arg^{265} in the third extracellular loop (EL3) to Trp. Neither mutation interfered with receptor surface expression but both altered function, since ADP inhibited the forskolin-induced increase of cAMP markedly less in cells transfected with either mutant $P2Y_{12}$ than cells with the wild type receptor.

Heterozygous $P2Y_{12}$ defect

The study of the son of patient MG allowed the characterization of a heterozygous $P2Y_{12}$ defect.[4] The son's platelets bound intermediate levels of [^{33}P]2MeS-ADP and underwent a normal first wave of aggregation after stimulation with ADP, but did not secrete normal amounts of ATP after stimulation with different agonists. This secretion defect was not caused by impaired production of thromboxane A2 or low concentrations of platelet granule contents, and is therefore very similar to that described in patients with an ill-defined and probably heterogeneous group of congenital defects of platelet secretion, sometimes referred to by the general term *primary secretion defect* (PSD). This defect, which is the most common congenital abnormality of platelet secretion, is characterized by abnormal/borderline-low platelet secretion induced by different

agonists, a normal primary wave of aggregation induced by ADP, normal granule stores and normal arachidonate metabolism. The results of this study, therefore, confirm our previous hypothesis that (some) patients with PSD are heterozygous for a severe defect of $P2Y_{12}$.[8] The important role of ADP interaction with its $P2Y_{12}$ receptor in primary hemostasis is emphasized by the finding that the patient, like others with the platelet PSD, in spite of the mild defect of $P2Y_{12}$, has a mild prolongation of the bleeding time (13 min).

Diagnosis

Based on the hypothesis that the most common congenital defect of platelet function, PSD, is due to heterozygous $P2Y_{12}$ deficiency, it is likely that the severe defect is relatively common and that, due to its characteristics and to the fact that it is not yet well known, it is currently under-diagnosed, being confused with other platelet function abnormalities.[9] It is therefore important to underline that this condition should be suspected when ADP, even at relatively high concentrations (≥ 10 μM), induces a slight and rapidly reversible aggregation which is preceded by normal shape change. Of the two possible confirmatory diagnostic tests, measurement of the platelet binding sites for radiolabeled 2MeSADP and inhibition of stimulated adenylyl cyclase by ADP, the latter one should be preferred because it is easier to perform, cheaper, more specific, and sensitive not only to quantitative abnormalities of the receptor but also to functional defects.

Therapy

The intravenous infusion of the vasopressin analog DDAVP (0.3 μg/kg) shortened the prolonged bleeding time of patient VR from 20 min to 8.5 min.[10] After the infusion of DDAVP, which was repeated twice at 24 hour intervals, the patient underwent a surgical intervention for disc hernia repair, which was not complicated by excessive bleeding. Although the efficacy of DDAVP in reducing bleeding complications of patients with defects of primary hemostasis is anecdotal, its administration is generally without serious side effects. Therefore, DDAVP can be recommended for the prophylaxis and treatment of bleeding episodes in these patients.

References

1. Cattaneo M, Lecchi A, Randi AM, McGregor JL, Mannucci PM. Identification of a new congenital defect of platelet function characterized by severe impairment of platelet responses to adenosine diphosphate. Blood 1992; 80:2787-96.
2. Gachet C, Cattaneo M, Ohlmann P, Hechler B, Lecchi A, Chevalier J, et al. Purinoceptors on blood platelets: further pharmacological and clinical evidence to suggest the presence of two ADP receptors. Br J Haematol 1995; 91:434-44.
3. Nurden P, Savi P, Heilmann E, Bihour C, Herbert JM, Maffrand JP, et al. An inherited bleeding disorder linked to a defective interaction between ADP and its receptor on platelets. Its influence on glycoprotein IIb-IIIa complex function. J Clin Invest 1995; 95:1612-22.
4. Cattaneo M, Lecchi A, Lombardi R, Gachet C, Zighetti ML. Platelets from a patient heterozygous for the defect of P2CYC receptors for ADP have a secretion defect despite normal thromboxane A2 production and normal granule stores: further evidence that some cases of platelet 'primary secretion defect' are heterozygous for a defect of P2CYC receptors. Arterioscler Thromb Vasc Biol 2000; 20:E101-E106.
5. Hollopeter G, Jantzen HM, Vincent D, Li G, England L, Ramakrishnan V, et al. Identification of the platelet ADP receptor targeted by antithrombotic drugs. Nature 2001; 409:202-7.
6. Conley PB, Jurek MM, Vincent D, Lecchi A, Cattaneo M. Unique mutations in the P2Y12 locus od patients with previously described defects in ADP-dependent aggregation. Blood 2001; 98:43b[abstract].
7. Cattaneo M, Zighetti ML, Lombardi R, Martinez C, Lecchi A, Conley PB, et al. Phenotypic and Genotypic characterization of a congenital bleeding disorder caused by a dysfunctional P2Y12 ADP receptor. Blood 2001; 98: 443a [abstract].
8. Cattaneo M, Lombardi R, Zighetti ML, Gachet C, Ohlmann P, Cazenave JP, et al. Deficiency of ^{33P}2MeS-ADP binding sites on platelets with secretion defect, normal granule stores and normal thromboxane A2 production. Evidence that ADP potentiates platelet secretion independently of the formation of large platelet aggregates and thromboxane A2 production. Thromb Haemost 1997; 77:986-90.
9. Cattaneo M, Lecchi A. Patients with congenital abnormality of platelet aggregation induced by $Ca^{(2+)}$ ionophores may have a defect of the platelet $P2Y_{12}$ receptor for ADP. Br J Haematol 2001; 115:485-7.
10. Cattaneo M, Zighetti ML, Lombardi R, Mannucci PM. Role of ADP in platelet aggregation at high shear: studies in a patient with congenital defect of platelet responses to ADP. Br J Haematol 1994; 88:826-9.

Unique mutations in the $P2Y_{12}$ locus of patients with previously described defects in ADP-dependent aggregation

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Pharmacological evidence suggests that ADP-dependent platelet aggregation requires activation

of two G protein-coupled receptors (GPCRs): the $P2Y_1$ receptor coupled to Gq which mediates intracellular calcium mobilization and platelet shape change, and the second receptor, coupled to inhibition of adenylate cyclase through Gi and the target of antiplatelet agents (ticlopidine and clopidogrel), recently cloned by our group and designated $P2Y_{12}$.[1] Three different studies have characterized patients with apparent defects in $P2Y_{12}$,[2-4] one of whom (ML) has been previously studied at the genetic level. We have now determined the molecular basis of the defect in ADP-dependent aggregation in these additional patients. Sequence analysis of the first patient, VR, revealed a homozygous mutation consisting of a two bp deletion in the $P2Y_{12}$ locus occurring 294 bp from the initiation methionine, resulting in a frame shift mutation and a premature truncation of the protein. Using peptide antisera raised to the carboxy-terminus of h $P2Y_{12}$, we demonstrated that platelet lysates from VR do not express wild-type $P2Y_{12}$ protein, and that a tagged-truncated protein can be detected. Similarly, for the second set of patients, two sisters[4] were found to each harbor an identical homozygous single bp deletion occurring just beyond TM3 in the $P2Y_{12}$ protein, again causing a frame shift mutation and premature truncation of the protein, and were also shown to have wild-type $P2Y_{12}$ in platelet lysates. Thus, in these additional patients, the lack of a functional $P2Y_{12}$ receptor can be attributed to a specific defect in the $P2Y_{12}$ gene. In conclusion, all the patients previously shown to be defective in ADP-dependent aggregation appear to have distinct mutations in the $P2Y_{12}$ locus.

References

1 Hollopeter G, Jantzen HM, Vincent D, Li G, England L, Ramakrishnan V, et al. Identification of the platelet ADP receptor targeted by antithrombotic drugs. Nature 2001; 409:202-7.

2. Cattaneo M, Lecchi A, Randi AM, McGregor JL, Mannucci PM. Identification of a new congenital defect of platelet function characterized by severe impairment of platelet responses to adenosine diphosphate. Blood 1992; 80:2787-96.

3. Nurden P, Savi P, Heilmann E, Bihour C, Herbert JM, Maffrand J, et al. An inherited bleeding disorder linked to a defective interaction between ADP and its receptor on platelets. Its influence on glycoprotein IIb-IIIa complex function. J Clin Invest 1995; 95:1612-22.

4. Cattaneo M, Lecchi A, Lombardi R, Gachet C, Zighetti ML. Platelets from a patient heterozygous for the defect of P2CYC receptors for ADP have a secretion defect despite normal thromboxane A2 production and normal granule stores: further evidence that some cases of platelet 'primary secretion defect' are heterozygous for a defect of P2CYC receptors. Arterioscler Thromb Vasc Biol 2000; 20:20:E101-6.

The platelet P2 receptors in experimental thrombosis

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Since the P2 receptors have distinct roles in platelet functions, they may have different roles in thrombosis. The $P2Y_{12}$ receptor has long been recognized as an attractive target for antithrombotic drugs. Ticlopidine and clopidogrel, as well as being direct $P2Y_{12}$ antagonists, have been shown to be efficient in animal models of thrombosis. These have included a collagen-coated arterioarterial shunt in the rat, stenosed and endothelium-injured coronary arteries in dogs and non-human primates, tPA-mediated thrombolysis of electrically-injured coronary thrombi in dogs, cyclic flow reduction in injured femoral arteries of dogs, electrically damaged stenosed carotid arteries of rabbits, and prosthetic grafts in femoral arterioarterial shunts in dogs. Clopidogrel has also been shown to have some effect in models of venous thrombosis in rats and rabbits. Concerning the $P2Y_1$ receptor, $P2Y_1$ knockout (KO) mice have been used in models of systemic thromboembolism induced by a mixture of collagen and adrenaline, or by tissue factor in which reduced mortality and/or platelet consumption was observed. Localized arterial thrombosis induced by ferric chloride in mesenteric arterioles was also studied and $P2Y_1$ KO mice had less thrombosis than did wild type mice. These studies were also performed using the competitive $P2Y_1$ antagonist MRS2179 which displayed a clear antithrombotic effect. In addition, this compound was found to be effective in the collagen-coated arterioarterial shunt and in venous thrombosis in the rat. These results indicate that the $P2Y_1$ receptor might also be a target for antithrombotic drugs. Platelets also express the $P2X_1$ receptor and its role is beginning to be better characterized, including shape change in human platelets and a contribution to collagen-induced platelet activation in mouse and human platelets. Using $P2X_1$ KO mice in systemic thromboembolism models no difference could be observed between KO and wild type mice suggesting that this receptor has only a minor role if any in thrombosis.

The role of aspirin in primary prevention

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Cardiovascular disease is the leading cause of death in Western countries. Low-dose aspirin administration has been shown to be effective in various high-risk conditions[1] and is currently recommended to prevent further clinical disease in subjects with a previous thrombotic history as well as in subjects with cerebrovascular or coronary instability. The role of aspirin in primary prevention is, however, less extensively investigated. Five randomized trials have tested the benefit/risk ratio of aspirin administration in the primary prevention of a cardiovascular event.[2-6] These studies tested aspirin regimens ranging from 75 to 500 mg once a day in low-risk, mostly male, subjects as well as in individuals with one or more risk factors. Meta-analysis of pooled data from these studies indicates that aspirin can reduce the risk of coronary artery disease by approximately one quarter while seemingly having no significant effect on mortality and stroke.[7] In high-risk patients this aspirin protection can be translated into a significant clinical benefit while, in subjects with a lower risk, treatment benefit may be offset by major bleedings caused by aspirin treatment. Excess bleeding from aspirin use has been consistently estimated, in both primary and secondary intervention trials, to be one bleed/1,000 patients per year. This largely consists of gastrointestinal bleeding but a moderate increase in intracranial hemorrhages has also been reported. The benefit/risk ratio of aspirin use thus progressively increases at higher levels of absolute risk. In evaluating aspirin use in primary prevention, physicians need to estimate the vascular risk in the individual patient using the available chart or scoring systems. These tools, although approximate and often based on epidemiological data gathered in populations with different characteristics, are becoming essential for tailoring an appropriate antithrombotic strategy. For practical purposes, it seems reasonable to consider aspirin administration in any subject older than 50 who has at least one major risk factor such as smoking, diabetes, hypertension and hypercholesterolemia. Most of these patients will have, according to the European Risk Chart, a vascular risk level ≥ 0.9% per year and /or a coronary risk, based on the Framingham risk score, > 0.6% per year. At these risk levels aspirin is potentially beneficial and accordingly, the U.S. Preventive Services Task Force has proposed considering these risk thresholds for aspirin use in primary prevention. In any case treatment must be discussed with the individual patient previously informed of current knowledge on the risk/benefit ratio of aspirin in primary prevention as well as on the essential role of other antithrombotic interventions such as the aggressive treatment of risk factors, attainment and maintenance of adequate body weight and leading a healthy life style. Future perspectives in this setting may include more precise estimates of aspirin's benefit in low-to moderate-risk subjects, the elucidation of mechanism(s) underlying the so-called *aspirin resistance* as well as the identification of patients at an increased risk of bleeding. These achievements, together with a better knowledge of the effect of combined antithrombotic approaches, may lead to optimization of aspirin use in primary prevention.

References

1. Anonymous. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. Antithrombotic Trialists' Collaboration. BMJ 2002; 12 324:71-86.
2. Peto R, Gray R, Collins R, Wheatley K, Hennekens C, Jamrozik K, et al. Randomised trial of prophylactic daily aspirin in British male doctors. BMJ 1988; 296:313-6.
3. Anonymous. Final report on the aspirin component of the ongoing Physicians' Health Study. Steering Committee of the Physicians' Health Study Research Group. N Engl J Med 1989; 321:129-35.
4. Anonymous. The Medical Research Council's General Practice Research Framework. Thrombosis prevention trial: randomised trial of low-intensity oral anticoagulation with warfarin and low-dose aspirin in the primary prevention of ischaemic heart disease in men at increased risk. Lancet 1998; 351:233-41.
5. Hansson L, Zanchetti A, Carruthers SG, Dahlof B, Elmfeldt D, Julius S, et al. Effects of intensive blood-pressure lowering and low-dose aspirin in patients with hypertension: principal results of the Hypertension Optimal Treatment (HOT) randomised trial. HOT Study Group. Lancet 1998; 351:1755-62.
6. de Gaetano G. Low-dose aspirin and vitamin E in people at cardiovascular risk: a randomised trial in general practice. Collaborative Group of the Primary Prevention Project. Lancet 2001; 357:89-95.
7. Hayden M, Pignone M, Phillips C, Mulrow C. Aspirin for the primary prevention of cardiovascular events: a summary of the evidence for the U.S. Preventive Services Task Force. Ann Intern Med 2002; 136:161-72.

Ongoing and future clinical trials with clopidogrel

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A wealth of evidence has demonstrated the benefits of long-term antiplatelet therapy for the secondary prevention of atherothrombotic events.[1] Clopidogrel, a specific, irreversible antagonist of the platelet $P2Y_{12}$ ADP receptor,[2] entered clinical development in 1985 and received its first marketing approval in 1998. The efficacy of clopidogrel was demonstrated in the landmark CAPRIE trial,[3] which showed that clopidogrel is superior to acetylsalicylic acid (ASA) in reducing the combined risk of ischemic stroke (IS), myocardial infarction (MI) and vascular death (overall relative risk reduction of 8.7%, $p = 0.043$) in patients with symptomatic atherothrombotic disease. Based on the results of the CAPRIE trial, clopidogrel is indicated for the reduction of IS, MI and vascular death in patients with a history of recent MI, recent IS, or established peripheral arterial disease (PAD). Subsequently, the results of the CURE study demonstrated the benefit of clopidogrel on top of other standard therapies including ASA in patients with acute coronary syndromes. In CURE, clopidogrel demonstrated short-term and long-term efficacy in preventing a composite endpoint of cardiovascular death, MI and stroke (relative risk reduction = 20%, $p = 0.00009$) in patients presenting with unstable angina and/or non-Q-wave MI.[4] Data from CURE formed the basis for a new indication for clopidogrel in patients with acute coronary syndrome without ST-segment elevation this year.

Ongoing trials in atherothrombosis

Although the benefit of clopidogrel on top of standard therapy (including ASA) has been demonstrated in patients with unstable angina/non-Q-wave MI,[4] this regimen has not yet been evaluated in patients with recent manifestations of cerebrovascular atherothrombosis. This is being addressed in MATCH, which is comparing clopidogrel on top of ASA versus clopidogrel alone in an international, randomized, double-blind trial in high-risk patients with recent transient ischemic attack or IS.[5] The planned duration of treatment and follow-up for each patient is 18 months, and the primary endpoint is a composite of IS, MI, vascular death or rehospitalization for acute ischemia. Patient recruitment (n = 7,601 patients) was completed earlier this year and initial results are expected in 2004. COMMIT/CCS-2 is evaluating the benefit of clopidogrel versus placebo for acute secondary prevention in patients presenting within 24 hours of acute MI and receiving standard therapy (including ASA).[6] Patients are randomized to study medication for up to 4 weeks or until hospital discharge; the two main study endpoints are death and major vascular events during the scheduled treatment period in hospital. To date, more than 20,000 patients have been recruited; the planned sample size is 40,000. Building on the large body of evidence of the efficacy and safety of clopidogrel on top of ASA after coronary stenting,[7,8] CREDO is a randomized, double-blind trial in more than 2,000 patients scheduled for percutaneous transluminal coronary angioplasty ± stent placement. The aim of CREDO is to evaluate the long-term (12-month) efficacy and safety of clopidogrel, as well as the benefit of pretreatment with a 300-mg clopidogrel loading dose on top of ASA.

Finally, the ongoing WATCH study is being performed to compare warfarin, clopidogrel and ASA in patients with congestive heart failure.

Future trials in atherothrombosis

Further trials of clopidogrel in atherothrombotic patients are planned. In cardiology, CLARITY (TIMI 28) will complement COMMIT/CCS-2, evaluating the benefit of clopidogrel on top of ASA in preventing re-occlusion in acute MI patients receiving thrombolytic therapy. CHARISMA is a major, double-blind, randomized trial that will evaluate the long-term efficacy and safety of clopidogrel in a broad spectrum of patients at high-risk of atherothrombosis. CHARISMA will compare clopidogrel versus placebo on top of standard therapy (including low-dose ASA) in over 15,000 high-risk patients (qualifying with a combination of atherothrombotic risk factors and/or documented cerebrovascular disease and/or documented coronary disease and/or symptomatic PAD). PAD patients, even if asymptomatic, are likely to have disseminated atherothrombosis and have an increased risk of MI and stroke. Moreover, in PAD patients undergoing peripheral interventions, prognosis is suboptimal, with a substantial proportion of patients experiencing graft occlusion or restenosis. These considerations form the basis of the CASPAR and CAP trials in patients who have undergone below-the-knee bypass surgery and peripheral angioplasty/stenting, respectively.

Perspectives in atrial fibrillation

It is also planned to evaluate clopidogrel in patients with atrial fibrillation (AF) receiving ASA, since there is a strong rationale to support the dual antiplatelet regimen in this population of patients. Antiplatelet therapy has been shown to be effective against thrombotic events related to stasis. Furthermore, the typical AF patient who requires antithrombotic therapy also has multiple risk factors for arterial vascular disease. Based on CURE, it is reasonable to hypothesize that clopidogrel on top of ASA would be superior to ASA alone in AF patients. It is also likely that clopidogrel plus ASA would have comparable efficacy to warfarin in patients with AF. These hypotheses will be tested in the ACTIVE trial.

Conclusions

A comprehensive program of ongoing and future clinical trials will evaluate the benefit of clopidogrel in a broad range of patients. These studies will evaluate acute and long-term therapy, and target patients with atherothrombotic manifestations affecting the different arterial beds (cerebral, coronary and peripheral). These trials will permit a more in-depth evaluation of the efficacy and safety of clopidogrel for the prevention of atherothrombotic events in different clinical settings, especially in high-risk patients, and also evaluate clopidogrel for the prevention of cardioembolic complications in patients with AF.

References

1. Anonymous. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. Antithrombotic Trialists' Collaboration. BMJ 2002; 324:71–86.
2. Savi P, Labouret C, Delesque N, Guette F, Lupker J, Herbert JM. $P2y_{12}$, a new platelet ADP receptor, target of clopidogrel. Biochem Biophys Res Commun 2001; 283: 379-83.
3. Anonymous. A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). CAPRIE Steering Committee. Lancet 1996; 348:1329–39.
4. Anonymous. The clopidogrel in unstable angina to prevent recurrent events trial investigators. Effects of Clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. N Engl J Med 2001; 345: 494-502.
5. Anonymous. Major ongoing stroke trials. Stroke 2002; 33:1728.
6. Anonymous. Rationale, design and organization of the Second Chinese Cardiac Study (CCS-2): a randomized trial of clopidogrel plus aspirin, and of metoprolol, among patients with suspected acute myocardial infarction. Second Chinese Cardiac Study (CCS-2) Collaborative Group. Second Chinese Cardiac Study (CCS-2) Collaborative Group. J Cardiovasc Risk 2000; 7:435–41.
7. Mehta SR, Yusuf S, Peters RJ, Bertrand ME, Lewis BS, Natarajan MK, et al. Effects of pretreatment with clopidogrel and aspirin followed by long-term therapy in patients undergoing percutaneous coronary intervention: the PCI-CURE study. Lancet 2001; 358:527–33.
8. Bhatt DL, Bertrand ME, Berger PB, L'Allier PL, Moussa I, Moses JW, et al. Meta-analysis of randomized and registry comparisons of ticlopidine with clopidogrel after stenting. J Am Coll Cardiol 2002; 39:9–14.

index of authors

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