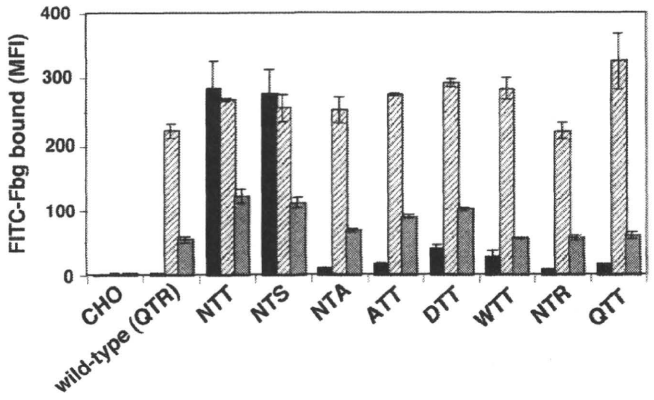

FIGURE 4. **Constitutive activation depends on the binding of *N*-glycan in the Q595N/R597T mutant.** Three amino acid residues starting from Gln-595 in the αIIb chain were mutated to various sequences. Fbg binding to the cells in the presence of 1 mM Ca^{2+}/1 mM Mg^{2+} with a control antibody (*solid column*) or with PT25-2 (*hatched column*) and in the presence of 1 mM Mn^{2+} with a control antibody (*gray column*) was examined. *MFI*, mean fluorescent intensity. *Error bars* indicate S.E.

comparable with that induced by NTT (Fig. 4). These results suggest that the introduction of virtually any sequence other than the N*X*(T/S) sequence fails to induce constitutive activation. Thus, these results indicate that the activating effect of these mutations actually depends on the attachment of a bulky *N*-glycan to these sites.

To confirm whether *N*-glycans are actually attached to the intended sites in these mutants, we next compared their molecular sizes using SDS-PAGE. If an extra *N*-glycan is indeed attached to these mutants, their molecular size should be larger than that of the wild-type. When surface-expressed αIIbβ3 was labeled with biotin and immunoprecipitated with anti-αIIb mAb, the β3 chain was always co-precipitated from the cells expressing wild-type or mutant αIIbβ3. However, the size of the mutant αIIb chain that carries an extra *N*-glycan-binding site (T478N, D589N/H591T, Q595N/R597T) was not remarkably different from that of the wild-type (supplemental Fig. S3*A*). As the size of the *N*-glycan was relatively small when compared with the entire αIIb chain, it was difficult to discriminate such small differences in molecular weight using SDS-PAGE. To circumvent this problem, the αIIb leg region encompassing amino acid residues 450–1008 was generated using a FLAG tag sequence on its N terminus. This fragment was surface-expressed and migrated as an 89-kDa band on SDS-PAGE when immunoprecipitated with PL98DF6. In contrast, a similar fragment carrying an extra *N*-glycan binding site migrated as a 91-kDa band, which is slightly larger than that of the wild type. The fragment that was not supposed to attach *N*-glycan (R597T) migrated as fast as the wild type (supplemental Fig. S3*B*). Similar results were obtained when anti-FLAG M2 was used instead of PL98DF6 (data not shown). This difference in apparent molecular weight was completely lost when the fragments were digested with peptide *N*-glycosidase F as all the fragments migrated as 69-kDa bands (supplemental Fig. S3*C*). These results clearly indicate that the mutant αIIb that carries an extra N*X*(T/S) motif indeed binds *N*-glycan to these sites.

αIIbβ3 Activation Induced by Integrin Extension Depends on the Swing-out of the Hybrid Domain—Takagi *et al.* (10) and Luo *et al.* (23) have reported that the outward swing of the hybrid

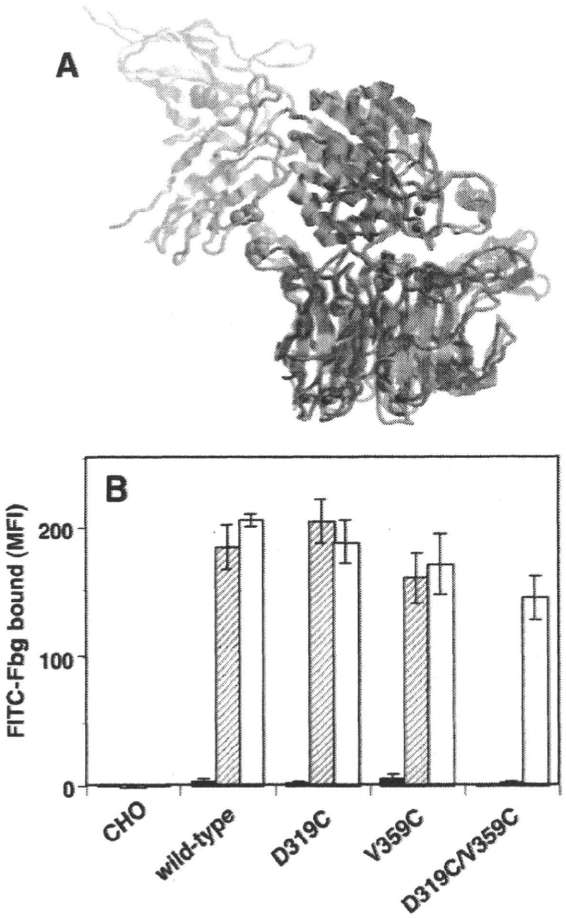

FIGURE 5. **Swing-out of the hybrid domain is required for integrin activation.** *A*, crystal structure of the αVβ3 head domains derived from the bent conformation complexed with RGD peptide is overlaid on the crystal structure of the αIIbβ3 head domains complexed with the ligand, which is shown as a *semitransparent ribbon*. In both parts of this graphic, the β-propeller domain in the α-chain is shown as a *blue ribbon*. The βA and the hybrid domains in the β3 chain are shown as *red* and *orange ribbons*, respectively. Note that Asp-319 in αIIb (Asp-306 in αV), shown as *cyan spacefill*, and Val-359 in β3, shown as *magenta spacefill*, are close to each other in the closed head (as in αVβ3) but are separated in the open head (as in αIIbβ3). *B*, to investigate the role of the swing movement of the hybrid domain in integrin activation, Asp-319 in αIIb and/or Val-359 in β3 were mutated to Cys to facilitate disulfide bridge formation between the two residues. Fbg binding to cells in the presence of 1 mM Ca^{2+}/1 mM Mg^{2+} is shown. The *solid column* represents binding in the presence of control antibody, and the *hatched column* represents binding in the presence of PT25-2. The *open column* represents binding to cells pretreated with DTT. *MFI*, mean fluorescent intensity. *Error bars* indicate S.E.

domain is the most critical step in integrin activation. To examine whether this step is truly required for activation, the swing-out of the hybrid domain was prevented by covalently ligating the β-propeller and the hybrid domains with a disulfide bridge. The amino acid residues Asp-319 of the αIIb β-propeller and Val-359 of the β3 hybrid domain are physically close in the closed head (swing-in) conformation, whereas they are separated in the open head (swing-out) conformation (Fig. 5*A*). If these residues are simultaneously mutated to Cys, a disulfide bridge will be formed between these domains, thereby fixing the angle between the βA and hybrid regions in the closed head conformation. The 2-3 loop in blade 5 of the αIIb propeller, where Asp-319 is located, was previously shown not to partic-

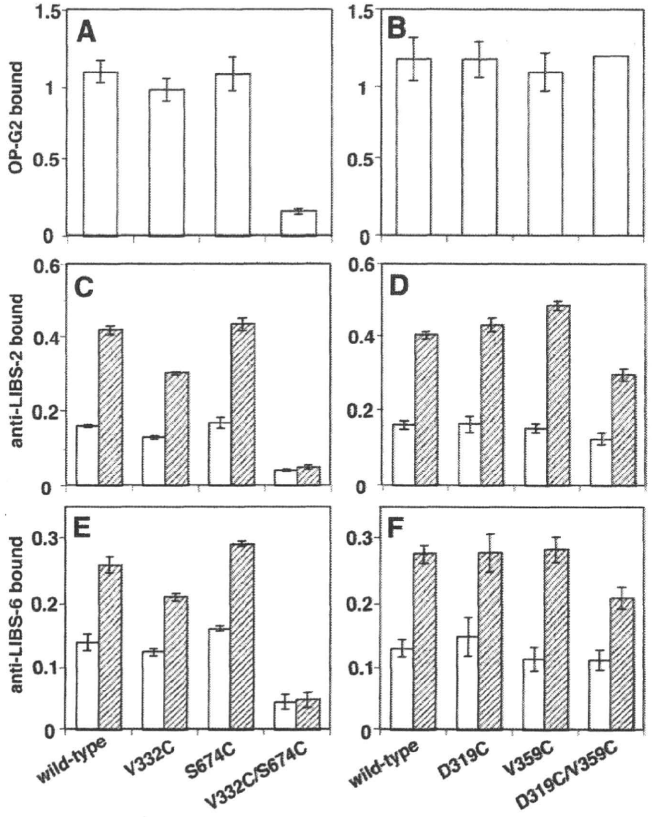

FIGURE 6. **mAb binding to cells expressing αIIbβ3 constrained in the bent conformation or in the closed head.** The binding of the ligand-mimetic mAb OP-G2 (*A* and *B*), anti-LIBS mAbs anti-LIBS2 (*C* and *D*), and anti-LIBS6 (*E* and *F*) to cells expressing αIIbβ3 constrained in the bent conformation (*A*, *C*, and *E*) or in the closed head (*B*, *D*, and *F*) was examined. Values in the *y* axis represent normalized mAb binding. The *open column* represents mAb binding in the presence of 1 mM Ca^{2+}/1 mM Mg^{2+}. The *hatched column* represents binding in the presence of 1 mM GRGDS peptide under the same cation conditions. *Error bars* indicate S.E.

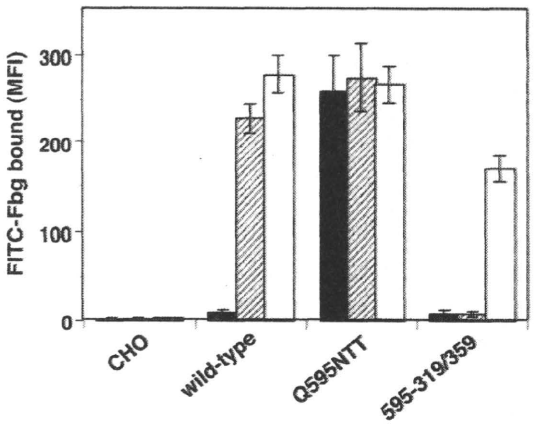

FIGURE 7. **Swing-out of the hybrid domain is required for activation induced by integrin extension.** The effect of the swing-out of the hybrid domain on extension-induced integrin activation was examined. To constrain αIIbβ3 in the extended conformation with a closed head, the Q595N/R597T and D319C/V359C mutations were combined (595-319/359). Fbg binding to the cells in the presence of 1 mM Ca^{2+}/1 mM Mg^{2+} and the control antibody (*solid column*) or PT25-2 (*hatched column*) and Fbg binding to cells pretreated with DTT (*open column*) is shown. *MFI*, mean fluorescent intensity. *Error bars* indicate S.E.

ipate in ligand binding (24). As shown in Fig. 5*B*, single D319C or V359C mutation did not significantly affect Fbg binding. However, D319C/V359C double mutation completely abolished the Fbg binding induced by PT25-2 unless the cells were pretreated with DTT. The binding of PT25-2 was unaffected by disulfide formation (supplemental Fig. S1). Next, we examined the binding of an activation-independent ligand-mimetic mAb, OP-G2. OP-G2 has an RGD-related RYD sequence in the CDR3 and binds αIIbβ3 in almost the same fashion as Fbg, although it does not require integrin activation for binding (25). Unlike the V332C/S674C mutation, which keeps integrin in a bent conformation, the D319C/V359C mutation did not affect OP-G2 binding (Fig. 6, *A* and *B*). To examine the effect on the conformational change induced by ligand binding, the binding of anti-LIBS mAb was examined. The binding of anti-LIBS2 and anti-LIBS6 increased significantly in the presence of RGD peptide in cells expressing wild-type αIIbβ3 as well as in cells expressing the single Cys mutation V332C or S674C. However, cells expressing V332C/S674C bound significantly less anti-LIBS mAb than cells expressing wild-type αIIbβ3, and these cells did not respond to RGD peptide (Fig. 6, *C* and *E*). In contrast, cells expressing D319C/V359C showed a basal binding comparable with that of the wild type or single Cys mutants, although the response to RGD peptide was slightly attenuated (Fig. 6, *D* and *F*). These results indicate that the swing-out of the hybrid domain is only required for high affinity ligand binding and that the prevention of the swing-out does not completely inhibit the conformational change associated with ligand binding. To examine whether the swing-out of the hybrid domain is required for the activation induced by integrin extension, we combined Q595N/R597T with D319C/V359C and examined its effect on Fbg binding. The resulting 595-319/359 mutant was expected to adopt an extended with a closed head conformation. The activating effect of the Q595N/R597T mutation was completely suppressed by the D319C/V359C mutation. PT25-2 was ineffective, unless the cells were pretreated with DTT (Fig. 7). These results suggest that integrin extension must be accompanied by the swing-out of the hybrid domain for it to induce activation.

DISCUSSION

By characterizing the recombinant αIIbβ3 integrin expressed in CHO cells, we established that αIIbβ3 constrained in its bent conformation represents a low affinity form, whereas αIIbβ3 constrained in its extended conformation represents a high affinity form. This constitutive activation depends on the swing-out of the hybrid domain because the prevention of this swing-out completely inhibited ligand binding regardless of the bent/extended state.

Integrin domains make large interdomain interfaces between the α-head and the β-head, the α-tail and the β-tail, and the β-head and the β-tail. Among these interfaces, the β-head/β-tail interface is presumed to play a central role in keeping integrin in its bent conformation because this is the only interaction that directly connects the head with the tail region in the bent conformer but not in the extended conformer. This interface is maintained by multiple interdomain interactions. The βA and the hybrid domains in the β-head make contact with the C-terminal βT domain. The hybrid domain also makes con-

tact with the EGF-3 and EGF-4 domains. We attempted to stabilize this interface by introducing artificial disulfide bridges between these domains. As previously reported, stabilizing the βA/βT interface (V332C/S674C) completely blocked Fbg binding (9). Likewise, stabilizing the hybrid/EGF-3 (S367C/S551C) or hybrid/EGF-4 (G382C/T564C) interface completely abolished Fbg binding. Regardless of the positions of the disulfide bridges that were introduced, stabilizing these interfaces prevented integrin from adopting the extended conformation. However, the S367C/S551C and G382C/T564C mutations not only prevented integrin extension but also restricted the relative movements of the hybrid and β-tail domains. For this reason, it might be premature to conclude that integrin extension is essential for activation. However, the fact that ligating the α-head with the β-tail or limiting αIIb extension using intrachain disulfide bridges that do not directly restrict hybrid/β-tail movement also prevented activation (9, 26) suggests that the completely bent conformer observed in the crystal structure represents the low affinity form rather than the high affinity form. These results also indicate that the β-head/β-tail interface must be disrupted all the way up to the linker region for the integrin to be activated.

The fact that the ligand-mimetic non-activation-dependent mAb OP-G2 did not bind to cells expressing the V332C/S674C mutant suggests that this mutant does not support low affinity ligand binding. In addition, anti-LIBS mAb binding to this particular mutant indicates that V332C/S674C is unable to undergo structural rearrangement in the presence of the RGD peptide. Taken together, these results suggest that the V332C/S674C mutant is not capable of binding either macromolecular ligands or ligands as small as the RGD peptide. Because the V332C/S674C mutation ligates the α7 helix in βA with the CD loop in βT, the possible downward movement of the α7 helix required for ligand binding in the integrin A domains would be inhibited. Thus, the effect of the V332C/S674C mutation is a combination of both β-head/β-tail stabilization and the inhibition of α7 helix movement. In agreement with these findings, the S367C/S551C mutation, which does not restrict α7 helix movement, only partially blocked OP-G2 binding (data not shown).

The contribution of each interface interaction in maintaining integrin in the bent conformation has not been clarified. It has been reported that replacing the β2 CD loop sequence with the homologous β3 sequence or inserting a *N*-glycan-binding site in the CD loop in αMβ2 integrin induced robust ligand binding (27). However, the fact that the deletion of the CD loop of the βT domain failed to activate αIIbβ3 in our experiment strongly argues against the deadbolt theory, in which an endogenous βA/βT interface interaction plays a critical role in maintaining integrin in its low affinity state. In addition, the insertion of a bulky *N*-glycan at the interface only slightly activated αIIbβ3 in the presence of Ca^{2+}/Mg^{2+}. These results suggest that hybrid/EGF-3, hybrid/EGF-4, and hybrid/βT interface interactions, rather than the βA/βT interface alone, play important roles in maintaining integrin in a low affinity state. In agreement with these conclusions, a computer-assisted approach has identified key interactions in the hybrid/β-tail interface in β3 integrin (28). Although the disruption of the hybrid/βT or the hybrid/EGF-3 interaction alone only produced weak activation, disrupting multiple interactions at the same time induced significant activation. Thus, the hybrid/β-tail interface seems to be maintained by a group of several key interactions that individually are not sufficiently strong to do so. The apparently distinct role of the βA/βT interface interaction in regulating activation in β2 and β3 integrins suggests that the contributions of each interdomain interaction to maintaining integrin in a low affinity state may differ among different integrin subfamilies.

A high resolution electron microscopic analysis of recombinant αVβ3 extracellular domains revealed that ligand-bound αVβ3 preferentially adopts an extended, rather than a bent, conformation (9). Based on these observations, it has been tentatively concluded that the extended conformer represents a high affinity form. The current study provides direct evidence that the highly extended conformer indeed has a higher ligand affinity than the completely bent conformer. Among the three mutants that showed constitutive Fbg binding in this study, β3S674N/K676T showed the lowest, αIIbQ595N/R597T showed the highest, and αIIbD589N/H591T showed an intermediate binding affinity. These results suggest that the degree of extension may be correlated with the extent of activation. These results also indicate that integrins are capable of assuming a wide range of affinity states depending on the degree of extension. Recently, it has been shown that integrin extension may not necessarily be accompanied by activation based on discrepancies between the expression of an extension-reporting epitope for KIM127 and an activation-reporting epitope for mAb24 on αLβ2 under flow conditions (29). It is possible that αA domain-containing integrin may require an additional step to achieve activation, unlike integrins without αA domains. It would be interesting to examine whether the introduction of a neoglycan that induces αL extension activates αLβ2.

Our results apparently contradict a report that αVβ3 is capable of binding fibronectin while in a bent conformation (14). However, it is not possible to tell to what degree integrin must extend to enable substantial ligand binding based on our experiments. The fact that αIIbβ3 can exist in a wide range of affinity states depending on the degree of the extension implies that as long as it is not completely bent, ligand binding could be observed to a varying extent. In other words, relaxation of the β-head/β-tail interface interaction, but not complete extension, may be sufficient for ligand binding to occur, especially in the presence of Mn^{2+}. As shown in Fig. 3*B*, Mn^{2+} seems to lessen the requirement for integrin extension for Fbg binding. Mn^{2+} activation alone has consistently been reported not to be accompanied by integrin extension (30). A recent report by Blue *et al.* (26) has provided a plausible explanation for the discrepancies in ligand binding observed under different cation conditions. Limiting αIIb extension using intrachain disulfides did not block Fbg binding in the presence of Mn^{2+}, although binding was blocked in the presence of Ca^{2+}/Mg^{2+}. In contrast, limiting β-head/β-tail movement using S367C/S551C, G382C/T564C, and V332C/S674C double mutations blocked Fbg binding significantly in the presence of Mn^{2+} as well as in the presence of Ca^{2+}/Mg^{2+} (data not shown). Taken together, these results may imply that it is not integrin extension *per se*, but

the relative β-head/β-tail movement (*e.g.* the swing-out of the hybrid domain), that is essential for activation in the presence of Mn^{2+}. These results may explain why ligand binding was observed for the bent conformer in some studies in which Mn^{2+} was utilized to induce ligand binding (13, 14). Further study is required to determine the differences in the structural requirements for activation under different cation conditions.

Springer and co-workers (10, 23) have shown that ligand binding induces swing-out of the hybrid domain and that this change induces strong activation by itself, regardless of the bent/extend conformation. Our results show that the swing-out of the hybrid domain is essential for activation and that extension-induced activation absolutely depends on this change. These results indicate that the affinity state of the extended conformer is controlled by the swing-out of the hybrid domain and that to down-regulate activation, integrin does not necessarily need to go back to its original bent conformation but that this can rather be accomplished by the swing-in of the hybrid domain. Interestingly, constraining the integrin head in a closed state did not prevent OP-G2 binding at all (Fig. 6*B*). Unlike PAC-1, which binds αIIbβ3 in an activation-dependent fashion, OP-G2 is less dependent on integrin activation (18). This difference indicates that the swing-out of the hybrid domain is required only for high affinity ligand binding but not for low affinity ligand binding. However, we are not sure at this point whether the swing-out of the hybrid domain alone is sufficient for high affinity ligand binding, as reported by Springer and co-workers (23). Our experiments using recombinant αIIbβ3 expressed on the CHO cell surface have shown that the swing-out of the hybrid domain only induced moderate activation. To induce full activation, integrin extension was required.[5] It is possible that the proximity of the integrin head domains to the plasma membrane in the bent conformation may limit the access of macromolecular ligands. Experiments utilizing cell-free binding studies should help to clarify these discrepancies. Interestingly, anti-LIBS mAb binding was still observed in the closed head mutant (D319C/V359C) in the presence of RGD peptide. Because ligand binding induces the outward swing of the hybrid domain and this movement would probably disrupt the β-head/β-tail interface, it is reasonable to assume that this swing-out triggers the structural transition from a bent to an extended conformation in outside-in signaling. However, our result suggests the possibility that a structural change in addition to the swing-out may trigger the conformational change upon ligand binding. A recent report also suggests that integrin affinity may be regulated independently from the swing-out of the hybrid domain based on the expression of anti-LIBS epitope located in the hybrid domain (31). Because we do not know the specific conformation to which each of the anti-LIBS mAbs binds, further analysis is needed to address this issue.

Then, what triggers the structural transition from the bent to the extended conformation during inside-out signaling? Numerous studies have suggested the importance of integrin cytoplasmic tails in regulating integrin activation. It has been shown that integrin cytoplasmic tails undergo structural rearrangement upon ligand binding (32). It was subsequently shown that the two cytoplasmic tails separate from each other upon ligand binding (3). On the other hand, the deletion of the entire α or β cytoplasmic tail at the membrane-proximal sites induced significant activation (33). NMR studies on recombinant αIIbβ3 cytoplasmic tails have shown that talin binding to the β3 cytoplasmic tail disrupts the endogenous interaction between the α and β cytoplasmic tails (2). Because talin binding to the β3 cytoplasmic tail activates αIIbβ3, it was concluded that the separation of the two cytoplasmic tails somehow induces structural rearrangement of the integrin extracellular domains (1). We have previously shown that stabilizing the α-tail/β-tail interface with artificial disulfide bridges completely abolished the activation induced by cytoplasmic tail deletion (22). Based on these observations, we hypothesized that the separation of the two extracellular tails following the cytoplasmic tail dissociation induces structural rearrangement from the bent to the extended conformation. Indeed, the separation of the α-tail/β-tail interface induced robust activation.[6] The α-tail/β-tail interface and the β-head/β-tail interface are located next to each other, flanking the β-tail. Because the β-head/β-tail interface, and not the α-tail/β-tail interface, maintains integrin in its bent conformation, it is reasonable to assume that the separation of one interface destabilizes the other. Further elucidation of the role of these interface interactions in integrin affinity regulation will facilitate understanding of integrin-mediated bidirectional signaling.

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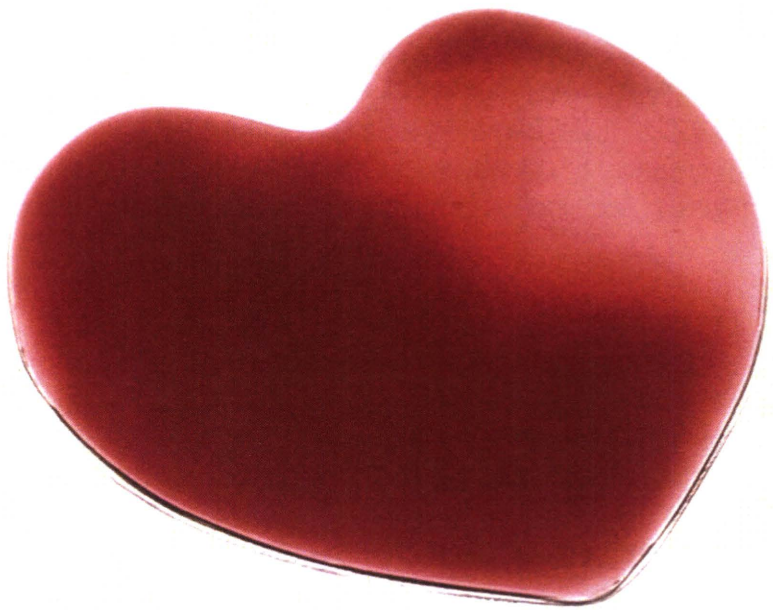
V. その他

平成22年度政策創薬総合研究推進事業研究成果等普及啓発事業　研究成果発表会

血液の不思議

人工血液はどこまで血液の代わりができるか？



2011年2月11日(金)
時間：午後1時30分～午後4時30分
慶應義塾大学信濃町キャンパス
医学情報センター　北里講堂
定員:250名

主　催：財団法人ヒューマンサイエンス振興財団
後　援：日本血液代替物学会

ご挨拶

このたびは厚生労働省科学研究・政策創薬創総合研究推進事業の平成22年度研究成果等普及啓発事業研究成果発表会「血液の不思議：人工血液はどこまで血液の代わりができるか?」にご参加をいただきましてありがとうございます。

20世紀は医学の進歩が著しく、輸血治療が確立し、多くの人々が輸血治療の恩恵を受けることができるようになりました。しかし、輸血治療が今日のように安全な治療手段となったのはつい20年前のことです。

輸血に関連した感染症については、たゆまぬ研究の結果1950年代の梅毒検査をはじめとし、現在ではB型肝炎ウィルス、C型肝炎、ヒト免疫不全ウィルス（HIV）のウィルス疾患については遺伝子増幅検査（NAT検査）を行って安全を確保しています。しかし、未知のウィルスやプリオン病に対する対策はまだ十分とはいえず、今後の研究に期待がかけられています。実際に輸血をする際には、血液型を合わせるために交差試験は必須で、迅速な治療の障壁となる場合があります。

また、少子高齢化の加速により、献血年齢の人口が急速に減少し、高齢者における使用が増える傾向は今後も続いてゆきます。また、大地震などの災害医療に用いる血液製剤の大量、迅速な供給も課題の一つです。

このような状況で輸血を補完する治療法として考えられているのが人工血液です。

本日は、まず献血で集められた血液がどのように使われているかについて分かりやすく説明していただきます。現在使用されている血液製剤としての輸血製剤、および血漿分画製剤についてご説明いただいたのち、血漿の役割を果たすために開発された人工血漿剤について現状と展望をお話しいただきます。

人工赤血球では血液型に関係なく、長期保存が可能で、感染症の危険のほとんどない製剤を目指して研究開発が進められています。われわれは厚生労働省の科学研究を通じて日本独自の人工酸素運搬体であるヘモグロビン小胞体を開発してきました。本日はこの物質の開発現状及び生体適合性について研究に携わっておられる先生方から研究成果について発表していただきます。

人工血小板の開発では、非常に巧みな粒子設計から生まれた日本独自の物質の開発が進んでいます。実験室での成果としては魅力のある成績を上げていますので、研究成果についてのご発表をいただきます。

この講演会を通じて血液の重要性、人工血液の必要性、開発状況についてご理解を深めていただければ幸いです。

平成23年2月11日

慶應義塾大学　医学部　呼吸器外科　准教授
堀之内宏久

プログラム

13:30	「開会のあいさつ」 慶應義塾大学　医学部　名誉教授	小林　紘一
第1部　司会	慶應義塾大学　医学部　名誉教授	小林　紘一
13:35~13:55	「血液の役割、献血と輸血」 北海道赤十字血液センター　所長	池田　久實
13:55~14:15	「血漿分画製剤の利用」 日本赤十字社　血液事業本部 血液事業経営会議委員	伴野　丞計
14:15~14:35	「人工血漿剤の利用」 川崎医科大学　名誉教授 東宝塚さとう病院　名誉院長	高折　益彦
14:35~14:55	「人工赤血球の開発現状」 慶應義塾大学　医学部　呼吸器外科　准教授	堀之内宏久
	14:55~15:05　休憩	
第2部　司会	川崎医科大学　名誉教授 東宝塚さとう病院　名誉院長	高折　益彦
15:05~15:25	「人工赤血球の体内での行方」 熊本大学　薬学部　客員教授 崇城大学　薬学部　教授	小田切優樹
15:25~15:45	「人工赤血球が体に与える影響（免疫系への作用）」 北海道赤十字血液センター　研究部　研究一課長	藤原　満博
15:45~16:05	「人工血小板の開発現状」 慶應義塾大学病院　輸血・細胞療法部　教授	半田　　誠
16:05~16:25	「人工血液のヒトでの利用は」 慶應義塾大学　医学部　名誉教授	小林　紘一
16:25~16:30	「閉会のあいさつ」 慶應義塾大学　医学部　呼吸器外科　准教授	堀之内宏久

発表概要と演者紹介

1 血液の役割、献血と輸血

北海道赤十字血液センター　所長　池田　久實

赤血球の輸血は、生命の維持に必要な酸素を体の隅々まで運ぶ赤血球の量の補充が目的です。輸血用血液の安定供給と安全性の確保が日赤血液センターの主な責務で、献血血液を製剤化し、輸血を必要とする患者に届けています。血液は「なまもの」であり、有効期間があるので、必要な量に見合う採血をすることが必須です。多すぎても少なすぎてもいけません。ABO型違い輸血をしないことも重要で、輸血された型違いの赤血球は壊され、重大な副作用を招くことがあるからです。

2 血漿分画製剤の利用

日本赤十字社　血液事業本部　血液事業経営会議委員　伴野　丞計

献血血液から製造される主要な血漿分画製剤の特徴とその利用について概説する。血液凝固因子製剤は、血友病の補充療法剤としての第Ⅷ、Ⅸ因子、及び血液凝固カスケードにおける役割、アルブミン製剤は等張製剤（5%）と高張製剤（20%, 25%）の臨床適応、そして免疫グロブリン製剤の種類と適応について紹介します。特に、適応拡大により使用量が急増した静注用人免疫グロブリン製剤（IVIG）については、その作用仮説を説明します。また、血漿分画製剤の国内自給率についても紹介します。

3 人工血漿剤の利用

川崎医科大学　名誉教授、東宝塚さとう病院　名誉院長　高折　益彦

血管内に電解質を維持できるのは血漿内にアルブミンのような膠質があるからです。このアルブミンの代用をするのが人工膠質であり、それを血漿類似の電解質液に溶解したのが人工血漿剤です。今回、この人工血漿剤がいかに臨床医療の現場で利用されているか解説します。

4 人工赤血球の開発状況

慶應義塾大学　医学部　呼吸器外科　准教授　堀之内　宏久

われわれは早稲田大学のグループとともに、期限切れで使用できなくなった献血血液より酸素を運ぶたんぱく質であるヘモグロビンを抽出、精製したのちに濃縮し、脂肪の膜で覆って、直径250ナノメーターの微小な球状の粒子を作成しました。ヘモグロビン小胞体と呼ばれるこの物質は、血液型がなく、2年間保存が可能です。生体内で赤血球に代わって酸素を運搬することができ、他の人工赤血球と比べ、副作用が少ないのが特徴です。臨床で使用する前に動物を用いての検討が進められ、その能力の高さに注目が集まっています。試験の成績を主に現在の開発状況を説明いたします。

5 人工赤血球の体内での行方

熊本大学　薬学部　客員教授、崇城大学　薬学部　教授　小田切　優樹

薬の開発において、その体内での動き（薬が体内のどこに、どれくらいの量と時間、存在するか）を知ることは重要なことです。薬は、作用部位に適切な時間に最適な量で存在しなければ効果を発揮することはできず（有効性）、反面、生体内で必要以上な量と期間存在すると、毒として作用する恐れがあります（副作用）。本講演では、人工赤血球の生体内での行方を追うことにより得られた情報をもとに、人工赤血球の有効性と安全性について分かりやすく紹介します。

6 人工赤血球が体に与える影響（免疫系への作用）

北海道赤十字血液センター　研究部　研究一課長　藤原　満博

ヘモグロビン小胞体は、脂質二重膜からなるリポソームの中にヘモグロビンを包みこんだ人工赤血球の一つです。人工赤血球として大量に投与されることから、ヘモグロビン小胞体そのものの安全性を調べる必要があります。ヘモグロビン小胞体を投与したラットから臓器を生体外にとり出した実験系では、一過性の免疫応答の低下がみられましたが、ラット個体での抗体産生は正常に起こることから、全身的な免疫応答へのヘモグロビン小胞体の影響は少ないと考えています。

7 人工血小板の開発現状

慶應義塾大学病院　輸血・細胞療法部　教授　半田　誠

血小板は破たんした血管を止血に導くうえで最も重要な血球成分です。大きさは大変小さく２ミクロン程度ですが、循環系の恒常性維持にはなくてはならない細胞です。血液の疾患や大量出血などで、血小板が極端に減ったりした際には血小板輸血を行います。保存期間が4日間と短く厳密な管理が必要な血小板輸血に代わっていつでもどこでも使える血小板代替物を開発しています。生理的な刺激成分を含み、特殊なペプチドでリポソーム表面を修飾したナノ粒子で、動物実験では実用化に向け有望な結果が得られています。

8 人工血液のヒトでの利用は

慶應義塾大学　医学部　名誉教授　小林　紘一

「血液は命」と表現されるように血液の働きは生命の維持にとって重要です。

安全で有効性が高く、長期間保存でき、いつでもどこでの使用可能な血液代替物に対する医学的、社会的要望は高まりつつあります。日本の研究グループは、厚生労働省のサポートを受け、世界の人工血液研究を牽引する成果を上げています。

酸素運搬をつかさどる人工赤血球に関してはヘモグロビンを脂質膜に内包したヘモグロビン小胞体（HbV）が血液型に関係なく使用できる物質として研究が進み、臨床応用へもうひと息というところまで来ています。人工血小板は血小板減少状態における止血時間の短縮や出血量の減少などを企図して研究が進んでいます。

ともに日本から世界に向かって発信できる研究成果として近い将来の臨床応用が期待されています。

第17回日本血液代替物学会年次大会
シンポジウム：人工血小板の現状と将来

平成22年10月18日　熊本

●人工血小板の必要性
- 輸血用血小板製剤：保存期間が短く（4日）、厳密な保存条件（22℃、振盪）⇒　緊急対応が困難

●人工血小板開発のコンセプト
- 血小板の機能を代替して、出血の予防や治療に用いる。
- しかし、血小板の機能をすべて備えることは不可能。
- 残存血小板数がゼロになる病態はない。

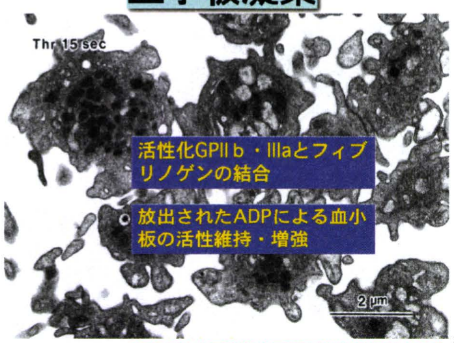
●人工血小板の要件
- 流血中では作用せず、止血局所でのみ働く

●人工血小板の標的
- 活性化血小板（リガンド型人工血小板）

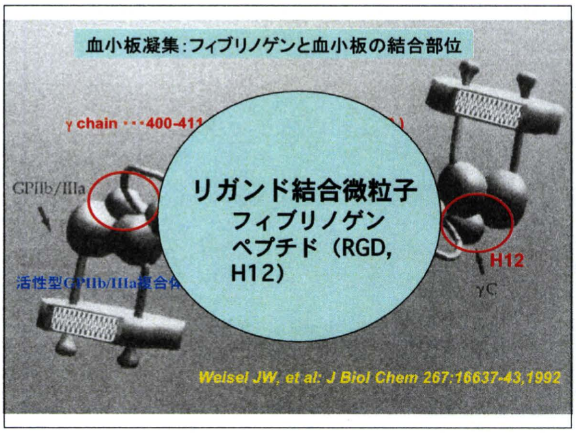
人工血小板

- **血小板由来産物**
  （Platelet Products）
  ヒト血小板あるいはその断片を固定、凍結乾燥
- **人工血小板（狭義）**
  （Platelet Substitutes）
  フィブリノゲンやそのペプチドを表面固定した微粒子（アルブミン微粒子やリポソーム）

血小板凝集



東京都臨床医学総合研究所鈴木英紀博士提供



第17回日本血液代替物学会年次大会
シンポジウム：人工血小板の現状と将来

オーガナイザ
丸山徹（熊本大学）
半田誠（慶応大学）

- 血小板の活性化機構：ADPの果たす役割
  冨山佳昭（大阪大学）
- 血小板αIIbβ3インテグリンの活性化メカニズム
  鎌田徹治（慶應大学）
- 人工血小板の開発状況
  半田誠（慶応大学）

第17回日本血液代替物学会年次大会
シンポジウム：人工血小板の現状と将来

平成22年10月18日　熊本
慶應義塾大学輸血・細胞療法センター　半田誠

- 人工血小板の種類
- 開発状況
- 実用化に向けた課題

人工血小板

- **血小板由来産物**
  (Platelet Products)
  ヒト血小板あるいはその断片を固定、凍結乾燥
- **人工血小板(狭義)**
  (Platelet Substitutes)
  フィブリノゲンやそのペプチドを表面固定した微粒子（アルブミン微粒子やリポソーム）

人工血小板代替物の開発状況

■ 血小板由来産物（凍結乾燥品）

Lyophilized whole platelets	(Stasix™:Entegrion)	前臨床
Platelet membrane fragments	Infusible Platelet Membrane (Cyplex™:Cypress Bioscience)	臨床I/II

■ 人工血小板：フィブリノゲンリガンド結合微粒子

Human Fibrinogen	Alb microcapsules	(Synthocytes™:ProFibrix)	臨床I/II
Human Fibrinogen	Alb microspheres	(Simplat™:Advanced Therapeutics)	臨床II/III
		(HaemoPlax™:Haemostatix)	前臨床
Human fibrinogen peptide (H12)	liposomes	(Y Okamura, et al, 2005-2009)	前臨床
Human fibrinogen peptide (RGD)	PLGA	(Bertram JP, et al, 2009)	前臨床

stasix

Entegrion 社の HP

凍結乾燥固定ヒト血小板 Stasix™

血中滞留時間：< 10 min

止血剤に特化

開発経過

Klein E et al, 1955

Allain & Brinkhous, 1975

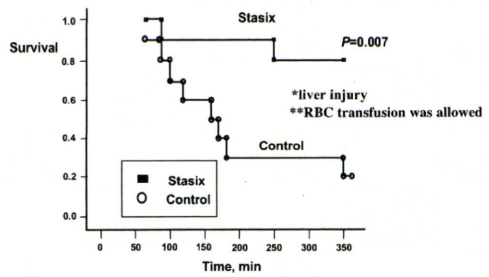
Read & Brinkhous, 1995

Bode & Fisher, 2001

Supported by Naval Research grants since 1989

Evaluation of lyophilized fixed platelets (Stasix) as an infusible hemostatic agent in experimental non-compressible hemorrhage* in swine

IV 15 min after injury/6 hr observation in hospital phase**



Hawksworth JS, et al: J Thromb Haemost 7:1663-71, 2009

Advanced Therapeutics 社 HP

フィブリノゲン結合アルブミン マイクロスフェア

Fibrinoplate-S is the premiere artificial platelet product from Advanced Therapeutics (ADVTX). It is intended for use in patients with an insufficient concentration of platelets, such as cancer patients, or in patients with inadequate platelet function, such as patients on anti-platelet medication who need emergency surgery.

As a third generation product, Fibrinoplate-S is cost-effective and available in a suspension formulation ready for intravenous administration. The Company intends to explore various indications for Fibrinoplate-S in the near future. More

Fibrinoplate™

ヒトフィブリノゲン結合アルブミンマイクロスフェア

（粒子径：1.1-1.3μm）

臨床試験結果（フェーズII/III）

4th Asian Pacific Cong on Thromb & Haemost, 2006

対象：血小板減少症（ITP、白血病、MDS、再生不良性貧血）
血小板数：＜3万／μl

方法：double blind（試験物：7.0 mg/kg vs Fg非結合Alb粒子）

結果： N=102,223

出血時間の短縮効果（秒）

	0 hr vs 8hr	0 hr vs 25hr
試験群	670.3 ± 564.5	459.2 ± 577.0
対照群	-55.5 ± 199.1	-8.8 ± 372.9
	p=0.000	*p*=0.042

RESEARCH ARTICLE

BLOOD CLOTTING

Intravenous Hemostat: Nanotechnology to Halt Bleeding

James P. Bertram,[1] Cicely A. Williams,[2] Rebecca Robinson,[1] Steven S. Segal,[3,4] Nolan T. Flynn,[5] Erin B. Lavik[4]*

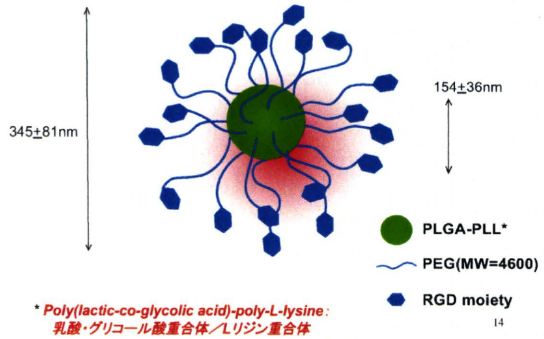
(Published 16 December 2009; Volume 1 Issue 11 11ra22)

Blood loss is the major cause of death in both civilian and battlefield traumas. Methods to staunch bleeding include pressure dressings and absorbent materials. For example, QuikClot effectively halts bleeding by absorbing large quantities of fluid and concentrating platelets to augment clotting, but these treatments are limited to compressible and exposed wounds. An ideal treatment would halt bleeding only at the injury site, be stable at room temperature, be administered easily, and work effectively for internal injuries. We have developed synthetic platelets based on Arg-Gly-Asp functionalized nanoparticles, which halve bleeding time after intravenous administration in a rat model of major trauma. The effects of these synthetic platelets surpass other treatments, including recombinant factor VIIa, which is used clinically for uncontrolled bleeding. Synthetic platelets were cleared within 24 hours at a dose of 20 mg/ml, and no complications were seen out to 7 days after infusion, the longest time point studied. These synthetic platelets may be useful for early intervention in trauma and demonstrate the role that nanotechnology can have in addressing unmet medical needs.

Science Translational Medicine 1:11-22, 2009

生体分解性RDG結合ナノ微粒子

Science Translational Medicine 1:11-22, 2009

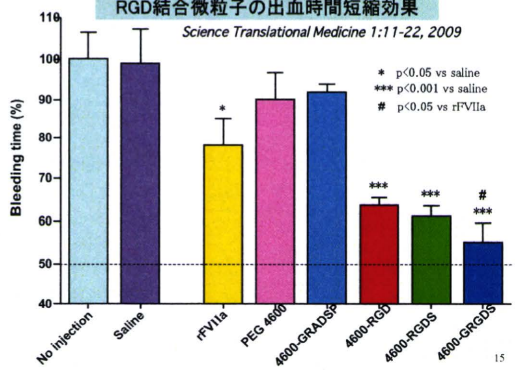


* *Poly(lactic-co-glycolic acid)-poly-L-lysine*:
乳酸・グリコール酸重合体／L-リジン重合体

14

ラット大腿動脈傷害モデル(N=5)における RGD結合微粒子の出血時間短縮効果

Science Translational Medicine 1:11-22, 2009



15

人工血小板／血小板代替物の開発

血液法制定：人工血液開発促進　　イノベーション２５：人工血液の開発

1997 — 2002 — 2007 — 2010 → 企業への技術移転（臨床試験、製剤化）

厚生労働科学研究費

＊1997-1999：高度先端医療研究
「人工血小板開発研究」、池田康夫

＊2000-2002：医薬安全総合研究
「人工血小板開発研究」、池田康夫

＊2003-2005：医薬品・医療機器等ＲＳ事業
「認識部位担持リポソーム・アルブミン重合体の安全性と止血効果の評価」、池田康夫

＊2006：医薬品・医療機器等ＲＳ事業
2007-2008：政策創薬総合研究事業
「臨床応用可能な人工血小板としてのH12結合微粒子のin vivo評価」、半田誠

＊2009-2011：創薬基盤推進研究事業
「H12(ADP)リポソームの人工血小板としての前臨床評価（効力と安全性）」、半田誠

受容体型微粒子

リガンド型微粒子

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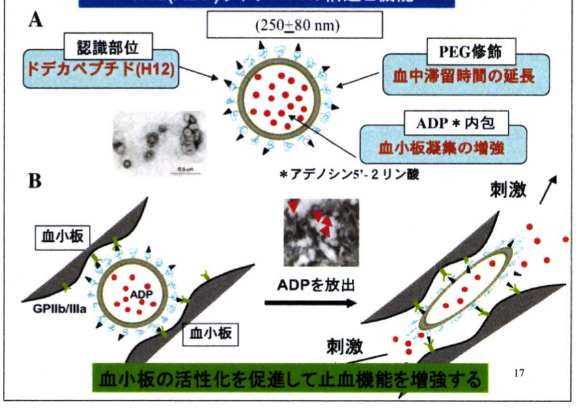
H12(ADP)リポソームの構造と機能



血小板の活性化を促進して止血機能を増強する

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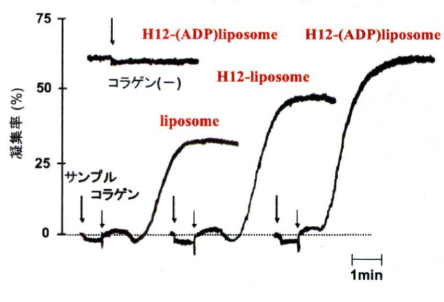
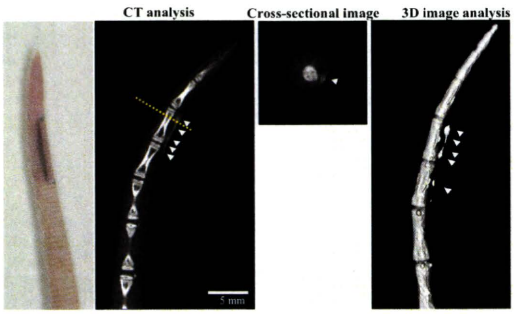
H12(ADP)リポソームの血小板凝集促進効果

[PLT]: 2.0 x 10^5 /μL, [collagen]: f.c. 0.4 μg/mL
[lipid]: f.c. 0.05 mg/mL.

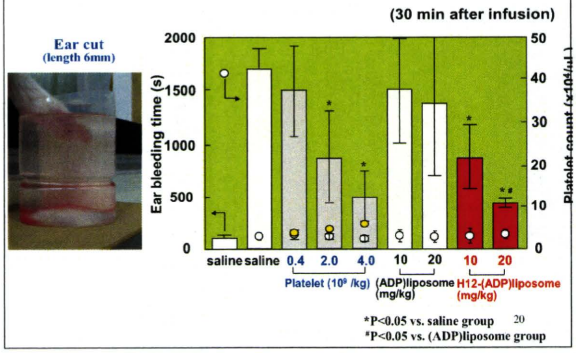


18

Specific accumulation of H12-(iopamidol)liposome at the site of vascular injury



Comparison of hemostatic effects of H12-(ADP)liposome with those of platelet rich plasma in severely thrombocytopenic rabbits



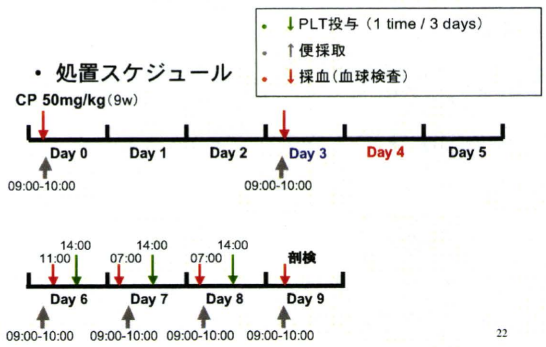
血小板減少ラットモデルの自然（内）出血評価系／予防投与

- 血小板減少を惹起する方法
  - カルボプラチン製剤 50(~ 40) mg/kg 単回腹腔内投与.
- 評価方法
  - 9日間観察（10日目以降回復傾向）
  - 経時的変化（非侵襲的検査）
    - 血液検査（赤血球数，ヘマトクリット値，血小板数など）
    - 便潜血検査（排泄前後の少数の直腸便）
  - 剖検（臓器出血定量）
    - 臓器内出血（肺，心，胃，皮膚など）
      - 臓器を凍結乾燥後，ヘモグロビン抽出し内出血の程度を定量化
- 出血予防評価系の検証
  - 血小板輸血／H12(ADP)リポソームによる各パラメータの有意な改善

慶応義塾大学／早稲田大学／田辺三菱製薬・リサーチパーク共同研究

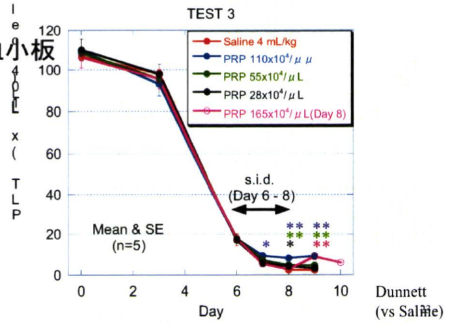
内出血モデルにおけるＰＲＰ投与の効果

- 処置スケジュール



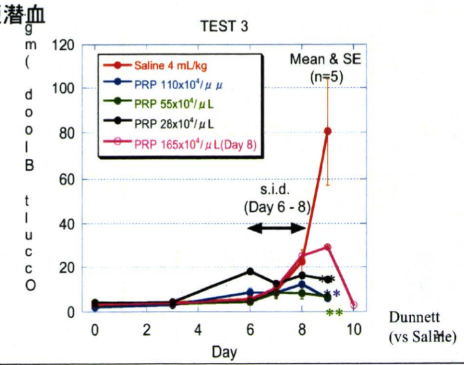
内出血モデルにおけるＰＲＰ投与の効果

- 血小板



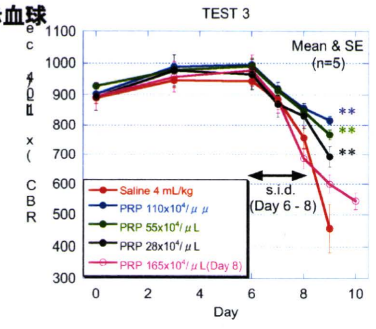
内出血モデルにおけるＰＲＰ投与の効果

- 便潜血



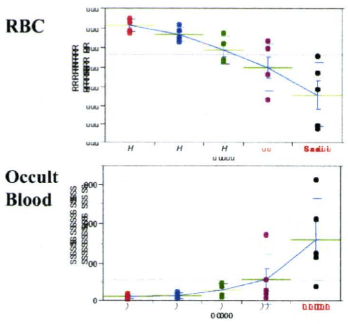
内出血モデルにおけるＰＲＰ投与の効果

- 赤血球



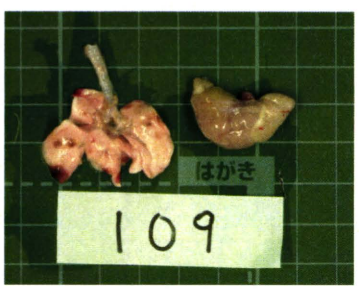
内出血モデルにおけるＰＲＰ投与の効果

– Day 9の赤血球数と便潜血 用量反応曲線の対比



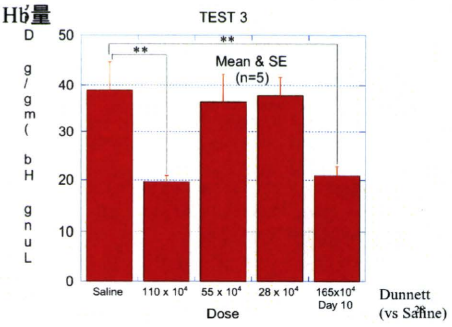
臓器出血：剖検所見（灌流後の肺・胃）

- Day 10 （生食） ： 出血斑とその消失像がみられた



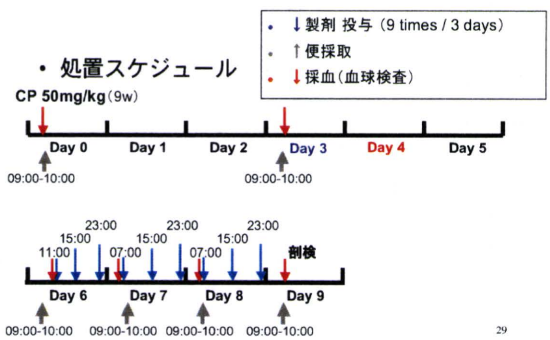
内出血モデルにおけるＰＲＰ投与の効果

- 肺 Hb量

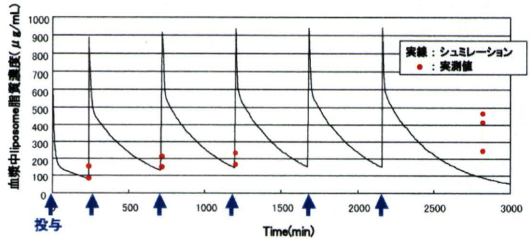


内出血モデルでのH12(ADP)リポソーム効力評価

- 処置スケジュール



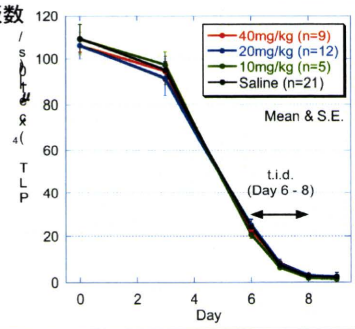
反復投与によるH12(ADP)リポソーム血中濃度（シミュレーション vs 実測値）



➢反復投与によって血中濃度が高濃度で維持されていることを確認した.
➢6回投与後の血中濃度は予想値よりも高く,投与ごとにクリアランスが減少する可能性が示唆された.

内出血モデルでのH12(ADP)リポソーム効力評価 用量反応性

- 血小板数



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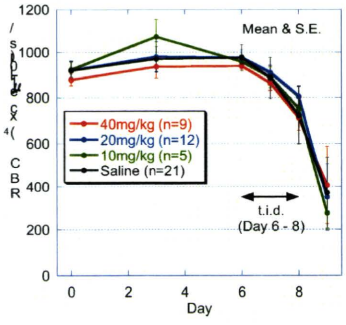
内出血モデルでのH12(ADP)リポソームの効力評価 用量反応性

- 赤血球数



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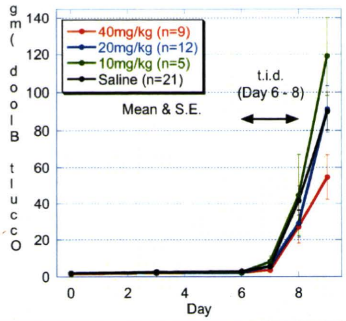
内出血モデルでのH12(ADP)リポソーム効力評価 用量反応性

- 便潜血



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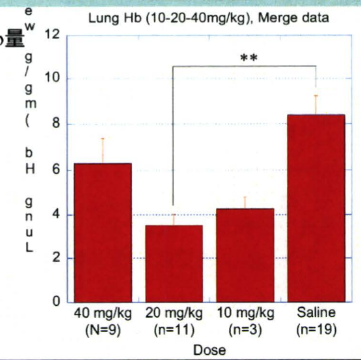
内出血モデルでのH12(ADP)リポソーム効力評価 用量反応性

- 肺Hb量



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H12(ADP)リポソームの人工血小板としての効力

現在までのまとめ

- 外出血モデル（ラット、ウサギ）評価系（事前の単回投与）
  - 10/20 mg/kg で有意な出血時間短縮作用を示した（ラット、ウサギ）
  - 20/40 mg/kg で有意な腹部術創からの出血量低減効果を示した（ウサギ）
  - 20 mg/kg で、大量出血に伴う血小板減少モデルにおいて、有意な出血量低減効果および救命率向上効果を示した（ウサギ）
- 内出血モデル（ラット）評価系（10 - 20 - 40 mg/kg, 3日間 t.i.d. の反復投与）
  - 20 mg/kg で統計学的に有意な肺出血発症抑制効果がみられた.
  - 消化管出血（便潜血）に有意差は見られなかった.

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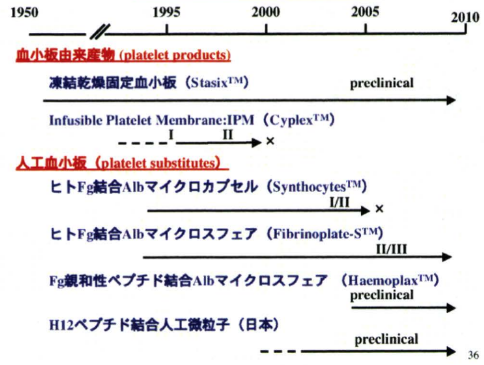
人工血小板開発の変遷



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NewsRx™ http://www.newsrx.com/newsletters/Blood-Weekly/

Expanded Phase II Cyplex Platelet Alternative Trial Launched

April 13th, 1998

Cypress Bioscience Inc., San Diego, California, announced that it has initiated a double-blinded, controlled clinical trial of Cyplex™ (Infusible Platelet Membranes), as an alternative to traditional platelet transfusions.

A smal n 1997, suggeste when given intravenously to patients with low levels of circulating platelets (thrombocytopenia) to control bleeding. In addition, the trial demonstrated the efficacy of Cyplex (Infusible Platelet Membranes) even in patients who were resistant to platelet transfusions.

しかし、予防的投与の効果が証明できなかった

Cypress BIOSCIENCE, INC.

人工血小板（リガンド結合微粒子）の開発推進のポイント/課題

1） 前提
- 血小板活性化依存性に血小板機能を補助する

2） 使用目的
- 血小板減少症における出血の予防・治療
  - → 十分な生体内寿命（リポソームのABC現象）
    残存血小板数を考慮した最適投与用量の設定
- 制御不能な出血の治療（止血剤）
  - → 生体内寿命は重要ではない
    血栓症のリスク

3） 効果の評価マーカー（Surrogate／True）の設定
- Surrogate：出血時間
  - → 出血の予想因子ではない
- True：便Hb定量、出血症状（他覚的/WHO grading）

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血小板の体内動態

1） 定常状態*
- 最大寿命：10.5日＝10%／日
- 統合性維持：0.71万*／μｌ／日
- 細胞回転：18%（4.12万／μｌ／日）
  - → 血小板輸血：10単位／毎日

2） 消費増大
- 出血、発熱、感染症、ＤＩＣ
  （血小板輸血不応因子）
  - → 血小板輸血：量／回数を追加

* Hanson SR & Slichter SJ: Blood 66:1105, 1985

*脾臓への停留量：34%を除外した場合は0.47万

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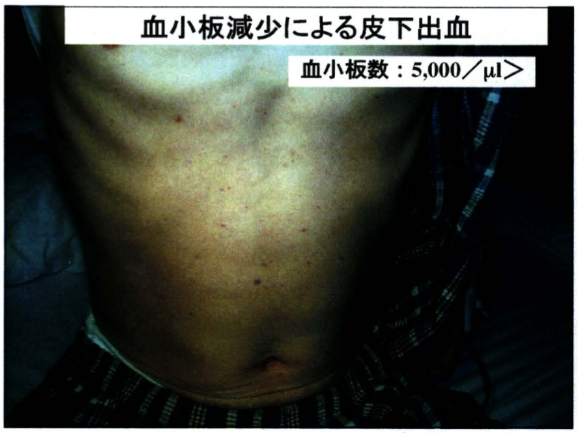
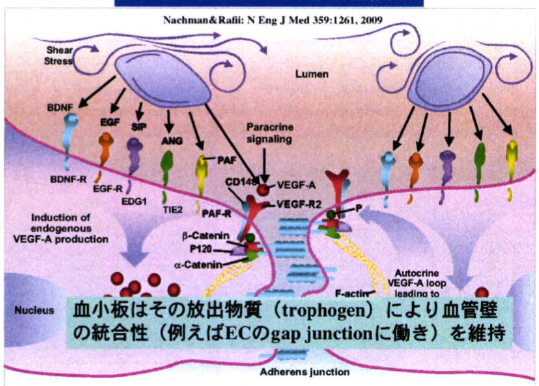
血小板減少による皮下出血

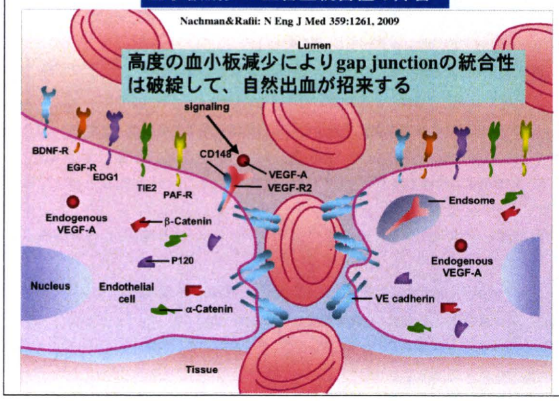


血小板の役割：血管壁統合性維持



血小板はその放出物質（trophogen）により血管壁の統合性（例えばECのgap junctionに働き）を維持

血小板減少：血管壁統合性の障害



高度の血小板減少によりgap junctionの統合性は破綻して、自然出血が招来する

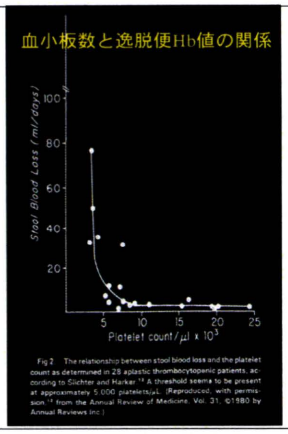


Fig 2. The relationship between stool blood loss and the platelet count as determined in 28 aplastic thrombocytopenic patients, according to Slichter and Harker.[14] A threshold seems to be present at approximatery 5,000 platelets/μL. [Reproduced, with permission[14] from the Annual Review of Medicine, Vol. 31, ©1980 by Annual Reviews Inc.]

血小板予防投与基準

血小板数：1万/μl

⇒ 便潜血量と血小板数とはNon-linear

人工血小板開発の将来展望：まとめ

その必要性

危機管理面ばかりでなく技術革新（ナノテク、DDSなど）の面からも医療に恩恵をもたらす。

その有望性

リガンド（フィブリノゲンとその関連ペプチド）結合微粒子（構造体）の開発が欧米で企業化されている。

将来展望

止血薬に特化する方向性が示されている。一方、血小板減少症への予防、治療目的への使用は幾つかの基本的な課題を克服しなくてはいけない。

謝辞

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H12(ADP)リポソームの人工血小板としての前臨床評価（効力と安全性）

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●慶応義塾大学・田辺三菱製薬リサーチパーク共同研究
（平成19年2月1日―22年1月31日）
血栓指向性／集積性ナノ粒子の創製および機能性評価