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研究テーマ　ベラプロストの血小板機能抑制機序:2種類の血小板凝集計を用いて。

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3．研究報告

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研究テーマ

ベラプロストの血小板機能抑制機序：２種類の血小板凝集計を用いて。

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プロスタサイクリンの安定化誘導体であるベラプロストの血小板凝集能に対する作用を、２種類の血小板凝集計を用いて判定した。一つの凝集計は従来の吸光度法を用いたものであり、広く日本で用いられているが小凝集塊の検出ができない、また凝集の程度と吸光度法の変化との相関が悪い等の弱点がある。一方、最近開発された散乱光を用いた血小板凝集能は　数個程度の血小板凝集塊も測定できること、また凝集塊のサイズと個数が定量的に判定できることなどの利点を有している。この新しい血小板凝集計を用いると、以前より低濃度の刺激剤でおきる血小板凝集が測定でき、それ故低濃度の血小板機能阻害剤の効果が判定できるのではないかとの仮定の下、今回の実験を行った。結果として、トロンボキサンA2類似体であるU-46619およびコラーゲン刺激の場合、散乱光を用いた血小板凝集計により判定されたベラプロストのED50は従来の吸光度法より数倍から１０倍程度低い値を取った。一方、エピネフリンおよびADPでは従来法との間に大きな差は認められなかった。ベラプロストは細胞内cAMPを増加させることにより血小板機能を抑制させるが、トロンボキサンA2による血小板活性化をおそらくこれに鋭敏に反応することが示唆された。ADPやエピネフリンの小凝集塊の形成はトロンボキサンA2非依存性であることが明らかにされており、おそらくこの現象がADPやエピネフリン刺激の場合のベラプロストの有効性を落としていることが示唆された。

Key words:

platelet aggregation, light scatter, optical density, sodium beraprost, collagen, U-46619, ADP, epinephrine

Introduction

Platelets play a key role in physiological hemostasis, and also participate in the pathogenesis of thrombosis which occurs with various disorders including diabetes mellitus and ischemic heart diseases. Platelets appear to be activated *in vivo* in these disorders, and the modification of platelet hyperfunction has been one of the therapeutic aims for a long time. A number of agents have been developed to attenuate platelet hyperfunction, and several drugs have been already introduced into market with limited success. Hence, accurate measurement of platelet function is a prerequisite for the evaluation of efficacy of anti-platelet agents.

The most widely applied method of platelet function today is platelet aggregometry based on changes in light transmission of platelet suspensions, developed by Born *et al.* in 1962. Although most, if not all, of clinical evaluations of platelet aggregation have been performed with this conventional platelet aggregometry, it is now realized that there is only a little, if not none, correlation between the extent of aggregation assessed by this method and clinical responses. This discrepancy may be attributed to the poor correlation between changes in light transmission and the formation of platelet aggregates, and to the low sensitivity of the conventional method to detect small aggregates. We have recently developed a platelet aggregometer based on a particle counting method using light scattering. This new method can quantitatively assess the number and size of platelet aggregates in a suspension. It is also sensitive enough to detect aggregates consisting of several platelets, and therefore can evaluate platelet activation of a weak magnitude. This device is now commercially available, and several clinical trials as well as fundamental evaluation have been reported.

Prostaglandin I_2 (PGI_2) is synthesized and released by endothelial cells. PGI_2 by interacting with its receptors on platelets elevates the intracellular level of cAMP in platelets, and this process is considered to play an important role in preventing platelet adhesion to endothelium and subsequent aggregate formation. While administration of PGI_2 should

prove useful in the therapeutic regimen for thromboembolism, its instability precluded its wide clinical application. Recently, several stable analogues of PGI_2 have been developed, and their clinical efficacy has met considerable success. They should also prove to be potent inhibitors of platelet aggregation in *in vitro* studies, but previous reports using the conventional platelet aggregometry have found inhibitory effects of these agents only at high concentrations which cannot be achieved *in vivo*. In this study, we evaluated the effects of sodium beraprost, a stable analogue of PGI_2 synthesized by Toray Co. Ltd., on platelet aggregation, as assessed by the new aggregometry based on light scattering. Changes in light transmission, used in the conventional aggregometry, were simultaneously measured along with light scattering, to delineate the comparative efficacy of these two methods in detecting changes in platelet aggregation.

Materials and Methods

1. Agents: Sodium Beraprost was a generous gift from Toray Co. Ltd., Tokyo, Japan. It was dissolved in distilled water at a concentration of 0.1 mM just prior to use. ADP and epinephrine were obtained from Sigma (St. Louis, MO, U.S.A). U46619 was purchased from Funakoshi (Tokyo, Japan). Collagen was obtained from Form-Chemie.
2. Platelets: Venous blood was drawn from healthy volunteers who denied uptake of any drug for a minimum of two weeks prior to experiments. Blood was immediately mixed with 3.8% sodium citrate (1 volume of citrate/9 volumes of blood), and the mixture was centrifuged at 160 x g to obtain platelet-rich plasma (PRP). For the measurement of changes in light transmission, PRP was further centrifuged at 2000 x g to obtain platelet-poor plasma, which was used to calibrate 100% light transmission.
3. Measurement of platelet aggregation: We developed a platelet aggregometer that simultaneously measures platelet aggregation by two means. One is the conventional method based on changes in the

light transmission (LT) of a platelet suspension, developed by Born. The other is based on the particle counting method using light scattering (LS). Briefly, a diode laser light beam (40 μm in width, wavelength 675 nm) was passsed through PRP in a cylindrical glass cuvette maintained at 37℃. An optical device focuses on a limited area of the platelet suspension, and measures the intensity of light scattered by particles passing through the area, thus minimizing multiple light scattering. The intensity of light scattering detected by this device provides information on the number and size of aggregates in the suspension. It has been demonstrated that this device is particularly sensitive in detecting platelet aggregates of small size.

Results

1. Dose-response curves of ADP-induced platelet aggregation assessed by LT or LS.

Assessed by LT, ADP at the range of 0.5 and 1.5 μM induced a transient increase in light transmission, which has been defined as the "primary aggregation". At higher concentrations of ADP, the primary aggregation is followed by the secondary aggregation, which represents irreversible platelet aggregation. Since the primary aggregation is of small magnitude and varies between measurements, the secondary aggregation has conventionally been used to evaluate the effects of various inhibitors on platelet aggregation.

On the other hand, LS which can evaluate the size and number of platelet aggregates demonstrates that the formation of small aggregates occurs in the phase of the primary aggregation, as defined by LT. LS also shows that platelet aggregates of small size precede the formation of larger aggregates, which coincides with the phase of the secondary aggregation as detected by LT. The ED50 value of ADP assessed by LS for inducing platelet aggregation is approximately

0.75 μM, whereas that of LT is approximately 2.0 μM. These findings clearly demonstrate that LS which can detect the formation of small aggregates can evaluate platelet activation more sensitively than LT. Other agonists, including epinephrine, collagen, and U46691 gave results essentially similar to that of ADP (data not shown).

In experiments thereafter, two concentrations were determined for each agonist. One was the minimal concentration of the agonist which induced the secondary aggregation assessed with LT, and the other was the maximum concentration of the agonist that induced the formation of small aggregates as assessed by LS but not that of larger aggregates or substantial changes in light transmission. The effects of sodium beraprost were determined on platelet aggregation induced by the agonists in the concentration range thus determined.

2. Effect of beraprost on platelet aggregation induced by ADP.

Beraprost in a dose-dependent manner inhibited ADP-induced platelet aggregation assessed by LT as well as LS. At concentrations higher than 10 nM, beraprost almost completely inhibited the formation of platelet aggregates induced by ADP. The ID50 values of beraprost were approximately 5 nM with LT, and 2.5 nM with LS.

3. Effect of beraprost on platelet aggregation induced by epinephrine.

Beraprost in a dose-dependent manner inhibited epinephrine-induced platelet aggregation assessed by LT as well as LS. At concentrations higher than 10 nM, beraprost almost completely inhibited the formation of platelet aggregates induced by epinephrine. The ID50 values of beraprost were approximately 7 nM with LT, and 5 nM with LS.

4. Effects of beraprost on platelet aggregation induced by U46619.

Beraprost up to the concentrations of 3 nM virtually had no effects on U46619-induced platelet aggregation, when assessed with LT. At concentrations higher than 10 nM, however, platelet aggregation was almost completely inhibited. Thus, although the ID50 value falls between 3 and 10 nM, its value could not be determined with accuracy. Assessed with LS, beraprost in a dose-dependent manner inhibited platelet aggregation with the ID50 value of 0.2 nM.

5. Effects of beraprost on platelet aggregation induced by collagen

Beraprost up to the concentrations of 3 nM had virtually no effects on collagen-induced platelet aggregation, assessed with LT. At concentrations higher than 10 nM, platelet aggregation was completely inhibited by beraprost. Thus, although the ID50 value falls between 3 and 10 nM, its value could not be determined with accuracy. When collagen-induced platelet aggregation was assessed with LS, the inhibitory effects of beraprost could be observed at concentrations lower than those required for LT. The ID50 value for beraprost was approximately 0.5 nM, when assessed with LS.

Discussion

In the process of platelet aggregation, aggregates consisting of a small number of platelets are initially formed. Platelet stimulation of a minor degree can only reach this phase, called the "primary aggregation", and eventually the aggregates dissociate into single platelets. With platelet activation of higher degree, the primary aggregation is followed by the secondary phase, in which small aggregates fuse to form aggregates of larger size. The newly developed platelet aggregometer based upon the particle counting method (LS) can sensitively detect the formation of small aggregates in the phase of the "primary aggregation".

The "primary aggregation" can also be detected by the conventional platelet aggregometry (LT) as a small deflection in light transmission. However, the changes are generally minimal, and variable in magnitude. Moreover, changes in light transmission in this phase reflect shape change as well as the formation of small aggregates, rendering the quantitative assessment of aggregate formation difficult. Thus, the conventional aggregometry mainly assesses the changes in the "secondary aggregation" which corresponds to the formation of large aggregates. Our findings clearly demonstrate that the agonist concentrations required for the "secondary aggregation" or the formation of large

aggregates, which allows the assessment with LT, are greater than those required for the formation of small aggregates that can be quantitatively measured by LS. Thus, LS can evaluate platelet aggregation of a minor degree induced by agonists at concentrations lower than those required for the assessment by LT.

Anti-platelet agents which have clearly proved useful in clinical trials are often known to lack inhibitory effects at physiologic concentrations on platelet aggregation *in vitro*. Most of these studies have used the conventional platelet aggregometry, which requires the formation of large aggregates for assessment, and the concentrations of the agonists used in these studies were much higher than could be generated *in vivo*. Platelet aggregate formation induced by potent agonists at high concentrations may be resistant to anti-platelet agents or requires higher concentrations of these inhibitors. It is possible that anti-platelet agents at concentrations closer to the physiologic range may prove effective if they are tested for platelet aggregation induced by agonists at concentrations closer to those *in vivo*. This hypothesis led us to perform the comparative study on the effects of beraprost on platelet aggregation, as assessed by LT and that of LS.

In the present study, we have demonstrated that sodium beraprost in a dose-dependent manner inhibited platelet aggregation induced by all the four agonists, ADP, collagen and U46619, irrespective of the methods for measuring platelet aggregation. However, there appear to be differences in the ID50 values of beraprost between the agonists. With ADP or epinephrine, the ID50 values determined by LS were relatively close to those of LT (2.5 nM vs. 5 with ADP stimulation, and 5 nM vs. 7 nM with epinephrine). On the other hand, the ID50 values determined by LS were much lower than those of LT with U-46619- or collagen-induced platelet aggregation (0.2 nM vs. 3-10 nM with U-46619, and 0.5 nM vs. 3-10 nM with collagen). These data raise several interesting notions. First, the formation of small aggregates induced by U-46619, a thromboxane A2 mimetic, and by collagen appears to be highly sensitive to the elevated intracellular cAMP level induced by beraprost.

Since the formation of small aggregates in collagen-induced platelet activation is known to be dependent upon thromboxane A2 formation, these findings suggest that cAMP potently inhibits the formation of small aggregates, which is the initial phase of platelet activation induced by thromboxane A2. It is also suggested that the intracellular cAMP level is relatively less potent in suppressing the transformation of small aggregates into large aggregates.