

Raloxifene-Induced Acceleration of Platelet Aggregation

Chiho Minamitani^{1,3}, Shinji Takai², Rie Matsushima-Nishiwaki², Yoshiteru Hanai^{1,2},
Takanobu Otuka³, Osamu Kozawa² and Haruhiko Tokuda^{1,2}

Abstract

A 59-year-old postmenopausal woman diagnosed to have primary osteoporosis began to take 60 mg daily of oral raloxifene. The platelet aggregation induced by 1 μ M adenosine diphosphate (ADP) and the α 2-antiplasmin activity were accelerated significantly after 8 weeks from the beginning of raloxifene-treatment, and gradually deteriorated up to 24 weeks. ADP markedly caused the phosphorylation of Akt in the platelets obtained at 24 weeks. Although there were no subjective complaints at 24 weeks, the medication was stopped with her consent to avoid any adverse effects due to thrombus formation. The platelet hyper-aggregability and Akt phosphorylation induced by ADP disappeared at 4 weeks after the cessation of medication. These results strongly suggest that raloxifene caused the acceleration of platelet aggregation and subclinical thrombus formation through the Akt signal pathway in this case.

Key words: raloxifene, osteoporosis, Akt, platelet aggregation

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Introduction

Raloxifene, a selective estrogen receptor modulator (SERM), has been well recognized to be an efficient therapeutic agent for postmenopausal osteoporosis (1). Raloxifene reportedly increases the vertebra and femoral neck bone mineral density, and reduces the risk of vertebral fracture (1). In addition, raloxifene has been reported to significantly reduce the incidence of breast cancer (1). Raloxifene is thought to be a preferable medicine for postmenopausal women. However, the use of this drug as well as estrogen is associated with an increased risk of developing venous thromboembolus (2). Platelet aggregation and the coagulation system are important for the formation of the thrombus. Although significant attention has been paid to the coagulation system, the influence of raloxifene on platelet aggregation is not known.

Platelet aggregation is usually measured using either the optical density (OD) method (3) or the impedance method (4), both of which are indispensable for the clinical evaluation of platelet function. However, these methods provide

little information about subtle but crucial changes in the number of platelet microaggregates in response to small changes in the aggregating stimuli or the proaggregatory status. The light-scattering (LS) method, which has primarily been used for experimental research, provides a tool with a greater sensitivity for detecting microaggregates of platelets than the conventional light transmittance method (4). A particle-counting method that employs LS has recently been developed (5), thus allowing identification of particle size in terms of light intensity and minimizing the interference by neighboring platelets, which may attenuate the high intensity light scattered by larger particles (6). Recently, an important role of platelet microaggregation in the circulation and a significant alteration of platelet microaggregation in pathogenesis were reported by the creation of a laser LS system for microaggregates (7-9). Therefore, an LS system was employed for the detection of microaggregates of platelets in our clinical study (10).

Adenosine diphosphate (ADP) is considered to be a weak physiological agonist for platelet aggregation by itself in comparison, for example, to thrombin or collagen (11). However, ADP is a necessary cofactor for normal activation

¹Department of Clinical Laboratory, National Hospital for Geriatric Medicine, National Center for Geriatrics and Gerontology, Obu, ²Department of Pharmacology, Gifu University Graduate School of Medicine, Gifu and ³Department of Orthopedic Surgery, Nagoya City University Graduate School of Medical Sciences, Nagoya

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Correspondence to Dr. Haruhiko Tokuda, tokuda@ncgg.go.jp

of platelets by other agonists. Even if it is weak agonist for platelet aggregation, low-concentration ADP amplifies the effects (12). A recent investigation revealed that purinoreceptor P2Y₁₂ is a principal receptor for mediating ADP-induced platelet aggregation and the thromboembolism (13, 14). Phosphatidylinositol 3-kinase (PI3K) effectors, such as the serine/threonine kinase Akt, play important roles in platelet aggregation as an intracellular signal system downstream of the P2Y₁₂ receptor (14). The current report describes the acceleration of low-dose ADP-induced platelet aggregation with the increasing Akt activation in a patient who was treated with a SERM, raloxifene.

Case Report

A 59-year-old postmenopausal woman, who had suffered from intermittent lumbago for several years, was referred to the outpatient service for osteoporosis. X-ray examination showed a vertebral fracture on T-12. Bone marrow depression (BMD) determined by dual-energy X-ray absorptiometry (QDR-2000) was: lumbar spine 0.803 g/cm² (T-score, -2.6); total femur 0.598 g/cm² (T-score, -2.8; Table 1). The blood analysis appeared to be in normal range except the elevation of alkaline phosphatase (ALP) and bone type ALP (Table 2). According to the diagnostic criteria for primary osteoporosis in Japan (15), she was diagnosed to have primary osteoporosis with a high turnover bone metabolism, and then was administered 60 mg daily of oral raloxifene. She agreed to undergo an assessment of her platelet function before and after the treatment, and signed an informed consent agreement as approved by the local institutional review board after receiving a detailed explanation of the protocol. Platelet-rich plasma (PRP) was chronologically obtained from freshly drawn venous blood samples. Platelet aggregation was assessed by PA-200 aggregometer (Kowa, Tokyo, Japan), which can determine the size of platelet aggregates based upon particle counting using light scatter (13). The platelets were preincubated for 2 minutes, then platelet aggregation was monitored for 5 minutes after the addition of various doses of ADP (0.3-30 μ M). The percentage of transmittance of the isolated platelets was recorded as 0%, and that of the appropriate platelet-free plasma (blank) was recorded as 100%. ADP concentration that caused 50% of transmittance of the isolated platelets was considered as ED 50. Just after evaluating platelet aggregation, the remaining PRP was sufficiently washed with phosphate buffered saline, separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and a Western blot analysis was performed using antibodies against phospho-specific Akt (Thr308) to detect the activated form and with Akt antibodies for determination of the total amount, as previously described (16).

Figures 1, 2 show that the ED₅₀ of ADP for the platelet aggregation gradually decreased after treatment with raloxifene. After 8 weeks after the initiation of treatment, less than 1 μ M ADP caused the significant acceleration of

Table 1. Clinical Feature

Age	59 years
Sex	female
Height	152.9 cm
Weight	56.4 kg
Body mass index	24.12 kg/m ²
Lumber spine L2-L4 BMD	0.803 g/cm ²
YAM	72%

Footnote: YAM: young adult mean

the platelet aggregation, and it deteriorated up to 24 weeks. Considering these results, although she did not show any symptoms of thrombosis, the administration of raloxifene was terminated to avoid major clinical problems. Four weeks after the cessation of raloxifene, the level of the platelet aggregation was markedly improved to the level before the raloxifene-treatment. According to the analysis of the size of platelet aggregates, a decrease of small aggregates (9-25 μ m) and the increase of large aggregates (50-70 μ m) were observed after 2 weeks, and an increase of medium aggregates (25-50 μ m) was subsequently observed after 4 weeks (Fig. 2), suggesting that the pathological change of platelet function occurred within at least 2 weeks of the raloxifene treatment. In addition, ADP (1 μ M) markedly caused the phosphorylation of Akt in the platelets obtained at 24 weeks, but the ADP-induced Akt phosphorylation disappeared in the platelets obtained at 4 weeks after the cessation of raloxifene (Fig. 3).

It is well known that a lack of α 2-antiplasmin (α 2AP), a major component of fibrinolytic system, causes an excess of plasmin, and that plasmin plays an important role in the formation of platelet aggregates. Consequently, the reduction of α 2AP could be a risk factor for the activation of platelets resulting in thrombus formation (9). The α 2AP activity was examined in this case to determine the effect of raloxifene treatment on the fibrinolytic system. The α 2AP activity gradually increased from the beginning of raloxifene-treatment, and it was sustained up to 4 weeks after the cessation of the medication (Fig. 4).

Discussion

This report presents a case of primary osteoporosis showing platelet hyper-aggregability to ADP, closely related to the administration of the standard dose of raloxifene. This is probably the first report clearly showing raloxifene-induced unusual platelet aggregation, which was detectable using the LS method. Interestingly, the hyper-sensitivity was correlated with the enhancement of Akt phosphorylation induced by ADP in platelets. The platelets play an important role in normal homeostasis and abnormal activation of platelets thus leading to thrombosis. During vascular injury or conditions of high shear, exposure of the collagen-rich subendothelium

Table 2. Laboratory Data

Parameters	normal range	
Peripheral Blood		
WBC	4600/ μ L	3500-9200/ μ L
RBC	431 x 10 ⁴ / μ L	388-488 x 10 ⁴ / μ L
Hb	13.3 g/dL	11.3-15.5 g/dL
Hct	39.9%	34.4-45.6%
Plt	16.4 x 10 ⁴ / μ L	15.5-36.5 x 10 ⁴ / μ L
Blood Chemistry		
Total protein	7.7 g/dL	6.3-8.1 g/dL
Albumin	4.7 g/dL	3.7-4.9 g/dL
AST	24 IU/L	9-38 IU/L
ALT	33 IU/L	4-36 IU/L
ALP	361 IU/L	60-201 IU/L
Urinary nitrogen	13 mg/dL	9-21 mg/dL
Creatinine	0.5 mg/dL	0.4-0.9 mg/dL
Serum calcium	9.5 mg/dL	8.4-9.7 mg/dL
Serum iP	3.5 mg/dL	2.5-4.5 mg/dL
Endocrinology		
free T ₃	2.79 pg/mL	2.84-4.45 pg/mL
free T ₄	1.19 pg/mL	0.82-1.72 pg/mL
TSH	0.73 mIU/mL	0.49-3.83 mIU/mL
Cortisol	14 ng/mL	7-25 ng/mL
High sensitivity PTH	212 pg/mL	90-270 pg/mL
Bone Metabolic Markers		
BAP	46.4 IU/L	7.9-29.0 IU/L
Serum NTx	13.3 nmolBCE/L	10.7-24.0 nmolBCE/L

Footnote: RBC; Red blood cell, WBC; White blood cell, Hb; Hemoglobin, Hct; Hematocrit, Plt; Platelet, AST; L-asparatate aminotransferase, ALT; L-alanine aminotransferase, ALP; Alkaline phosphatase, free T₃; free tri-iodothyronine, free T₄; free thyroxine, TSH; thyroid-stimulating hormone, PTH; parathyroid hormone, BAP; bone type alkaline phosphatase, Serum NTx; Serum cross-linked N-telopeptides of I type collagen.

activates the platelets and results in the formation of a stable thrombus due to the combined action of ADP secreted from platelet-dense granules and generated thrombin (14, 17). The present results suggest that raloxifene could induce hyper-reactivity of platelet aggregation by increased sensitivity to ADP stimulation and by Akt activation. The hyper-sensitivity of platelets shown here might lead us to pay attention to the risk of arterial thrombosis, in addition to the increased hazard ratio of venous thromboembolism, under standard raloxifene treatment for osteoporosis.

Prior studies have shown that PI3K is a necessary component which plays a pivotal role in the signaling of purinore-

ceptor P2Y₁₂ in platelets (14, 18). A potential downstream effector for PI3K is Akt, which contributes to fibrinogen receptor activation and platelet aggregation. In addition, PI3K/Akt signaling is also reportedly involved in the release of ADP-containing granules (18). The platelet-aggregated form consists of two types of platelet membrane receptors, glycoprotein (GP) IIb/IIIa and GPIb/V/IX. GPIIb/IIIa not only binds fibrinogen and von Willebrand factor (vWF) to mediate platelet aggregation and adhesion, but it also serves as a signaling receptor. ADP induces the signaling, which activates the receptor function of GPIIb/IIIa for soluble fibrinogen, and ADP-induced platelet aggregation promotes the

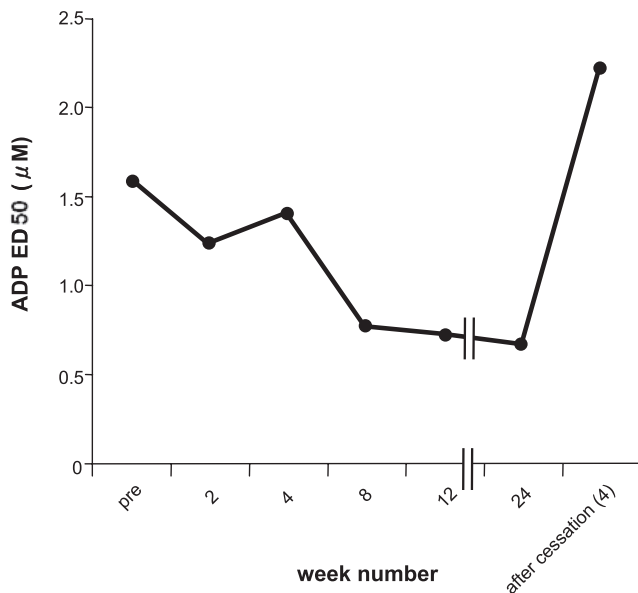


Figure 1. The change in ED50 for ADP-induced platelet aggregation. PRP samples were obtained during the treatment and 4 weeks after the cessation of raloxifene administration. PRP was chronologically obtained from freshly drawn venous blood samples. The ED50 for ADP was determined.

binding of fibrinogen to GPIIb/IIIa (19). The P2Y₁₂ receptor is involved in the constitution of stable macroaggregates (irreversible change) through full activation of the GPIIb/IIIa (13, 20). In the present case, raloxifene probably increased the platelet sensitivity to ADP and induced the enhancement of Akt activity in platelets, thus suggesting that the up-regulation in signaling events of P2Y₁₂ receptor occurred under raloxifene administration. Blockade of P2Y₁₂ receptor signaling might be a possible therapeutic strategy to avoid the adverse effects of this useful agent. The accumulation of more such cases and further investigations will thus be required to clarify the above mechanism of action in detail.

On the other hand, an increase of α 2AP activity was also observed in this case. Plasmin plays a crucial role in the formation of platelet aggregation. It is generally recognized that increase of α 2AP, which is produced by the liver, causes the reduction of plasmin, resulting in the inhibition of fibrinolytic system. Thus, it seems that an inhibition of the fibrinolytic system occurred in parallel with the increase of platelet aggregation under the treatment with raloxifene. As plasmin is known to play an important role in the formation of platelet aggregation, it is possible that the increase of α 2AP activity resulting in the reduction of plasmin in this case is a patho-physiological defensive mechanism against the raloxifene-induced platelet hyper-aggregability. In addition, the inhibition of fibrinolytic system might reduce the capacity of thrombolysis, resulting in the thrombus formation. In the present case, raloxifene treatment was continued for 6 months and the increased activity of α 2AP sustained after the cessation of raloxifene. It seems that the 4 weeks cessation of raloxifene after 6 months administration is not sufficient for the recovery of fibrinolytic system. The moni-

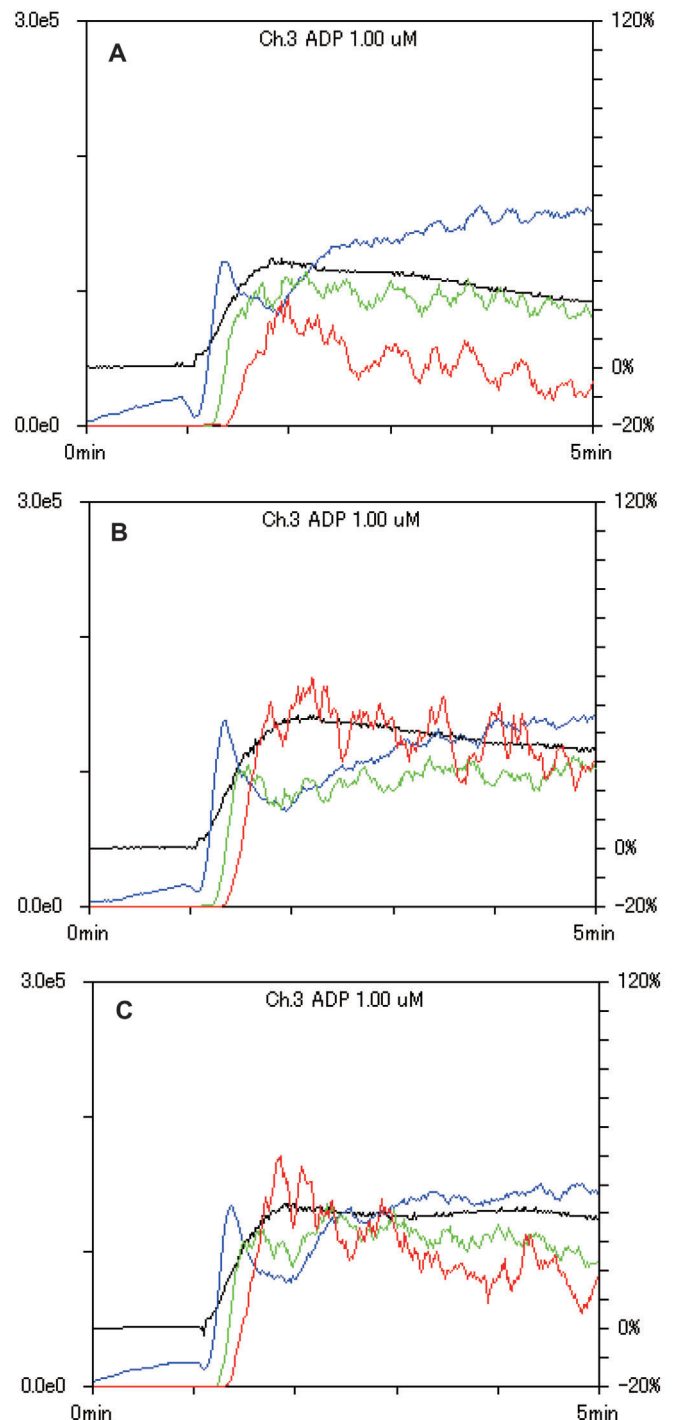


Figure 2. Platelet aggregability after the addition of ADP measured with the laser-light scattering method. Blue line indicates small aggregates; green line, medium aggregates; red line, large aggregates; and black line, optical density (%T). PRP samples were obtained before (A), after 2 weeks (B) and after 4 weeks (C) of raloxifene administration, and the ADP (1 μ M)-induced platelet aggregation was determined using a PA-200 aggregometer.

toring of α 2AP, in addition to the platelet microaggregates by a LS system, is probably a useful tool for the detection of the subclinical thrombus formation in the clinical use of raloxifene.

In conclusion, a standard dosage of raloxifene, one type

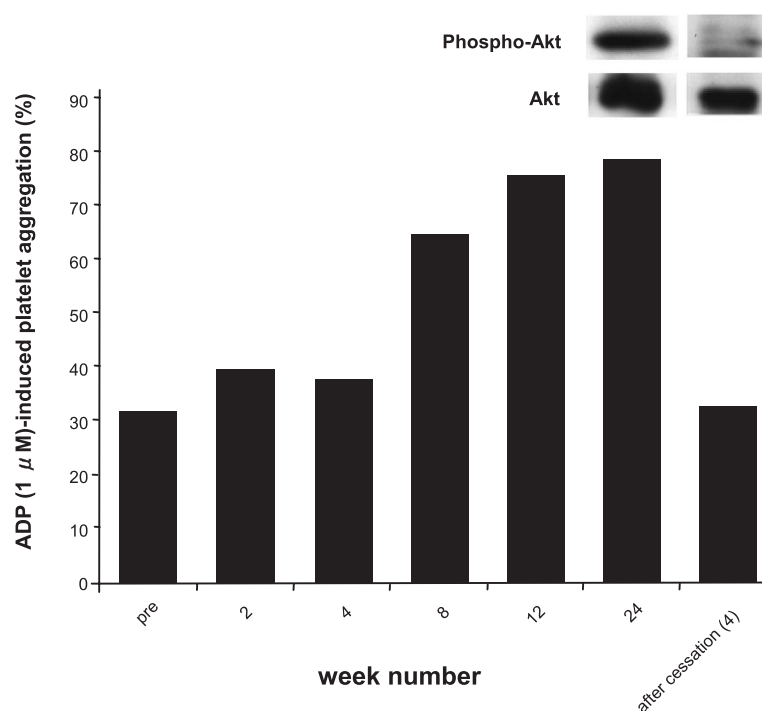


Figure 3. The change in ADP-induced platelet aggregation and the phosphorylation of Akt in the platelets under the treatment of raloxifene. Under the treatment of raloxifene and 4 weeks after the cessation, PRP sample were obtained at the indicated time. The platelet aggregation (%) induced by ADP (1 μ M) was determined using a PA-200 aggregometer, and then with the same samples, the phosphorylation of Akt induced by ADP (1 μ M) was examined at 24 weeks and 4 weeks after the cessation of the treatment. The platelet extracts were separated by SDS-PAGE and a Western blot analysis was performed with antibodies against either phospho-specific Akt or Akt.

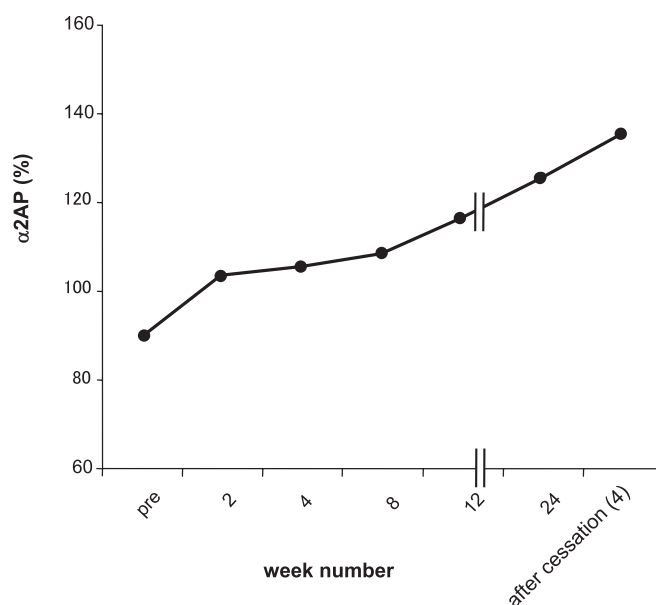


Figure 4. The change in the α 2-antiplasmin (α 2AP) activity. Under the treatment of raloxifene and 4 weeks after the cessation, PRP samples were obtained at the indicated week. PRP was chronologically obtained from freshly drawn venous blood samples.

of SERM, was found to induce platelet hyper-aggregability during the conventional therapeutic management for post-menopausal osteoporosis. The clinicians using SERM must be aware of the acceleration of platelet aggregation in addition to that of coagulation which may occur during the ad-

ministration of this regimen.

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