Platelet Size and Age Determine Platelet Function Independently

By Craig B. Thompson, Joseph A. Jakubowski, Patrick G. Quinn, Daniel Deykin, and C. Robert Valeri

This study was undertaken to examine the interaction of platelet size and age in determining in vitro platelet function. Baboon megakaryocytes were labeled in vivo by the injection of ^{75}Se-methionine. Blood was collected when the label was predominantly associated with younger platelets (day 2) and with older platelets (day 9). Size-dependent platelet subpopulations were prepared on both days by counterflow centrifugation. The reactivity of each platelet subpopulation was determined on both days by measuring thrombin-induced aggregation. Platelets were fixed after partial aggregation had occurred by the addition of EDTA/formalin. After removal of the aggregated platelets by differential centrifugation, the supernatant medium was assayed for remaining platelets and ^{75}Se radioactivity. Comparing day 2 and day 9, no significant difference was seen in the rate of aggregation of a given subpopulation. However, aggregation was more rapid in the larger platelet fractions than in the smaller ones on both days. A greater percentage of the ^{75}Se radioactivity appeared in the platelet aggregates on day 2 than on day 9. This effect was independent of platelet size, as it occurred to a similar extent in the unfractionated platelets and in each of the size-dependent platelet subpopulations. The data indicate that young platelets are more active than older platelets. This study demonstrates that size and age are both determinants of platelet function, but by independent mechanisms.

DESPITE YEARS OF STUDY, the role played by platelet size and age in determining platelet function remains a controversial issue.[1-9] Several authors[10-12] have reported that platelets released into the circulation in response to thrombocytopenia are larger and more functional than normal. Because platelets have been reported to deteriorate in functional ability with both decreasing size[2] and increasing age,[13] it has been suggested that size and age are dependent determinants of platelet activity. In this proposed model, young platelets are large, and senescence in the circulation results in a simultaneous decrease in both platelet size and function.[2] Studies by George et al.,[14,15] demonstrating that platelets lose surface glycoproteins as they age, appear to support this hypothesis.

However, using animals in steady-state thrombopoiesis, we have shown that platelets of different sizes are produced at similar rates and have similar lifespans.[16] While a number of studies have demonstrated that large platelets are more active than small ones, as measured by in vitro tests of platelet function,[17-19] we have shown that these differences are accounted for by quantitative differences based on size[20,21] rather than qualitative differences that might be expected if the differences were a result of aging.

To date, no study has been conducted in which platelet size and age have been examined independently as determinants of platelet function. Using the recently described method of counterflow centrifugation, which isolates platelets of differing size, but of similar density, the present study was undertaken in order to delineate the relationship of platelet size and age in determining in vitro platelet activity. By studying the reactivity of young and old labeled platelets, separated into size-dependent subpopulations, we have demonstrated that, although both size and age influence platelet activity, they act independently of each other in affecting platelet function.

MATERIALS AND METHODS

Three adult baboons (*Papio cynocephalus*, 22–25 kg) were housed in conformance with NIH guidelines and fed a routine diet. The animals were anesthetized with ketamine hydrochloride (5 mg/kg, i.m.) prior to venesection. All blood samples were obtained by direct puncture of a femoral vein, using a 20-gauge needle without a tourniquet.

Platelet Labeling

Baboon megakaryocytes were labeled by the intravenous injection of 5.5 μCi/kg of ^{75}Se-methionine (3.3 μCi/μg, Amersham Corp., Arlington Heights, IL) on day 0. Blood samples were taken on day 2, a time interval that previous studies have shown to be approximately the 50% point on the ascending slope of the ^{75}Se-methionine incorporation curve and when the label was predominantly in young platelets.[16] Subsequent samples were taken on day 9, a time interval that previous studies have shown to be approximately the 50% point on the descending slope of the ^{75}Se-methionine incorporation curve and a time at which reutilization was minimal and the label was predominantly in old platelets.[16]

Platelet Subpopulations

The method for isolation of size-dependent platelet subpopulations by counterflow centrifugation has been described in detail

From the Naval Blood Research Laboratory, Boston University School of Medicine, and the Department of Medicine, Veterans Administration Medical Center, Boston, MA.

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Address reprint requests to Dr. Craig B. Thompson, Naval Blood Research Laboratory, Boston University School of Medicine, 615 Albany Street, Boston, MA 02118.

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previously.[19] In brief, counterflow centrifugation opposes an outwardly directed centrifugal force with an inwardly directed force generated by the flow of fluid through the centrifuge separation chamber. Separation is achieved on the basis of platelet size and the effect of differing platelet density is minimal.[19]

In these experiments, 4–7 × 10^9 platelets derived from 21.25 ml of whole blood with a size distribution representative of 93.7% ± 3.3% of circulating platelets, were separated into 7 sequential size-dependent fractions free of plasma proteins and white blood cells.[16] In order to obtain sufficient and comparable quantities of platelets in the subpopulations used for subsequent functional studies, the platelets in fractions 1 and 2 and fractions 6 and 7 were pooled. The platelet count in all of the final suspensions, which included a sample of the original unfractionated population, was adjusted to approximately 2 × 10^8/ml, as previously described.[20] Bovine serum albumin and $CaCl_2$ were added to a final concentration of 0.05% and 3 m*M*, respectively. Platelet counts and volumes were determined as described previously.[19]

Platelet Aggregation

Platelet aggregation was measured in an aggregometer (Chronolog Corp., Havertown, PA). Four hundred and fifty microliters of the platelet suspension was equilibrated at 37°C in a siliconized glass cuvette for 2 min prior to stimulation. The platelets were then stimulated with 50 μl of either human α-thrombin (2 U/ml) (Dr. John Fenton, New York State Department of Health) or buffer. Aggregation was measured as the percent change in light transmission (% ΔT) over time. Previous studies in our laboratory have shown thrombin to be the most reproducible aggregating agent for baboon platelets. A concentration of 2 U/ml was established to consistently give greater than 20% ΔT in each of the platelet subpopulations.

Platelet aggregation was arrested, and platelet aggregates were fixed at 20% ΔT by the addition of 50 μl of 0.1 *M* EDTA in 2.5% formalin.[20] Large aggregates were removed by a modification of the method of Haver and Gear[22] by centrifuging the sample at 500 *g* for 10 sec in a Sorvall GLC-2 centrifuge. A control platelet suspension, to which 50 μl of buffer was added, was centrifuged simultaneously. After centrifugation, 450 μl of supernatant from both the aggregated sample and the control were removed. Fifty microliters from each sample was counted for the number of residual single platelets. The percent of platelets remaining unaggregated was determined by dividing the platelet count in the aggregated sample by the count in the control. This method of analysis was chosen in order to eliminate the potential error resulting from the nonspecific removal of single platelets by brief centrifugation. Four hundred microliters of the supernatant from both the aggregated sample and the control was counted for ^{75}Se radioactivity. The percent of ^{75}Se radioactivity remaining with the platelet suspension was determined by dividing the specific activity of the platelets remaining in the aggregated sample by the specific activity of the platelets of the control sample. In separate experiments, prolonged centrifugation of the aggregated samples resulted in virtually complete removal of ^{75}Se radioactivity from the supernatant. These data suggest that the majority of ^{75}Se radioactivity was platelet associated, as it remained with the particulate fraction after aggregation. Each sample was processed in duplicate and the mean value used for data analyses.

Statistical Analysis

Statistical analyses were performed using either Student's t tests for paired data or best linear fit correlation coefficients.[23]

RESULTS

The size characteristics of the ^{75}Se-labeled platelet subpopulations examined 2 and 9 days after injection of ^{75}Se-methionine are presented in Fig. 1. No significant difference in the mean platelet volumes was observed between day 2 and day 9 for either the unfractionated platelet suspensions or for any given size-dependent subpopulation. The distribution of the platelets in the subpopulations is also shown and corresponds to the relative frequency of similar-sized platelets in unfractionated platelets. On both days, the specific activity of the platelets (Table 1) correlated closely with their mean platelet volume (Fig. 1) (r = 0.96, $p < 0.01$ for both days). On each day, the platelet fractions were stimulated with 2 U/ml of thrombin, allowed to aggregate to 20% ΔT, and the aggregates removed. No significant difference in the percent of single platelets remaining after partial aggregation was noted for any given subpopulation on day 2 or 9 (Table 1). There was a slightly lower percentage of single platelets remaining in the unfractionated suspension on both days relative to the fractions. This was possibly due to a small amount of red blood cell contamination in the unfractionated platelets, which contributed to sample opacity, thus causing a 20% ΔT to represent a greater degree of aggregation than it did in the individual subpopulations. Such an effect has

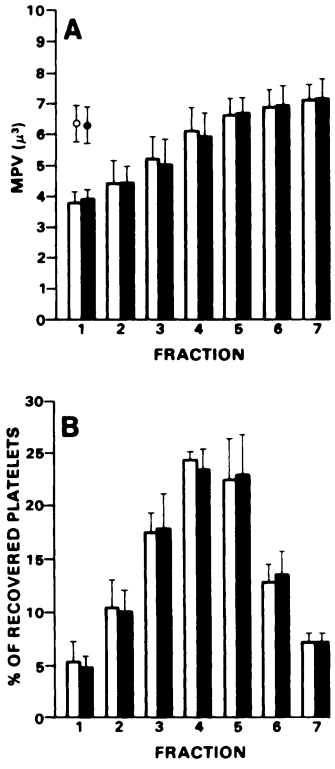

Fig. 1. Characteristics of the size-dependent platelet subpopulations isolated and studied 2 days (open bars) and 9 days (closed bars) after ^{75}Se-methionine injection. Results are expressed as mean ± SD, n = 3. ○ Unfractionated platelets on day 2, ● unfractionated platelets on day 9. (A) Mean platelet volume. (B) Percent of recovered platelets in each subfraction.

Table 1. ^{75}Se-Specific Activity and Response to Thrombin (2 U/ml) of Size-Dependent Platelet Subpopulations and Original Unfractionated Platelet Population (Mean ± SD, n = 3)

Fraction	^{75}Se Radioactivity (cpm/10^9 Platelets)	Single Platelets Remaining After Aggregation (% of Total)	Time to 20% ΔT (min)
Day 2			
1/2	50.6 ± 15.0	68.3 ± 6.6	1.97 ± 0.22
3	61.4 ± 13.7	63.6 ± 5.9	1.20 ± 0.19
4	70.4 ± 16.8	60.4 ± 3.1	0.98 ± 0.16
5	85.5 ± 18.2	59.7 ± 5.5	0.89 ± 0.12
6/7	100.0 ± 14.2	63.7 ± 13.2	0.73 ± 0.18
Unfractionated	80.8 ± 15.7	47.7 ± 7.1	1.12 ± 0.24
Day 9			
1/2	68.9 ± 5.5	60.1 ± 10.8	2.04 ± 0.72
3	97.4 ± 12.5	67.3 ± 7.6	1.31 ± 0.51
4	113.3 ± 30.9	67.0 ± 8.2	0.97 ± 0.18
5	125.6 ± 14.7	62.6 ± 12.7	0.84 ± 0.20
6/7	148.8 ± 13.6	56.5 ± 8.7	0.85 ± 0.15
Unfractionated	109.7 ± 10.6	50.4 ± 11.5	1.06 ± 0.34

Size-dependent platelet subpopulations were prepared 2 and 9 days after ^{75}Se-methionine injection, and their specific activity was determined. After adjusting the platelet count, aggregation was induced by the addition of 2 U/ml thrombin. After a 20% ΔT, platelet aggregates were fixed by the addition of EDTA/formalin, removed by differential centrifugation, and the number of single platelets remaining determined. The percent of platelets remaining is expressed as a percent of the control value as described in Materials and Methods.

been observed after the addition of red blood cells to suspensions of human platelets (C. B. Thompson, unpublished data). The average percentage of platelets lost from the supernatant after aggregation and brief centrifugation, when averaged for all the fractions and unfractionated platelets, was comparable on both days (39.4% ± 7.0% on day 2 and 39.3% ± 6.5% on day 9). Despite the similarities in the extent of aggregation in the subpopulations, there was a marked difference in the rate of aggregation among them. On days 2 and 9 there was a significant trend toward more rapid aggregation with increasing mean platelet volume ($r = 0.95$, $p < 0.01$ and $r = 0.92$, $p < 0.01$, respectively).

The loss of ^{75}Se radioactivity in platelet aggregates is shown in Table 2. There was a greater loss of ^{75}Se radioactivity on day 2 than on day 9 for each of the subpopulations and the original unfractionated platelets. This difference, when taken for all of the samples together, was statistically significant ($p < 0.001$). Although not statistically significant, there does appear to be a greater loss of ^{75}Se radioactivity in the larger subpopulations as compared to the smaller subpopulations on both days. This may simply reflect the fact that large platelets are more reactive than the smaller ones. Alternatively, it may be accounted for by the fact that differential centrifugation was performed for the same time period on all samples. Under such conditions, aggregates of large platelets may be removed more efficiently, due to their greater sedimentation velocity. To account for this possibility, the ratio of loss of ^{75}Se radioactivity between the 2 days was calculated (Table 2, column 3). No trend in the ratios of loss of ^{75}Se radioactivity with platelet aggregates on day 2 versus day 9 occurred in the size-dependent subpopulations ($r = -0.15$, $p > 0.5$).

Table 2. ^{75}Se Radioactivity Removed With Platelet Aggregates After Thrombin Stimulation (Mean ± SD, n = 3)

Fraction	Day 2 (% Loss)	Day 9 (% Loss)	Ratio of Loss (Day 2/Day 9)
1/2	16.3 ± 6.5	11.1 ± 6.5	1.57 ± 0.29
3	19.7 ± 8.0	9.4 ± 2.9	2.06 ± 0.27
4	26.4 ± 5.0	12.1 ± 7.3	2.18 ± 0.68
5	21.7 ± 5.0	13.8 ± 7.3	1.78 ± 0.59
6/7	24.4 ± 7.1	18.0 ± 1.5	1.34 ± 0.28
Unfractionated	39.0 ± 7.9	20.8 ± 12.5	2.28 ± 1.08

The percent of ^{75}Se radioactivity removed with platelet aggregates after partial aggregation of platelet subpopulations was determined 2 and 9 days after ^{75}Se-methionine injection. See legend to Table 1.

DISCUSSION

The use of ^{75}Se-methionine is an established and accepted means of platelet cohort labeling.[24,25] The close correlation between the incorporation of ^{75}Se-methionine and mean platelet volume documented in this study confirms our earlier findings.[16] The similarity in the distribution of ^{75}Se radioactivity in size-dependent platelet subpopulations 2 and 9 days after ^{75}Se-methionine infusion indicates that the size spectrum of platelets is maintained independently of age. If size and age were related, one would have expected a shift in the ^{75}Se-specific activity to the smaller platelet subpopulations. Our present data are in accordance with our previous data[16] and suggest that platelet size heterogeneity is determined during megakaryocyte development rather than during aging in the circulation. This interpretation is supported by Evatt and Levin,[24] who showed that when plasma from thrombocytopenic rabbits was transfused into normal rabbits it induced an increase in platelet protein incorporation at the megakaryocyte level, but not in the number of platelets released from the bone marrow. Their data suggest that the increase in circulating platelet size observed following thrombocytopenia may be due to a shift in the size of platelets produced by the megakaryocyte rather than a change in the age spectrum of the circulating platelets.

Our observation that, on both days of study, the larger platelets aggregated more rapidly than the smaller platelets is in agreement with previous reports.[1,18,20] This difference cannot be accounted for by differences in the frequency of collisions of dif-

ferent-sized platelets, as we have previously demonstrated that small platelets agglutinate at the same rate as large platelets in response to ristocetin.[20] The close correlation between the rate of aggregation and the mean platelet volume of the baboon platelet subpopulations suggests that the differences in function are quantitative ones, based on the differences in size of the platelets. The similar percentage of single platelets remaining in each fraction after partial aggregation confirms that 20% ΔT is a valid indication of the extent of aggregation in each fraction. These data agree with our previous study of functional differences in human platelet subpopulations.[20,21]

We have observed that, during aggregation, more labeled platelets appeared in the aggregates when the labeled platelets were predominantly young (day 2) compared to when the label was associated with older platelets (day 9). These results suggest that age is a determinant of platelet functional ability, as evidenced by the fact that young platelets are more likely to aggregate than older platelets. Haver and Gear[22] also showed that young platelets were functionally more active than old platelets and suggested that the differences were related to their larger size. However, we found that the increased activity occurred to a similar extent in each of the aging size-dependent platelet subpopulations. Thus, aging does lead to functional deterioration of platelets, but the decrement is independent of platelet size. The mechanism for the age-related decrease in activity is unknown, but may reflect a loss of glycoproteins from the platelet surface over time in the circulation, as suggested by George et al.[14] Such loss can occur in the absence of a change in platelet volume, as platelet volume is primarily determined by the platelet cytosol and its constituents. Indeed, George et al. have shown that the rate of loss of membrane glycoproteins exceeds the rate of loss of cytosol during aging in the circulation.[14] Although this may be related to elution of the label from the platelet surface, it may also correlate with elution of glycoproteins needed for platelet adhesion during aging in the circulation.

Our data show that both platelet size and age are significant determinants of platelet function. However, there is no correlation between platelet size and age. Thus, the effects of size and age on the functional ability of platelets are independent.

REFERENCES

1. Karpatkin S: Heterogeneity of human platelets. I. Metabolic and kinetic evidence suggestive of young and old platelets. J Clin Invest 48:1073, 1969
2. Karpatkin S: Heterogeneity of human platelets. II. Functional evidence suggestive of young and old platelets. J Clin Invest 48:1083, 1969
3. Corash L, Tan H, Gralnick HR: Heterogeneity of human whole blood platelet subpopulations. I. Relationship between buoyant density, cell volume, and ultrastructure. Blood 49:71, 1977
4. Mezzano D, Hwang K, Catalano P, Aster RG: Evidence that platelet buoyant density, but not size, correlates with platelet age in man. Am J Hematol 11:61, 1981
5. Minter FM, Ingram M: Platelet volume: density relationships in normal and acutely bled dogs. Br J Haematol 20:55, 1971
6. Paulus JM: Platelet size in man. Blood 46:321, 1975
7. Penington DG, Lee NLY, Roxburgh, AE, McGready JR: Platelet density and size: the interpretation of heterogeneity. Br J Haematol 34:365, 1976
8. Penington DG, Streatfield K, Roxburgh AE: Megakaryocytes and the heterogeneity of circulating platelets. Br J Haematol 34:639, 1976
9. Rand ML, Greenberg JP, Packham MA, Mustard JF: Density subpopulations of rabbit platelets: Size, protein, and sialic acid content, and specific radioactivity changes following labeling with ^{35}S-sulfate in vivo. Blood 57:741, 1981
10. McDonald TP, Odell TT, Gosslee DG: Platelet size in relation to platelet age. Proc Soc Exp Biol Med 115:684, 1964
11. Ginsburg AD, Aster RH: Changes associated with platelet aging. Thromb Diath Haemorrh 27:407, 1972
12. Blajchman MA, Senyi AF, Hirsh J, Genton F, George JN: Hemostatic function, survival, and membrane glycoprotein changes in young versus old rabbit platelets. J Clin Invest 68:1289, 1981
13. Hirsh J, Glynn MF, Mustard JF: The effect of platelet age on platelet adherence to collagen. J Clin Invest 47:466, 1968
14. George JN, Lewis PC, Sears DA: Studies on platelet plasma membranes. II. Characterization of surface proteins of rabbit platelets in vitro and during circulation in vivo using diazotized (^{125}I) diiodosulfanilic acid as a label. J Lab Clin Med 88:247, 1976
15. George JN, Lewis PC: Studies on platelet and plasma membranes. III. Membrane glycoprotein loss from circulating platelets in rabbits: inhibition by aspirin-dipyridamole and acceleration by thrombin. J Lab Clin Med 91:301, 1978
16. Thompson CB, Love DG, Quinn PG, Valeri CR: Platelet size does not correlate with platelet age. Blood 62:487, 1983
17. Mannucci PM, Sharp AA: Platelet volume and shape in relation to aggregation and adhesion. Br J Haematol 13:604, 1967
18. Karpatkin S: Heterogeneity of human platelets. VI. Correlation of platelet function with platelet volume. Blood 51:307, 1978
19. Thompson CB, Eaton KA, Princiotta SM, Rushin CA, Valeri CR: Size dependent platelet subpopulations: Relationship of platelet volume to ultrastructure, enzymatic activity, and function. Br J Haematol 50:509, 1982
20. Thompson CB, Jakubowski JA, Quinn PG, Deykin D, Valeri CR: Platelet size as a determinant of platelet function. J Lab Clin Med 101:205, 1983
21. Jakubowski JA, Thompson CB, Vaillancourt R, Valeri CR, Deykin D: Arachidonic acid metabolism by platelets of differing size. Br J Haematol 53:503, 1983
22. Haver VM, Gear ARL: Functional fractionation of platelets. J Lab Clin Med 97:187, 1981
23. Bahn AK: Basic Medical Statistics. Orlando, FL, Grune & Stratton, 1972, p 45
24. Evatt BL, Levin J: Measurement of thrombopoiesis in rabbits using ^{75}selenomethionine. J Clin Invest 48:1615, 1969
25. Ardaillou N, Najean Y, Eberlin A: Measurement of platelet survival and production using methionine radioisotopes, in Paulus JM (ed): Platelet Kinetics. Amsterdam, North-Holland, 1971, p 327