Hemostatic Function, Survival, and Membrane Glycoprotein Changes in

Young versus Old Rabbit Platelets

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A B S T R A C T   Although in vitro studies have demonstrated functional differences between young and old platelets, in vivo differences have not been precisely established. Therefore the in vivo hemostatic function of young and old platelets and the survival time have been examined in rabbits. The hemostatic function was measured by performing serial ear bleeding times in irradiation-induced thrombocytopenic rabbits. After irradiation with 930 rad the platelet count gradually diminished reaching a nadir ($\sim 20 \times 10^3/\mu l$) at 10 d. The platelets present in the circulation, 7–10 d after irradiation, were considered old platelets, and the platelets present after recovery, 11–14 d postirradiation, young platelets. The measurement of platelet size was consistent with the hypothesis that platelets become smaller with age: the mean size was 3.84 μm^3 for old platelets and 5.86 μm^3 for young platelets. Regression analysis of the relationship between the bleeding time and the platelet count in 18 rabbits showed a significantly different slope for rabbits with predominantly old platelets compared with rabbits with predominantly young platelets ($P < 0.001$). Young platelets were more effective giving much shorter bleeding times than old platelets at comparable platelet counts. Survival times of young and old platelets were measured using platelets harvested on day 8 postirradiation (old platelets) and day 12 postirradiation (young platelets) that were labeled and then reinjected into normal recipient animals. The mean platelet survival time, calculated by gamma function, of old platelets

was 28.8 h; of young platelets, 87.4 h; and of normally circulating heterogeneous platelets, (normal platelets) 53.0 h. Notably, the survival of old platelets was found to be exponential, and of young platelets, linear. Analysis of the membrane glycoproteins in young, old and normal platelets indicated that there was no qualitative difference amongst the young, normal, and old platelets. The relative relationship among all the glycoprotein peaks was equal and the only changes observed were quantitative, with young platelets having significantly more membrane glycoprotein per cell than old platelets and normal platelets. Normal platelets had intermediate concentrations of each glycoprotein. These results demonstrate that young platelets are hemostatically more effective in vivo than old platelets. The data are compatible with the hypothesis that platelets age in the circulation by losing membrane fragments and then after becoming senescent, are removed from the circulation by a random process.

INTRODUCTION

A number of in vitro studies have demonstrated that platelets are heterogeneous with respect to their physical, biochemical, and functional characteristics (1–25). Platelets have been separated by differential centrifugation into light small, and heavy large platelets. The heavy large platelets are metabolically more active and in vivo studies in experimental animals, using ^{75}Selenomethionine incorporation, indicate that these heavy large platelets are younger than the small light platelets (11, 16, 22), and that they lose membrane glycoprotein as they age in the circulation (22, 26). The results of studies in man also suggest that young platelets are both larger and hemostatically more effective than old platelets (6, 8, 11, 12, 14, 23). These observa-

tions have led to the hypothesis that platelets become smaller and hemostatically less effective as they age in the circulation. In this paper we report on studies in rabbits that directly examine the differences in the hemostatic function, survival, and membrane glycoproteins in young and old platelets from rabbits during marrow suppression and recovery.

METHODS

Preparation of rabbits with predominantly young or old platelets. New Zealand white rabbits weighing between 2.5 and 3 kg were irradiated with 930 rad for 30 min using a cesium source. During irradiation the rabbits' ears were shielded with a lead ribbon to avoid damage to the ear vessels which were used for bleeding time experiments. After irradiation, the platelet count began to fall after 4 d, usually reached a nadir at 10 d, and recovered to pretreatment levels at ~day 14 (Fig. 1). Platelets present in the circulation 6–8 d after irradiation were considered old platelets. To obtain young platelets, heterologous platelet antiserum produced in sheep (27) was injected on day 8. This resulted in a sharp fall in the platelet count to <10,000/μl, which slowly began to recover after day 10. Platelets from normal unirradiated rabbits, with a heterogeneous distribution of platelets of various age served as a control and were considered "normal" platelets.

Ear bleeding time determinations. Hemostatic function in thrombocytopenic anesthetized rabbits was evaluated using a standardized ear bleeding time technique (27). The bleeding time was performed by warming the rabbit's ear for 5 min in a saline bath maintained at a temperature of 37°C. The ear was then removed from the bath and a standard incision was made through the ear avoiding any macroscopically obvious vessels. The incised ear was then immediately replaced into the saline bath, which was gently agitated with a magnetic stirrer. The bleeding time result was taken as the time required for blood flow from the incision to cease. Each result represents the mean of two determinations. Previous studies have shown that the bleeding time is highly reproducible in both normal and thrombocytopenic rabbits (27).

Platelet size determination. Blood for platelet size determination was collected by venipuncture from the marginal ear vein of unanesthetized rabbits into a tube containing liquid EDTA in a proportion of blood/2% EDTA, 0.9:0.1 ml. The measurements were made within 2 h of collection. Erythrocytes were sedimented at 1 g for 30 min, then 3.3 μl of platelet-rich plasma were diluted in 10 ml of Isoton (Coulter Electronics Inc., Hialeah, Fla.). The sizing experiments were performed using a Coulter Channelizer model 1,000, which had been standardized with 2.02 and 3.4 μm latex particles. The window width was set at 100 to allow only particles sizing from 1.4 to 28 fl. Each window was made to equal 0.27 fl. Electrical noise was minimized by using a base channel threshold of four. After 1,000 platelets were counted within one channel, a histogram was produced, and from this the median platelet volume was determined. All platelet counts were done using phase contrast microscopy (28).

Platelet survival measurements. Platelet survival of the young, normal, and old platelet populations were performed using standard techniques from blood samples taken from the marginal ear vein (29). Old platelets were harvested 8 d after irradiation, labeled with ⁵¹Chromium, and then infused into normal rabbits. Young platelets were harvested 12 d after irradiation and 4 d after injection of heterologous platelet antiserum. These were then labeled with ⁵¹Cr and infused into

normal rabbits. To obtain sufficient platelets for calculation of platelet survival, young or old platelets were obtained from nine donor animals, pooled, and then divided into three equal parts and reinfused into three recipient normal rabbits. The survival of normal platelets was performed using standard methods (29). Before ⁵¹Cr labeling, aliquots of each sample were taken for platelet size determination. The mean platelet survival time was calculated using a standard-assisted computer technique that determined, by the least squares method, the mean platelet survival time as a linear, exponential, and γ-function (29, 30).

Quantitative analysis of rabbit platelet membrane glycoprotein. Young, old, or normal rabbit platelets were prepared as described for human platelets (31). Platelets washed three times in Ringer's citrate-dextrose buffer containing 50 ng/ml PGE$_1$ were then resuspended in the buffer containing 5 mM EDTA, 0.4 mM phenylmethyl sulfonyl fluoride, 0.5 mM N-carbobenzoxyl-L-glutamyl-L-tyrosine, and 50 ng/ml PGE$_1$ to a concentration of 10^9/ml. They were then solubilized with 3% sodium dodecyl sulfate (SDS) and 40 mM dithiothreitol (DDT)[1], immediately frozen in dry ice, and shipped to San Antonio, Tex. 50 μl (5 × 10⁷ platelets) or 100 μl (10⁸ platelets) were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) using 5% acrylamide and 0.25% N,N'-methylene bisacrylamide (31). Each sample contained 4 μg of orosomucoid (alpha$_1$-acid glycoprotein, kindly provided by Dr. G. A. Jamieson, American Red Cross, Bethesda, Md.). After electrophoresis, the gels were stained for carbohydrate by the periodic-acid-Shiff (PAS) reaction and scanned at 550 nm (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). The PAS bands were then quantitatively analyzed by planimetry and the data expressed as arbitrary units, using the orosomucoid band as an internal standard for the PAS reaction in each gel (31). Molecular weights were estimated from known standards analyzed by a parallel SDS-PAGE gel in each experiment (31, 32). Although whole platelets were analyzed in these experiments, the glycoproteins are referred to as membrane proteins because previous studies demonstrated that all PAS bands present in whole platelet samples were also present in isolated membranes (32).

RESULTS

Validation of Methods for Preparing Young and Old Platelets

Effect of heterologous platelet antiserum. To confirm that the rise in platelet count after day 10 was due to platelets newly released from the bone marrow, and not to reappearance of platelets sequestered after exposure to antiserum, experiments were performed with ⁵¹Cr-labeled normal rabbit platelets. Normal ⁵¹Cr-labeled platelets were injected into rabbits that had been irradiated 8 d previously; an injection of heterologous platelet antiserum was then administered. Serial measurements of platelet radioactivity and platelet count revealed that the ⁵¹Cr-labeled platelets were removed from the circulation within 30 min. Radioactivity did not reappear in the circulation during recovery of the platelet count (data not shown) indi-

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[1] *Abbreviations used in this paper*: DTT, dithiothreitol; PAGE, polyacrylamide gel; PAS, periodic-acid-Schiff.

cating that the rise in platelet count reflected platelets newly released from the marrow.

Effect of irradiation on platelet survival. To confirm that the fall in platelet count following irradiation was not caused by either vascular damage or platelet damage, which would lead to the premature destruction of platelets, two series of experiments were performed. In the first, platelets harvested from donor rabbits that had been irradiated 12 h previously were compared with the survival of platelets derived from nonirradiated animals. The platelet survival of ^{51}Cr-labeled platelets in both groups of rabbits was normal and identical. In the second, the survival of normal platelets was studied in animals that had been irradiated 12 h earlier and compared with their survival in nonirradiated animals. Again, the platelet survival was identical (and normal) in both groups of rabbits (data not shown).

Effect of irradiation on platelet count and platelet size. The effect of irradiation (930 rads) on the platelet count in 36 rabbits is shown in Fig. 1. The platelet count, which was ~250,000/μl before irradiation, started to fall on day 4, and by day 8 had dropped > 70% of the initial count. After the injection of heterologous platelet antiserum (shown by the arrow in Fig. 1) there was a sharp fall in the platelet count to <10,000/μl. The count then began to rise slowly and steadily on day 10 and by day 14 had returned to preirradiation levels.

There was a progressive decrease in median platelet size after irradiation and a significant increase during recovery of the platelet count. The mean median platelet size preirradiation and on day 1 after irradiation was 4.1 μm^3, and on day 8, 3.7 μm^3. After recovery, the mean platelet size reached 5.9 μm^3 on day 11. These mean platelet sizes are all statistically significantly different from each other ($P < 0.025$).

The bleeding times in rabbits with predominantly young or old platelets. The relationship between the

platelet count and the bleeding time in 18 animals with predominantly young or old platelets is shown in Fig. 2. Bleeding times in animals with predominantly old platelets were performed on days 6, 7, and 8 post-irradiation, and bleeding times in animals with predominantly young platelets were performed on days 11, 12, and 13. The slopes of the regression lines relating the bleeding time and the logarithm of the platelet count for old and young platelets are significantly different from each other ($P < 0.001$). Thus, at any given platelet count the bleeding time was shorter in the animals with circulating young platelets.

The difference between the bleeding time produced by old and young platelets was also seen in individual rabbits. In 13 of 18 rabbits comparable platelet counts (within 10,000/μl) were obtained when the circulating platelets were predominantly old or young. The difference in bleeding time for each individual rabbit at a comparable platelet count thus reflected the difference in hemostatic function between young and old platelets. In these animals young platelets were consistently more effective at reducing the bleeding time than old platelets at comparable platelet counts ($P < 0.001$). The mean bleeding time $\pm$1 SD for old platelets was 587$\pm$148 s, and for young platelets 210$\pm$29 s. The corresponding mean platelet counts were 62 and 64 $\times$ 10^3/μl, respectively.

To exclude the possibility that irradiation influenced platelet function and hence the results of the bleeding time experiments, bleeding times were performed on animals with normal platelets or young
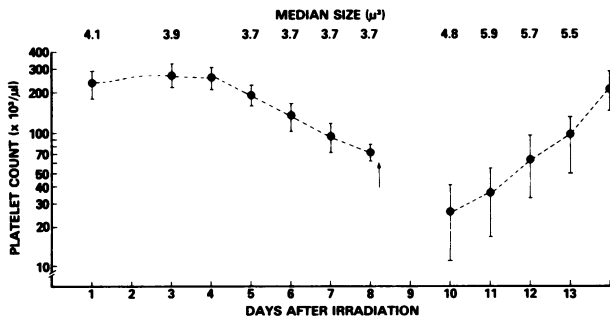

FIGURE 1 Mean platelet count ($\pm$SD) in 36 rabbits after 930 rad of irradiation. The arrow shown (day 8) indicates the administration of heterologous platelet antiserum. The median platelet size corresponding to the day after irradiation is also shown.
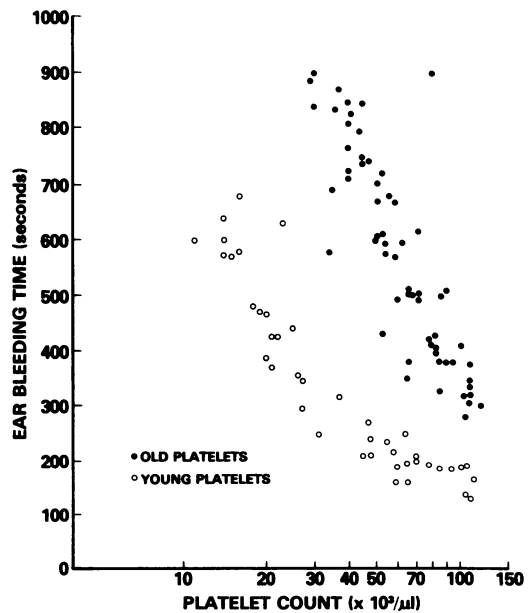

FIGURE 2 The relationship between the bleeding time and the platelet count for old and young platelets in 18 rabbits.

platelets both before and 12 h after irradiation. As seen in Table I, the platelet counts were similar before and after irradiation in both groups and there was no difference between the pre- and postirradiation bleeding times in either the normal rabbits or those with circulating young platelets.

Platelet survival times of young, old, and normal platelets. Results of the platelet survival studies are shown in Fig. 3. The survival time was expressed as a percentage of the 2-h sample, which was designated as being 100%. The survival time of young, normal, and old platelets was significantly different from each other, young platelets having the longest survival and old platelets the shortest. Survival times were calculated as a linear, exponential, and gamma function model (29). Results are shown in Table II. For young platelets, the results obtained using the linear and gamma function models were similar, suggesting that platelet lifespan most closely approximated linear survival. In contrast, for old platelets, the survival time obtained with the gamma function model most closely approximated the exponential model, suggesting that the decay of old platelets followed an exponential pattern.

Quantitative analysis of membrane glycoproteins in young, old, and normal platelets. The pattern of the PAS reaction of normal rabbit platelets solubilized in SDS-DTT and separated by SDS-PAGE is shown in Fig. 4. Six PAS-positive bands were consistently identified. The quantitative analysis of the membrane glycoproteins is summarized in Table III. Young platelets contain significantly more membrane glycoprotein per cell than old platelets, and the results for normal platelets were intermediate between the two. There were, however, no qualitative changes of the platelet membrane glycoprotein related to cell age.

DISCUSSION

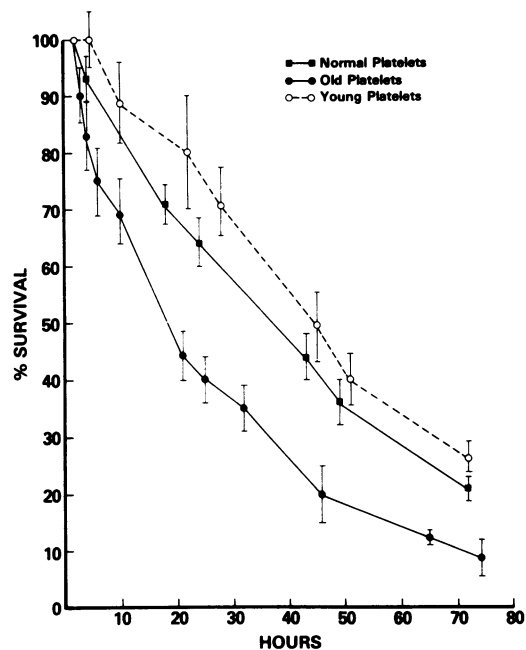By using a combination of x-irradiation and platelet antiserum, it has been possible to investigate the func-

FIGURE 3 Platelet survival of young, old, and normal platelets. The percentage survival ($\pm$SD) at each time is expressed as a percentage of the count at 2 h. The number of determinations for each point was as follows: normal platelets, 26, old platelets, 9, and young platelets, 12.

tion and structure of circulating platelets in rabbits with a mean age that is either older or younger than the mean age of a normal heterogeneous population of platelets. The rabbits with predominantly young platelets represent rabbits with "stress" platelets in their circulation because the platelets were formed and released under the influence of a marked thrombocytopenic stimulus. A number of experiments were performed to validate our contention that animals investigated 6–8 d after irradiation had a predominantly old population of platelets in their circulation and those investigated 12 d after irradiation and 4 d after the injection of heterologous platelet antiserum had predominantly young platelets in their circulation. Cross transfusion experiments using either irradiated donor platelets or irradiated recipient rabbits demonstrated that neither platelet survival time nor platelet function were affected by the irradiation procedure, per se. Experiments with ^{51}Cr platelets demonstrated that the injection of heterologous antiserum was associated with permanent platelet destruction and that the rise in platelet count that subsequently followed irradiation was due to newly formed platelets.

Our studies, therefore, confirm results of a number of less direct experiments that young platelets are hemostatically more competent than old platelets and that young platelets are larger than old platelets. The results

TABLE I
*The Effect of Irradiation on the Hemostatic Function of Normal and Young Platelets**

Platelet population		Mean platelet count ($\pm$SD)	Mean bleeding time ($\pm$SD)
		$\times 10^3/\mu l$	s
Normal	Preirradiation	279$\pm$40.6	90.3$\pm$19.7
	Postirradiation	289.2$\pm$56.6	94.3$\pm$17.3
Young	Preirradiation	127.2$\pm$20.0	112.4$\pm$8.0
	Postirradiation	134.3$\pm$19.5	108.4$\pm$6.7

* The number of rabbits in each experiment was 12.

TABLE II

Platelet Survival of Young, Old, and Normal Platelets Estimated as a Gamma, Linear, or Exponential Function and Expressed as a Mean ±SD*

	Gamma function	Linear function	Exponential function
	h	h	h
Young (12)	85.8±15.3	89.7±8.8	51.9±5.2
Old (9)	28.3±3.3	67.1±2.2	30.9±3.6
Normal (26)	58.5±8.3	84.7±3.7	46.9±4.3

* The number of determinations is shown in parentheses.

of these experiments also confirm that young platelets survive longer in the circulation than normal platelets, which in turn survive longer than old platelets (20, 22, 33). The pattern of survival of young platelets was essentially linear, indicating that their survival in the circulation was relatively unaffected by a random destructive process. In contrast, the platelet survival pattern of old platelets showed an exponential decay, suggesting that after platelets have circulated for a period of time their removal from the circulation is random.
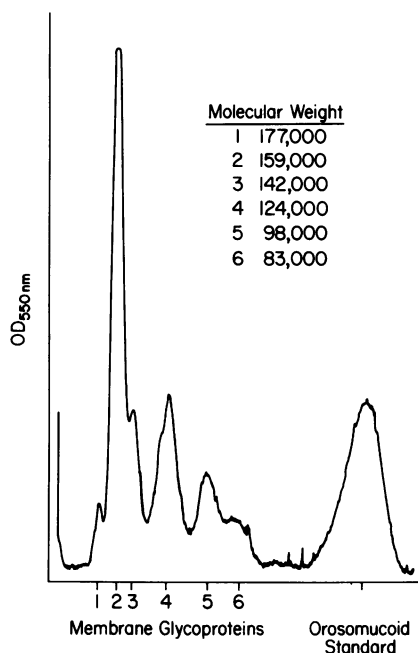
FIGURE 4 Analysis of rabbit platelet glycoproteins by SDS-PAGE. Normal rabbit platelets were isolated and solubilized in SDS and DTT and 5×10^7 platelets plus 4 μg of orosomucoid were applied to the polyacrylamide gel. After electrophoresis the gels were stained for carbohydrate by the PAS reaction. Six PAS-positive bands were identified by densitometer scanning and their molecular weights are shown.

TABLE III

Quantitative Analysis of Platelet Glycoproteins from the Membranes of Young, Normal, and Old Rabbit Platelets

Membrane glycoprotein band*	Young (10)	Normal (9)	Old (6)
GP 1	0.19±0.11	0.12±0.07	0.05±0.02
GP 2	1.77±0.55	1.08±0.31	0.56±0.23
GP 3	0.56±0.27	0.20±0.07	0.20±0.10
GP 4	0.82±0.30	0.48±0.15	0.34±0.15
GP 5	0.67±0.23	0.32±0.09	0.20±0.08
GP 6	0.30±0.07	0.24±0.06	0.15±0.03

* Data are mean values±SD of arbitrary "PAS units," as defined in methods, for the number of samples shown in parentheses. Platelet glycoproteins were analyzed by planimetry of the densitometer scans of PAS-stained SDS-PAGE gels. All values for young platelets were significantly greater than the values for old platelets ($P < 0.01$). All values for normal platelets were significantly less than the values for young platelets ($P < 0.05$) except for GP 1, and were greater than the values for old platelets ($P < 0.02$) except for GP 3 and GP 4.

The results of recent studies using the membrane-label ^{125}I-diazotized diiodosulfanilic acid suggest that platelets lose membrane fragments as they circulate, possibly as a result of reversible contact interaction with the vessel wall and/or other intravascular particles (27, 32, 34). It is possible that the loss of these surface glycoproteins contribute to platelet senescence as well as the structural and functional changes that occur as platelets age. The present studies demonstrate directly that old platelets have considerably less membrane glycoprotein than normal platelets, which in turn have less membrane glycoprotein than young "stress" platelets. The decrease in membrane glycoproteins was symmetrical, not selective, suggesting the loss of intact pieces of membrane.

The findings in these experiments are consistent with the hypothesis that young platelets are hemostatically most effective when they are released from the bone marrow, and that they undergo significant changes in the circulation as they age: decreased size, symmetrical loss of membrane glycoprotein, and decreased hemostatic effectiveness.

ACKNOWLEDGMENTS

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