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BRIEF REPORT

Lipid Peroxidation in Aging Platelets

By Minoru Okuma, Manfred Steiner and Mario Baldini

CONSIDERABLE EVIDENCE has accumulated which suggests that platelets undergo progressive functional impairment as they age in the circulation. In 1942, Wright observed that young platelets were more adhesive than aged platelets[1] and, recently, adhesiveness to collagen was shown to be decreased in aged rabbit platelets.[2] Aging was implicated in the demonstration of morphologically different populations of platelets on exposure to osmotic stress.[3] Size,[4] content of ATP[5] and protein synthesis[6] are decreased in senescent platelets. Lipid deterioration can now be added to the list of known functional, morphological and biochemical parameters affected by aging.

Lipid peroxidation is important to cell function. It appears to produce widespread damage to membranes of cells and subcellular particles leading to inactivation and destruction of enzymes, disruption of metabolic pathways and inhibition of cell division.[7-10] Indication for an age-dependent difference in the formation of lipid peroxides has been found in regenerating rat livers.[11] Increased resistance of young red cells to damage by H_2O_2 has recently been demonstrated in vitamin E-deficient rats.[12]

The interest in investigating lipid peroxidation in aging blood platelets does not derive only from the fact that no studies on lipid peroxides of platelets are available in the literature, but more from the idea that lipid peroxidation could be a physiologic mechanism of platelet senescence and death. In the experiments to be presented here, the lipid peroxide content of platelets and the rate of lipid peroxidation by the action of heavy metal were studied in platelet cohorts of different ages obtained in rats. It was shown that lipid peroxidation is much more pronounced in old than in young platelets and that it may be a cause of platelet aging.

Thirty five male Sprague-Dawley rats (280–330 Gm.) were kept at least 4 weeks on a standard Purina laboratory chow before the experiment. Platelet cohorts of different age were obtained from animals recovering from thrombo-

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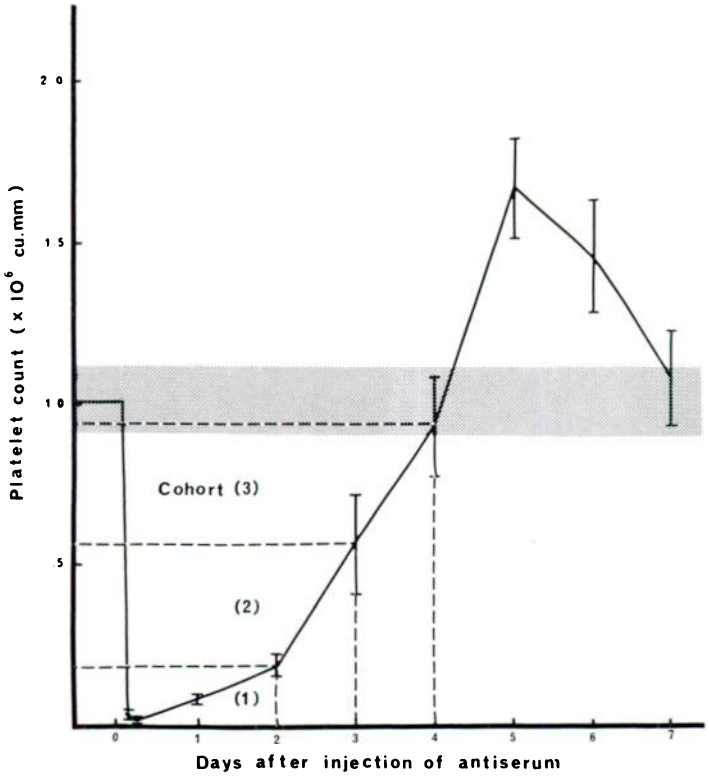

Fig. 1.—Changes in platelet count after injection of specific anti-platelet serum. Each point of the curve represents the mean platelet count of 5–10 rats and the vertical bars denote the extent of ± 1 S.D. The shaded area includes the normal mean count ± 1 S.D. The interrupted lines demarcate cohorts 1, 2 and 3 consisting of platelets collected 2, 3 and 4 days after injection of antiserum.

cytopenia induced by intravenously injected specific anti-rat-platelet immune rabbit serum. Figure 1 shows the response to the injection of 0.1 ml. platelet antiserum. The platelet count[13] before injection of the antiserum was 1,009,400 ± 114,400 per cu. mm. (mean ± 1 S.D.). Severe thrombocytopenia was produced within 1 hour after injection of the antiserum and the lowest count (8,600 ± 3,000) was observed 5 hours after injection. Thereafter the platelet count increased progressively with time reaching the initial level 4 days after injection of the antiserum and the peak of the rebound on the fifth day. Blood was drawn from the abdominal aorta of anesthetized rats into a 20 ml. plastic syringe containing 2 ml. ACD solution. Platelets were separated by differential centrifugation and were washed once with 0.9 per cent NaCl. Contamination with red and white cells was kept below 1/40,000 platelets for the former and 1/120,000 platelets for the latter by repeated centrifugations at 4 C. Lipid peroxides were measured by a modification of the method of Placer et al.[14] The term "lipid peroxides" is used in this report with the understanding that products of lipid peroxidation and not lipid peroxides themselves were measured. The thiobarbituric acid (TBA) reaction was

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Table 1.—Lipid Peroxide Values in Rat Platelets

Time After Injection of Antiserum (days)	Lipid Peroxides* (nanomoles malonaldehyde/1 × 10^9 platelets) Control	+$FeCl_3$
0	0.38 ± 0.03	3.17 ± 0.27
2	0.22 †	1.05 †
3	0.25 ± 0.02	2.05 ± 0.25
4	0.27 ± 0.04	2.66 ± 0.31
5	0.36 ± 0.03	2.98 ± 0.30
6	0.37 ± 0.04	3.20 ± 0.29

* Each value was obtained from 5 rats (Mean ± 1 S.D.).

† Values were obtained from platelets pooled from 5 animals.

Control refers to platelets suspended in 0.2 M trismaleate buffer (pH 5.9) without addition of metal salt. Lipid peroxides were measured without delay in this group.

+$FeCl_3$ refers to platelets suspended in the same buffer as the control groups but with addition of $FeCl_3$ (1×10^{-4}M). Lipid peroxides were measured following 1 hour incubation at 37 C. In both groups each determination was done in duplicate.

selected as the method of choice for measuring the products of lipid peroxidation because of its sensitivity and its direct applicability to biologic tissues without preceeding extraction of lipids. Substances other than malonaldehyde, the cleavage product of hydroperoxides of unsaturated fatty acids, will give a similar red color with TBA reagent. Such chromophores as aromatic aldehydes, ketones and deoxyribose, however, are either unstable, have different absorption maxima or lower optical absorbance than malonaldehyde.[15] Another peculiarity which must be realized in the interpretation of this reaction is the fact that only a small portion of the total peroxidized unsaturated fatty acids will yield malonaldehyde, particularly those containing 2 or more double bonds, i.e., linolenate and arachidonate.[16] In spite of all these shortcomings, the TBA reaction has been widely applied to the measurement of lipid peroxidation products in a variety of tissues and cell fractions under a multitude of different experimental conditions.

To a suspension of 1×10^9 platelets in 0.5 ml. 0.2 M trismaleate buffer (pH 5.9) was added an equal volume of TBA reagent. This mixture was heated in a boiling water bath for 10 minutes and after cooling to room temperature 0.5 ml. Bio-solv (BBS-3, Beckman Instruments Inc., Calif.) was added and mixed thoroughly. Complete clearing of the solution was obtained by addition of 0.5 ml. IN NaOH. The optical absorbance was then read at 548 nm. A standard absorption curve for malonaldehyde was prepared using 1,1,3,3-tetraethoxypropane.[17] The molar absorptivity was calculated as $E = 1.45 \times 10^5$.

Table 1 shows the lipid peroxide values in platelets in relation to age. Platelets representing a random population were prepared from rats not treated with platelet antiserum. Lipid peroxides were highest in this group, 0.38 nanomoles malonaldehyde/1 × 10^9 platelets. The cohort of platelets collected 2 days after injection of the antiserum (cohort 1) had the lowest accumulation of lipid peroxides. Approximately 60 per cent of these platelets were less than 24 hours old and the majority of the remainder less than 2 days of age. As the fraction of older platelets (more than 2 days old) increased

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in platelet cohorts 2 and 3 collected on days 3 and 4 and in those collected 5 and 6 days following the immunologically induced thrombocytopenia, the content of lipid peroxides also showed a roughly proportional increase. The platelets produced after the injection of the antiserum were normal, young platelets. It is known that an antibody to platelets would disappear within minutes from the circulation.[18] The number of platelets started to increase in our rats one day after injection of the antiserum and the first cohort was separated after two days. These platelets were presumably normal also with respect to their structure because they were produced at a normal (not supranormal) rate by the megakaryocytes in the bone marrow. In fact, it took four days for the circulating platelets to reach the normal level, and this period of time corresponds to the normal life span of rat platelets.[19]

Heavy metals, in particular iron, have been shown to catalyse both chemically and enzymatically induced lipid peroxidation.[20] The extent of this catalysis depends amongst other factors upon the content of unsaturated fatty acids in the lipids of the tissue and the presence of mechanisms neutralizing the catalytic effect of the heavy metal. As shown in the table, iron (1×10^{-4}M $FeCl_3$) produced a marked increase in lipid peroxides of platelets. Young platelets subjected to iron again demonstrated considerably lower lipid peroxidation than aged ones. As the cohort age increased, the metal-catalysed formation of lipid peroxides approached that found in a platelet population of random age as obtained in rats not treated with the platelet antiserum.

The accumulation of products of lipid peroxidation in senescent platelets may lead to a cumulative damage of membranous structures of the platelets themselves and of their subcellular particles, such as the alpha granules, mitochondria and endoplasmic reticulum. Lipid peroxidation may affect metabolic pathways directly by reducing the activity of lipid dependent enzymes such as NADH- or succinate- cytochrome c reductase or indirectly through release of lysosomal enzymes from alpha granules.

The results presented suggest the existence in young platelets of mechanisms of defense against oxidative destruction of unsaturated fatty acids. Young platelets appear to be much more effective in this regard than old platelets. The mechanisms involved may concern antioxidant activity, the relative proportion of unsaturated fatty acids in the lipids and the content of catalysts of lipid peroxidation in the platelets. Which of these mechanisms is more efficient in platelets or what factors are primarily responsible for the increase in lipid peroxides in senescent platelets remains to be elucidated.

Summary

Blood platelets showed an age dependent accumulation of lipid peroxides. Lipid peroxidation stimulated by heavy metal also increased with platelet age and rose at a much faster rate than the spontaneous peroxide formation. This suggests the loss of a cellular defense mechanism against lipid peroxidation in aging blood platelets.

SUMMARIO IN INTERLINGUA

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