Platelet derived malonyldialdehyde production in patients with thalassaemia major

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SUMMARY Malonyldialdehyde, a product of membrane lipid peroxidation, was measured in the platelets of 16 normal subjects after stimulation with a variety of aggregating and stimulating agents. Nethylmaleimide and hydrogen peroxide generated the largest amounts of malonyldialdehyde. These agents were used to stimulate platelets from 11 patients with thalassaemia major suffering from iron overload due to repeated transfusion. Mean malonyldialdehyde concentrations were the same in normal subjects as in thalassaemic patients, but high concentrations were recorded in patients with severe iron overload. There was a highly significant correlation between malonyldialdehyde and serum ferritin concentrations in all thalassaemic patients. Platelet derived malonyldialdehyde may be a useful test of continuing membrane damage in patients with iron overload.

Lipid peroxidation of cell membranes is associated with a number of pathological conditions, such as radiation damage to the lungs, liver injury, atherosclerosis, and thrombosis¹⁻⁴ and many inflammatory processes.^{5,6} Malonyldialdehyde, a stable secondary product of lipid peroxidation,^{7,8} can be measured after oxidant stress.⁹ With this technique red cells have been shown to be more susceptible to autoxidation in autoimmune haemolytic anaemia and β -thalassaemia major.¹⁰ Iron salts are among the most potent catalysts of lipid peroxidation,^{11,12} and high red cell malonyldialdehyde concentrations found in patients who have had multiple transfusions may reflect the damage to other organs caused by excess iron. In patients with β -thalassaemia major it is difficult to correlate red cell malonyldialdehyde concentrations with membrane damage in other tissues as many of these cells are transfused, producing inappropriately low values, and thalassaemic red cell membranes contain large amounts of lipid susceptible to autoxidation compared with normal cells.¹³

Malonyldialdehyde is also formed in platelets during the non-enzymatic peroxidation of polyunsaturated fatty acids¹⁴ and enzymatically during prostaglandin production.¹⁵ There has been one previous report of abnormal platelet function in β -thalassaemia major.¹⁶ We have therefore investigated malonyldialdehyde generation in the platelets of normal subjects and repeatedly transfused

patients with β -thalassaemia major on exposure to oxidant stress with hydrogen peroxide and on activation with nethylmaleimide, an agent used to assess platelet activation in vitro.^{17,18} We have also correlated platelet malonyldialdehyde production with the degree of iron overload assessed by serum ferritin.

Material and method

Twenty millilitres of venous blood was collected into 3.13% sodium citrate and centrifuged at 150 g for 15 min at 20°C. The supernatant platelet poor plasma was then inverted for 2 min to remove all plasma, and the platelet buttons were resuspended in 0.9 ml of phosphate buffered saline (PBS). Malonyldialdehyde generation was measured after stimulation with adenosine diphosphate, adrenaline, collagen, ristocetin, nethylmaleimide, and hydrogen peroxide.

To four of the tubes we added 100 μ l of one of the stimulating agents and to the fifth we added 100 μ l of PBS as a blank. The suspensions were then incubated for 60 min at 37°C in a waterbath. After incubation 1 ml of the 2-thiobarbituric acid solution was added to each tube, which was vortex mixed to terminate the reaction and then incubated for 30 min at 80°C. The tubes were then removed, cooled on ice, and centrifuged at 2000 g for 15 min at 4°C. The optical density of the supernatant was read at 532 nm against the appropriate blank, and the concentration of malonyldialdehyde was calculated as follows:

$$\text{nmol malonyldialdehyde per } 10^9 \text{ platelets} = \frac{A.V_f}{N.V_i.E} \times 10^9$$

where A = optical density at 532 nm, V_f = final volume (1 ml), V_i = initial volume (0.9 ml), N = platelet count (platelet rich - platelet poor plasma), and E = molar extinction coefficient of malonyldialdehyde (1.55×10^5).

PATIENTS

Malonyldialdehyde concentrations were measured in 16 healthy volunteer subjects (age range 18–30 years) and 11 patients with transfusion dependent thalassaemia major (age range 16–25 years) who were attending the thalassaemia clinic at University College Hospital. Serum ferritin concentrations¹⁹ were measured in samples taken on the same day as the malonyldialdehyde measurements were performed.

Results

Malonyldialdehyde generation from platelets was measured in 16 normal volunteers after stimulation with one of the above agents. These induced different concentrations in normal subjects: adenosine diphosphate, adrenaline and collagen induced low concentrations; ristocetin gave higher values; and hydrogen peroxide and nethylmaleimide induced the highest values (Table 1).

Nethylmaleimide and hydrogen peroxide were then used to generate malonyldialdehyde in 11 patients with β -thalassaemia major who had had multiple transfusions. Both agents stimulated malonyldialdehyde production in all patients and there was no significant difference between mean malonyldialdehyde concentrations derived from thalassaemic and normal platelets, but the range of values for the patients with thalassaemia was increased (Table 2).

The level of nethylmaleimide and hydrogen peroxide stimulated malonyldialdehyde production was then compared with serum ferritin concentrations in 10 patients (Figure). There was a highly significant correlation between malonyldialdehyde concentrations and serum ferritin concentrations when either stimulant was used: for nethylmaleimide $r = 0.84$ ($p < 0.001$) and for hydrogen peroxide $r = 0.90$ ($p < 0.01$). Three patients with substantially raised serum ferritin concentrations had malonyldialdehyde values outside the normal range ($> \pm 2$ SD for 16 normal subjects). These patients were all heavily iron overloaded and complied poorly with desferrioxamine. All had strikingly abnormal liver function tests, were heavily pigmented, and two had signs of compromised cardiac function.

Table 1 Effects of various stimulating agents on malonyldialdehyde generation from platelets from 16 normal subjects

Stimulating agent (concentration)	Malonyldialdehyde concentration (nmol/ 10^9 platelets) ± 2
Nethylmaleimide (1 mM)	1.05 $\pm$ 0.25*
Hydrogen peroxide (10 mM)	0.85 $\pm$ 0.33
Ristocetin (12 mg/ml)	0.46 $\pm$ 0.04
ADP (10 μ M)	0.14 $\pm$ 0.02
Adrenaline (1 mg/ml)	0.08 $\pm$ 0.02
Collagen (1 μ g/ml)	0.05 $\pm$ 0.01
	n = 16

*Values given as mean $\pm$ 2 SD

Table 2 Malonyldialdehyde (nmol/ 10^9 platelets) generated from platelets from patients with β -thalassaemia major

Patient no	Nethylmaleimide (1.0 mM)	Hydrogen peroxide (10 mM)
1	1.09 $\pm$ 0.06*	0.78 $\pm$ 0.15
2	1.78 $\pm$ 0.11	1.10 $\pm$ 0.23
3	2.61 $\pm$ 0.02	1.75 $\pm$ 0.11
4	0.66 $\pm$ 0.05	ND
5	1.15 $\pm$ 0.13	ND
6	0.44 $\pm$ 0.04	0.62 $\pm$ 0.32
7	0.25 $\pm$ 0.03	0.10 $\pm$ 0.07
8	1.77 $\pm$ 0.04	ND
9	0.57 $\pm$ 0.14	ND
10	ND	0.23 $\pm$ 0.09
11	0.40 $\pm$ 0.02	0.34 $\pm$ 0.17
Normal subjects (mean)	1.05 $\pm$ 0.25	0.85 $\pm$ 0.33
Thalassaemia major (mean)	1.07 $\pm$ 0.73	0.70 $\pm$ 0.53

*Values given as mean $\pm$ 2 SD.

ND = not done.

Discussion

In this study we assessed the ability of several platelet stimulating agents to induce malonyldialdehyde release from the platelets of normal subjects and of patients suffering from thalassaemia major. In platelets from patients with β -thalassaemia, mean malonyldialdehyde concentrations were normal, but there was a close correlation between platelet

Concentrations of platelet derived malonyldialdehyde and serum ferritin in patients with thalassaemia major.

malonyldialdehyde values and serum ferritin concentrations whether hydrogen peroxide or nethylmaleimide was used as the stimulating agent. The highest malonyldialdehyde concentrations were recorded in patients who not only had considerably raised serum ferritin concentrations but also had other signs of iron overload. As platelet associated malonyldialdehyde provides a measure of lipid peroxidation of cell membranes, the raised concentrations found in the most heavily iron overloaded patients may therefore reflect increased iron mediated cell membrane damage; such high values may be associated with increased quantities of free iron or that loosely bound in the postulated toxic iron pool.²⁰

Serum ferritin concentrations closely correlate with total and hepatic iron stores,²¹ and in the well chelated, iron overloaded patient much of the transfused iron is sequestered in a relatively inert form^{22, 23} and plays a comparatively small part in continuous iron mediated membrane damage. In grossly iron overloaded patients, however, or in those patients who comply poorly with desferrioxamine treatment, there may be sufficient iron in an active form to cause increased membrane lipid peroxidation and consequent tissue damage. High concentrations of platelet malonyldialdehyde may be recorded only in these patients, and our data suggest that values outside the normal range are indeed obtained in patients who show signs of iron toxicity in addition to raised serum ferritin concentrations.

Clinical improvement in iron overloaded thalassaemic patients may be brought about by increasing the dosage of desferrioxamine²⁴ and deterioration may follow administration of large amounts of vitamin C,²⁵ with comparatively minor changes in serum ferritin; this suggests that the serum ferritin concentration is not an adequate measure of continuing organ damage.

We conclude that the use of stimulated platelet malonyldialdehyde concentrations provides a useful, simple measure of lipid peroxidation and consequent cell membrane damage in chronically iron overloaded patients and that serial measurements may be a useful guide to clinical progress.

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