Platelet serotonin in systemic sclerosis

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SUMMARY Platelet serotonin concentrations were measured in 43 patients with systemic sclerosis, in 11 patients with primary Raynaud's phenomenon, and in 38 normal controls. Patients with the CREST variant (calcinosis, Raynaud's phenomenon, oesophageal dysmotility, sclerodactyly, telangiectasia) had significantly lower platelet serotonin concentrations than normal controls. Patients with diffuse systemic sclerosis had normal platelet serotonin concentrations. In patients with CREST treatment with ketanserin, a specific serotonin antagonist, normalised platelet serotonin concentrations. These data provide further evidence suggesting that in systemic sclerosis, particularly the CREST variant, there is widespread platelet activation.

Microvascular disease is a major feature of systemic sclerosis, with Raynaud's phenomenon occurring in most of the patients. The episodic nature of the ischaemia is suggestive of active vasoconstriction, though structural vessel wall changes have been clearly shown.¹

Several observations have implicated serotonin as a possible pathogenetic factor in the development of the microvascular disease. Serotonin can produce digital ischaemia and constriction of isolated human digital arteries.²⁻⁴ In patients with Raynaud's phenomenon vasoconstriction after infusions of serotonin is abnormally prolonged. There is circulatory improvement with serotonin antagonists.⁵ Furthermore, ketanserin, a specific S₂ receptor serotonin antagonist, improves digital blood flow and clinical features in systemic sclerosis.^{6,7}

Almost all circulating serotonin is contained within platelets in specialised granules. During platelet aggregation serotonin is released.⁸ In systemic sclerosis platelet activation has been clearly shown.⁹⁻¹¹

To study circulating serotonin concentration in systemic sclerosis we developed a simple and reliable method of harvesting intact platelets and adapted our high performance liquid chromatography catecholamine method¹² to the measurement of serotonin, the serotonin precursor 5-hydroxytryptophan, and the breakdown product 5-hydroxy-

indoleacetic acid in plasma and platelets. We compared the results for patients with systemic sclerosis, patients with primary Raynaud's phenomenon, and normal subjects.

Patients and methods

PATIENT GROUPS

The group with systemic sclerosis were 43 patients (34 women, nine men) with a mean age of 46 years (range 22-72) who met the American Rheumatism Association criteria.¹³ Mean disease duration was 9 years. Thirty six patients had the CREST variant with at least three of the five criteria calcinosis, Raynaud's phenomenon, oesophageal involvement, sclerodactyly, and telangiectasia. Seven patients had diffuse systemic sclerosis with proximal scleroderma and major organ involvement. The group with primary Raynaud's phenomenon consisted of 11 patients (10 female, one male) with a mean age of 44 years (range 31-60). These patients had had Raynaud's phenomenon (biphasic or triphasic colour changes with numbness or paraesthesiae in response to cold, or both) for at least three years with no clinical or immunological evidence of connective tissue disease. There were 38 normal subjects (23 female, 15 male) with a mean age of 42 years (range 20-73). Fifteen patients with the CREST variant were treated with ketanserin, a serotonin antagonist (40 mg orally three times a day). In 10 of these patients platelet serotonin was measured before the start of ketanserin treatment and then after two months treatment.

Accepted for publication 7 December 1988.

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Fig. 1 Platelet and plasma separation.

BLOOD SAMPLING

Venepuncture was performed with an indwelling catheter with minimal occlusion. The first 5 ml of each sample was discarded. All patients and normal subjects were bled after an overnight fast in a temperature and humidity controlled room (19.5–20.5°C, 40–50%) after 20 minutes' equilibration. Smokers were asked to abstain overnight.

SAMPLE PROCESSING

Venous blood (8.5 ml) was anticoagulated with 1.5 ml of acid citrate dextrose (NIH A formula) containing 300 nmol prostacyclin analogue ZK36374 (Schering AG, Berlin). Platelet rich plasma and platelet poor plasma were obtained as outlined in Fig. 1 and were immediately frozen.

ASSAY

Platelets were disrupted by thawing samples. Additional sonication of the platelet rich plasma did not improve serotonin yield. To 250 µl of plasma was added 250 µl of 0.02 M HCl, 100 µl 2.8 nM ascorbic acid, 50 µl of 4 µM 3,4-dihydroxybenzylamine (internal standard). Proteins were precipitated by the addition of 50 µl of 7 M perchloric acid. All tubes were vortex mixed for two minutes. Clear supernatants were transferred to autosampler vials and 10 µl injected into the high performance liquid chromatography system.

The chromatographic system consisted of a Kratos Spectroflow 400 solvent delivery system (Kratos

Analytical Instruments, Ramsey, NJ 07446) with an ESA Coulochem electrochemical detector (model 5100A; Environmental Sciences Associates Inc, Bedford, MA 01730) fitted with a conditioning cell (model 5021) and a dual analytical cell (model 5011). The 250×4.6 mm column was packed with 5 µm particles of Spherisorb ODS-2 protected by an SSI 0.5 µm precolumn filter and a Brownlee MPLC guard column fitted with an RP-8 Spheri-5 cartridge (Brownlee Labs Inc, Santa Clara, CA 95050). Samples were injected with a Gilson autosampling injector, model 231-401 (Anachem, Luton, Bedfordshire, UK). Results were calculated with a Pye Unicam computing integrator (Pye Unicam, Cambridge, UK). The mobile phase consisted of 1 litre of 0.1 M citrate/phosphate buffer (pH 3.8) containing 4 mmol 1-heptane sulphonic acid and 0.2 mmol disodium edetate, to which was added 200 ml methanol and 40 ml acetonitrile. The mobile phase was recycled at a flow of 1.2 ml/min. Samples were injected every 20 minutes. Serotonin, 5-hydroxyindoleacetic acid, and 5-hydroxytryptophan were obtained from Sigma (Poole, Dorset BH17, UK). Weighed amounts of these compounds were added to indole free plasma to produce a standard curve. Plasma serotonin was expressed as nmol/l and platelet serotonin concentration as nmol/10⁹ platelets.

STATISTICAL METHODS

All results were analysed by non-parametric statistical tests. Patient group comparisons were made with the Mann-Whitney U test. Correlations were carried out using Spearman correlation coefficients.

Results

The concentrations of 5-hydroxytryptophan in the plasma and platelets were similar in all the groups studied. There were no significant age or sex effects on either the plasma or platelet serotonin, 5-hydroxyindoleacetic acid, or 5-hydroxytryptophan. For all the analytes measured there were no significant differences between the patients with primary Raynaud's phenomenon, those with diffuse systemic sclerosis, and the controls. The patients with CREST, however, had significantly lower platelet serotonin concentrations than the controls (1.50 v 3.22 nmol/10⁹ platelets; $p < 0.0001$). Platelet serotonin concentrations in patients with CREST were also significantly lower than in patients with primary Raynaud's phenomenon (3.46 nmol/10⁹ platelets) and patients with diffuse systemic sclerosis (4.25 nmol/10⁹ platelets); $p < 0.001$ and $p = 0.007$ respectively) (Fig. 2). Platelet serotonin concentration in the patients with CREST who were treated

was significantly higher than in the untreated patients (Table 1). In those patients whose platelet serotonin was measured before and during treatment there was significant increase in platelet serotonin after ketanserin treatment ($p<0.01$) (Fig. 3).

The mean plasma serotonin concentration in the patients with CREST was 84 nmol/l (range 8–239 nmol/l), which was greater than for the control subjects (62 nmol/l (range 10–262 nmol/l)). This difference did not reach statistical significance. The

Fig. 2 Platelet serotonin concentrations in normal controls and disease groups. Horizontal bars indicate mean values for each group. Diffuse=diffuse systemic sclerosis; PRP=primary Raynaud's phenomenon.

Table 1 Effect on ketanserin treatment on platelet and plasma serotonin concentrations in patients with CREST

	Mean (SEM) platelet serotonin (nmol/10 ⁹ platelets)	Mean (SEM) plasma serotonin (nmol/l)
Ketanserin treated patients with CREST (n=15)	3.68 (0.33)	65 (14)
Untreated patients with CREST (n=36)	1.50 (0.09)	84 (9)
p Values	<0.001	NS

PLATELET SEROTONIN nmol/10⁹ platelets

Fig. 3 Effect of ketanserin on platelet serotonin.

mean plasma 5-hydroxyindoleacetic acid, however, was significantly raised in the patients with CREST (73 nmol/l (range 10–204 nmol/l)) compared with controls (35 nmol/l (range 11–56 nmol/l); $p=0.014$).

There was an inverse relation between platelet serotonin and both plasma serotonin and plasma 5-hydroxyindoleacetic acid. This did not, however, reach significance ($p=0.14$ and $p=0.17$ respectively).

Discussion

We have shown that in the CREST variant of systemic sclerosis platelet serotonin is significantly reduced compared with normal controls, patients with primary Raynaud's phenomenon, and patients with diffuse systemic sclerosis. The possible mechanisms involved include a diminished absorption of serotonin precursor, decreased platelet uptake of serotonin, or increased platelet release. The normal plasma concentration of 5-hydroxytryptophan in the patients with CREST suggests that absorption of serotonin precursors is normal.

It seems unlikely that serotonin uptake and storage are abnormal. Treatment with ketanserin, an S_2 receptor antagonist, normalises the platelet serotonin concentration. This effect was present after two weeks of ketanserin treatment and was still present after 12 months' treatment.

Our data can be explained on the basis of widespread platelet activation, aggregation, and serotonin release in patients with CREST. The precise pathogenetic role of platelets in systemic

sclerosis is not known. Platelet activation is clearly recorded.⁹⁻¹¹ It has also been shown that there are extensive vascular abnormalities.¹⁴ The initial changes affect the endothelium, with a variety of mechanisms being involved in the endothelial cell injury.¹⁵ The resultant exposure of the subendothelial collagen is a most potent trigger of platelet activation. Thus the platelet involvement may be secondary to endothelial damage.

Possibly this platelet activation plays an important contributory part in the development of the microvascular disease of systemic sclerosis. It is known that in the microcirculation, where the flow is slow and non-pulsatile, high concentrations of serotonin (10^{-7} mol/l) occur at sites of platelet activation.¹⁶ Serotonin has diverse effects on blood vessels. The end result is increased vessel permeability, oedema, microcirculatory impairment, and tissue underperfusion. These features are commonly seen in patients with CREST. A weak inverse relation was found between platelet serotonin and both plasma serotonin and plasma 5-hydroxyindoleacetic acid. These observations suggest that serotonin concentrations in the microcirculation are indeed high and derived from platelets. It is also clear that the patients with CREST studied do not represent a strictly homogeneous group of patients with identical disease severity. It is, therefore, possible that there are variable degrees of endothelial cell damage, with variable peripheral serotonin clearance. This is in part an endothelial function.^{17,18} Furthermore, serotonin and platelet derived growth factors may contribute to the vascular and dermal fibrosis.^{19,20} Platelet serotonin concentrations were not reduced in those patients with diffuse disease. It is possible that there is a fundamental clinical difference between the two disorders. Raynaud's phenomenon and digital ischaemia are much more prominent in the CREST syndrome. More severe peripheral microcirculatory disease in the CREST syndrome may cause more extensive platelet activation and the subsequent release of serotonin.

Our data suggest that ketanserin can antagonise platelet aggregation and, therefore, serotonin release. Interestingly two clinical studies have shown that ketanserin is of benefit in the treatment of Raynaud's phenomenon and digital ulceration in systemic sclerosis.^{6,7}

This study provides further evidence of platelet activation in systemic sclerosis. Increased local concentrations of serotonin may contribute to the microcirculatory impairment, which is a major feature of the CREST syndrome. Our observations suggest that specific S_2 serotonin antagonists may be useful in the treatment of digital ischaemia seen in patients with CREST. Significant platelet serotonin

release does not appear to occur in patients with diffuse systemic sclerosis or with primary Raynaud's phenomenon.

We thank Mrs T Pearce for typing this manuscript. We are also indebted to Dr R Baker for providing statistical assistance.

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