RESONANCE RAMAN SPECTROMETRIC STUDY OF HUMAN BLOOD PLATELETS

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1. Introduction

Spectrometric methods have been introduced to monitor structural features and detect changes which accompany biological functions. Among them vibrational spectrometry offers high promise [1] since vibrational frequencies, available from Raman and infrared spectra, are sensitive to geometric and bonding arrangements of localised groups of atoms in a molecule [2,3].

As a part of our spectrometric investigations on intact platelet and isolated platelet membranes [4] we report here Raman spectrometry data of human blood platelets. Results indicate the presence of carotenoid(s) in normal human platelets.

2. Materials and methods

2.1. *Platelet isolation*

Human platelets were isolated at 15°C from acid–citrate–dextrose (NIH formula A) normal whole blood and washed free of plasma by differential centrifugation procedure [5].

2.2. *Protease treatment of washed platelets*

Washed platelets (1.5–2.5 mg protein/ml) were treated for 20 min at 37°C with 250 μg α-chymotrypsin (Worthington) in 500 μl 0.05 M Tris–HCl (pH 7.4) buffer containing 0.145 M NaCl (TS). After digestion, the mixture was cooled at 0°C and platelets were sedimented at 4°C at 3000 × *g* for 20 min in a Sorvall RC2B centrifuge. The chymotrypsin-treated platelets were twice washed with the above buffer by centrifugation and finally resuspended in TS buffer pH 7.4 for Raman analysis.

2.3. *Platelet lipid extraction*

Washed platelets were treated with 2 vol. chloroform:methanol, 2:1 (v/v) at 20°C for 60 min with stirring. The mixture was centrifuged for 15 min at 3500 × *g*; the delipidated platelets and the combined organic phases including 2 washes were taken to dryness under reduced pressure and stored at −20°C until use.

Protein was measured by the Lowry method [6] with bovine serum albumin as standard. Other methods are described in the figure legends. Absorption spectra of platelets were recorded with a Cary spectrophotometer.

2.4. *Raman spectrometry*

The sample (1.5 cm^3) was introduced into a continuous flow system with a syringe. A peristaltic pump forced it to circulate through the silicon rubber tube of the pump, Raman cell (a pyrex tube, i.d. 1 mm) and the teflon tubing. A large part of this tubing, in the vicinity of the Raman cell, was immersed in a constant temperature bath. A thermocouple placed inside the Raman cell, near the laser beam, was used to measure temperature which was maintained at about 10°C.

Spectra were recorded by a Coderg PH 1 spectrometer. An argon laser (Spectra Physics Model 164 AC) with a 600 mW power, tuned at 488 nm and 514.5 nm was used. Spectral slits were between 2 cm^{-1} and 8 cm^{-1}. Peak frequencies were considered to be accurate to ± 2 cm^{-1}.

3. Results

3.1. *Intact washed platelets*

Raman spectra of human blood intact washed platelets were recorded in the frequency range of 1000–1600 cm^{-1} using a laser beam with 514.5 nm (fig.1I). They exhibit only three prominent peaks near 1010, 1160 and 1530 cm^{-1}. Their intensity depends on the frequency of the incident light showing that resonance enhancement of Raman bands comes into play when samples were illuminated by a 488 nm laser beam (fig.1II). Indeed the absorption spectrum of the extract of normal human blood platelets in chloroform–methanol mixture possesses three maxima at 475, 450 and 435 nm. These three bands are highly characteristic of the vibrational structure of the carotenoid $^1B_u \leftarrow {}^1A_g$ transition [7]. Moreover the three intense Raman peaks (fig.1I,II) might be assigned to the vibrations of conjugated double-bond carotenoid systems [8–10]: the 1160 cm^{-1} and 1530 cm^{-1} correspond to ν(=C–C=) and ν(–C=C–) vibrations while the less intense peak at 1010 cm^{-1} corresponds to the stretching of the C–CH_3 bond perpendicular to the molecular axis [9]. Because of their similar electronic and Raman spectra [9,10] we did not discriminate more specifically between β-carotene, lutein and lycopene, all of them present in human plasma [12].

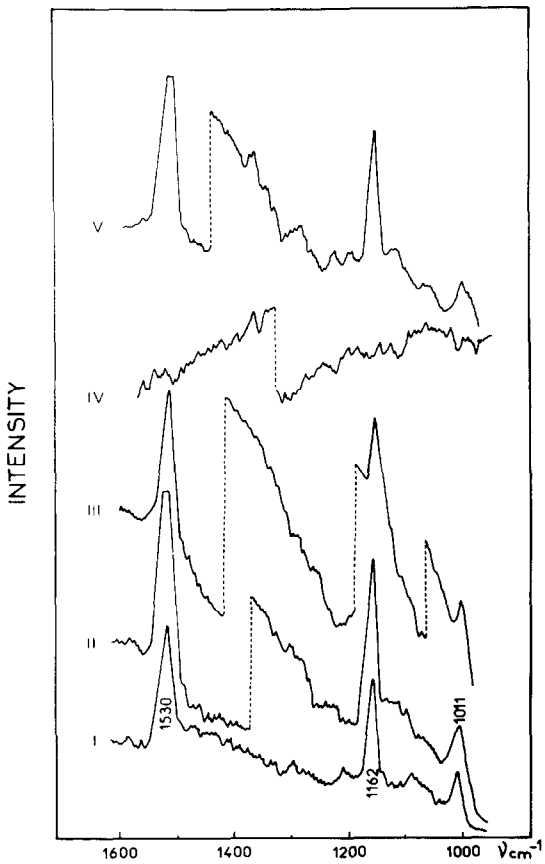

Fig.1. Resonance Raman spectra of isolated human blood washed platelets in 10 mM Tris–HCl–145 mM NaCl (pH 7.4) $T \sim 10°C$: (I, II) intact washed platelets with excitation wavelengths of 514.5 nm and 488 nm, respectively; (III) chymotrypsin-treated platelets; (IV) chloroform–methanol-treated platelets; (V) lipid extract. Dotted vertical lines indicate change in the background.

3.2. *Chymotrypsin-treated platelets*

We used chymotrypsin as an agent to study spectra of washed platelets without peripheral proteins because its large molecule normally does not penetrate biological membranes and does not induce platelet release reactions [13]. Figure 1III shows that platelet treatment with chymotrypsin did not cause significant change of the resonance Raman (RR) spectra.

Aliquots of chymotrypsin-treated platelets were electrophoresed on polyacrylamide gel microdisc. The separated protein and glycoprotein were visualized as in [14]. No selective differences were noted between normal and chymotrypsin-treated platelet protein patterns (fig.2a,b). As can be seen (fig.2c,d) the protease-digested platelets present a decreased amount of the major platelet surface glycoproteins [14].

3.3. *Chloroform–methanol-treated platelets*

When platelets were treated with a chloroform–methanol mixture the characteristic peaks disappear completely (fig.1IV). Examination of the concentrated lipid extract reveals the presence of the three RR peaks (fig.1V).

4. Discussion

In the course of our studies on the molecular characteristics of blood platelet and isolated platelet membrane constituents able to participate in the platelet specific function [15] we observed that RR

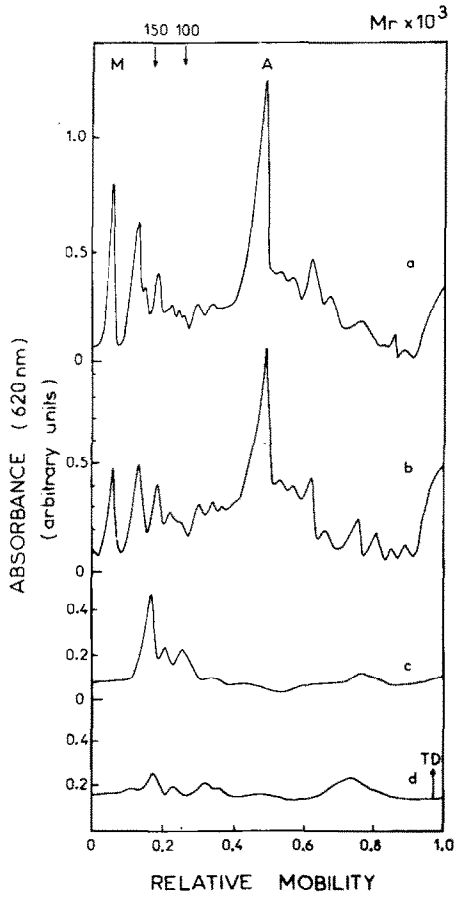

Fig.2. SDS–polyacrylamide gel electrophoresis patterns of human blood washed platelets: (a, b) Coomassie brillant blue stain; (c, d) periodic acid Schift (pAS) stain. Electrophoresis was performed in Tris–glycine buffer (pH 8.6) plus 0.5% acrylamide, 0.2% *N,N'*-methylene bis acrylamide, 0.5% SDS and Tris–glycine buffer were used for the preparation of gels. Sample solubilisation, relative mobilities determination and molecular weight estimation were described [14]. (M) myosin area; (A) actin area; (TD) tracking dye.

spectra of all normal human platelet preparations presented intense peaks near 1160 cm^{-1} and 1530 cm^{-1}. The analysis of this zone allowed us to identify the spectral characteristics of platelet carotenoid(s).

Carotenoid pigments are rather sparingly mentioned among the platelet constituents: their intracellular presence in human platelets has been indicated [16,17]. Although higher amounts of carotenoids were estimated in platelets than in plasma [16], the possibility that platelets might have internalized the plasmatic carotenoid pigments cannot be excluded.

Whatever its origin might be, enough strong bonds seem to exist between the hydrophobic molecule of carotenoid(s) and some unidentified platelet constituent, to enable its detection even when washed platelets were used for the RR spectra.

In order to get some insight on the carotenoid(s) location in platelets, chymotrypsin-digested platelets were studied. This enzyme is considered to be a good cell surface modifier agent which, presumably, is neither able to traverse the cell membrane barrier nor to induce platelet secretory activity [13]. The RR spectra of chymotrypsin-treated platelets are similar to those of untreated platelets (fig.1II,III). Thus it appears that no binding of carotenoid(s) to chymotrypsin-sensitive platelet surface-exposed constituents could be detected. On the contrary, the altered RR spectra of chloroform–methanol-treated platelets show that the carotenoid characteristic peaks completely disappeared (fig.1IV). These peaks reappeared at the same frequencies as in RR spectra of intact platelets when the lipid extract was analyzed (fig.1V). Thus the removal of almost all the platelet lipids and some lipid–protein complexes is accompanied by the loss of carotenoid(s) due to its extraction in the organic phase of the platelet–solvent mixture. Altered profiles of delipidated platelets electrophoresed on microdisc acrylamide gels were obtained (not shown); however, we did not succeed to pursue the possible selective extraction of the (glyco)proteins in the aqueous phase with chloroform–methanol mixture; these (glyco)-proteins are accessible at the outer platelet surface and span the plasma membrane [18]. The possibility thus exists that the carotenoid(s) might be associated or bound with/to the integral proteins of the plasma membrane.

Erythrocyte membrane, sarcoplasmic reticulum membrane as well as frog sciatic nerve also exhibit carotenoid Raman patterns [19–21]. Neither the structure nor the role of carotenoid binding in these membranes is yet known.

The interest in the study of platelet carotenoid(s) comes from its possible involvement in the platelet function and particularly of β-carotene as a potential precursor of retinol–vitamin A_1. It has been shown that vitamin A-deficient rats have an increased platelet adhesiveness [22]. A possible role of vitamin A in

platelet–collagen adhesion involving glycosyl transferases has been suggested [23] where the glycose transfer to the collagen–sugar acceptor is preceded by the formation of a complex glycolipid involving retinol.

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