ULTRASONIC EXPOSURE MODIFIES PLATELET MORPHOLOGY AND FUNCTION *IN VITRO*

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Abstract—Samples of human platelet rich plasma (PRP) exposed to 1 MHz ultrasonic irradiation contained more cellular debris than their controls, indicating that a small population of the cells had been disrupted, possibly by some form of cavitation-like activity. The surviving cells appeared undamaged under the electron microscope and functioned as well as their controls in forming a platelet thrombus when tested immediately after the ultrasonic exposure but not after a 30 min incubation at 22°C. Parallel studies showed that incubation of control platelets with substances released from damaged or disrupted platelets could mimic the changes seen in incubated samples which had previously been exposed to ultrasound.

Key words: Platelets, Ultrasound, Coagulation, Recalcification, Shear stress, Biological effects.

INTRODUCTION

It has been shown that when fresh human platelet rich plasma (PRP) is exposed to ultrasound, a time-dependent traumatic insult to the platelet population results (Williams *et al.*, 1976). Immediately following exposure to ultrasound no changes were detected in the recalcification process as assayed by the recalcification time (i.e., the time taken to "clot" following the restoration of calcium ions). Incubation of the exposed samples at room temperature, however, resulted in a time-dependent decrease in the recalcification time to an asymptotic value. This value was invariably less than that of its control and was attained within approx. 30 min. The decrease was interpreted as an increased availability or expression of platelet procoagulant PF-3 activity which under certain circumstances could result in an accelerated rate of blood coagulation. It was postulated that hydrodynamic shear stresses were generated during the ultrasonic exposure, and that these stresses initiated a sequence of events culminating in the platelet release reaction and an increased availability of PF-3.

The effects of hydrodynamic shear stresses on the morphology and function of fresh human platelets have been extensively investigated by Brown *et al.* (1975). They showed that platelets exposed for 5 min in a couette cylinder apparatus to shear stresses between 50 and 100 dyn cm^{-2} caused damage to the platelet plasma membrane and the release of cytoplasmic materials not contained within the secretory granules while platelets exposed to shear stresses greater than about 250 dyn cm^{-2} underwent fragmentation. Simultaneously, the sheared platelets underwent changes in functional properties: platelets exposed to shear stresses greater than 100 dyn cm^{-2} lost their ability to aggregate in response to added ADP or collagen. Similarly, it was demonstrated (Glover *et al.*, 1974, 1975) that a shear stress between 100 and 150 dyn cm^{-2} caused acceleration of the rate at which a clot attained a given strength, but, on the other hand, its

maximum strength was less than the clot which resulted from unsheared platelets. It was suggested that shear stress accelerates the rate of clot initiation or its early growth and development and that the final clot had a reduced number of cross links between adjacent fibrin strands, thereby inhibiting the phenomenon of clot retraction.

This extreme shear sensitivity of human platelets renders them liable to damage by any cavitation-induced forces. Williams (1974) showed that human platelets exposed to the second order (d.c.) acoustic microstreaming field, generated around a transversely oscillating wire, released serotonin after each cell had been adjacent to the wire surface for approximately one millisecond at an estimated shear stress of only 150 dyn cm^{-2}.

This paper examines the effect of ultrasonic irradiation on the morphology and function of fresh human platelets following clot formation. Studies were also performed with platelet suspensions subjected to hydrodynamic shear stresses in a cone and plate viscometer to test the hypothesis that the changes observed following ultrasonic exposure could have been initiated by cavitation-induced hydrodynamic trauma.

MATERIALS AND EXPERIMENTAL METHODS

Blood was withdrawn from an antecubital vein of healthy adult volunteers in 20 ml aliquots by an experienced technician into a disposable plastic syringe fitted with a 20 gauge needle and anticoagulated with 0.4 ml of 20% trisodium citrate (this essentially removes the calcium ions). The blood was transferred to polycarbonate centrifuge tubes within 10 min and spun at 1,000 g at room temperature for 5 min to sediment the erythrocytes. Approximately 7–9 ml of PRP was removed with a siliconized Pasteur pipette and placed in another polycarbonate tube and kept at room temperature. This tube was then gently inverted for about 10 sec at 5 min intervals to prevent the platelets from settling.

The equipment and dosimetric details of the ultrasonic exposure system have been described by O'Brien *et al.* (1974) and the exact exposure procedure relative to this work has been described by Williams *et al.* (1976). Briefly, 1.5 ml samples of PRP were placed in a siliconized,

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cylindrical (1 cm dia. × 1.7 cm long) glass vessel with acoustically transparent windows (Saran Wrap$^{(R)}$) each end. The vessel was immersed in a tank of isotonic saline, maintained at $30 \pm 0.2°C$, and positioned on axis at a distance of 24 cm from the 1 MHz ultrasonic transducer. The two ultraonsic free field intensities, 0.2 and 0.6 W cm^{-2} (accuracy $\pm 30\%$), were determined by spatially averaging over the irradiation vessel's window but without the vessel in place. The peak intensity was approximately 10% higher. The ultrasonic measurements utilized two primary techniques, the bouyant float, for total power and the suspended ball radiometer for spatial intensity, along with beam plots at the site where the vessel is positioned.

Following a 5 min ultrasonic exposure, the sample was divided into two aliquots. One was immediately recalcified, as described below, and the second aliquot was placed in a polycarbonate tube to incubate at room temperature (occasionally inverted to prevent settling) for 30 min before being recalcified.

Hydrodynamic shear stresses were produced in a Ferranti–Shirley cone and plate viscometer fitted with a 7 cm dia. steel cone ground to an angle of 0.0059773 radians. A 0.7 ml sample of PRP, maintained at $25 \pm 0.3°C$, was exposed to a uniform velocity gradient of $1.714 \times 10^4 \text{ sec}^{-1}$ for 5 min at a cone speed of 1,000 rpm (this subjected the PRP sample to a uniform shear stress of $215 \pm 5 \text{ dyn cm}^{-2}$). The sheared PRP sample was removed with a clean disposable plastic syringe. A sample of this sheared PRP was either (1) recalcified immediately, (2) incubated for 30 min at room temperature before recalcification, (3) diluted with an equal volume of control PRP for immediate recalcification, or (4) diluted and incubated.

The recalcify, 0.2 ml of 0.0375 M calcium chloride was placed in a prewarmed polyethylene vessel, positioned on

a constant temperature heating block, and allowed to warm up to 37°C. At time zero, 0.4 ml of PRP was added to the vessel, the sample mixed for 10 sec and then left undisturbed for 15 min at 37°C. The clot was tipped out onto a white tile, cut into 1 mm cubes, immersed in fixative (2% gluteraldehyde in 0.1 M Sorenson's phosphate buffer pH 7.4, plus 0.1 M Sucrose) at 4°C. The clots were dehydrated in acetone and embedded in Epon$^{(R)}$. Thin sections were cut and stained with uranyl acetate and lead citrate and examined in a Siemens Model 1A electron microscope. The initial observations of the electron micrographs were done without the observer having knowledge whether the sample had been irradiated or not.

RESULTS AND DISCUSSION

PRP samples which were exposed to ultrasound and recalcified immediately (Cases 3 and 5 of Table 1) appeared to become liquid again within 15 min. When these clotted samples were removed from the polyethylene reaction vessels, this apparent re-liquification was found to have been caused by a contraction of the platelet/fibrin clot and the consequent expulsion of clear yellow serum. This was also true for the controls (Case 1). Both the control sample and PRP samples exposed to ultrasound gave small dense jelly-like clots which could be cut with relative ease using a razor blade. The volume of clear yellow serum expelled during clot contraction was typically about 0.3 ml.

PRP samples exposed to ultrasound and incubated (Cases 4 and 6) before recalcification were morphologically and functionally different from their control (Case 2). Visual examination of these incubated samples before recalcification showed evidence of the spontaneous formation of weak floccules, i.e. larger unstable accretions of platelets with a low density of cell packing. These floc-

Table 1. Summary of experimental observations

Case No.	Fig. No.	Treatment	Ultrasonic intensity (W cm^{-2})	Dilution with control PRP	Incubation at 22°C for 30 min	Flocculation during incubation	Approx. volume of expelled serum (ml)	Thrombosthenin contractile system	Clot description	Electron micrographic description
1		C	---	---	No	---	0.3	Normal	Dense jelly-like	Normal
2		C	---	---	Yes	No	0.3	Normal	Dense jelly-like	Normal
3		U/S	0.2	---	No	---	0.3	Normal	Dense jelly-like	Appeared normal Slight cellular debris
4	2	U/S	0.2	---	Yes	Yes (+)	0.2	Abnormal	Larger and less rigid than control Retraction reduced	More aggregates Fewer platelets per aggregate
5	1	U/S	0.6	---	No	---	0.3	Normal	Dense jelly-like	Appeared normal Slight cellular debris
6		U/S	0.6	---	Yes	Yes (++)	0.1	Abnormal	Larger and less rigid than control Retraction reduced	More aggregates Fewer platelets per aggregate
7		HS	---	No	No	---	0	Abnormal	No retraction Mechanical integrity low	Platelets suffered irreversible damage Platelets swollen (up to 5 μm dia.)
8		HS	---	No	Yes	Yes (+++)	0	Abnormal	No retraction	Platelets suffered irreversible damage Platelets swollen (up to 5 μm dia.)
9	3	HS	---	Yes	No	---	0.2	Normal	Dense jelly-like	Mixture of normal and damaged platelets
10	4	HS	---	Yes	Yes	Yes (+++)	0	Abnormal	No retraction	Many small loose aggregates of vacuolated platelets

C = control, U/S = ultrasonic exposure, HS = exposure to hydrodynamic shear, +'s = degree of flocculation.

cules were easily dispersed by agitation or stirring, and the rate and extent of flocculant formation appeared to increase with both the time of incubation at room temperature and the ultrasonic intensity to which the PRP had been exposed. Following the addition of calcium ions, these incubated samples formed a solid clot more rapidly than their controls which is in agreement with the recalcification time measurements of Williams et al. (1976). However, these clots did not appear to become liquid again, and when examined visually were larger and less rigid than control clots. A form of dose–effect relationship seemed to exist in that incubated PRP samples exposed at 0.2 and 0.6 W cm^{-2} for 5 min expelled about 0.2 and 0.1 ml of serum, respectively, after 15 min in the presence of calcium. The design of the experimental system, the uncertain "end points" and the "loose" consistency of the formed clots prevented better quantification of these observations.

Figure 1 shows a thin section through a representative platelet aggregate from a typical platelet–fibrin clot in a sample of PRP exposed to ultrasound at an intensity of 0.6 W cm^{-2} and recalcified immediately (Case 5). This aggregate is almost indistinguishable from similar aggregates within control samples (Cases 1 and 2) and consists of a number of platelets in close opposition, tightly bound together within a meshwork of fibrin.

Both ultrasonically exposed samples which were immediately recalcified (Cases 3 and 5) appeared to contain slightly more cellular debris (labelled D) than the control (Case 1). This suggests that one or more forms of cavitation-like activity had occurred which had disrupted those platelets in the immediate vicinity of each of the cavitation events. The proportion of the cell population disrupted in this manner was probably less than about 3% (as estimated from the electron micrographs) and this small quantity of debris had a negligible effect on the recalcification time of PRP when measured immediately following exposure to ultrasound (Williams et al., 1976).

Figure 2 shows a representative platelet aggregate from a PRP sample exposed to ultrasound at 0.2 W cm^{-2} and incubated before recalcification (Case 4). It differs from the sample shown in Fig. 1 in that more aggregates were

Fig. 1. A representative electron micrograph of a platelet thrombus formed following the recalcification of PRP immediately after it had been exposed to ultrasound at an intensity of 0.6 W cm^{-2}. With the exception of the peripheral cell debris (D), this sample is indistinguishable from its unexposed controls. 22,700×.

Fig. 2. A representative platelet thrombus from a PRP sample exposed to ultrasound at an intensity of 0.2 W cm^{-2} but incubated at 22°C for 30 min in a polycarbonate tube before recalcification. In addition to the expected cell debris (D), this sample differed from its incubated controls and the non-incubated sample shown in Fig. 1 in that each platelet thrombus contains fewer cells, is less tightly packed, and contains a higher proportion of multivacuolated cells (V). 22,800×.

present and that each individual aggregate contained few platelets. In general, each aggregate was well permeated with fibrin and was characterized by a lower density of packing of constituent platelets, i.e. corresponding to a "looser weave" in the fibrin/platelet meshwork. In addition, each "loose" clot appeared to contain a higher proportion of multivacuolated cells.

PRP samples exposed to hydrodynamic shear stress and recalcified immediately (Case 7) formed a solid clot within a minute and showed no visual evidence of clot retraction. This resembles the accelerated rate of clot formation noted by Glover *et al.* (1974, 1975) in recalcified PRP which had previously undergone platelet damage and the induction of the release reaction following exposure to hydrodynamic shear stress. They observed that the clot exhibited an impaired ability to retract. When the clot was removed from the polyethylene reaction vessel, no serum was exuded until the clot was compressed with a razor blade while trying to cut it. The mechanical integrity of

these clots was low. Electron microscopic examination showed that the surviving platelets had suffered what appeared to be irreversible damage in that portions of the cell membrane were broken and the cells had swollen to gigantic proportions (up to 5 times the normal dia). Electron micrographs showed numerous damaged cell remnants such as those shown in Figs. 3 and 4. This cell swelling following partial membrane rupture has also been observed in canine platelets exposed for a short time to hydrodynamic shear stresses of approx. 3000 dyn cm^{-2} for at least 5 passes of 5×10^{-5} sec near a transversely oscillating wire (Williams, unpublished observations). This effect appears to be similar to the cell swelling seen in Ehrlich ascites cells when a region of the plasma membrane was ruptured and the intracellular organelles were exposed to the extracellular environment while still being strongly bound together with cytoplasmic material (Williams, 1972).

Of special interest in comparing Figs. 1 and 2 were the

Fig. 3. A typical platelet thrombus formed by the immediate recalcification of a 50:50 mixture of control PRP and PRP which has suffered membrane damage during exposure to hydrodynamic shear stress. With the exception of the obviously membrane damaged cells (R), which are found at the periphery of the aggregates, these platelet thrombi are virtually indistinguishable from their controls. 13,200×.

fragments of cellular debris (D) seen in association with many platelet aggregates. The quantity of cellular debris seen in irradiated samples exceeded that of their controls and is indicative of some form of traumatic exposure to a small proportion of the platelet population. Most of the platelet population immediately after their removal from the ultrasonic exposure system looked undamaged under the electron microscope and appeared to be functionally indistinguishable from their controls.

Aggregating agents such as ADP were undoubtedly released by that small proportion of the platelet population which were disrupted or otherwise damaged by the large hydrodynamic shear forces adjacent to each of the postulated cavitation events. It is probable that, during the incubation of ultrasonically exposed PRP samples, these aggregating agents initiated the self perpetuating cycle of platelet aggregation followed by the release of more ADP which in turn induced more aggregation. Alternatively, the slow induction of platelet aggregation may have been initiated by the release of enzymes (such as neuraminidase) from those platelets damaged during the ultrasonic exposure. These enzymes would remove the surface electrostatic charges which provide the mutually repulsive force between the platelets and result in aggregation and perhaps the subsequent triggering of the release reaction (Massini and Luscher, 1971).

These time-dependent morphological and functional changes seen in platelets exposed to ultrasound (Figs. 1 and 2) appear to provide a plausible explanation for the time-dependent changes in recalcification time measurements reported by Williams et al. (1976), since aggregation and the induction of the release reaction are intimately associated with the initiation of blood coagulation. The hypotheses proposed to explain these changes are based on the assumption that "agents" released from damaged or disrupted platelets are capable of inducing morphological and functional changes of a similar nature in normal undamaged platelets within the same time scale. This
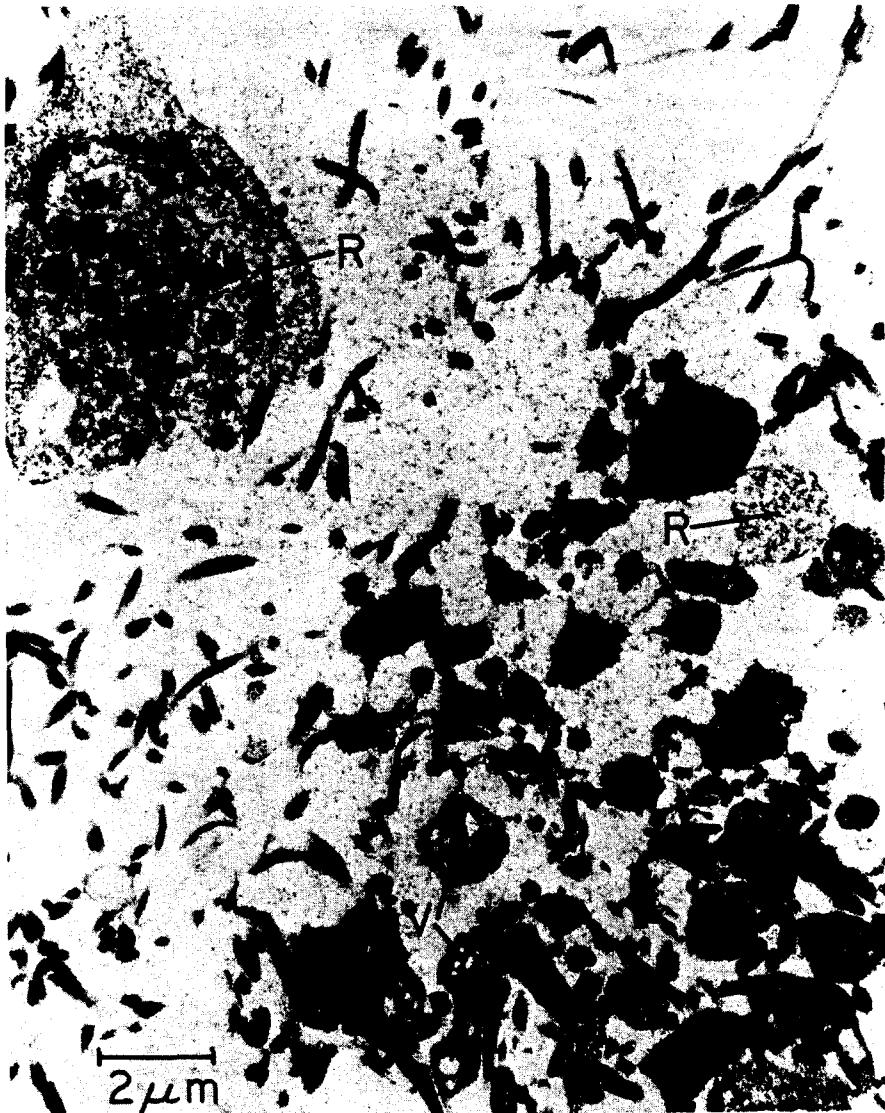
Fig. 4. A representative platelet thrombus formed by recalcification of the same 50:50 mixture of control and damaged PRP described in Fig. 3, but after it had been incubated for 30 min at 22°C in a polycarbonate tube in the absence of calcium ions. Damaged cell remnants (R) are present at the periphery of each small aggregate, which typically exhibits a more extreme version of the differences already noted between Figs. 1 and 2, i.e. each aggregate is composed of fewer cells which have little or no cell contact but contain a higher proportion of multivacuolated cells (V). 13,200×.

assumption was tested by adding aliquots of platelet suspensions which had been damaged by various means to suspensions of control PRP and noting the nature, extent and time course of any observed morphological and functional changes.

Figure 3 presents a representative platelet aggregate from the clot obtained by the immediate recalcification of a 50:50 mixture of control PRP and PRP exposed to hydrodynamic shear stress (Case 9). This clot formed rapidly and contracted leaving about 0.2 ml of clear serum. The aggregate in Fig. 3 is virtually indistinguishable from its controls in that it consists of a large, well adhered mass of apparently normal platelets (except for the presence of damaged cells and giant cell remnants (labelled R) at the periphery of the aggregate). This shows that the materials released by damaged platelets have no detectable immediate effects upon the morphology or functioning of undamaged platelets.

Figure 4 presents a representative portion of the clot obtained by the recalcification of the 50:50 mixture of control and sheared PRP, but after it had been incubated (Case 10). This sample also clotted rapidly, but showed no evidence of clot retraction indicating that the thrombosthenin contractile system had been seriously impaired. Comparison of Fig. 3 and 4 shows more extreme versions of the same sort of changes already noted between Figs. 1 and 2, i.e. many more but smaller "loose" aggregates of platelets having little cell contact but containing a higher proportion of vacuolated cells. Before recalcification, this incubated sample had spontaneously formed weak, flocculent aggregates, while control PRP samples incubated under identical conditions remained as discrete cells and, following recalcification, the incubated control formed large dense aggregates which contracted strongly.

Thus, "agents" released by damaged or disrupted platelets had no detectable immediate effects on the morphology or functioning of control platelets; however, during incubation these materials caused the spontaneous

formation of unstable flocculent accretions of platelets, inhibited the platelet thrombosthenin contractile system, and mimicked all the morphological changes seen in platelet thrombi from PRP which had been exposed to ultrasonic irradiation before being incubated at room temperature. It is therfore highly probable that the time-dependent changes in recalcification time measurements observed following exposure of PRP to ultrasonic irradiation (Williams et al., 1975) were due to the time-dependent action of materials released by that small fraction of the platelet population which was adjacent to each cavitation event.

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