Platelet-Aggregating Activity of Type I and Type III Collagens from Human Aorta and Chicken Skin

By MICHAEL J. BARNES,* JOHN L. GORDON† and D. EUAN MACINTYRE

Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, U.K.

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Human or chicken type III collagen dissolved in 0.1 M-acetic acid was much more potent than type I collagen at inducing platelet aggregation. After incubation in 0.38 M- Na_2HPO_4 to promote fibrillogenesis, the platelet-aggregating activity of both collagen types increased, and type I was then virtually equiactive with type III. Preincubation in cell-free plasma increased the activity of chicken but not that of human collagen. The platelet-aggregating activity of type III collagen did not appear to depend on the integrity of the intrachain disulphide bonds.

The interaction of blood platelets with vascular collagen results in the secretion of platelet constituents and the formation of platelet aggregates; these reactions are vitally important in haemostasis and thrombosis, and also possibly in atherosclerosis and inflammation. Investigations into the nature of platelet-collagen interaction have shown that collagen must be in fibrillar form to stimulate platelets (Muggli & Baumgartner, 1973; Brass & Bensusan, 1974; Gordon & Dingle, 1974; Jaffe & Deykin, 1974), and the discovery of different types of collagen in the vascular wall (Chung & Miller, 1974; Trelstad, 1974) has led to suggestions that type III collagen may be of prime importance in initiating platelet aggregation (Balleisen *et al.*, 1975; Hugues *et al.*, 1976).

The results of the present study indicate that type III collagen dissolved in 0.1 M-acetic acid can (unlike type I collagen) induce platelet aggregation in platelet-rich plasma at concentrations around 5 $\mu\text{g}/\text{ml}$ and above, after a delay consistent with fibrillogenesis *in situ*. If the collagens are preincubated under conditions that promote fibrillogenesis, however, aggregating activity increases, and type I collagen is almost as active as type III. Human plasma apparently inhibits fibrillogenesis of human collagen.

Materials and Methods

Preparation of collagen

Type I and type III collagens were prepared from human aorta and chicken skin by procedures based on those of Chung & Miller (1974) and Epstein (1974).

* Member of External Scientific Staff of the Medical Research Council.

† Present address: Cell Biology Group, A.R.C. Institute of Animal Physiology, Babraham, Cambridge CB2 4AT, U.K.

Aortas were collected from necropsy cases aged 40-70 years. In the studies described here, collagen was prepared only from the intimal portion, but similar results were obtained when whole aorta was used. Nine intimal samples were pooled, and the tissue was homogenized ($4 \times 15\text{s}$) at 4°C in 5 vol. of 1 M-NaCl in Tris/HCl buffer (0.05 M; pH 7.1 at 23°C) by using an Ato-Mix blender. Skin from six chickens (Rhode Island $\times$ Light Sussex cross, about 2 months old), previously plucked free of feathers, was scraped to remove underlying fat and muscle, macerated in a Spong mincer, and homogenized as described above.

Homogenates were diluted with 10 vol. of 1 M-NaCl in 0.05 M-Tris/HCl buffer, pH 7.1, and the tissue was extracted by stirring the mixture slowly for 3 days at 4°C . After centrifugation ($25000g_{av.}$; 30 min; 4°C) the residue was similarly extracted with 0.5 M-acetic acid. It was then suspended in 20 vol. of 0.5 M-acetic acid containing 1 mg of pepsin (twice-crystallized, from Worthington Biochemical Corp., Freehold, NJ, U.S.A.)/ml and incubated at 4°C for 18 h. The incubation mixture was then centrifuged ($80000g_{av.}$; 1 h; 4°C) and the soluble collagen was precipitated from the supernatant by addition of solid NaCl to 0.9 M. After centrifugation (as above), the collagen was redissolved in 0.5 M-acetic acid, and dialysed ($2 \times 4\text{h}$; $1 \times 16\text{h}$) successively against 0.15 M-acetic acid, distilled water, 0.56 M-NaCl and 1 M-NaCl in 0.05 M-Tris/HCl buffer (pH 7.1 at 23°C). Insoluble material was removed by centrifugation (as above), and the supernatant dialysed (as above) against 1.71 M-NaCl in 0.05 M-Tris/HCl buffer (pH 7.1 at 23°C) to precipitate type III collagen. The precipitate was collected by centrifugation (as above), and the supernatant (after dialysis as above against 2.14 M-NaCl in 0.05 M-Tris/HCl buffer, pH 7.1, and removal of insoluble material) was dialysed against 2.56 M-NaCl in 0.05 M-Tris/HCl buffer, pH 7.1, to precipitate type I collagen. Type III and type I precipitates

were redissolved in 0.5 M-acetic acid and the solutions subjected to the same dialysis sequence as above. Material from the initial 1.71 M-NaCl-precipitated fraction, reprecipitated at 1.71 M-NaCl, and material from the original 2.56 M-NaCl-precipitated fraction, reprecipitated at 2.56 M-NaCl, represented respectively the type III and type I collagen preparations used in this study. Examination of such preparations by CM-cellulose chromatography under denaturing conditions (Barnes *et al.*, 1976) indicated that, in accord with published data (Chung & Miller, 1974; Epstein, 1974), preparations designated type III collagen contained a small amount of type I (estimated at 5–10%), and preparations designated type I collagen contained no detectable type III collagen.

Collagen preparations were dissolved in 0.1 M-acetic acid at approx. 400 µg/ml and centrifuged (80000 g_{av} ; 1 h; 4°C) to remove fibrillar material. The concentration of collagen in these solutions was obtained from the hydroxyproline content (Bergman & Loxley, 1963) assuming the following proportions of hydroxyproline: human type I 14%, type III 18% (Epstein *et al.*, 1971; Chung *et al.*, 1974); chicken type I 15%, type III 19% (Kang *et al.*, 1969a,b; Herrmann & von der Mark, 1975).

Treatment of collagen with mercaptoethanol

A solution of chicken type III collagen in 0.1 M-acetic acid was dialysed (2 × 4 h; 1 × 16 h) at 4°C initially against water (to decrease the acidity), then against 0.56 M-NaCl in 0.05 M-Tris/HCl buffer, pH 7.6 at 23°C. After dialysis, β -mercaptoethanol (final concentration 10 mg/ml) was added for 6 h at 16°C and for 16 h at 4°C. Under these conditions the collagen triple helix remains intact. β -Mercaptoethanol was removed by dialysis (1 h; 4°C) against four changes of 0.56 M-NaCl in 0.05 M-Tris/HCl buffer, pH 7.6 at 23°C. The sample was then centrifuged as described above to remove any fibrils, and subsamples were taken for hydroxyproline determination. The remainder was tested for platelet-aggregating activity.

After treatment with β -mercaptoethanol and dialysis as described above, a separate sample was incubated at 45°C for 30 min with sodium dodecyl sulphate (20 mg/ml) to denature the collagen triple helix, and then dialysed (1 × 2 h) against sodium dodecyl sulphate (1 mg/ml of 0.1 M-sodium phosphate buffer) before chromatography on Bio-Gel A5M (Barnes *et al.*, 1976). This demonstrated that treatment with β -mercaptoethanol had ruptured the intrachain disulphide bonds.

Measurement of platelet aggregation

Platelet aggregation was measured photometrically in 0.1 ml volumes of human citrated platelet-rich

plasma (Born, 1962; Gordon & Drummond, 1974). Cell-free plasma was prepared by centrifuging platelet-rich plasma at 14700 g_{av} for 120 s. The ability of the collagen preparations to induce platelet aggregation was studied by adding various amounts of the solutions to platelet-rich plasma, either directly or after incubation of the solutions at 37°C for up to 120 min in an equal volume of cell-free plasma, iso-osmotic NaCl or 0.38 M-Na₂HPO₄.

Results and Discussion

The addition of chicken skin type I collagen in 0.1 M-acetic acid directly to stirred platelet-rich plasma induced no detectable platelet aggregation at concentrations up to 60 µg/ml, whereas type III collagen induced a dose-dependent aggregation response at concentrations of 5 µg/ml and above. The responses were characterized by a lag period, which was inversely related to the collagen concentration, followed by a transient decrease in light transmission (apparently associated with a change in platelet shape), then by a progressive increase in light transmission (associated with platelet-aggregate formation). Responses were measured as the maximum rate of increase in light transmission. After incubation with an equal volume of cell-free plasma for 30 min at 37°C, the aggregation response to type III collagen showed a shortened lag phase and an increased rate of aggregation, and type I collagen was at least as active as type III collagen. Fig. 1 shows dose-response curves for platelet aggregation induced by chicken collagen types I and III before and after incubation, and Fig. 2(a) shows representative aggregation responses. Aggregation responses to chicken skin collagen treated with β -mercaptoethanol were indistinguishable from those induced by untreated collagen. Incubation with 0.38 M-Na₂HPO₄ (final pH 7.2) had the same effect as incubation in cell-free plasma, whereas both type I and type III collagen were inactive after incubation in iso-osmotic NaCl (Fig. 2a).

These results are consistent with the concept that collagen must be in fibrillar form before it can stimulate platelets (Muggli & Baumgartner, 1973; Brass & Bensusan, 1974; Gordon & Dingle, 1974; Jaffe & Deykin, 1974), since optimal activity was generated by preincubating the collagen under conditions that promote fibrillogenesis (Gross & Kirk, 1958). Further, it appears that differences in platelet-aggregating activity between soluble collagens of different types could be largely due to variations in fibrillogenesis. Neither type I nor type III collagen could induce platelet aggregation after incubation with iso-osmotic NaCl at acid pH, and since collagen is known to form precipitates rather than fibrils under these conditions (Gross & Kirk, 1958), this suggests

Fig. 1. Platelet-aggregating activity of chicken skin collagen type I and type III

(a) Samples (0.1 ml) of human platelet-rich plasma were preincubated for 3 min at 37°C before the addition of chicken skin collagen type I (●) or type III (○) in 0.1 M-acetic acid. Platelet-aggregation responses were measured as the maximum rate of increase in light transmission (units/min), with the apparatus adjusted to give maximum transmission (100 units) through cell-free plasma. (b) Samples of chicken skin collagen type I (●) and type III (○) in 0.1 M-acetic acid were incubated for 30 min at 37°C with an equal volume of human cell-free plasma, then tested for platelet-aggregating activity. Points shown are mean values of triplicate determinations.

that collagen must not only be in polymeric form to initiate platelet aggregation, but that the polymers must have an ordered structure similar to that in the native collagen fibril.

The results obtained with human collagens were similar to those of the chicken in as much as addition of acid-soluble type III collagen induced platelet aggregation at concentrations around 5 µg/ml and above, whereas type I collagen was inactive at 60 µg/ml. Incubation of human collagens with an equal volume of 0.38 M-Na₂HPO₄ increased platelet-aggregating activity and made type I collagen almost as active as type III collagen, but the incubation period necessary was 120 min at 37°C (compared with only 30 min at 37°C for chicken collagens). Incubation in cell-free plasma was, however, ineffective; indeed, the aggregating potency of type III collagen decreased slightly. Like the chicken collagens, human type I and type III collagens were inactive after incubation in iso-osmotic NaCl at acid pH. Fig. 2(b) shows representative aggregation responses induced by human type I and type III collagens. The minimum concentrations required to induce platelet aggregation were 2–3 µg/ml and 0.5–1.5 µg/ml respectively for human type I and type III collagen after incubation in 0.38 M-Na₂HPO₄ for 120 min at 37°C.

These results with human aorta collagens are somewhat similar to those reported by Balleisen *et al.* (1975) with human and calf skin collagens, but differ in certain important respects. They found that the

minimal effective concentration for human type III collagen after incubation was 1–2 µg/ml, whereas that for type I collagen was 8–10 µg/ml. They, however, incubated collagen in cell-free plasma for only 15 min at 36°C, and it is possible that incubation in buffer under optimal conditions for fibrillogenesis could have lowered the minimal effective concentration for type I collagen to the value for type III collagen. Our results suggest that human plasma contains an inhibitor of fibrillogenesis which preferentially affects human collagens; Balleisen *et al.* (1975) found that pepsin-solubilized type I collagen from calf-skin increased up to 20-fold in platelet-aggregating activity after incubation in cell-free plasma, compared with only a threefold increase for human type I collagen. It is not clear why these workers found an increase in platelet-aggregating activity of human collagen after incubation in plasma, whereas we did not, but the effect of the putative fibrillogenesis inhibitor could presumably be overcome by increasing the collagen concentration, as Gross & Kirk (1958) showed for urea, and Balleisen *et al.* (1975) used concentrations of collagen up to 4 mg/ml in their incubations (tenfold higher than in the present study), which might explain the discrepancy in results. Nossel *et al.* (1971) showed that human plasma contained an inhibitor of collagen-induced platelet aggregation, but did not consider the possibility that their inhibitor might be influencing collagen fibrillogenesis.

Fig. 2. Platelet-aggregation responses induced by type I and type III collagen from human aorta and chicken skin

Aggregation responses to collagen (added at the arrows to a final concentration of $20 \mu\text{g/ml}$) were measured as described in Fig. 1. (a) Chicken collagen. 1. Untreated. 2. Preincubated for 30 min at 37°C with an equal volume of iso-osmotic NaCl . 3. Preincubated as above, but with cell-free plasma or $0.38\text{M-Na}_2\text{HPO}_4$. (b) Human collagen. 1. Untreated. 2. Preincubated for 120 min at 37°C with an equal volume of cell-free plasma. 3. Preincubated as above, but with iso-osmotic NaCl . 4. Preincubated as above, but with $0.38\text{M-Na}_2\text{HPO}_4$.

Hugues *et al.* (1976) showed that soluble calf skin type III collagen was much more effective than type I collagen at inducing platelet aggregation, and suggested that the activity of type I preparations could be due to contamination by type III. They did not, however, test the platelet-aggregating activity of these collagens in a preformed fibrillar state. It seems unlikely that our results could be explained by contamination of type I collagen with type III, since the

great difference in activity of the soluble collagens cannot be reconciled with their being virtually equiactive in fibrillar form. The simplest explanation for our results is that type III collagen forms fibrils more readily than type I collagen *in vitro*, and indeed such a difference in fibrillogenesis has been reported by Hugues *et al.* (1976).

In summary, our results indicate that, although there may be differences between collagens with

respect to fibrillogenesis, when collagens are in fibrillar form there is little difference between type I and type III collagen of human or chicken origin in platelet-aggregating potency. This implies that the platelet-collagen interaction depends more on an ordered physical form than on particular chemical groupings in the individual collagen molecules. This conclusion is entirely consistent with our knowledge of the platelet-collagen interaction *in vivo*, where to initiate haemostasis the platelets must interact with vascular collagen, which is, of course, in fibrillar form.

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