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PLATELET ADHESION AND AGGREGATION ON SURFACES COATED WITH HUMAN COLLAGENS OF TYPES I, III, IV, AND V

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Interaction between platelets and the collagen components of the connective-tissue matrix of the injured vessel wall is the key factor in initiation of hemostasis and thrombosis. Four genetic types of collagen, namely I, III, IV, and V (CI, CIII, CIV, and CV) are found in arteries [5, 10]. These collagens differ in the composition of the chains, location in the vessel wall, and capacity for fibrillogenesis [2, 5, 10]. CV is located on the endothelial surface facing the lumen of the vessel, CIV and CV, which do not form fibrillary structures in vivo, in the basement membrane. CI and CIII form fibrils in the intima, media, and adventitia [2, 5, 10]. The ability of different collagens to induce platelet aggregation in suspension depends on the form in which the collagens are present – monomeric (triple-chain) and fibrillary, for only fibrillary collagens induce aggregation [3, 4, 7, 11, 14].

In this investigation scanning electron microscopy (SEM) and radioisotopic methods were used to study platelet adhesion on surfaces coated with monomeric human collagens CI, CIII, CIV, and CV, polymeric-fibrillary CI and CIII, or polymeric-amorphous CIV and CV. The collagen substrates differ considerably in their general level of adhesion and in their ability to induce changes in shape of adherent platelets and the formation of large stratified (thrombus-like) aggregates. These differences are entirely due to the genetic type of collagen and are independent of whether the immobilized collagen is in the polymeric or monomeric form. Brief details about these cultures were published in the form of abstracts [9].

EXPERIMENTAL METHOD

CI, CIII, CIV, and CV were isolated from a pepsinized homogenate of human placenta by differential salting out with 0.7-4.5 M NaCl at neutral and acid pH values, followed by chromatography on DEAE-cellulose [6, 13]. Wells (16.4 mm in diameter) in "Multiwell" No. 3008 cultural plates (Falcon Plastics, USA) were coated with monomeric CI, CIII, CIV, and CV, polymeric fibrillary CI and CIII, polymeric amorphous CIV and CV, gelatin-denatured CI or CIII, and ovalbumin. The collagens were dissolved in 100 mM acetic acid (pH 2.8) in a concentration of 1-4 mg/ml. Gelatin was prepared by heating solutions of collagens in acetic acid for 1 h at 56°C. To prepare the monomeric coating an aliquot of an acid solution of collagen or gelatin was diluted with 200 mM carbonate buffer (pH 9.6) to a final concentration of 10 μg/ml, poured into the wells in a volume of 0.5 ml, and incubated for 12-18 h at 4°C. Wells coated with monomeric collagen and gelatin were treated additionally with ovalbumin to bind with areas of the polystyrene that were not coated with collagen [12]. The ovalbumin was dissolved in 30 mM phosphate buffer containing 150 mM NaCl (pH 7.4) in a concentration of about 1 mg/ml, poured into the wells in a volume of 0.5 ml, and incubated for 30 min at 22°C [12]. To obtain a polymeric coating 0.3-

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TABLE 1. Adhesion of ^{51}Cr-labeled Platelets on Various Collagen Substrates

Substrate	Adherent platelets, %	P
Ovalbumin	7,4±0,6	—
Collagens:		
gelatin	6,5±0,5	H
CV	5,9±0,9	<0,05
CIV	9,0±1,0	<0,05
CIII	16,4±1,0	<0,001
CI	18,3±1,3	<0,001

Legend. Platelet adhesion determined in five donors in three parallel measurements and expressed as a percentage of the number of platelets added. Significance of differences shown relative to ovalbumin substrate. Differences between groups CI/CIII and CIV, P < 0.001. N) Not significant.

TABLE 2. Platelet Adhesion to Surfaces of Monomeric and Polymeric (Amorphous) Collagen of Types IV and V

Platelets	Monomeric collagen		Amorphous collagen	
	CV (12)	CIV (13)	(CV 6)	CIV (8)
A_t	3970±1450	32 590±5 460**	764±180	8 460±2 770**
A_c	3890±1460	17 940±2 820**	760±180	6 380±1 770**
Discoid, %	53,3±7,4	7,5±2,5**	57,2±11,7	7,1±4,7**
Spherical, %	44,4±6,8	18,6±5,5*	38,2±10,9	42,7±3,7
Spreading, %	2,3±2,1	73,9±7,1**	4,6±1,9	50,2±4,4**

Legend. Significance of differences between groups CV and CIV: *P < 0.01, **P < 0.001. A_c and A_t on amorphous (polymeric) CIV and CV 3-5 times less than on monomeric CIV and CV respectively (P < 0.001). Here and in Table 3, number of determinations shown in parentheses.

0.5 ml (400-800 μg) of a solution of collagen in 30 mM sodium-phosphate buffer (pH 7.4), was introduced into the wells, heated for 6 h at 37°C, and dried [1]. Under these conditions a fibrillary substrate is formed from CI and CIII, and an amorphous substrate from CIV and CV.

Unlabeled and ^{51}Cr-labeled platelets were obtained by gel-filtration on Sepharose 2B [1]. The platelet suspension ($3 \cdot 10^7$ cells per well) was introduced in a volume of 250 μl into the wells coated with collagen, gelatin, or ovalbumin, and mixed for 40 min at 36 rpm and 37°C in a horizontal incubator-shaker.

Platelet adhesion was determined by SEM using a Phillips PSEM 500× microscope (the Netherlands) [1, 8]. No fewer than 100 platelets in 50 scanning fields under a magnification of 5000 were counted on CIV and CV, and the total number of adherent platelets was calculated by the equation $A_t = U_C + S_C + U_S$; the number of platelets adherent to the collagen substrate (initial adhesion) was given by $A_C = U_C + S_C$, where U_C denotes the number of nonspreading (discoid and spherical) platelets, S_C the number of spreading platelets adherent to collagen, and U_S the number of nonspreading platelets adherent to spreading platelets (all values calculated per square millimeter) [8]; the percentage of discoid, spherical, and spreading platelets was calculated relative to A_C. On CI and CIII at least 100 thrombus-like aggregates were counted in 10-20 scanning fields under a magnification of 320. Stratified (at least two layers of nonspreading platelets) aggregates with a base area of not less than 75 μm^2, were taken as thrombus-like aggregates. To construct a distribution frequency polygon of the thrhombus-like aggregates by size, the area of the bases of 400 thrombus-like aggregates was measured by means of a semiautomatic MOP-2 system (from Reichert-Jung, Austria). The experimental results are presented in the M ± m form. The significance of differences between means was calculated by Student's t test.

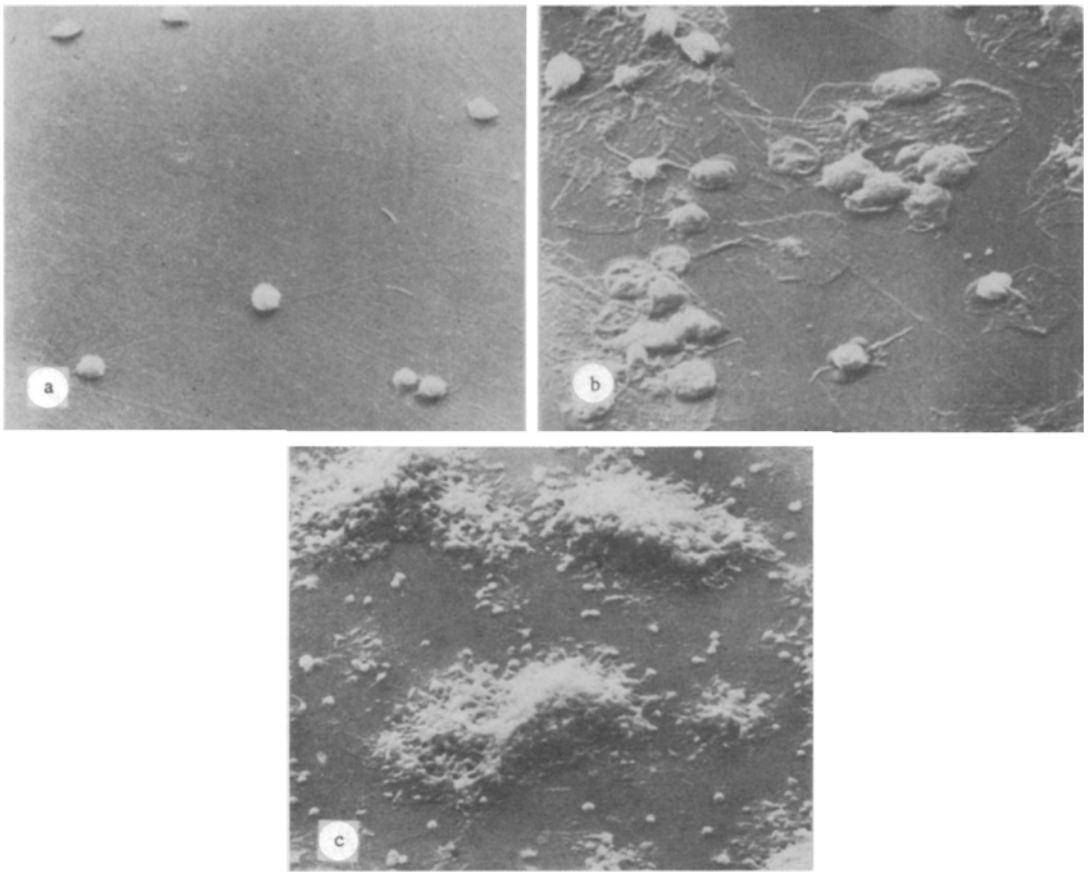

Fig. 1. Platelet adhesion and aggregation on surface coated with monomeric collagens of different genetic types: a) CV: adherent platelets are discoid and spherical. Scale 10 μm; b) CIV: adherent platelets are actively spreading, spherical forms with pseudopodia more numerous than discoid forms, nonspreading platelets adherent to spreading platelets. Scale 10 μm; c) CIII: stratified thrombus-like aggregates alternate with single spreading and nonspreading platelets. Scale 50 μm.

EXPERIMENTAL RESULTS

The general level of platelet adhesion (deposition) on monomeric collagen substrates was determined by the use of ^{51}Cr-labeled platelets. Adhesion of platelets on CV was about one-third of that on CI and CIII, and two-thirds of that on CIV, and negligibly less than on gelatin and ovalbumin (Table 1). Collagen substrates, gelatin, and ovalbumin did not induce liberation of ^{51}Cr from the platelets. The level of free ^{51}Cr, not bound with platelets, after incubation of the platelets with these substrates (from 2.1 ± 1.1 to 2.5 ± 1.1%, n = 5) did not exceed the level of free ^{51}Cr in the original preparation of gel-filtered platelets (2.9 ± 1.7%, n = 5).

The morphological picture of platelet adhesion on the different collagen substrates was studied by SEM and a stage by stage morphometric analysis of the adhesion process was undertaken. Platelets adherent to CV were mainly single discoid and spherical platelets (Fig. 1a). Not more than 5% of the adherent platelets were spreading (Table 2).

Adhesion of platelets to CIV took place more actively: The general level of adhesion was much higher than on CV (Table 2); the substrate induced spreading of adherent platelets (Fig. 1b; Table 2); single platelets from the suspension adhered to the upper surface of the spreading platelets (Fig. 2a). Adhesion on monomeric CV and CIV was significantly more marked than on polymeric amorphous CV and CIV. However, regardless of whether these collagens were in monomeric or amorphous forms, CIV was a more adhesive substrate than CV (Table 2). SEM revealed much greater differences between levels of platelet adhesion to CIV and CV compared with radioisotopic method (Tables 1 and 2). This disparity can evidently be attributed to the relatively more rigorous procedure of rinsing during preparation of specimens for SEM, as a result of which mainly the weakly adherent nonspreading platelets were removed.

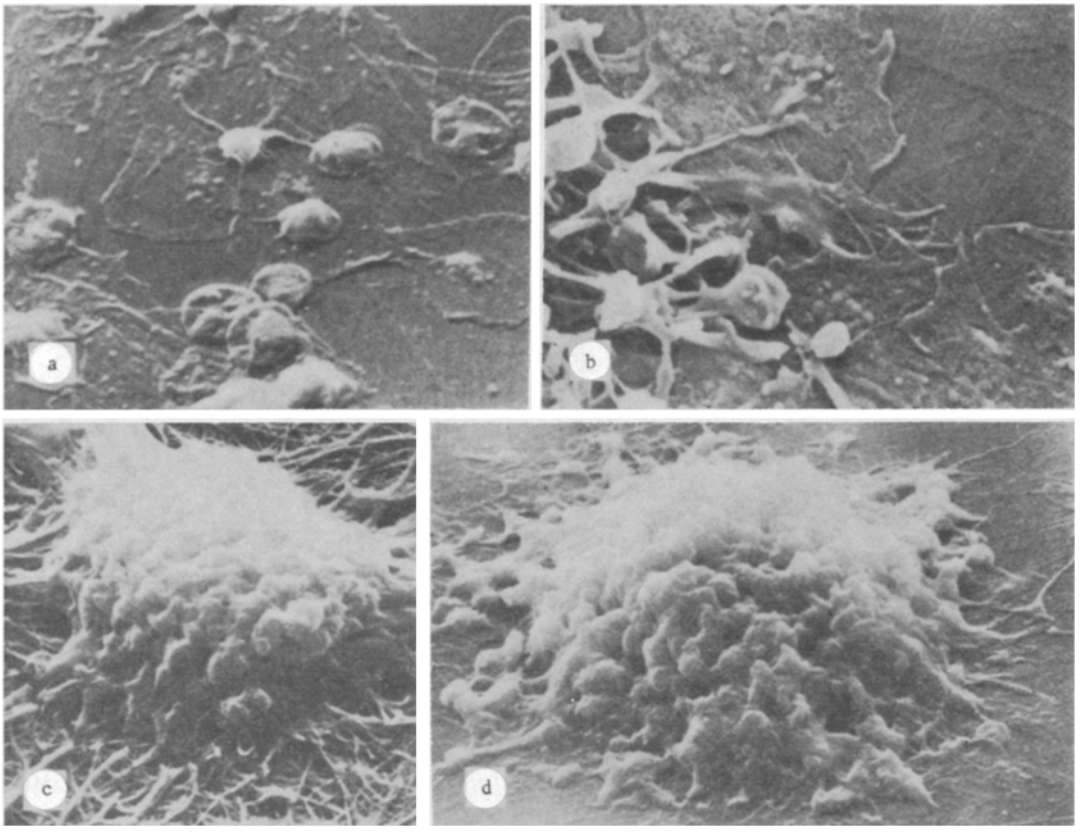

Fig. 2. Platelet adhesion from suspension to upper surface of platelets spreading on monomeric CIV (a) and CI (b), and thrombus-like aggregates adherent to fibrillary (c) and monomeric (d) CI (spreading platelets can be seen at the base of the aggregates). Scale: a, b) 5 μm; c, d) 10 μm.

CI and CIII are thrombogenic. This means that besides active spreading and adhesion of single nonspreading platelets to spreading platelets (Fig. 2b) which are processes characteristic of platelet adhesion to CIV (Figs. 1b and 2a), large stratified (thrombus-like) aggregates, with spreading platelets at their base (Fig. 1c, Fig. 2c, d) were formed on CI and CIII. The dimensions of the thrombus-like aggregates varied within wide limits (Fig. 3). No difference was found between the thrombogenicity of CI and CIII and the thrombogenicity of monomeric and fibrillary forms of these collagens (Fig. 2c, d; Table 3). Thrombus-like aggregates never formed on CIV and CV (Table 3).

Differences in principle were thus found between platelet adhesion on substrates formed by different genetic types of collagen. On CV only the stage of initial adhesion was realized, but no spreading of the platelets. On CIV the spreading process took place actively, and single platelets from the suspension adhered to the upper surface of the spreading platelets. On CI and CIII, besides the stages of adhesion characteristic of CIV and CV, large, thrombus-like aggregates also formed on layers of spreading platelets. The ability of collagen substrates CI and CIII to induce platelet aggregation is entirely determined by the genetic type of collagen and is independent of whether the collagen is monomeric or fibrillary in form. Platelet aggregation in suspension was induced only by CI and CIII, organized into fibrillary structures [3, 4, 7, 11, 14]. Evidently to induce platelet aggregation by means of CI and CIII simultaneous multivalent platelet – collagen interaction is required, and it is realized either by immobilization of collagen on the surface or by the formation of fibrils in suspension. CIV and CV are incapable in principle of inducing platelet aggregation.

Surfaces coated with different types of collagen are convenient models with which to study the various stages of platelet – substrate interaction: initial adhesion (CV), spreading and adhesion on spreading platelets (CIV), the formation of thrombus-like aggregates bound to the surface (CI and CIII).

These results suggest that exposure of CI and CIII in the vessel lumen in the presence of severe injuries or pathological states of the vessel wall is more likely to lead to the formation of juxtamural thrombi and throm-

TABLE 3. Formation of Thrombus-like Aggregates on Surfaces Coated with Monomeric and Polymeric CI, CIII, CIV, and CV

Collagen substrate	Number of thrombus-like aggregates	
	monomeric collagen	polymeric collagen*
CI	276±56 (12)	388±138 (5)
CIII	330±68 (10)	327±92 (3)
CIV	0 (18)	0 (7)
CV	0 (18)	0 (6)

*CI and CIII – fibrillary collagen, CIV and CV – amorphous collagen.

Legend. Differences between groups CI and CIII and groups of monomeric and polymeric collagens are not significant.

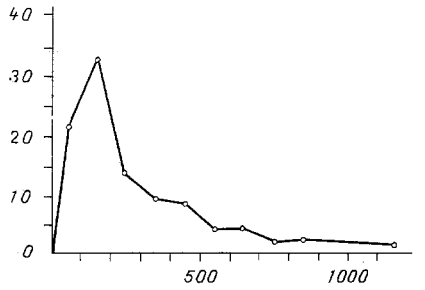

Fig. 3. Frequency polygon of distribution of thrombus-like aggregates adherent to monomeric CIII by size. Abscissa, area of base of thrombus-like aggregates (in μm^2); ordinate, percentage of thrombus-like aggregates with given base area (in % of total number of adherent aggregates). Results of one typical experiment for 400 thrombus-like aggregates are given. Mean area of base 260 μm^2; 17% of surface area of substrate covered by thrombus-like aggregates. Distribution is approximated best of all by a lognormal distribution with parameters $\mu = 5.249$ and $\sigma = 0.794$. Significance of difference by ω^2 criterion 0.128, by Kolmogorov–Smirnov criterion 0.326.

boembolic compositions than exposure of CIV in the presence of desquamation of the endothelium or surface injuries. Interaction of platelets with CV localized on the surface of the endothelium of the vascular wall facing the lumen [10] is not accompanied by spreading of platelets or by the formation of thrombus-like aggregates.

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