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Short Communication

Anti-CD31 Delays Platelet Adhesion/Aggregation at Sites of Endothelial Injury in Mouse Cerebral Arterioles

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The arterioles on the surface of the mouse brain (pial arterioles) were observed by* in vivo *microscopy. A focus of minor endothelial damage was produced in a single pial arteriole in each mouse by briefly exposing the site to a helium neon laser after an intravenous injection of Evans blue. Mice were injected 10 minutes before injury with a monoclonal antibody (MAb) to mouse CD31, also known as platelet endothelial cell adhesion molecule. This treatment doubled ($P < .01$) the time required for the laser to produce a recognizable platelet aggregate. In additional experiments, an MAb to mouse CD61 and an MAb to mouse intercellular adhesion molecule 1 had no effect. The data support previous observations indicating that platelet adhesion/aggregation in this model is induced by endothelial injury without exposure of basal lamina. The data are consistent with the hypothesis that the endothelial injury exposes or activates a platelet endothelial cell adhesion molecule on the endothelium which is blocked by the MAb directed against CD31. This may be the first demonstration of an effect of an anti-platelet endothelial cell adhesion molecule on platelet adhesion/aggregation* in vivo. *(Am J Pathol 1994, 145:33–36)

Local platelet adhesion and aggregation has been reported in arterioles without denudation of endothelium or exposure of basal lamina.[1,2] One means of reliably producing such adhesion/aggregation is to produce endothelial injury with a "light/dye" technique that employs a source of radiant energy and an intravascular vital dye that acts as an energy-absorbing target.[3–6] The present investigation employed one such technique, which uses a helium neon (HeNe) laser and intravascular Evans blue.[4,5] The laser energy is converted to heat when absorbed by the dye, and the endothelial injury is thought to be caused by the heat.[5] Prolonged exposure to the laser results in endothelial denudation and exposure of the basal lamina. However, local platelet adhesion/aggregation occurs long before this point is reached, after much shorter exposure to the laser. Exhaustive examination of the arterioles by transmission electron microscopy reveals very minimal or no abnormality of the endothelium at this stage[4,5] without exposure of basal lamina. Similar findings have been reported after an equally exhaustive electron microscopic study of another "light/dye" model of endothelial injury and associated platelet adhesion/aggregation.[3]

The local platelet adhesion/aggregation produced by the HeNe laser/Evans blue model can be enhanced or inhibited by pharmacological treatments thought to alter local levels of an endothelium-derived relaxing factor (EDRF).[7] The data from these studies suggest that loss of EDRF at the damaged site may account for the platelet adhesion/aggregation; EDRF is known from *in vitro* or *ex vivo* studies to be a potent inhibitor of platelet adhesion.[8] However, additional factors that might account for the local platelet

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adhesion/aggregation could include exposure of adhesion molecules on the damaged endothelium and/or on the platelets.

There is little reason to believe that the laser/dye directly alters the platelets. The arteriolar blood flows at a rate of several mm/second or faster, and the laser beam affects a zone less than 200 μ long.[4,5] Therefore, a passing platelet would be exposed to the laser for less than a tenth of a second and perhaps for only a hundredth of a second. Thus, if the light/dye injury acts at least in part by causing the exposure of or "activation" of adhesion molecules, it is probable that this occurs on the endothelial cell surface rather than on the platelet. The present study tested this hypothesis by employing a monoclonal antibody (MAb) to murine CD31, also known as platelet endothelial cell adhesion molecule-1. The study was carried out *in vivo* using pial arterioles on the surface of the mouse brain. These were selected because they were the object of previous studies of platelet adhesion/aggregation by this laboratory.[3–7]

Materials and Methods

The preparation for this study has been exhaustively described.[3–5,7,9] In brief, a craniotomy was performed in the mouse anesthetized with urethane, the pial vessels were exposed by stripping the dura from the site, and the surface of the brain was continuously suffused with a mock cerebrospinal fluid (Elliott's solution) at a constant pH (7.35). The arterioles were observed under a microscope with epi-illumination from a Halogen lamp and fiberoptic probe. The mouse was injected via tail vein with 25 mg/kg Evans blue (0.5% solution in 0.9% NaCl). Thirty minutes later a segment of a preselected arteriole 30–50 μ in diameter was exposed to the beam of a 6-mW HeNe laser (Spectra-Physics, Mountain View, CA), which was directed downward through the objective lens of a Leitz (Rockleigh, NJ) metallurgic illuminator. The study employed a 20-power infinity-corrected objective lens, and the laser beam was 18 μ wide at the focal plane.

The laser was kept on until platelet adhesion/aggregation ("white body" formation)[10] was noted at the exposed site. Propensity for adhesion/aggregation was defined as the time (seconds) required to elicit the first noticeable platelet aggregate. Ten minutes before laser challenge each mouse was injected via tail vein with 2 mg/kg anti-mouse CD31 (MEC 13.3,[11] PharMingen, San Diego, CA) or with the diluent (0.9% NaCl). The MAb-treated and diluent-treated mice were alternated. The number of mice treated with MAb were determined by the amount of MAb available in two consecutive purchases from the same lot. This proved to be 16 mice, which were compared with 16 contemporary saline-injected controls alternated with the MAb-injected mice.

The anti-mouse CD31 was characterized by indirect immunofluorescent staining and flow cytometric analysis of a murine endothelioma, by immunoprecipitation, and by immunohistochemical staining of frozen sections utilizing a three-step indirect immunoperoxidase technique. Indirect immunofluorescent staining was carried out on cell suspensions of freshly isolated tissues from BALB/c or C3H mice and on suspensions of the murine endothelioma, eEnd.2[12] after detachment by brief exposure to trypsin/EDTA. Approximately 1×10^6 cells per sample were incubated with purified MEC13.3 or hybridoma supernatant for 30 minutes on ice, washed with flow diluent (0.5% bovine serum albumin, 0.1% sodium azide in phosphate-buffered saline), similarly incubated with fluoresceinated mouse-anti-rat immunoglobulin G (Jackson Immunoresearch Laboratories, West Grove, PA), then washed with and resuspended in flow diluent. Relative fluorescent staining was measured using a FACScan (Becton Dickinson, San Jose, CA).

Immunoprecipitation was carried out after labeling cell-surface antigens with biotin as previously described.[13] For immunoprecipitation, protein G-Sepharose (Pharmacia, Piscataway, NJ) was incubated with purified MAb (50 μg/ml), washed, then incubated with an extract from the biotinylated eEnd.2 cells. The bound material was eluted with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, run in SDS-PAGE, transferred to nitrocellulose and detected with avidin-HRPO.

The anti-mouse CD31 MAb reacted with murine endothelium, as observed by the immunohistochemical staining of arterioles in frozen sections of spleen, and by indirect immunofluorescent staining and flow cytometric analysis of the murine endothelioma. In addition to reaction with endothelial cells, weak surface staining of murine platelets, splenocytes and thymocytes was also consistently observed in flow cytometric analysis. Further, the MAb immunoprecipitated an antigen of approximately 130 kd from extracts of surface-biotinylated eEnd.2 cells. These observations are in agreement with the published tissue distribution and molecular size of murine CD31.[14,15]

In a separate experiment an additional group of 10 mice was treated with an MAb directed against mouse CD61 (PharMingen, San Diego, CA), a locus

of GpIIIa activity on platelets.[16,17] These were compared with their own contemporary control group. In still another experiment 13 mice were treated with an MAb against mouse intercellular adhesion molecule-1 (ICAM-1, 2 mg/kg) and compared with 14 controls. The anti-ICAM-1 was supplied by Dr. Szuzsa Fabry (Dept. of Pathology University of Iowa).

The data are expressed as the mean (M) number of seconds required for the laser to induce the first noticeable aggregate. Standard deviations (SDs) rather than standard errors are given so that the scatter in the data is easily seen. The experimental data points are compared with the control using the nonparametric Mann-Whitney test rather than the parametric *t*-test, because the distribution of the data in the control and/or experimental populations is unknown and the populations may not have a normal distribution. Also, there could be significant differences between the variances of each population.

Results

In 16 control mice in arterioles 34 ± 5 μ in diameter it took 76 ± 35 seconds (mean ± SD) for the laser to produce endothelial damage sufficient to induce a recognizable platelet aggregate at the damaged site. In the mice injected 10 minutes earlier with anti-CD31 it took almost twice as long (133 ± 71 seconds, $P < 0.01$, Mann-Whitney test) to induce aggregation in arterioles 34 ± 3 μ in diameter (mean ± SD).

In contrast to these results there was no significant effect of anti-mouse CD61 also given at a dose of 2 mg/kg, 10 minutes before laser-induced endothelial injury. In the controls (n = 10) it took 77 ± 54 seconds (mean ± SD) to induce a noticeable aggregate in arterioles 35 ± 3 μ in diameter. This compared with 93 ± 31 seconds in arterioles 35 ± 2 μ in diameter from 10 mice treated with anti-CD61 ($P \gg 0.05$).

Similarly, anti-ICAM-1 had no effect. The aggregation was initiated in 100 ± 57 seconds (mean ± SD) in the treated mice and 100 ± 81 seconds in the controls.

Discussion

The new information provided by the data is that anti-CD31 inhibits platelet adhesion/aggregation *in vivo* at the site of endothelial injury in brain arterioles. Unfortunately, our model does not permit us to separate adhesion to the endothelium, which is the prelude to aggregation, from platelet-platelet interaction that begins aggregation. The effect of the anti-CD31 could have been caused by an inhibitory action on either platelet-endothelial cell or on platelet-platelet interaction, inasmuch as the MAb detects platelet endothelial cell adhesion molecule-1 on both platelets and endothelial cells. However, an MAb directed against the CD61 or GpIIIa sites on the platelet and endothelial cells had no effect, even though these sites are thought to play a key role in platelet adhesion and aggregation.[16–18]

This failure of anti-CD61 may simply represent a failure of the anti-CD61 to be a functional blocking antibody; that is, it may be directed against epitopes that do not regulate function. On the other hand, the regulation of platelet adhesion/aggregation in our model may be more dependent upon platelet GpIIIa, and the endpoint we use may not be sensitive to blockade of platelet GpIIIa. We are monitoring the appearance of the first recognizable adhering aggregate and not its subsequent growth. The GpIIIa which helps platelets adhere to each other might be more important as the aggregate grows. In addition to modulating platelet-platelet interaction, the GpIIIa molecule is part of a GpIIIaIIb complex that binds to components of basal lamina.[16,17] Anti-CD61 may be ineffective in our model, because we do not have exposed basement membrane at the time of initial aggregation.[4,5] Whatever the explanation, the failure of the anti-GpIIIa to work shows that the inhibitory action of anti-CD31 was not merely a nonspecific effect caused by injection of an antibody. Similarly, the failure of anti-ICAM-1 also indicates absence of nonspecific effects.

It should be noted that there is a large interanimal variation in the aggregation latency. Therefore, a comparison of experimental and control mice must be made between absolutely contemporary groups, or else the values in one group might, by chance, be significantly different from values in the other group. For this reason, each of the three MAbs was evaluated in separate experiments at three different points of time. During each experiment the MAb-treated and control mice were alternated on each test day so that the experimental and control mice in each study were completely contemporary.

The present data do not rule out an effect of anti-CD31 on the platelet as an explanation for the delay in platelet adhesion/aggregation at the site of endothelial injury. However, the data are consistent with the hypothesis that the laser/dye injury induces local platelet adhesion/aggregation by exposing or somehow "activating" a constitutive CD31 site on the endothelial cells. It is also possible that the effect of the MAb is not caused by blockade of endothelial CD31 sites "activated" or exposed by the laser, but instead by blockade of CD31 sites that were already fully "ac-

tivated" or exposed on the endothelial cells before laser injury, and which play an important role in facilitating platelet adhesion triggered by some as-yet-unidentified action of the laser injury on that endothelium. Until more is known about CD31, a choice cannot be made between these alternatives. However, the importance of CD31 for platelet adhesion/aggregation on injured endothelial cells is indicated by the present study, which is to our knowledge the first *in vivo* demonstration of an anti-CD31 effect on platelet adhesion/aggregation.

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