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Immunologic Injury to Vascular Endothelial Cells: Effects on Release of Prostacyclin

By Jonathan C. Goldsmith and John J. McCormick

Prostacyclin is released from cultured and ex vivo bovine vascular endothelium following sublethal immunologic injury by a heterologous antibody to endothelial cells developed in rabbits. This release was dependent on calcium and complement and was not enhanced by the presence of platelets. Prostacyclin release was diminished 1–2 hr after the injury, but recovered fully following reculture of the endothelial cells for 72 hr.

ENDOTHELIAL CELL INJURY may represent the initial event in the pathogenesis of atherosclerosis, vasculitis, and hemorrhagic and thrombotic disorders.[1] Dysfunction of endothelial cells as a consequence of injury could lead to impaired expression of endothelial properties, such as prostacyclin release and availability of angiotensin-converting enzyme activity. Endothelial cells can be injured by both physical and chemical mediators, such as oxygen radicals and homocysteinemia.[2,3] A specific form of chemical injury of the vascular endothelium mediated by immunoglobulins might be operative in atherosclerosis occurring after renal transplantation[4,5] and in the pulmonary hemorrhage of Goodpasture's syndrome.[6]

Alteration in endothelial cell function or change in structure after immunologic injury and the time course of recovery from such injury could affect maintenance of the fluidity of blood through loss of endothelial properties and enhanced platelet–vessel wall interactions. In the present studies, we have examined the effect of antibodies and complement on prostacyclin release from cultured and ex vivo bovine vascular endothelium.

MATERIALS AND METHODS

Materials

Hanks' balanced salt solution, pH 7.4, was modified by the substitution of 15 m*M* HEPES for bicarbonate. Endothelial cells were cultured in modified media 199 (Grand Island Biological Company, Grand Island, NY) with supplemental vitamins, amino acids, 18% fetal bovine serum, and antibiotics.[7] ^{3}H-6-keto-PGF$_{1\alpha}$ (New England Nuclear, Boston, MA), Ionophore A23187 (CalBiochem Behring, LaJolla, CA), arachidonic acid (Nu-Check Prep, Elysian, MN), and FITC-conjugated goat anti-rabbit immunoglobulin and rabbit complement (Cappel Laboratories, Cochranville, PA) were purchased. Rabbit complement was either reconstituted from lyophilized rabbit plasma or thawed from rabbit plasma stored at −80°C immediately prior to use. Chemicals were of reagent grade.

Cell Culture

Primary monolayer cultures of bovine aortic and pulmonary arterial endothelium were initiated from collagenase digests of the luminal surface of vessels, as previously described.[7] Cells were plated onto 24-mm diameter culture plates (Linbro, Flow Laboratories, Hamden, CT) and were confluent 4–6 days after plating. Media were changed every other day.

Template Device

Endothelium from the aorta and pulmonary artery was also studied in a lucite template device that permitted evaluation of the endothelium in apposition to medial elements of the vessel wall.[7]

Preparation of Antiendothelial Cell Antibody

A heterologous IgG antibody was developed in rabbits. The immunogen employed was derived from pure strains of bovine pulmonary arterial or aortic endothelial cells. The cells were the progeny of a single cell cloned by dilution from a collagenase digest of either pulmonary artery or aorta. These cell strains retained properties of the vascular endothelium through culture, such as the production of factor VIII antigen, prostacyclin, angiotensin-converting enzyme activity, and plasminogen activator activity, and maintained the growth characteristic of strict contact inhibition.

Four T-75 flasks (Falcon Plastics Company, Los Angeles, CA) containing confluent endothelial cell monolayers were rinsed 3 times with protein-free culture media. The cells were removed from the flasks with a rubber policeman, then disrupted in 0.02 *M* Tris–0.01 *M* EDTA, pH 7.0, and homogenized until the cells were broken when observed under phase microscopy. Nuclear material was removed by centrifugation at 4,000 *g* for 10 min. Membranes were collected from the supernatant by a second centrifugation at 100,000 *g* for 60 min and then applied to a discontinuous sucrose gradient of 15%, 30%, and 45% for 16 hr at 100,000 *g*.[8] The 15%–30% band was chosen as the immunogen because it contained the greatest amount of angiotensin-converting enzyme activity, a cell membrane-associated activity.[9]

This material was combined with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, MI) and was injected intradermally into New Zealand white rabbits on a weekly schedule for 1 mo. Rabbits were boosted at 8 and 12 wk. Bleedings were begun after 5 wk by collecting whole blood into 1/10 volume 3.8% sodium citrate. Plasma from immunized and control animals was obtained by an initial centrifugation at 3,000 *g* for 10 min to remove

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red cells and rendered platelet poor by a second centrifugation at 3,000 *g* for 20 min.

The rabbit plasma was heated to 56°C for 45 min to inactivate complement. The heated plasma was raised to a 20% concentration of ammonium sulfate and the precipitate discarded. The supernatant was increased to 45% ammonium sulfate concentration. The resultant precipitate was dissolved in a minimum volume of distilled water and was applied to a DEAE-Sephadex A-50 column (Pharmacia, Uppsala, Sweden) containing 1 ml of gel for each initial 1 ml of rabbit plasma. The column was equilibrated and developed with 0.01 *M* Tris-HCl, 0.15 *M* NaCl, pH 7.0. Antibody employed in these studies failed to bind to the column. Control rabbit immunoglobulin was similarly prepared.

The resultant antibodies bound to bovine aortic, pulmonary arterial, and pulmonary venous endothelium in a homogeneous pattern. This was demonstrable by indirect immunofluorescence with FITC-conjugated goat anti-rabbit IgG (Fig. 1). However, the antibodies were not specific for endothelial cells, as bovine fibroblasts and smooth muscle cells of vascular origin also fluoresced.

Experimental Incubations

Following three initial washes with buffer, cultured endothelial monolayers or endothelial-lined vascular segments were incubated for 30 min with either antiendothelial or control rabbit immunoglobulin diluted in Hanks'-HEPES buffer, pH 7.4. Immune antibody and control rabbit immunoglobulin were used at a concentration of 0.203 mg/ml. All buffer solutions contained 0.1% bovine serum albumin (BSA), essentially fatty acid free (Fraction V, Sigma, St. Louis, MO) to minimize nonspecific antibody or control immunoglobulin–endothelial cell interactions. After 3 washes with BSA-containing buffer, the endothelial cell preparations were exposed for 15 min to rabbit complement diluted in buffer (1:30), buffer alone, or complement inactivated for 45 min at 56°C in buffer (1:30). In some experiments, 100,000–150,000 human washed platelets/μl[10] were present in the complement solutions. When cells were recultured, all reagents were filtered with a 0.45-μ filter (Nalge Company, Rochester, NY). Prostacyclin was quantitated in the supernatants by a radioimmunoassay for its stable hydrolysis product 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$).[11] Protein concentrations found in the supernatant did not interfere with the radioimmunoassay. Results are given for the 15-min incubation period for 2×10^5 cells. The number of cells was determined by collagenase treatment of monolayers, dispersion of cells in 0.1% trypsin–0.05% EDTA, and enumeration in a hemocytometer. Reported concentrations of 6-keto-$PGF_{1\alpha}$ are equivalent to pmole/15 min/8×10^5 cells.

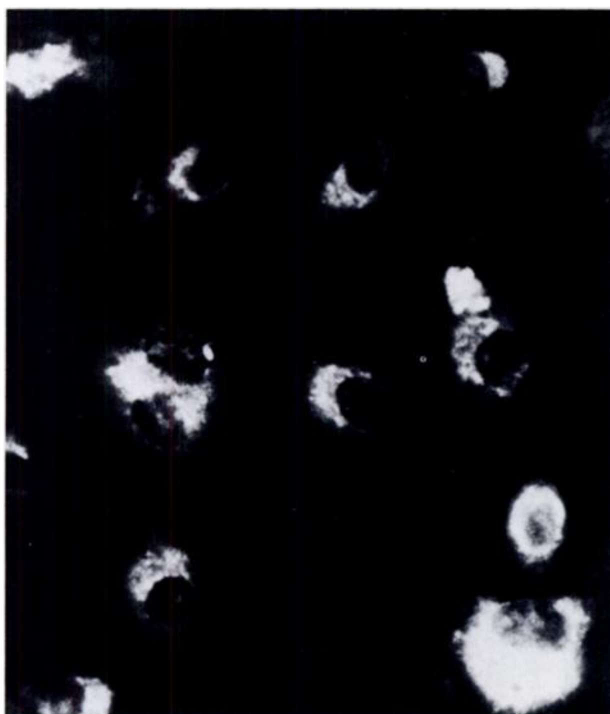

Fig. 1. Photomicrograph of bovine pulmonary artery endothelial cells exposed to antiendothelial cell antibody and FITC-conjugated goat anti-rabbit IgG (260× magnification).

Angiotensin-Converting Enzyme Activity

This was measured as previously reported.[7]

LDH Activity

Lactic dehydrogenase (LDH) was determined in duplicate spectrophotometrically in monolayer supernatants (a-gent, Abbott Laboratories, South Pasadena, CA).

RESULTS

Exposure of cultured pulmonary arterial endothelial cell monolayers to sublethal concentrations of antiendothelial antibody followed by complement resulted in the release of large quantities of prostacyclin, equivalent to an eightfold increase over nonimmune IgG controls (black bars) (Fig. 2). Data shown are the

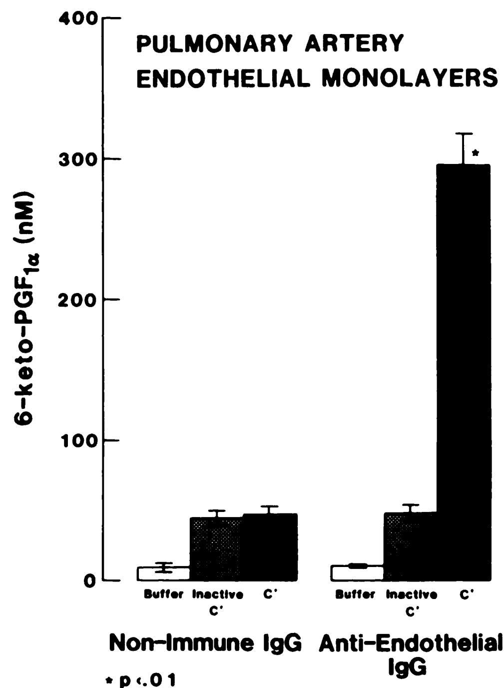

Fig. 2. Release of prostacyclin, quantitated as 6-keto-prostaglandin $F_{1\alpha}$, from pulmonary arterial endothelial cell monolayers exposed to control rabbit IgG or antiendothelial cell rabbit IgG followed by buffer, heat-inactivated complement, or complement solutions. Data are the means of triplicate observations from six separate cultures.

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mean ± SEM from 6 separate endothelial cell cultures. Differences were significant at the $p < 0.01$ level (Student's t test). Addition of heat-inactivated complement (stippled bars) to monolayers exposed to antiendothelial antibody enhanced 6-keto-$PGF_{1\alpha}$ release 2–3-fold over buffer baselines (open bars).

Monolayers of cultured aortic endothelium behaved similarly, with a marked release of 6-keto-$PGF_{1\alpha}$ after exposure to antiendothelial cell antibody and complement (Fig. 3) that was 13-fold greater than with nonimmune IgG (black bars) and 40-fold greater than buffer (open bars). Complement-dependent, antibody-induced prostacyclin release was also observed with ex vivo vascular segments. Data for aortic segments (Fig. 4) demonstrated a 3–5-fold, statistically significant increase in 6-keto-$PGF_{1\alpha}$ over baseline values (black bars). Pulmonary arterial segments also released concentrations of 6-keto-$PGF_{1\alpha}$ severalfold over baseline when antibody and complement were included in the incubation buffer (data not shown).

Evidence suggesting injury of monolayers of aortic and pulmonary artery endothelium was obtained in some experiments by the determination of LDH content of supernatants. Sonicates of monolayers (2×10^5 cells) released 465 ± 40 U/liter (mean ± SEM) of

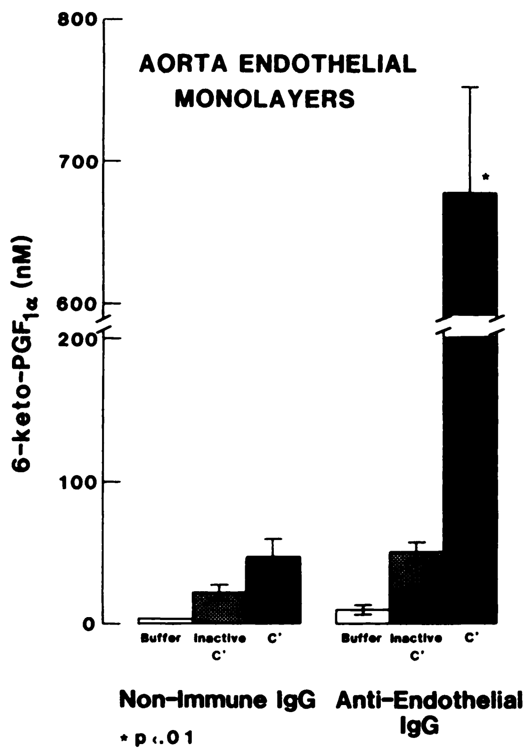

Fig. 3. Prostacyclin release from aortic endothelial cell monolayers exposed to nonimmune or antiendothelial cell antibody followed by buffer, inactivated complement, or complement. Data are the means of triplicate observations from six distinct cultures.

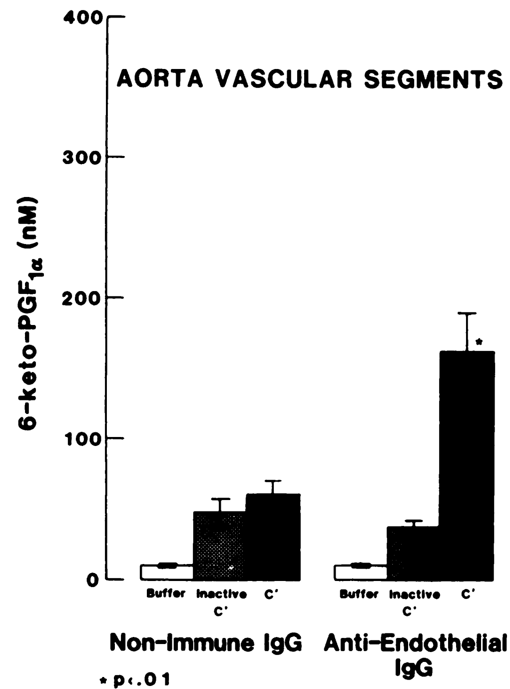

Fig. 4. Prostacyclin release from aortic vascular segments under conditions outlined for monolayers of aortic or pulmonary endothelial cells. Data are the means of triplicate observations from ten vascular segments.

LDH activity into supernatants. Cells exposed to antiendothelial antibody and active complement released 22.6%–36.6% of maximal LDH activity (Table 1). Release of LDH activity correlated with 6-keto-$PGF_{1\alpha}$ release: $r = 0.985$ ($p < 0.001$) for aortic endothelium and $r = 0.958$ ($p < 0.001$) for pulmonary artery endothelium.

The sublethal nature of the immunologic injury in monolayers and segments was confirmed by exclusion of 0.5% trypan blue dye by >99% of cells following experiments. Viability of monolayers was also demonstrated by their continued growth for 72 hr in culture when incubation solutions were removed and replaced by culture media containing fresh fetal bovine serum.

Heat-inactivated complement released small quantities (20–50 nM) of 6-keto-$PGF_{1\alpha}$ from control monolayers that were not previously exposed to antibody (stippled bars in Figs. 2–4). Complement behaved similarly in monolayers that were not exposed to antiendothelial antibody (black bar, left panel of Figs. 2–4). Endothelial monolayers exposed only to buffer for the second incubations released 3–15 nM 6-keto-$PGF_{1\alpha}$ in response to changing solutions (open bars in Figs. 2–4). 6-keto-$PGF_{1\alpha}$ released was manyfold greater from monolayers exposed to antiendothelial antibody and complement. However, the observations with heat-inactivated complement, when compared to buffer controls, suggested an interaction of the rabbit

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Table 1. LDH Release From Cultured Bovine Aortic and Pulmonary Artery Endothelial Cell Monolayers

Lactic Dehydrogenase (U/Liter) (% of Maximal Release)		
Initial Incubation	Second Incubation	Percent
Buffer	Buffer	0.3–0.4
	Inactive complement	0.7–0.9
	Complement	1.7–2.0
Nonimmune immunoglobin	Buffer	0.7–0.8
	Inactive complement	0.7–0.8
	Complement	1.5–2.0
Antiendothelial cell antibody	Buffer	0.5–0.8
	Inactive complement	0.5–1.2
	Complement	22.6–36.6

Results are based on the means of triplicate observations from five separate experiments with both cell types.

Table 2. Prostacyclin Release From Cultured Bovine Pulmonary Artery Endothelial Cell Monolayers After Exposure to Antiendothelial Cell Antibody and Complement in the Presence or Absence of Blood Platelets

Initial Incubation Solution	Second Incubation Constituents	6-keto-PGF$_{1\alpha}$ (nM/2 × 10^6 cells/15 min) (mean ± SEM)
Antiendothelial cell immunoglobulin G	Heat-inactivated complement	57 ± 5
	Heat-inactivated complement + human washed platelets	68 ± 7
	Complement	426 ± 63
	Complement + human washed platelets	375 ± 42

Data are the means of triplicate determinations from three primary cultures.

complement source with endothelial cells. Several experiments were performed to examine this possibility.

Antiendothelial cell antibody, control immunoglobulin, complement, and heat-inactivated complement were adsorbed at 0°C with saline-washed bovine red blood cells.[12] These adsorbed reagents released quantities of 6-keto-PGF$_{1\alpha}$ similar to unadsorbed solutions. Preformed anti-bovine antibodies adsorbable with red cells did not enhance prostacyclin release.

The increased 6-keto-PGF$_{1\alpha}$ release after exposure to heat-inactivated complement appeared to be partially a consequence of protein concentration, as high concentrations of essentially fatty acid-free BSA (2.23 mg/ml) increased 6-keto-PGF$_{1\alpha}$ release twofold over buffer controls. Heating of BSA at this concentration for 45 min at 56°C had similar effects.

That complement was important for prostacyclin release was further shown by experiments in which fresh bovine serum was employed as a source of complement. After exposure to antiendothelial antibody, bovine serum released 6-keto-PGF$_{1\alpha}$ (72 ± 8 nM, mean ± SEM). Whole heated bovine serum released quantities equivalent to those from buffer controls. This release of 6-keto-PGF$_{1\alpha}$ with bovine sera was less than with rabbit complement and could reflect species specificities.

While humorally mediated injury had significant effects on the vascular endothelium, under whole blood conditions there may be a cellular contribution to the process of injury. Granulocytes or blood platelets attracted by endothelial bound antibody and/or complement may release enzymatic activities, adding to endothelial injury. The blood platelet that bears Fc receptors was investigated as a contributor to immunologic injury. Human washed platelets at a final concentration of 100,000–150,000/μl were incorporated into the second incubation solutions (Table 2). Three different primary pulmonary artery cultures were examined. 6-keto-PGF$_{1\alpha}$ release was not increased above concentrations obtained with antibody to endothelial cell membranes and complement when washed platelets were included. These results suggest that there is maximal effect due to the presence of antibody and complement.

Calcium was necessary to support complement-dependent, antibody-induced prostacyclin release from bovine endothelium. When experiments were conducted in calcium-free Hanks'-HEPES buffer, prostacyclin release was not increased from pulmonary arterial or aortic cell monolayers or vascular segments. The lack of calcium affected antibody:complement interaction. The omission of calcium did not affect antibody attachment to endothelial cells.

One question of significant interest was whether the sublethal immunologic injury altered endothelial cell function following the insult. That is, could endothelial cells quantitatively respond with prostacyclin release after sublethal injury. Primary bovine pulmonary arterial endothelial cell monolayers were exposed to either immune or control immunoglobulin plus complement. Following the experimental protocols, culture media with fetal bovine serum were replaced, and the monolayers returned to a 5% CO_2 atmosphere incubator for 72 hr. At the end of the time period, the monolayers were washed 3 times with buffer containing 0.1% BSA and then incubated for 15 min with buffer alone, buffer containing 10 μM arachidonic acid, or buffer containing 10 μM ionophore A23187 (Table 3). Three independently derived primary cultures exposed to antibody and complement or control solutions released similar amounts of prostacyclin under the stimulating conditions. These results were in contrast to stimulated prostacyclin release 1–2 hr after the control or antibody and complement incubation, when concentrations of 6-keto-PGF$_{1\alpha}$ recovered from injured mono-

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Table 3. Prostacyclin Release From Bovine Pulmonary Arterial Endothelial Monolayers Exposed to Nonimmune or Immune IgG With Complement and Then Recultured for 72 Hours

	6-keto-PGF$_{1\alpha}$ (n*M*)		
Prior Exposure	Buffer	Arachidonic Acid (10 μ*M*)	A23187 (10 μ*M*)
Nonimmune IgG + complement	15 ± 5	555 ± 32	225 ± 37
Antiendothelial IgG + complement	12 ± 4	516 ± 40	256 ± 17

Mean ± SEM for quadruplicate observations from three cultures.

layers were only 60%–80% of those in supernatants from control monolayers.

DISCUSSION

The biochemical and anatomic integrity of the vascular endothelium are important but not absolute requirements for maintaining fluidity of blood and permeability characteristics of the vessel wall. Injury of the endothelium can be a consequence of numerous pathophysiologic mechanisms with potentially deleterious effects on vascular integrity.

The present studies on early events after sublethal immunologically mediated injury indicate that the vascular endothelium responds by releasing a burst of prostacyclin. Previously, prostacyclin release from human and animal systemic and umbilical endothelium has been demonstrated following stimulation with thrombin, ionophore A23187, trypsin, bradykinin, arachidonic acid, and cyclic endoperoxides.[13-15] The injury induced in our system was mediated by heterologous antibody to normal bovine pulmonary arterial or aortic endothelium in the presence of complement. The complement source employed in these studies released small amounts of 6-keto-PGF$_{1\alpha}$ and LDH from endothelium in the absence of antiendothelial antibody. Heat-inactivated complement had a similar effect. This effect was present after adsorption of complement with bovine red blood cells, suggesting the presence of nonadsorbed, heat-stable injurious substances in the complement. Studies with a species-specific complement source confirmed the importance of complement as a mediator of 6-keto-PGF$_{1\alpha}$ release. It is unlikely that endothelial cell–antibody interactions were consequences of viral induced expression of endothelial Fc and C3b receptors, as our cultures are periodically monitored for viral infection.[16] Therefore, the injury employed in these studies mimics postulated in vivo events in disorders such as Goodpasture's syndrome.

High concentrations of prostacyclin, found after injury, produce local and downstream vasodilation as well as inhibiting platelet aggregation. In effect, the endothelium counters thrombotic tendencies induced by humoral immune mechanisms.

The release of prostacyclin required calcium, as there was no augmentation of prostacyclin production from immunologically injured monolayers in calcium-free buffers. Calcium was essential for antibody:complement interaction. In red cell systems, complement increases intracellular calcium.[17] If similar circumstances are operative in endothelium, then increased prostacyclin release from immunologically injured monolayers could be a consequence of calcium movement into or within the endothelial cell.[15]

We have also demonstrated that the endothelium can recover from sublethal injury and release normal quantities of prostacyclin, especially if favorable growth conditions are restored. Under our experimental conditions at least, one cellular element of blood—platelets—does not appear to enhance humorally mediated endothelial cell injury.

Immunologic injury of the endothelium has important implications in the pathogenesis of thrombotic and hemorrhagic disorders and acceleration of atherosclerosis.[18] Our studies indicate that immunologic injury of the vascular endothelium causes a burst in prostacyclin production that may influence the response of blood and the vessel wall to this injury. Further, we have shown that the endothelium can recover some normal biochemical functions if the injurious stimulus is removed and a more favorable environment restored.

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