Cultured Human Endothelial Cells Generate Tissue Factor in Response to Endotoxin

M. COLUCCI, G. BALCONI, R. LORENZET, A. PIETRA, D. LOCATI, M. B. DONATI, and N. SEMERARO, *Istituto di Ricerche Farmacologiche Mario Negri, 20157 Milano, Italy*

ABSTRACT Bacterial infection is associated with disseminated intravascular coagulation and fibrin deposition in the microcirculation; the mechanism of these effects in humans is still unclear. We have studied the generation of procoagulant activity (PCA) by cultured human endothelial cells (EC) in response to endotoxin. Cells from umbilical cord veins were grown in Eagle's minimum essential medium with 20% fetal calf serum till confluence. Absence of fibroblasts and macrophages was carefully checked. Endotoxin (*Salmonella enteritidis* lipopolysaccharide (LPS) W or *Escherichia coli* 0111:B4 LPS W, 0.01–1.0 μg/ml) was added to culture dishes for 4–6 h. PCA of EC was measured by a one-stage clotting assay and/or a two-stage amidolytic assay with the chromogenic substrate S-2222. In the absence of endotoxin, EC generated little, if any PCA (2–5 units/10^5 cells). In contrast, the addition of endotoxin resulted in generation of strong PCA that reached a maximum within 4–6 h (185–241 units/10^5 cells) and was dose-dependent between 1 and 0.01 μg endotoxin/ml of culture medium. The generation of PCA required RNA and protein synthesis but did not require the presence of serum. No activity was found in the culture medium. The activity was of tissue thromboplastin type, as indicated by biological and immunological criteria. These endotoxin effects were observed in the absence of endothelial damage, as shown by phase-contrast microscopy and lack of ^{51}Cr release. These data could contribute to elucidate the pathogenesis of vascular complications associated with endotoxemia in man.

INTRODUCTION

Endotoxin-producing bacteria cause disseminated intravascular coagulation (DIC),[1] shock, and ultimate death of an estimated 25% of patients with bloodstream infections (1, 2). The mechanism of endotoxin action in man is still unclear and difficult to study. In animals endotoxin induces injury of endothelial cells (EC) and this is thought to play an important role in blood clotting activation (3, 4).

We describe here experiments indicating that endotoxin induces the generation of tissue factor procoagulant by cultured human EC. This hitherto undescribed effect of endotoxin could play a key role in the generation and deposition of fibrin in blood vessels.

METHODS

EC. Human EC were obtained from umbilical cord veins according to Gimbrone et al. (5) as previously described (6). The collected cells were equally distributed in flat-bottom, 16-mm diam, four-well plates (Nunc, Roskilde, Denmark) and grown at 37°C in Eagle's minimum essential medium

Dr. Semeraro's current address is Istituto di Patologia Generale, Università di Bari, Italy.

Received for publication 4 February 1983 and in revised form 28 March 1983.

[1] *Abbreviations used in this paper:* DIC, disseminated intravascular coagulation; EC, endothelial cells; LPS, lipopolysaccharide; PCA, procoagulant activity.

on Hanks' balanced salt solution supplemented with Hepes (20 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 20% fetal calf serum (Flow Laboratories, Irvine, Ayrshire, Scotland). The medium was replaced every second day. In this study 5-d-old confluent cultures were used. EC were characterized by positive immunofluorescent staining with a specific anti-Factor VIII antibody (Behringwerke Scoppito, Italy). No obvious contamination with fibroblast-like cells was detected in the EC cultures used. These cultures contained no macrophages as judged morphologically or by nonspecific esterase staining (7, 8).

Experimental procedure. In each experiment a series of EC cultures derived from the same umbilical cord vein was used. The culture medium was removed and the cell monolayers were washed three times with serum-free culture medium. Then 1 ml of fresh culture medium with or without absorbed human AB serum containing 1 μg/ml endotoxin (*Salmonella enteritidis* lipopolysaccharide (LPS) W or *Escherichia coli* 0111:B4 LPS W, Difco Laboratories, Detroit, MI), or saline (as a control) was added to the wells and the cultures were incubated at 37°C. At predetermined intervals, the supernatant was removed and the EC were washed three times with serum-free culture medium before evaluating their procoagulant activity (PCA). The cell number was estimated by counting in a hemocytometer (Electronic Coulter Counter, model B, Culture Electronics Ltd., Luton, England) the cells released by 0.25% trypsin from a companion set of washed monolayers. Virtually all the cells were released into suspension. Cell counts in all wells varied by <10%. The average number was 2×10^5/well. Cell viability was always >90% by the trypan blue dye exclusion test.

In some experiments endotoxin-stimulated and control EC were detached from the plastic wells by brief exposure to a 0.25% trypsin–0.02% EDTA solution or to a 0.02% EDTA solution alone, washed extensively and suspended in culture medium before testing for PCA.

Evaluation of PCA. PCA of EC that had been disrupted by freezing and thawing directly in the wells or of undisturbed EC fixed to the bottom of the wells was measured by a one-stage clotting assay and/or by a two-stage amidolytic assay (9). The freeze-thaw procedure was performed by placing the cell plates on top of a dry ice ethanol mixture for freezing and by shifting them to a water-bath at 37°C for thawing. The clotting time was determined in duplicate in prewarmed plastic tubes using the following test system: 0.1 ml test sample, 0.1 ml citrated normal or coagulation factor-deficient plasma, and 0.1 ml 0.025 M $CaCl_2$. In the amidolytic assay (9) PCA was measured by incubating the test sample with a $BaSO_4$ serum eluate (as source of Factor X and VII) and $CaCl_2$. After 30 min the reaction was stopped by EDTA and the activated Factor X (Xa) present was assayed by measuring its amidolytic activity on the chromogenic substrate S-2222 (Kabi Diagnostica, Stockholm, Sweden).

Results were expressed in arbitrary units by comparison of the clotting times of EC with a standard curve of clotting times produced by dilutions of rabbit brain thromboplastin. 1,000 units of thromboplastin cause normal plasma to clot in 20 s. Similarly, the EC PCA measured in the amidolytic assay was expressed in arbitrary units by comparison of Factor Xa activity generated by the EC with Factor Xa activity obtained in the same system by testing dilutions of thromboplastin.

Determination of EC damage. Injury to EC was evaluated by phase-contrast microscopy and by a ^{51}Cr-release assay as described (10).

RESULTS

Fig. 1 shows that after 5-d culture human EC had little if any PCA, which did not increase significantly during 6-h incubation with fresh, endotoxin-free culture medium (2.1±0.5 vs. 5.3±2.1 units; $n = 5$, $P > 0.1$ using the Student's t test). However, when endotoxin was added to the medium strong PCA could be detected in EC after 3-h incubation, reaching a maximum within 4–6 h. No activity was recovered in the culture medium (clotting time always >300 s). The endotoxin effect was dose dependent between 1 and 0.01 μg/ml culture medium. The generation of EC PCA induced by endotoxin was completely abolished by cycloheximide (1 μg/ml culture medium) suggesting that RNA and protein synthesis are required for this phenomenon (Fig. 1); cycloheximide did not affect cell viability. The observed PCA generation by EC did not require the presence of serum in the cell culture environment and, in fact, in parallel experiments in the absence of serum, EC generated comparable levels of PCA in response to endotoxin (data not shown). Table I shows that EC PCA induced by endotoxin is identical to tissue thromboplastin on the basis of two criteria: (*a*) it failed to shorten the coagulation time of Factor VII-deficient plasma but did shorten the coagulation time of normal, Factor VIII- or Factor IX-deficient plasma to similar extents; (*b*) it was neutralized by a goat antiserum against the apoprotein III component of human brain thromboplastin (kindly provided by Prof. Prydz, Research Institute for Internal Medicine, Rikshospitalet, Oslo, Norway).

To determine whether undisturbed EC have PCA available on their surface, endotoxin-stimulated and control cell monolayers were washed and incubated with a source of Factor X and VII ($BaSO_4$-serum eluate) and $CaCl_2$ (Methods). The apparent PCA level in these undisturbed cells was compared with that of the same number of cells disrupted by freezing and thawing (three times) directly in the well before addition of the clotting zymogens (serum eluate) and $CaCl_2$.

Fig. 2 shows that undisturbed endotoxin-stimulated EC did express some PCA. This activity represented approximately one-fourth that of the disrupted cells (total PCA).

In some experiments EC, after 4-h incubation with endotoxin or saline, were detached from the plastic plates by exposure to trypsin-EDTA or EDTA alone before testing. Again, in all instances, only endotoxin-stimulated cells expressed PCA. Most of this activity was detected in intact cells, suggesting that the detachment procedure causes unmasking of PCA (Fig. 3). No difference was found between cells detached with trypsin-EDTA and those detached by EDTA

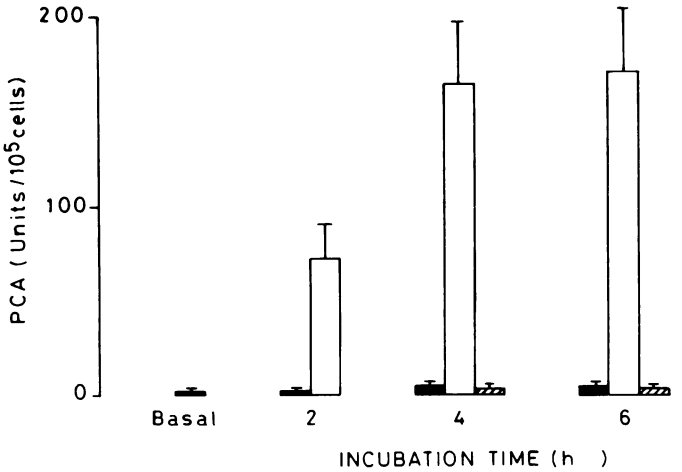

FIGURE 1 PCA generated by human EC during incubation with endotoxin. PCA was measured by the clotting assay on disrupted cells (Methods). Basal, PCA of unincubated EC cultures. Results are the mean±SE of five separate experiments. Saline, (■); endotoxin, (□); cyclohexiimide, + endotoxin (▨).

alone, suggesting that trypsin per se had no effect on cellular PCA availability.

Failure of endotoxin to induce endothelial damage. No changes were observed by phase-contrast microscopy in the morphology of EC monolayers during the 6-h incubation with endotoxin (1–10 μg/well). In addition, endotoxin (up to 10 μg/well) did not induce ^{51}Cr release (95–107%, range of four experiments, compared with respective controls set to 100%).

TABLE I
Characterization of PCA

Sample	PCA
	units/10⁵ cells
I EC tested in normal plasma	185.4–241.7
II EC tested in Factor VIII-deficient plasma	193.8–245.3
III EC tested in Factor IX-deficient plasma	195.2–240.9
IV EC tested in Factor VII-deficient plasma	2.1–3.9
V EC treated with antiapoprotein III	3.1–4.7
VI EC treated with normal goat serum	198.0–254.5

EC cultures were stimulated with endotoxin for 4 h and disrupted as described in Methods. Samples V and VI were incubated with equal volumes of goat antiserum to apoprotein III or normal goat serum (both diluted 1:40) for 1 h at room temperature before testing in normal plasma. Results are the range of three separate experiments.

DISCUSSION

One of the main effects of endotoxin both in humans and experimental animals is the production of DIC. Endotoxin-induced endothelial damage is considered one of the main pathogenetic factors in laboratory animals (3, 4). However, there is no evidence, so far, that cultured human EC are directly injured by endotoxin (10–13). In this study we also failed to demonstrate endothelial damage as assessed by ^{51}Cr release.

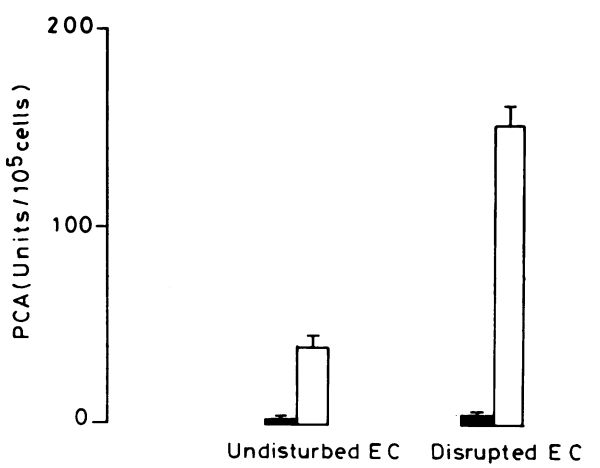

FIGURE 2 PCA expressed by undisturbed EC monolayers and by the same number of disrupted cells after stimulation with endotoxin for 4 h. For details see text. Results are the mean±SE of four separate experiments. Saline, (■); endotoxin, (□).

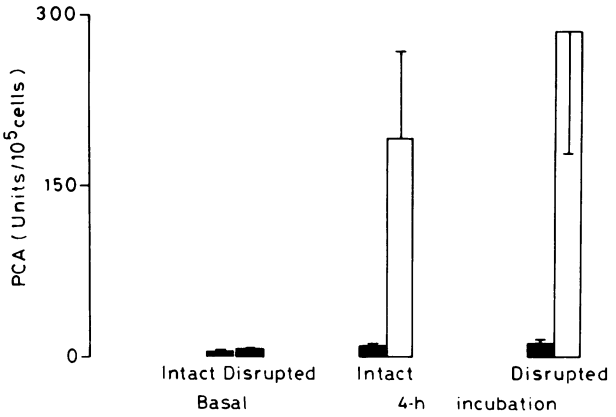

FIGURE 3 PCA of endotoxin-stimulated EC detached from the plastic wells with trypsin-EDTA before testing in the clotting assay. The assay was performed with intact and disrupted (by freezing and thawing) cells. Basal, PCA of unincubated EC cultures. Results are the mean±SE of three separate experiments. Saline (■); endotoxin (□).

Nevertheless, endotoxin is capable of inducing the generation in human cultured EC of tissue factor procoagulant, a potent trigger of blood coagulation. Interestingly, some endotoxin-induced PCA is also expressed by undisturbed monolayers, suggesting that it is available, to some extent, at the cell surface. The inhibitory effect of cycloheximide suggests that messenger RNA and protein synthesis are necessary for tissue factor generation by human EC in response to endotoxin. Maynard et al. (14) and Smariga and Maynard (15) showed that after subculture the EC tissue factor increased, reaching a maximum after 5–8 h and that most of the activity became available after freeze-thaw disruption of cells. In our study EC cultures incubated for 4–6 h in the absence of endotoxin had very low PCA, similar to that of unincubated cells. Moreover, their disruption did not result in a significant increase in PCA. However, no direct comparison can be made between our data and those of Maynard et al. (14), since our EC were not subcultured.

Endotoxin-induced DIC is characterized by fibrin deposition in the microcirculation. The generation of PCA by EC reported in this study could help explain fibrin generation and deposition in the microcirculation.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Elisabetta Dejana for stimulating discussions. Judy Baggott, Graziella Scalvini, Felice and Vincenzo de Ceglie, and Ivana Garimoldi helped prepare this manuscript.

This work was partially supported by Italian National Research Council (82.02038.56).

REFERENCES

1. Wolff, S. M., and J. V. Bennett. 1974. Gram-negative rod bacteremia. *N. Engl. J. Med.* **291:** 733–734.
2. Young, L. S. 1979. Gram-negative sepsis. *In* Principles and Practice of Infectious Diseases. G. L. Mandel, R. G. Douglas, Jr. and J. E. Bennett, editors. John Wiley & Sons, Inc., New York. **1:** 571–608.
3. Morrison, D. C. and R. J. Ulevitch. 1978. The effects of bacterial endotoxins on host mediation systems. A review. *Am. J. Pathol.* **93:** 526–617.
4. Semeraro, N. 1980. Interactions of platelets, leukocytes, and endothelium with bacterial endotoxins: possible relevance in kidney disorders. *In* Hemostasis, Prostaglandins, and Renal Diseases. G. Remuzzi, G. Mecca, and de Gaetano G., editors. Raven Press, New York. 99–116.
5. Gimbrone, M. A., Jr. 1976. Culture of vascular endothelium. *In* Progress in Hemostasis and Thrombosis. T. H. Spaet, editor. Grune & Stratton, Inc., New York. 1–28.
6. Barbieri, B., G. Balconi, E. Dejana, and M. B. Donati. 1981. Evidence that vascular endothelial cells can induce the retraction of fibrin clots. *Proc. Soc. Exp. Biol. Med.* **168:** 204–207.
7. Koski, I. R., D. G. Poplack, and R. M. Blaese. 1976. A nonspecific esterase stain for the identification of monocytes and macrophages. *In* In Vitro Methods in Cell-Mediated and Tumor Immunity. B. Bloom and J. R. David, editors. Academic Press, Inc., New York. 359–362.
8. Mantovani, A., T. R. Jerrells, J. H. Dean, and R. B. Herberman. 1979. Cytolytic and cytostatic activity on tumor cells of circulating human monocytes. *Int. J. Cancer.* **23:** 18–27.
9. Colucci, M., L. Curatolo, M. B. Donati, and N. Semeraro. 1980. Cancer cell procoagulant activity: evaluation by an amiloyditic assay. *Thromb. Res.* **18:** 589–595.
10. Galdal, K. S., and S. A. Evensen. 1981. Effects of divalent cations and various vasoactive and haemostatically active agents on the integrity of monolayers of cultured human endothelial cells. *Thromb. Res.* **21,** 273–284.
11. Wall, R. T., J. M. Harlan, L. A. Harker, and G. E. Striker. 1977. Endotoxin-mediated vascular injury. *Blood.* **50**(Suppl. 1): 286.
12. Harlan, J. N., L. A. Harker, G. E. Striker, and R. B. Counts. 1981. Endotoxin-mediated endothelial injury in vitro. *Thromb. Haemostasis.* **46:** 200.
13. Yamada, O., C. F. Moldow, T. Sacks, P. R. Craddock, M. A. Boogaerts, and H. S. Jacob. 1981. Deleterious effects of endotoxin on cultured endothelial cells: an in vitro model of vascular injury. *Inflammation.* **5:** 115–126.
14. Maynard, J. R., B. E. Dreyer, M. B. Stemerman, and F. A. Pitlick. 1977. Tissue-factor coagulant activity of cultured human endothelial and smooth muscle cells and fibroblasts. *Blood.* **50:** 387–396.
15. Smariga, P. E., and J. R. Maynard. 1982. Platelet effects on tissue factor and fibrinolytic inhibition of cultured human fibroblasts and vascular cells. *Blood.* **60:** 140–147.