

## Activated Protein C Directly Activates Human Endothelial Gelatinase A\*

(Received for publication, December 21, 1999, and in revised form, January 14, 2000)

Minh Nguyen, Jacky Arkell, and  
Christopher J. Jackson‡

From the Sutton Arthritis Research Laboratory, Royal North Shore Hospital, St. Leonards, New South Wales 2065, Australia

**Angiogenesis (formation of new blood vessels) occurs in a number of diseases such as cancer and arthritis. The matrix metalloproteinase (MMP), gelatinase A, is secreted by endothelial cells and plays a vital role during angiogenesis. It is secreted as a latent enzyme and requires extracellular activation. We investigated whether activated protein C (APC), a pivotal molecule involved in the body's natural anti-coagulant system, could activate latent gelatinase A secreted by human umbilical vein endothelial cells (HUVEC). APC induced the fully active form of gelatinase A in a dose (100–300 nM)- and time (4–24 h)-responsive manner. The inactive zymogen, protein C, did not activate gelatinase A when used at similar concentrations. APC did not up-regulate membrane type 1 MMP (MT1-MMP) mRNA in HUVEC. In addition, the MMP inhibitor, 1,10-phenanthroline (10 nM), was unable to inhibit APC-induced activation. These results suggested that MT1-MMP was not involved in the activation process. APC activation of gelatinase A occurred in the absence of cells, indicating that it acts directly. APC may contribute to the physiological/pathological mechanism of gelatinase A activation, especially during angiogenesis.**

Angiogenesis is a prominent feature of cancer and arthritis. The matrix metalloproteinase (MMP),<sup>1</sup> gelatinase A, plays a vital role during angiogenesis by degrading the collagens present in the basement membrane (1) and allowing the endothelial cells to invade the stroma. The enzyme is constitutively expressed by human endothelial cells in a latent form and can be activated by membrane-type MMPs (MT-MMPs) on the cell surface (2). Activation can be induced in endothelial cells by non-physiological agents, such as phorbol myristate acetate, resulting in the generation of the intermediate active 62-kDa and the fully active 59-kDa species (3). Recently, two physiological agents, thrombin and type I collagen, have been shown to activate gelatinase A in human and rat endothelial cells,

\* This work was supported by the Arthritis Foundation of Australia, Northern Sydney Area Health Service, Rebecca L. Cooper Medical Research Foundation, and the Henry Langley Fellowship (University of Sydney). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 612-99266043; Fax: 612-99266269; E-mail: cjackson@med.usyd.edu.au.

<sup>1</sup> The abbreviations used are: MMP, matrix metalloproteinase; MT-MMP, membrane-type MMP; APC, activated protein C; HUVEC, human umbilical vein endothelial cells; TIMP2, tissue inhibitor of metalloproteinase-2.

respectively (4, 5).

Activated protein C (APC) is a serine protease that plays a central role in physiological anticoagulation. The inactive precursor, protein C, is a vitamin K-dependent glycoprotein synthesized by the liver and found in the plasma. Activation of protein C occurs on the endothelial cell surface and is triggered by a complex formed between thrombin and thrombomodulin (6). APC functions as an anticoagulant by binding to the cofactor, protein S, which inactivates the clotting factors Factor VIIIa and Factor Va. The importance of APC as an anticoagulant is reflected by the findings that deficiencies in this molecule result in familial disorders of thrombosis (7). In addition to its anti-coagulant activity, APC has been reported to have an anti-inflammatory effect (8). In the current report we describe a new role for APC, demonstrating that it can activate gelatinase A in human endothelial cells.

### EXPERIMENTAL PROCEDURES

**Materials**—Human APC and human protein C were obtained from ICN Biomedicals (Aurora, OH). TIMP2 was purchased from Oncogene Science (Uniondale, NY). 1,10-Phenanthroline was obtained from Sigma.

**Cells**—Human umbilical vein endothelial cells (HUVEC) were isolated and maintained as described previously (9). HUVEC were grown in Biorich containing 20% fetal calf serum plus 50  $\mu$ g/ml endothelial cell growth supplement (Sigma) and 50  $\mu$ g/ml heparin (Sigma). Cells were used at passage four.

**Experimental Protocol**—Cells were plated down at 30,000 cells/well in 96-well plates in growth medium for 5 days. They were washed twice with Hanks' balanced salt solution and preincubated for 6 h in basal medium (Biorich plus 1% normal pooled human serum, which was stripped of gelatinases by running through a gelatin-Sepharose column) (Amersham Pharmacia Biotech). The culture medium was then replaced with fresh basal medium, and test agents were added for 24 h. The conditioned media were collected for analysis. To ensure that the results were standardized between wells, the cell numbers were quantitated using the CellTiter One Solution cell proliferation assay (Promega, Madison, WI). The cell numbers did not differ between any of the treatments used in the experiments (data not shown).

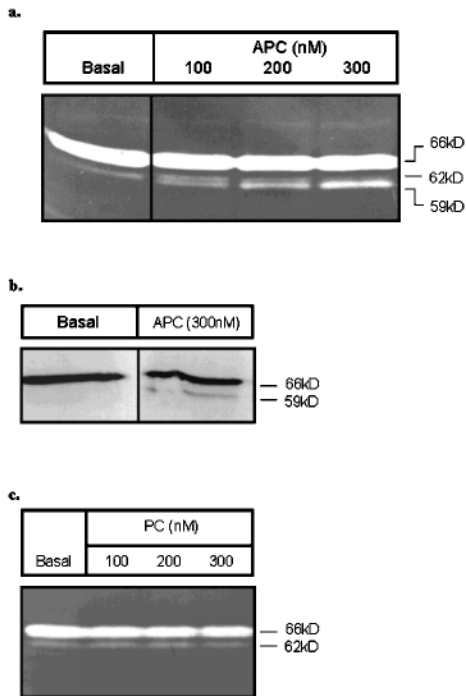
**Gelatin Zymography**—Gelatinase A was detected using gelatin zymography under non-reducing conditions as described previously (10). The gels were scanned into an IBM PC, and the intensity of the bands was semi-quantitated using Scion Image (Meyer Instruments, Houston, TX).

**Western Blotting**—Latent and active gelatinase A were detected by Western analysis after SDS-polyacrylamide gel electrophoresis. A monoclonal antibody to gelatinase A (Oncogene Science) was used at 2  $\mu$ g/ml.

**Northern Blotting**—The extraction of total RNA was performed using the acid guanidine thiocyanate/phenol/chloroform method of Chomczynski and Sacchi (11). Ten  $\mu$ g of total RNA was run on a 1% agarose gel containing 1.25 M formaldehyde. The RNA was transferred to a Hybond-N+ nylon membrane (Sigma) and cross-linked by ultraviolet irradiation. Northern analysis for MT1-MMP was performed as described previously (4). The MT1-MMP probe was generously provided by Prof. Paul Basset (Illkirch, France).

### RESULTS AND DISCUSSION

**APC Activates Gelatinase A in Human Endothelial Cells**—HUVEC were treated with human APC or no test agent for 24 h, and the conditioned media were analyzed for gelatinase A by zymography. Results are shown in Fig. 1a. Consistent with our previous report (12), under basal conditions, HUVEC expressed a prominent latent form, a 62-kDa intermediate form (~3% total gelatinase A activity, as determined by scanning densitometry) and a barely detectable level of the 59-kDa fully active form of gelatinase A (<1% total protein). Treatment of



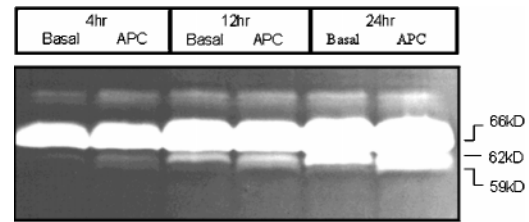
**FIG. 1. Gelatinase A activation in response to APC.** Confluent HUVEC were preincubated in basal medium (Biorich plus 1% normal pooled serum) for 6 h, followed by incubation for 24 h in fresh basal medium in the presence of no test agent (*Basal*) or 100, 200, or 300 nM human APC (*a*) or 100, 200, or 300 nM human protein C (*PC*) (*c*). The conditioned medium was collected and measured for gelatinase A using zymography (*a* and *c*) or Western analysis (*b*) as described under "Experimental Procedures."

cells with 100 nM APC substantially enhanced the amount of the 59-kDa fully active form to ~8% of total gelatinase A activity. Western analysis confirmed the activation of gelatinase A by APC (Fig. 1*b*).

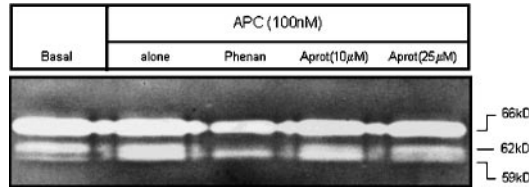
APC was dose-responsive in its activation of gelatinase A (Fig. 1*a*). When used at 200 nM, APC increased the amount of the 59-kDa fully active enzyme by approximately 2.1-fold, compared with 100 nM. Interestingly, there was a concomitant decrease in the amount of the intermediate species generated as the concentration of APC increased. At 300 nM, APC converted almost all the intermediate form to the fully active form. In contrast, the inactive zymogen, protein C, did not activate gelatinase A when used at similar concentrations to APC (Fig. 1*c*). Time course experiments revealed that APC induced the fully active form as early as 4 h, the levels of which progressively increased after 12 and 24 h of exposure to APC (Fig. 2).

Previous workers have shown that plasmin (13, 14) can activate gelatinase A to the fully active form in HT1080 cells. To determine whether contaminating plasmin was contributing toward endothelial gelatinase A activation by APC, we tested whether the serine protease inhibitor, aprotinin, was able to inhibit activation. Aprotinin inhibits plasmin, trypsin, and kallikrein but not APC (15). The inhibitor was added to HUVEC in the presence of 100 nM APC for 16–20 h at concentrations of 10 or 25  $\mu$ M. Aprotinin did not inhibit APC-induced gelatinase A activation at either concentration (Fig. 3). This suggests that activation of endothelial gelatinase A is attributable to APC and is not due to plasmin.

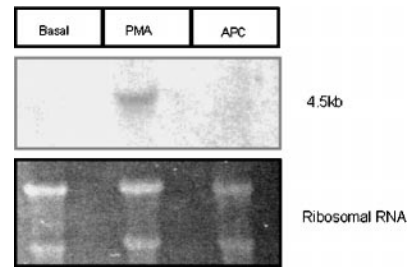
**MT1-MMP Is Not Required for APC-Induced Gelatinase A Activation**—We examined whether the activation by APC was mediated through the well described MT1-MMP pathway. First, we measured the levels of mRNA for MT1-MMP by Northern analysis. HUVEC did not up-regulate MT1-MMP mRNA after stimulation with 100 nM APC for 24 h (Fig. 4). To



**FIG. 2. Time course of gelatinase A activation by APC.** Confluent HUVEC were preincubated in basal medium (Biorich plus 1% normal pooled serum) for 6 h, followed by incubation in fresh basal medium in the presence of 100 nM APC for 4, 12, or 24 h. The conditioned media were assessed for gelatinase A activity by zymography.



**FIG. 3. Effect of 1,10-phenanthroline and aprotinin on gelatinase A activation.** Confluent HUVEC were preincubated in basal medium (Biorich plus 1% normal pooled serum) for 6 h, followed by incubation for 24 h in fresh basal medium in the presence of 100 nM APC alone or with 1,10-phenanthroline (*Phenan*) (10  $\mu$ g/ml) or aprotinin (*Aprot.*) (10 and 25  $\mu$ M). The conditioned medium was collected and measured for gelatinase A using zymography.



**FIG. 4. Northern analysis of MT1-MMP mRNA.** Confluent HUVEC were preincubated in basal medium (Biorich plus 1% normal pooled serum) for 6 h, followed by incubation for 24 h in fresh basal medium in the presence of 100 nM APC, 100 ng/ml phorbol myristate acetate (*PMA*) or no test agent (*Basal*). Total RNA was extracted and hybridized with a  $^{32}$ P-labeled MT1-MMP probe. The 4.5-kilobase (*kb*) transcript represents MT1-MMP. Ribosomal RNA was used to verify equal sample loading.

confirm that MT1-MMP was not involved, we tested the effects of the MMP inhibitor, 1,10-phenanthroline, at 10  $\mu$ g/ml. Previous workers have shown that MT1-MMP-mediated activation of gelatinase A is blocked by 1,10-phenanthroline at this concentration (4). HUVEC were stimulated with APC (100 nM) for 24 h in the presence of 1,10-phenanthroline, and the conditioned medium was analyzed using zymography. Results are shown in Fig. 3. As expected, phenanthroline (shown as *Phenan* in Fig. 3) inhibited the production of the 62-kDa intermediate form, which has previously been shown to be generated by constitutively expressed MT1-MMP (12). In contrast, the generation of the 59-kDa fully active form by APC was not affected by phenanthroline. Together, these results suggested that activation of gelatinase A by APC does not require active MT1-MMP.

**APC Directly Activates Gelatinase A**—To determine whether APC was dependent upon MT1-MMP or another endothelial membrane protein(s), we examined its effect on gelatinase A in the absence of cells. HUVEC-conditioned medium, which contains latent gelatinase A, was incubated in the presence or absence of 100 nM APC for 24 h at 37  $^{\circ}$ C. The samples were then measured for gelatinase A activity using zymography

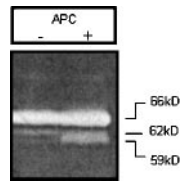


FIG. 5. **Gelatinase A activation by APC in the absence of cells.** Conditioned media were collected from HUVEC, which had been incubated in basal medium (Biorich plus 1% normal pooled serum) for 24 h. The media were then incubated in the absence (–) or presence (+) of 100 nM APC for 24 h at 37 °C. The media were measured for gelatinase A activity using zymography.

(Fig. 5). In the absence of APC, conditioned medium contained an intermediate band and barely detectable fully active band of gelatinase A. In response to APC, the levels of the fully active band were markedly elevated, indicating that APC directly activated gelatinase A and did not require the presence of cells.

**TIMP2 Partially Inhibits APC-induced Gelatinase A Activation**—TIMP2 has several functions, which are independent of its inhibition of MMPs. For example, TIMP2 binds to the C-terminal domain of gelatinase A and at low concentrations promotes activation via MT1-MMP (16, 17). We tested the effect of TIMP2 on APC-induced activation of gelatinase A. TIMP2 (10 or 25 nM) was added to HUVEC in the presence of 100 nM APC for 24 h. The results of zymographic analysis of the conditioned medium are shown in Fig. 6. When used at 10 and 25 nM, TIMP2 completely inhibited the generation of the intermediate form. This observation is in agreement with previous workers who reported that excess TIMP2 inhibits the formation of the 62-kDa intermediate species generated by MT1-MMP (18, 19). Interestingly, at both concentrations, TIMP2 partially prevented the formation of the 59-kDa fully active species generated by APC. At 25 nM, TIMP2 inhibited the fully active form by  $83 \pm 1.7\%$  as determined by scanning densitometry (mean of 3 cell lines). Since we have shown that MT1-MMP is not involved in this process (Figs. 3 and 4), it appears that TIMP2 is playing an independent role during APC-induced gelatinase A activation. It is feasible that excess TIMP2 may interfere with an interaction between APC and gelatinase A (and possibly other molecules) and thus partially reduces activation. The mechanism of TIMP2 inhibition needs to be further explored.

Our report is the first to show that the serine protease, APC, activates gelatinase A. Two other serine proteases, plasmin and thrombin, have previously been shown to activate gelatinase A. In contrast to our results for APC, activation by plasmin is fully inhibited by TIMP2 in HT1080 cells (14). In addition, in the absence of cell membranes, APC activates gelatinase A, whereas plasmin does not activate but rapidly generates degradation products that lack catalytic activity. Thus, it appears that APC works via a different mechanism to plasmin. Zucker *et al.* (20) first reported that thrombin can induce gelatinase A activation in human endothelial cells. We have recently shown that activation of gelatinase A by thrombin is rapid, efficient, and independent of MT1-MMP (4). Similarly, we have shown here that APC generates the fully active form within 4 h and does not require MT1-MMP. Thrombin, through its interaction with thrombomodulin on the endothelial cell surface, is a physiological activator of protein C (21). It is feasible that thrombin-induced activation is mediated through APC. This is currently under investigation.

MT1-MMP has recently been implicated as the key participant in physiological activation of gelatinase A in most cell types, including human endothelial cells (3). HUVEC express MT1-MMP under basal conditions, which can be up-regulated by the potent tumor-promoting chemical, phorbol myristate

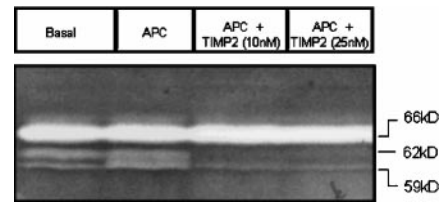


FIG. 6. **Effect of TIMP2 on gelatinase A activation by APC.** Confluent HUVEC were preincubated in basal medium (Biorich plus 1% normal pooled serum) for 6 h, followed by incubation for 24 h in fresh basal medium in the presence of 100 nM APC alone or with TIMP2 (10 and 25 nM). The conditioned medium was collected and measured for gelatinase A using zymography. Scanning densitometry was used to semi-quantitate the activity of the 59-kDa fully active form, and the results are shown as the mean  $\pm$  S.D. from three different HUVEC cell lines.

acetate (3). Surprisingly, there have been no reported physiological agents that activate gelatinase A via MT1-MMP in human endothelial cells. Prior to the current report, thrombin, which acts via a mechanism independent of MT1-MMP (4), was the only known physiological substance that can activate gelatinase A in these cells. The contribution of MT1-MMP, thrombin, or APC in physiological/pathological activation of endothelial gelatinase A is unknown. It is possible that these molecules act synergistically to generate active gelatinase A.

Whereas the biological actions of plasmin and thrombin are multifactorial, APC is thought to be a relatively selective enzyme. In the presence of its cofactor, Protein S, it inactivates Factors Va and VIIIa, which leads to anti-coagulation (6). The reason(s) that this pivotal molecule involved in physiological anti-coagulation activates gelatinase A is unclear. It is unlikely that the active gelatinase A directly contributes to fibrinolysis, as Bini *et al.* (22) have shown that gelatinase A does not cleave fibrin. However, there is ample evidence to show that gelatinase A plays a vital role during angiogenesis. It induces vascular network formation when added to endothelial cells cultured on Matrigel (23). Brooks *et al.* (24) demonstrated that a fragment of the hemopexin-like domain of gelatinase A, termed PEX, significantly disrupted angiogenesis in the CAM system. Itoh *et al.* (25) have recently reported a substantial reduction in both angiogenic activity and tumor progression in gelatinase A-deficient mice. Our finding that APC activates gelatinase A suggests that a link exists between anticoagulation and angiogenesis. This is supported by the recent findings of O'Reilly *et al.* (26) who demonstrated that a cleaved conformation of anti-thrombin III has potent anti-angiogenic activity. They concluded that the clotting and fibrinolytic pathways are directly involved in the regulation of angiogenesis. It is tempting to speculate that APC activates gelatinase A in angiogenic diseases such as cancer and arthritis, where clotting abnormalities are present. The inhibition of APC-induced gelatinase A activation may prove useful as a potential therapeutic target in angiogenic diseases.

**Acknowledgments**—We thank Dr. Paul Basset for providing the cDNA for MT1-MMP, Professor Phillip Sambrook, Dr. Ross Davey, and Dr. Jim Melrose for helpful comments, Amanda Burke for expert technical assistance, and Eddie Jozefiak and Paula Ellis for photography.

#### REFERENCES

1. Basbaum, C. B., and Werb, Z. (1996) *Curr. Opin. Cell Biol.* **8**, 731–738
2. Sato, H., and Seiki, M. (1996) *J. Biochem. (Tokyo)* **119**, 209–215
3. Foda, H. D., George, S., Conner, C., Drews, M., Tompkins, D. C., and Zucker, S. (1996) *Lab. Invest.* **74**, 538–545
4. Nguyen, M., Arkell, J., and Jackson, C. J. (1999) *Lab. Invest.* **79**, 467–476
5. Hass, T. L., Davis, S. J., and Madri, J. A. (1998) *J. Biol. Chem.* **273**, 3604–3610
6. Esmon, C. T. (1999) *FASEB J.* **9**, 946–955
7. Baker, W. F., and Bick, R. L. (1999) *Semin. Thromb. Hemostasis* **25**, 387–405
8. Cicala, C., and Cirino, G. (1998) *Life Sci.* **62**, 1817–1824
9. Jaffe, E. A., Nachman, R. L., Becker, C. G., and Minick, C. R. (1973) *J. Clin. Invest.* **52**, 2745–2756

10. Herron, G. S., Banda, M. J., Clark, E. J., Gavrilovic, J., and Werb, Z. (1986) *J. Biol. Chem.* **261**, 2814–2818
11. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
12. Jackson, C. J., and Nguyen, M. (1997) *Int. J. Biochem. Cell Biol.* **29**, 1167–1177
13. Mazzieri, R., Masiero, L., Zanetta, L., Monea, S., Onisto, M., Garbisa, S., and Mignatti, P. (1997) *EMBO J.* **16**, 2319–2332
14. Baramova, E. N., Bajou, K., Remacle, A., Lhoir, C., Krell, H. W., Weidle, U. H., Noel, A., and Foidart, J. M. (1997) *FEBS Lett.* **405**, 157–162
15. Gebhard, N., Tschesche, H., and Fritz, H. (1986) in *Biochemistry of Aprotinin and Aprotinin-like Inhibitors in Protease Inhibitors* (Barrett, A. J., and Salvesen, G., eds) pp. 375–388, Elsevier Science Publishers B.V., Amsterdam
16. Zucker, S., Drews, M., Conner, C., Foda, H. D., DeClerck, Y. A., Langley, K. E., Bahou, W. F., Docherty, A. P., and Cao, J. (1998) *J. Biol. Chem.* **273**, 1216–1222
17. Cao, J., Drews, M., Lee, H. M., Conner, C., Bahou, W. F., and Zucker, S. (1998) *J. Biol. Chem.* **273**, 34745–34752
18. Kinoshita, T., Sato, H., Okada, A., Ohuchi, E., Imai, K., Okada, Y., and Seiki, M. (1998) *J. Biol. Chem.* **273**, 16098–16103
19. Will, H., Atkinson, S. J., Butler, G. S., Smith, B., and Murphy, G. (1996) *J. Biol. Chem.* **271**, 17119–17123
20. Zucker, S., Conner, C., Dimasmo, B. I., Ende, H., Drews, M., Seiki, M., and Bahou, W. F. (1995) *J. Biol. Chem.* **270**, 23730–23738
21. Esmon, C. T., Fukudome, K., Mather, T., Bode, W., Regan, L. M., Stearns-Kurosawa, D. J., and Kurosawa, S. (1999) *Haematologica* **84**, 254–259
22. Bini, A., Itoh, Y., Kudryk, B. J., and Nagase, H. (1996) *Biochemistry* **35**, 13056–13063
23. Schnaper, H. W., Grant, D. S., Stetlerstevenson, W. G., Fridman, R., Dorazi, G., Murphy, A. N., Bird, R. E., Hoythya, M., Fuerst, T. R., French, D. L., Quigley, J. P., and Kleinman, H. K. (1993) *J. Cell. Physiol.* **156**, 235–246
24. Brooks, P. C., Silletti, S., Schalscha, T., Friedlander, M., and Cheresch, D. A. (1998) *Cell* **92**, 391–400
25. Itoh, T., Tanioka, M., Yoshida, H., Yoshioka, T., Nishimoto, H., and Itoharu, S. (1998) *Cancer Res.* **58**, 1048–1051
26. O'Reilly, M. S., Pirie-Shepherd, S., Lane, W. S., and Folkman, J. (1999) *Science* **285**, 1926–1928