Activated Protein C Decreases Plasminogen Activator–Inhibitor Activity in Endothelial Cell-Conditioned Medium

By V.W.M. van Hinsbergh, R.M. Bertina, A. van Wijngaarden, N.H. van Tilburg, J.J. Emeis, and F. Haverkate

Confluent cultures of endothelial cells from human umbilical cord were used to study the effect of activated human protein C (APC) on the production of plasminogen activators, plasminogen activator–inhibitor, and factor VIII–related antigen. Addition of APC to the cells in a serum-free medium did not affect the production of tissue-type plasminogen activator (t-PA) or factor VIII–related antigen; under all measured conditions, no urokinase activity was found. However, less plasminogen activator–inhibitor activity accumulated in the conditioned medium in the presence of APC. This decrease was dose dependent and could be prevented by specific anti-protein C antibodies. No decrease was observed with the zymogen protein C or with diisopropylfluorophosphate-inactivated APC. APC also decreased the t-PA inhibitor activity in endothelial cell-conditioned medium in the absence of cells, which suggests that the effect of APC is at least partly due to a direct effect of APC on the plasminogen activator–inhibitor. High concentrations of thrombin—that not of factor Xa or IXa—had a similar effect on the t-PA inhibitor activity. The effect of APC on the plasminogen activator–inhibitor provides a new mechanism by which APC may enhance fibrinolysis. The data suggest that activation of the coagulation system may lead to a secondary increase of the fibrinolytic activity by changing the balance between plasminogen activator(s) and its (their) fast-acting inhibitor.

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PROTEIN C is the zymogen of a vitamin K-dependent serine protease, present in human plasma. A congenital deficiency of protein C is associated with recurrent thromboembolic disease in heterozygotes. Activated protein C (APC), which is identical to autoprothrombin II, markedly retards the coagulation process. It prolongs the partial thromboplastin time, probably by inactivation of the coagulation factors Va and VIIa. A role of APC in fibrinolysis has also been suggested. APC shortens the clot lysis time in vitro. In dogs, but not in monkeys, plasma plasminogen activator activity has been reported to be increased after the intravenous administration of APC. However, the precise role of APC in the regulation of fibrinolysis still is not understood.

Human endothelial cells produce tissue-type plasminogen activator (t-PA) and, according to Booyse et al., also urokinase-type plasminogen activator (u-PA). However, the latter finding was not confirmed by other investigators. In conditioned medium from cultured human endothelial cells, t-PA is present as an inactive t-PA-inhibitor complex. This complex probably has been formed by reaction of t-PA with the fast-acting inhibitor of plasminogen activator, which we and others recently identified in conditioned media of endothelial cells. An APC-dependent increase in plasminogen activator activity, as reported by Comp and Esmon, thus might be the result of either an increase of plasminogen activator concentration or a decrease in the concentration of its specific inhibitor or both.

The present article describes the influence of human APC on the production of fibrinolytic proteins by cultured human endothelial cells. Because thrombin, a related serine protease, induces release of factor VIII–related antigen by endothelial cells, we also investigated the effect of APC on the release of factor VIII-related antigen by endothelial cells. APC decreased the amount of plasminogen activator inhibitor, whereas no changes in secretion of t-PA and factor VIII-related antigen were observed.

MATERIALS AND METHODS

Endothelial cell growth factor (ECGF) was extracted from bovine hypothalamus as described by Maciag et al. Bovine serum albumin was purchased from Prowit (Organon-Teknika, The Netherlands); urokinase was purchased from Leo Pharmaceuticals (Copenhagen). Human plasmin was purified from Cohn fraction III by lysine-Sepharose chromatography. Human plasmin (prepared by activation of plasminogen with immobilized urokinase) was prepared by Dr D.W. Traas (Gaubius Institute TNO, Leiden, The Netherlands). Soluble fibrinogen digest was prepared as previously described. Two-chain t-PA was purified from supernatant of Bowe's melanoma cells. Anti-protein C IgG was prepared from rabbit antiserum raised against purified human protein C. Anti-t-PA IgG and anti-urokinase IgG were prepared by Drs D. Rijken and G. Wijngaards (Gaubius Institute TNO). Tween 80 was purchased from Sigma Chemical Co (St Louis), H-pyro-Glu-Pro-Arg-pNA (5-2337), and b-Ile-Glu(Pip)-Glu-Arg-pNA (S-2366) were purchased from Kabi (Stockholm). M-199 medium (Earle's base) supplemented with 20 mmol/L HEPES was obtained from Flow Laboratories (Irvine, Scotland).

Isolation of Coagulation Factors

Human protein C and human thrombin were isolated as previously described. The concentration of protein C was estimated.
After addition of anti-protein C IgG (0.26 mg/mL), no APC activity was recovered by use of the chromogenic assay throughout the incubation period. From the E280 after correction for Rayleigh scattering, APC preparations contained 0.17% of purified APC and the anticoagulant activity in an assay, using an E = 14.5 and a molecular weight of 62,000.

Activated Protein C

APC was prepared by activation of human protein C (4.8 μmol/L) with 1 μmol/L human thrombin for one hour at 37 °C. After completion of the activation—as estimated from the APC-associated amidolytic activity toward S-2366—the reaction was stopped by the addition of 20 mmol/L benzamidin. APC was separated from thrombin by chromatography on DEAE-Sephadex and stored at −20 °C in 50% glycerol. The final preparation (nonreduced) migrated as one band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). APC preparations contained ≤ 0.17% (mol/mol) thrombin and no detectable factor Xa (as measured with the specific chromogenic substrate S-2366). When added to normal plasma, the APC preparation greatly prolonged the activated partial thromboplastin time (cf. 1). APC activity was estimated from its amidolytic activity toward S-2366 in a buffer containing 25 mmol TRIS-HCl (pH 8.3), 150 mmol/L NaCl, 0.02% ovalbumin, and 0.8 mmol/L S-2366. The amidolytic activity of APC toward S-2366 was insensitive to antithrombin III-heparin or hirudin. Two APC batches were used in this study. The experiments described in Table 1 and Figs 1 and 2 were done with one APC preparation. A second preparation of human APC was used for the experiments shown in Figs 3 to 6 and Table 2.

DIP-APC

Diisopropylfluorophosphate-inactivated (DIP)-APC was prepared by incubation of purified APC with 5 mmol/L diisopropylfluorophosphate for three hours and subsequent dialysis against 25 mmol/L TRIS-HCl, 50 mmol/L NaCl, and 50% glycerol (pH 7.5). Residual APC activity, as measured from the amidolytic activity toward S-2366 and the anticoagulant activity in an APTT assay, was less than 1%. Molar concentrations of APC and DIP-APC were calculated from the E360 after correction for Rayleigh scattering using an E360 = 14.5 and a molecular weight of 62,000.

Radiolabeled APC

125 I-APC was prepared by iodination of purified APC using the iodogen method. Excess free 125 I was removed by extensive dialysis.

Recovery of functional activity, as evaluated by an APTT assay, was > 60%. The partial loss of activity was associated with a partial reduction of the APC, as apparent from the iodination heavy chain after SDS-PAGE of nonreduced 125 I-APC (specific activity 0.3 × 106 dpm/nmol).

Endothelial Cells

Endothelial cells from human umbilical cord arteries and veins were isolated by the method of Jaffe et al and cultured as previously described. Cells were grown on fibronectin-coated T25 flasks (Costar, Cambridge, Mass) in M-199 medium supplemented with 20% human serum (not heat-inactivated), 200 μg/mL ECGF, 100 IU/mL penicillin, and 100 μg/mL streptomycin under 5% CO2/95% air atmosphere. Confluent cultures (0.5 to 0.7 mg cell protein per 25-cm² flask), obtained after two passages, were washed by Laurell assay, where 1 U equals the amount of protein C antigen in 1 mL of pooled normal plasma (64 pmol). Human factor IXa and factor Xα were prepared as previously described. Concentrations of these proteins were determined by active site titration.

Table 1. Effect of APC, Protein C, and DIP-APC on the Accumulation of Plasminogen Activator and Its Inhibitor in ECCM

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Condition</th>
<th>t-PA Antigen (pmol/mL)</th>
<th>t-PA Inhibitor Activity (pmol/mL)</th>
<th>u-PA Inhibitor Activity (pmol/mL)</th>
<th>Factor VIII-Related Antigen (mU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Control</td>
<td>0.02</td>
<td>0.83</td>
<td>0.72</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>+ anti-protein C IgG</td>
<td>0.04</td>
<td>1.02</td>
<td>1.02</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td>+ APC (60 nmol/L)</td>
<td>0.03</td>
<td>0.58</td>
<td>0.50</td>
<td>10.4</td>
</tr>
<tr>
<td></td>
<td>+ APC + anti-protein C IgG</td>
<td>0.03</td>
<td>0.58</td>
<td>0.50</td>
<td>10.4</td>
</tr>
<tr>
<td>2.</td>
<td>Control</td>
<td>0.19</td>
<td>0.26</td>
<td>ND</td>
<td>14.1</td>
</tr>
<tr>
<td></td>
<td>+ APC (67 nmol/L)</td>
<td>0.24</td>
<td>0.17</td>
<td>ND</td>
<td>13.2</td>
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<tr>
<td></td>
<td>+ protein C (64 nmol/L)</td>
<td>0.22</td>
<td>0.28</td>
<td>ND</td>
<td>14.5</td>
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</tbody>
</table>

APC, activated human protein C; DIP-APC, diisopropylfluorophosphate-inactivated-APC; ECCM, endothelial cell-conditioned medium; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; ND, not determined.
twice with prewarmed serum-free M-199 medium and then incubated at 37 °C under 5% CO₂/95% air atmosphere in 1.0 mL incubation medium, which consisted of 0.8 mL M-199 medium supplemented with penicillin/streptomycin and 0.2 mL of a buffer containing 150 mmol/L NaCl, 10 mmol/L Na₂HPO₄, 1.5 mmol/L KH₂PO₄ (pH 7.4), 0.5% bovine serum albumin, and the amount of APC indicated. No morphologic changes were observed during the incubation period. Endothelial cell-conditioned medium (ECCM) was centrifuged at 4 °C for two minutes at 10,000 g and stored at -20 °C.

Assays of Plasminogen Activator Activity

Plasminogen activator activity in the ECCM was assayed by the following two methods:

1. Quantitatively, in a two-step spectrophotometric assay. In brief, the following reagents were added to wells of a 96-well titertek plate: 65 μL of 0.10 mol/L TRIS-HCl (pH 7.5) containing 0.10% Tween 80, 20 μL of soluble fibrin digest (1 mg/mL), 40 μL of ECCM, 100 μL of 2251 (0.66 mmol/L), and 25 μL of plasminogen (1.11 mmol/L). The microtiter plates were incubated at 37 °C, and the absorption at 405 nm was read in a Titertek Multiscan spectrophotometer (Flow Laboratories, Irvine, Scotland) after 60, 120, and 180 minutes. Plasminogen activator activity was calculated according to Drapier et al. 2

2. Qualitatively, plasminogen activator activity was detected by a fibrin autograph technique after 8% SDS-PAGE of the ECCM. After electrophoresis, gels were washed for five minutes in 100 mmol/L NaCl and 50 mmol/L TRIS-HCl (pH 7.7), then washed.
Inhibition of Plasminogen Activator Activity

Inhibition of plasminogen activator activity was measured by a modification of the quantitative assay for plasminogen activator activity as described previously. In the assay mixture, 3 μL of t-PA (7 IU/mL) or 5 μL urokinase (21 IU/mL) also were present, together with varying amounts (usually 1 to 10 μL) of ECCM. The amount of t-PA or urokinase inhibition was calculated from the volume of ECCM that gave 50% inhibition of the added plasminogen activator activity and was expressed as IU of t-PA or u-PA activity neutralized by ECCM. For comparison of urokinase inhibition and t-PA inhibition values, units of activity were converted into molar concentrations (two-chain t-PA: mol wt 70,000, sp act 50,000 IU/mg; Dr J. Verheyen, Gauibius Institute, TNO, personal communication): urokinase: mol wt 35,000, sp act 218,000 IU/mg. The intraassay variation of this assay was 5% to 7%, and the interassay variation coefficient was 10%.

Inhibition of Plasmin Activity

Inhibition of plasmin activity by ECCM was measured as described above for the quantitative assay of plasminogen activator

| Table 2. Effect of Various Serine Proteases on t-PA Inhibitor Activity |
|----------------------------------|------------------|------------------|
| Addition | HUAEC (pmol/mL) | HUVEC (pmol/mL) |
| None | 0.32 | 1.11 |
| APC | 0.14 | 0.54 |
| Factor IXa | 0.35 | 1.03 |
| Factor Xa | 0.24 | 0.93 |
| Thrombin | 0.12 | 0.37 |
| Thrombin + hirudin | 0.32 | 1.09 |

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ml of pooled normal human plasma. The intraassay variation of this assay is 5%, and the interassay coefficient of variation is 10%.

RESULTS

Confluent endothelial cells from human umbilical cord artery and vein were incubated in serum-free medium. No production of plasminogen-activator activity was observed with the two-step spectrophotometric assay. Significant t-PA inhibition and urokinase inhibition could be demonstrated in the supernatant fluid (controls in Fig 1A, B; Table 1). No plasmin inhibition was found. After incubation of the ECCM with excess t-PA, the inhibition of urokinase had disappeared, whereas after incubation with excess urokinase, no inhibition of t-PA could be observed (not shown). This indicates that the same inhibitor reacts with both t-PA and urokinase. This is further suggested by the parallel changes in t-PA and urokinase inhibition (Table 1).

Because no plasminogen activator activity could be detected in ECCM, we investigated whether t-PA antigen could be detected with an enzyme immunoassay. Figure 1C demonstrates that there is production of t-PA antigen and that this production is linear with time. It was found that t-PA inhibitor is present in a twofold to 20-fold molar excess over t-PA antigen (Fig 1, Table 1). This explains why no plasminogen-activator activity was found in the untreated culture supernatants. However, some plasminogen-activator activity could be detected after treatment of the endothelial cell–conditioned medium with SDS. SDS-PAGE and fibrin autography revealed that this plasminogen-activator activity is associated with a protein with a molecular weight of 95,000 daltons (Fig 2A, lane 2), which is about 30,000 daltons larger than that of t-PA (Fig 2A, lane 1). This activity was inhibited by the inclusion of specific anti–t-PA IgG in the fibrin layer (Fig 2B), while anti-urokinase IgG had no effect (Fig 2C). Thus far, we have been unable to demonstrate urokinaselike activity in the conditioned media from human umbilical cord endothelial cells.

The production of t-PA antigen, t-PA–inhibitor activity, urokinase-inhibitor activity, and factor VIII–related antigen by endothelial cells was linear with time (Fig 1). Incubation of endothelial cells with serum-free medium containing increasing amounts of APC had no effect on the production of t-PA antigen and factor VIII–related antigen (Fig 1). No evidence for the induction by APC of urokinase activity was found (Fig 2A, B, lane 3). Incorporation of 35S-methionine into secreted proteins was also unchanged after addition of APC (1,204 ± 1,173 cpm/μg protein), demonstrating that protein synthesis was not influenced. However, APC decreased the activity of t-PA inhibitor and urokinase inhibitor in the endothelial cell–conditioned medium, especially after prolonged incubation (Fig 1). When five endothelial cell cultures from different umbilical cords were incubated for four hours with 0.06 μmol/L APC, t-PA inhibition and urokinase inhibition were 66% ± 13% and 65% ± 11% of the values obtained in the control incubations (mean ± SD). This difference from control incubations was highly significant (P < .01, Student’s t-test for paired data).

The effect of APC on t-PA and urokinase inhibition was due exclusively to the added APC, since pretreatment of the APC with specific anti-protein C IgG prevented this effect (Table 1). The proteolytic active site is required for this effect, since the zymogen protein C did not affect the production of the inhibition of t-PA (Table 1). Furthermore, 0.10 μmol/L DIP-APC did not decrease the production of t-PA inhibition (0.60 pmol t-PA inhibited/mL ECCM ± 0.65 pmol t-PA inhibited per milliliter of ECCM in control incubations).

To assess whether a change in endothelial cell metabolism is required for the decrease of t-PA–inhibitor activity by APC, endothelial cell–conditioned media obtained from control incubations were incubated with APC in the absence of cells for various time intervals. Without APC present, the t-PA–inhibitor activity decreased spontaneously with a half-life of about three hours (Fig 3). In the presence of 0.06 μmol/L APC, the half-life of the t-PA–inhibitor activity was considerably shortened. The degree of inactivation of the t-PA inhibitor was dependent on the concentration of APC (Fig 4). The observation that 0.10 μmol/L DIP-APC had no effect on the half-life of the t-PA inhibitor (Fig 4) again indicates the involvement of the active site of APC. Hirudin, which specifically inhibits thrombin and not APC, did not change the effect of APC on the t-PA inhibitor (not shown). These data indicate that, APC also decreases the t-PA–inhibitor activity in the absence of endothelial cells.

One of the explanations for the inhibitory action of APC on the t-PA inhibitor activity might be the slow formation of a stable inactive complex between the inhibitor and APC. To study this possibility, 58 nmol/L of 125I-APC was incubated with ECCM and control medium at 37 °C. After 1/2 hours, samples were taken and analyzed by SDS-PAGE (nonreduced samples) and subsequent autoradiography (Fig 5). Incubation of the 125I-APC with ECCM did not result in an increase in radiolabeled high molecular weight complexes when analyzed by autoradiography or by counting the radioactivity of gel slices of the various lanes (not shown). These experiments indicated that after incubation with ECCM, less than 1.5% of the
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APC was bound to other proteins. When 125I-APC was added to human serum under similar conditions, increasing amounts of radiolabeled complex (mol wt 100,000 daltons) could be demonstrated concomitantly with decreasing amounts of radiolabeled APC.

To evaluate whether the inactivation of t-PA–inhibitor activity by APC is specific for this protease, ECCM was incubated for 1/2 hours at 37 °C with several other related proteases (factor IXa, factor Xa, and thrombin). The concentration of t-PA inhibitor was assayed by adding various amounts of t-PA to 10 μL- aliquots of the incubation mixture and analyzing residual t-PA activity with a spectrophotometric assay (Fig 6). Factor IXa (60 nmol/L) had no effect on the t-PA–inhibitor activity, whereas factor Xa (60 nmol/L) and thrombin (6 nmol/L) caused a slight decrease; however, 60 nmol/L thrombin appeared to be equally effective as APC in the inactivation of the t-PA–inhibitor activity. The effect of thrombin, in contrast with that of APC, was sensitive to hirudin. When control culture medium was used instead of ECCM, 60 nmol/L of APC or thrombin had no effect on the activity of added t-PA.

DISCUSSION

Endothelial cells from human umbilical cord arteries and veins concomitantly produce t-PA and a component that inhibits both t-PA and urokinase activity. In ECCM, t-PA–inhibitor activity is present in an excess amount in comparison with the amount of plasminogen activator(s). The t-PA activity in ECCM is found as a molecular weight of about 100,000 daltons, probably representing a t-PA–inhibitor complex. A similar fast-acting t-PA–inhibitor activity has been found in human plasma. Its concentration in plasma is about 0.2 nmol/L, which is 500-fold lower than that of the plasma APC inhibitor.

The amount of t-PA or urokinase inhibition present in endothelial cell–conditioned medium was decreased after incubation of the cells in the presence of APC. This decrease in inhibition is not caused by an increased secretion of plasminogen activator, because the release of t-PA antigen was not influenced by APC, and induction of urokinase activity could not be detected. Under our experimental conditions, APC had no effect on the release of factor VIII-related antigen or on protein synthesis in general. Thus, it might be possible that APC affected the production of t-PA inhibitor by endothelial cells. However, APC also decreased the t-PA–inhibitor activity in the absence of endothelial cells, indicating that the effect of APC is at least partly due to a direct effect of APC on the plasminogen activator–inhibitor. This direct effect on the t-PA inhibitor was also found when APC was replaced by thrombin, but not with factor Xa or factor IXa. Low concentrations of thrombin (6 nmol/L) were not effective in this respect (Fig 6).

Because relatively high concentrations of APC were used, it is important to exclude contributions of contaminants, especially thrombin, which might be present in the APC preparation. Based on the following results, we conclude that the effect of APC on the t-PA inhibitor is a specific effect of APC: (1) The thrombin contamination in the APC preparation (as evaluated in a clotting assay using purified human fibrinogen) is less than 0.12%. Thus, thrombin contamination is less than 0.06 nmol/L in the experiment of Fig 6, whereas when 6 nmol/L thrombin was added to ECCM it did not markedly decrease the t-PA–inhibitor activity. (2) The effect of APC was blocked by preincubation with anti-protein C IgG. (3) The effect of APC was found to be insensitive to hirudin. The effect of 60 nmol/L thrombin on the t-PA–inhibitor activity, however, was prevented by hirudin.

The effects of thrombin and APC on the t-PA–inhibitor activity can be due either to proteolytic degradation or to the (slow) formation of stable inactive complexes. The proteolytic active site clearly is involved, since neither DIP-APC nor the zymogen protein C have any effect. However, this does not exclude complex formation, because blockage of the active site with diisopropylfluorophosphate also can prevent binding to the inhibitor (cf DIP-t-PA and t-PA inhibitor). Attempts to demonstrate the production of a stable APC-t-PA inhibitor complex by incubation of ECCM with radiolabeled APC and subsequent SDS-PAGE failed. Using the same technical approach, however, we were able to demonstrate the formation of the APC-inhibitor complex in serum (Fig 5). If the decrease in t-PA–inhibitor activity had been completely due to complex formation with APC (1:1 complex), 2.4% of the 125I-APC would have migrated as a high molecular weight complex. This is only slightly above the detection limit (1.5%). Better resolution could not be obtained because of the low concentration of t-PA–inhibitor activity in ECCM, which is 20- to 60-fold less than the added amount of APC. The formation of reversible APC-t-PA–inhibitor complexes is rather unlikely, because of the equal slopes of the t-PA titration curves of APC-treated media and control media (Fig 6).

In the alternative explanation, where APC inactivates the t-PA inhibitor by proteolytic cleavage, an explanation is needed for the extremely slow rate of inactivation (0.00014 mol inhibitor inactivated per mol APC per minute; Fig 3). First, we have to consider that APC was studied under rather unfavorable conditions: low Ca²⁺ concentration, no added phospholipids, no
protein cofactors (protein S\textsuperscript{42}), and a low substrate (t-PA inhibitor) concentration. Second, the aforementioned rate of t-PA–inhibitor inactivation is rather similar to those reported for factor Xa and IXa under similarly unfavorable conditions.\textsuperscript{43,44} At present, we favor the model in which APC inactivates t-PA–inhibitor activity by proteolytic degradation; however, final conclusions can only be made on the basis of experiments with a purified t-PA inhibitor.

The inhibitory effect of APC on the t-PA–inhibitor activity provides a new mechanism by which APC can enhance fibrinolysis. At present, it is not clear to what extent the reported effects of APC on clot lysis time\textsuperscript{5,8} and plasminogen–activator activity in dogs\textsuperscript{9} can be explained by this effect of APC on the t-PA inhibitor. Under our experimental conditions, APC had no effects on t-PA release from cultured endothelial cells. It cannot be excluded, however, that other, still unknown mediators are involved in the interaction between APC and endothelial cells. To our knowledge, the present study provides the first evidence that activation of the coagulation system might lead to a secondary activation of the fibrinolytic system by changing the balance between plasminogen activator(s) and its (their) fast-acting inhibitor(s). This regulatory mechanism might be even more pronounced in vivo, where the t-PA–inhibitor activity in plasma only slightly exceeds the amount of t-PA antigen.\textsuperscript{45}

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**ACKNOWLEDGMENT**

We thank Dr D. Rijken for stimulating discussion and assay of t-PA antigen, and C.M. van den Hoogen, M. Scheffer, and M.M. Barrett for excellent technical assistance. We also thank the co-workers of the Department of Gynaecology, St Elisabeth Hospital, Leiderdorp (headed by Dr H.A.L.M. Mudde) for regularly providing us with human umbilical cords.
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