Polar Secretion of Endothelin-1 by Cultured Endothelial Cells*

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The aim of this study was to determine the permeability of endothelial monolayers for endothelin-1 and a possible directionality of the endothelin-1 secretion process. Human umbilical vein endothelial cells were cultured on acellular amniotic membranes, dividing the tissue culture wells into an apical (luminal) and a basolateral (abluminal) compartment. Whereas in the absence of endothelial monolayers 44.9 ± 2.3 and 43.5 ± 2.0% of the unilaterally added endothelin-1 permeated from the apical to the basolateral side and from the basolateral to the apical side, respectively, only 6.5 ± 0.6 and 6.6 ± 0.4% diffused in the presence of endothelial cells. Analyzing endothelin-1 secretion, ~80% of the total amount of synthesized endothelin-1 was found in the basolateral compartment; thrombin (10 units/ml) stimulated the production of endothelin-1 ~2-fold, but did not change the relative distribution of endothelin-1 between the apical and basolateral compartments. In the presence of dexamethasone (10^{-7} M), a decrease in the level of endothelin-1 was found in the apical compartment, whereas the total amount of endothelin-1 produced was not affected. Dexamethasone did not influence the permeability of human umbilical vein endothelial cell monolayers for endothelin-1. These results strongly support the hypothesis that endothelin-1 is a local paracrine regulator of vasotone.

Endothelin-1 is a potent vasoconstrictor peptide isolated originally from the supernatant of porcine aortic endothelial cells (1). Comparing the sites of endothelin-1 messenger RNA production localized by in situ hybridization and exogenous 125I-endothelin-1 binding in rat tissues, it was reported that synthesis of messenger RNA occurs in close proximity to the binding sites of endothelin-1 in many organs, e.g. lung, kidney, intestine, and eye (2). This suggests that endothelin-1 acts locally in a paracrine fashion mediated by endothelin-1 receptors expressed closely to the sites of endothelin-1 production in these organs. However, in other organs, e.g. heart and renal cortex, endothelin-1-binding sites are present in the absence of endothelin-1 mRNA, indicating a humoral mode of endothelin-1 action (2). This key question concerning the role of endothelin-1 in cardiovascular control (paracrine versus endocrine mode of action) nevertheless is still in debate. A predominantly paracrine mode of action of endothelin-1 would imply a polarized secretion pattern; in fact, in 1989, Masaki (3) speculated that endothelial cells may release endothelin-1 predominantly into a paracrine fashion. In vitro experiments with endothelial cells grown to confluence on amniotic membranes were designed to test this hypothesis (4). The endothelial origin of the cultured cells was confirmed by typical cobblestone morphology (5), immunofluorescence staining with anti-von Willebrand factor antibodies (6), and uptake of acetylated low density lipoprotein (7). HUVECs from passage 2 were seeded at a high density onto acellular amniotic membranes prepared as described previously (8). Pieces of human amniotic tissue were fastened to Teflon rings, and the amniotic epithelium was lysed by incubation in 0.25 M NH4OH for 2 h. Teflon rings holding the membranes were placed into tissue culture wells. HUVECs were plated on the stromal surface of the prepared amniotic tissue and were grown to confluent monolayers. HUVECs reached confluence ~2-3 days after seeding. The endothelial monolayer grown on the amniotic membranes thus divided the culture wells into two compartments, an upper one (1 ml) on the apical side and a lower one (2 ml) on the basolateral side, thereby imitating in vivo conditions with a luminal and an abluminal side.

Acute Determination of Permeability of HUVEC Monolayers for Endothelin-1—
For analysis of permeability of endothelial cell monolayers for endothelin-1, confluent monolayers of HUVECs grown to confluence on amniotic membranes were rinsed twice with Hanks' balanced salt solution. Thereafter, the cells were incubated in Medium 199 with Earle's salts to which 125I-endothelin-1 (1145 Ci/mmol, Peninsula Laboratories, Inc.) was added on the apical or the basolateral side. HUVECs grown to confluence on amniotic membranes were rinsed twice with Hanks' balanced salt solution. Thereafter, the cells were incubated in Medium 199 with Earle's salts to which 125I-endothelin-1 (1145 Ci/mmol, Peninsula Laboratories, Inc.) was added on the apical or the basolateral side. The conditioned media from either side of the membrane were collected, and the activity was counted.

Hirayama (4) speculated that endothelial cells may release endothelin-1 in a polar fashion directed toward the underlying intimal smooth muscle. This hypothesis, however, has so far not been tested. The aim of this investigation therefore was to determine whether endothelin-1 produced by cultured endothelial cells is released preferentially toward one side. We found that HUVECs grown to confluent monolayers on amniotic membranes release endothelin-1 predominantly into the basolateral compartment.

EXPERIMENTAL PROCEDURES

Cell Culture—HUVECs were isolated essentially as described previously (4). The endothelial origia of the cultured cells was confirmed by typical cobblestone morphology (5), immunofluorescence staining with anti-von Willebrand factor antibodies (6), and uptake of acetylated low density lipoprotein (7). HUVECs from passage 2 were seeded at a high density onto amniotic membranes prepared as described previously (8). Pieces of human amniotic tissue were fastened to Teflon rings, and the amniotic epithelium was lysed by incubation in 0.25 M NH4OH for 2 h. Teflon rings holding the membranes were placed into tissue culture wells. HUVECs were plated on the stromal surface of the prepared amniotic tissue and were grown to confluent monolayers. HUVECs reached confluence ~2-3 days after seeding. The endothelial monolayer grown on the amniotic membranes thus divided the culture wells into two compartments, an upper one (1 ml) on the apical side and a lower one (2 ml) on the basolateral side, thereby imitating in vivo conditions with a luminal and an abluminal side.

Analysis of Permeability of HUVEC Monolayers for Endothelin-1—
For analysis of permeability of endothelial cell monolayers for endothelin-1, confluent monolayers of HUVECs grown to confluence on amniotic membranes were rinsed twice with Hanks' balanced salt solution. Thereafter, the cells were incubated in Medium 199 with Earle's salts to which 125I-endothelin-1 (1145 Ci/mmol, Peninsula Laboratories, Inc.) was added on the apical or the basolateral side. HUVECs grown to confluence on amniotic membranes were rinsed twice with Hanks' balanced salt solution. Thereafter, the cells were incubated in Medium 199 with Earle's salts to which 125I-endothelin-1 (1145 Ci/mmol, Peninsula Laboratories, Inc.) was added on the apical or the basolateral side. The conditioned media from either side of the membrane were collected, and the activity was counted.
RESULTS

Permeability of HUVEC Monolayers for Endothelin-1—Monolayers of HUVECs grown on amniotic tissue for 4–5 days prior to the experiments were used to examine the diffusion of endothelin-1. $^{125}$I-Endothelin-1 was added to the upper or the lower chamber at a concentration of 0.1 nM (1145 Ci/mmol) at 37 °C, and the amount of $^{125}$I-endothelin-1 was determined in the lower or the upper chamber after 1, 2, 3, and 4 h (Fig. 1, A and B). The diffusion of $^{125}$I-endothelin-1 was linear over the whole period of time, and there was no difference in regard to the diffusion from the upper to the lower chamber or from the lower to the upper chamber. Thus, within the first hour of incubation, 6.5 ± 0.6% of the added $^{125}$I-endothelin-1 diffused from the upper to the lower compartment as compared to 6.6 ± 0.4% in the reverse direction. In the absence of endothelial cells, 44.9 ± 2.3% of the total amount of $^{125}$I-endothelin-1 applied diffused from the apical to the basolateral side as compared to 43.5 ± 2.0% from the basolateral to the apical side within the first hour. Thus, almost complete equilibrium of concentrations was achieved within 2 h. The addition of cold endothelin-1 (1 μM) did not influence the diffusion of $^{125}$I-endothelin-1 (6.7 ± 1.0% transfer from the apical to the basolateral side and 6.3 ± 0.7% from the basolateral to the apical side within the first hour of incubation). Neither thrombin (10 units/ml) nor dexamethasone ($10^{-7}$ M) had any effect (Fig. 1, A and B). Recovery of $^{125}$I-endothelin-1 in either experiment was >95%.

Polarity of Secretion of Endothelin-1 by HUVEC Monolayers—To investigate whether endothelin-1 secretion proceeds in a polar fashion, HUVECs were grown to confluent monolayers on amniotic membranes. After 1, 2, 3, and 4 h of incubation, the conditioned media from the upper and the lower compartments were collected separately. Under basal conditions, a total amount of ~5.2 fmol of endothelin-1 was produced by 10$^5$ cells within 1 h. The increase in the total amount of endothelin-1 over time was linear for the whole observation period of 4 h. Approximately 80% of the overall amount of synthesized endothelin-1 was secreted into the lower (basolateral) compartment. The relative distribution of endothelin-1 between the upper and the lower compartments did not change over the observation time (Fig. 2).

When thrombin (10 units/ml) was added to the culture medium, an exponential increase in the total amount of endothelin-1 produced over time was observed (Fig. 2). After an incubation period with thrombin of 4 h, an ~2-fold increase in the level of endothelin-1 was found. The relative distribution of endothelin-1 between the upper and the lower compartments did not change over time and was not different from basal conditions. Again, ~80% of the synthesized endothelin-1 was found in the basolateral (abluminal) compartment at each time point (Fig. 2).

When HUVECs were incubated with dexamethasone ($10^{-7}$ M), the total amount of endothelin-1 produced was equal to control conditions at every time point (Fig. 2). In the presence of dexamethasone, only 5.6 ± 1.3 fmol of endothelin was found in the upper compartment after 4 h of incubation as compared to 10.4 ± 2.1 fmol in the absence of dexamethasone ($p < 0.05$). In the basolateral compartment, 46.8 ± 2.8 and 41.7 ± 5.4 fmol of endothelin-1 were found in the presence and absence of dexamethasone, respectively. As described above, dexamethasone had no effect on the diffusion of endothelin-1.

DISCUSSION

Since the first description of endothelin-1 (1), considerable attention has been focused on plasma concentrations of endothelin-1 in both healthy and diseased humans as well as on the pharmacological effects of the peptide. It was reported that endothelin-1 circulates in human plasma (9, 12) and is found to be increased in plasma under a variety of pathological circumstances including essential hypertension (13), uremia (14), and cardiogenic shock (15). Endothelin-1 exerts a vasoconstrictor potential in vivo when applied systemically in several species (1) including humans (10). However, because endothelin-1 may act locally on the underlying smooth muscle rather than as a circulating hormone and plasma endothelin-1 may represent merely the overflow of a local paracrine peptide system, the pathophysiological importance of these

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**Fig. 1.** Transfer of $^{125}$I-endothelin-1 across HUVEC monolayers. HUVECs were grown to confluent monolayers on prepared acellular amniotic membranes, thus dividing the culture well into an apical and a basolateral compartment. $^{125}$I-Endothelin-1 (1145 Ci/mmol) was added to the apical (A) or the basolateral (B) side at a final concentration of 0.1 nM. Aliquots of either compartment (30 μl) were collected at several time points; the activity was counted on a γ-counter; and the transfer of $^{125}$I-endothelin-1 through the endothelial monolayer was determined (○). The transfer of $^{125}$I-endothelin-1 in the presence of human thrombin (10 units/ml) (□) and dexamethasone ($10^{-7}$ M) (△) was studied in an analogous fashion. Alternatively, the transfer of $^{125}$I endothelin-1 through the membranes in the absence of endothelial cells was determined (△).
the endothelium was intact, however, vasocostriction was observed only when endothelin-1 was applied at the adventitial surface, whereas luminally applied endothelin-1 showed no vasomotor response, indicating that the endothelium forms a tight barrier to circulating endothelin-1. Alternatively, an increased endothelin-1-induced release of endothelin-derived relaxing factor or prostacyclin may also explain these results (21).

In this study, we investigated the hypothetical barrier function of an endothelium for exogenous endothelin-1 as well as the suggested polarity of endothelial cells concerning the secretion of endogenous endothelin-1. HUVECs cultured on amniotic membranes exhibited a significant barrier for the diffusion of endothelin-1 in either direction. This finding allowed us to study the polarity of the endothelin-1 secretion. Under basal conditions, ~80% of the total amount of endothelin-1 synthesized by the endothelial cells was found on the basal (abluminal) side of the endothelial monolayer. At least half of the amount of endothelin-1 found in the apical (abluminal) compartment may originate from diffusion from the abluminal side. The significant increase in the level of endothelin-1 upon stimulation with thrombin is consistent with the data of others (1, 22). Thrombin did not influence the polarity of the secretion. Dexamethasone treatment, however, which did not influence the total amount of secreted endothelin-1, led to an even more pronounced polarity.

In conclusion, an intact endothelium appears to be a major barrier for the diffusion of endothelin-1. The secretion of endothelin-1 is clearly polarized under basal as well as thrombin-stimulated conditions whereby the majority of the endothelin-1 is released into the basolateral compartment, where under in vitro conditions the smooth muscle cells would be located. These findings are in good agreement with the hypothesis that endothelin-1 acts in a local paracrine way, leading to constriction of the underlying vascular smooth muscle located close to the site where endothelin-1 is produced.

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