5-Hydroxytryptamine as a potent migration enhancer of human aortic endothelial cells

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Abstract The purpose of the present study was to investigate whether 5-hydroxytryptamine (5-HT, serotonin) affects migration of vascular endothelial cells. 5-HT significantly enhanced migration of human aortic endothelial cells (HAECs), and this enhancement was completely inhibited by GR 55562, a 5-HT₁ receptor antagonist, and fluoxetine, a 5-HT transporter inhibitor, but was not affected by ketanserin, a 5-HT₂ receptor antagonist. 5-HT stimulation increased RhoA and ERK activity of HAECs, and inhibitors of RhoA (Y-27632 and H-1152) abolished the 5-HT-induced increase in migration velocity. Inhibition of Rho kinase by Y-27632 blocked stress fiber formation and rear release of HAECs. Thus, 5-HT has a potent enhancing action on migration by Y-27632 blocked stress fiber formation and rear release of HAECs. Therefore, 5-HT greatly augments migration of HAECs through activation of RhoA and ERK-dependent pathways.

Keywords: ERK; 5-Hydroxytryptamine; Migration; Rho kinase; Vascular endothelium

1. Introduction

Migration of vascular endothelial cells is involved in angiogenesis [1], which is essential for wound repair and tumor growth and metastasis [2]. Humoral factors such as growth factors are known to regulate migration of vascular endothelial cells [3]. 5-Hydroxytryptamine (5-HT, serotonin) is released from aggregated platelets, and platelet activation is involved in angiogenesis [4,5]. However, there have been no studies on 5-HT action on migration of vascular endothelial cells except for one study that showed that 5-HT inhibited movement of bovine aortic endothelial cells as evaluated by cell phagokinesis [6]. On the other hand, 5-HT has been reported to potentiate proliferation of vascular endothelial cells [7,8], although the mechanism involved in this 5-HT action remains to be determined. The purpose of the present study was to investigate whether 5-HT affects movement of human vascular endothelial cells (HAECs) by using time-lapse microscopy, a sensitive method to evaluate cell motility. We also investigated the effects of 5-HT on cellular signaling that is involved in vascular endothelial cell migration. We found that 5-HT greatly augments migration of HAECs through activation of RhoA and ERK-dependent pathways.

2. Materials and methods

2.1. Cell culture

Human aortic endothelial cells (HAECs) (KURABO, Osaka, Japan) were cultured in HuMedia-EG2 medium (KURABO) in CO₂ (5%)-containing humidified air at 37 °C. The passage number of the cells used was between 5 and 9, and confluent cells were spread and cultured in a new dish every 3–4 days.

2.2. Cell migration assay

HAECs were incubated in RPMI1640 medium (Sigma, St. Louis, MO, USA) containing 5% fetal bovine serum (FBS) under CO₂ (5%)-containing humidified air at 37 °C for 24 h in a 35-mm plastic dish before each experiment. Cell movement on the dish was recorded using a time-lapse microscope for 90 min after stimulation with 5-HT and analyzed by an image analyzing software, MetaVue (Universal Imaging Corporation, PA, USA). Migration velocity of single HAECs was measured and the mean migration velocity of 20 cells for each experimental condition was calculated.

2.3. GST pull-down assay for activated RhoA

GST pull-down assay was performed as previously described [9]. Briefly, the cells lysates were incubated with GST-rhotekin Rho-binding domain (RBD) immobilized on glutathione agarose. The beads were incubated with the cell lysates, and the proteins on the beads were run on SDS–PAGE. GTP-bound RhoA was detected by immunoblotting with anti-RhoA polyclonal antibody and anti-GST-Tag antibody.

2.4. Immunostaining

Immunostaining was performed as previously described [10]. Briefly, the cells stained with Alexa Fluor 488-conjugated phalloidin on the culture slides were mounted with antifade-containing glycerol/phosphate-buffered saline, and cell movement was observed under a confocal laser-scanning microscope.

2.5. Western blotting

The cell lysates were separated by SDS–PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with skim milk and then incubated with anti-phospho-p44/p42 or anti-p44/p42 MAPK polyclonal antibody. The membrane was then incubated with horseradish peroxidase-conjugated goat anti-rabbit antibodies, and specific proteins were detected using an enhanced chemiluminescence immunoblotting system.

2.6. Statistical analysis

Statistical analysis was done using analysis of variance followed by Scheffe's F test. P values less than 0.05 were regarded as significant.

3. Results and discussion

Fig. 1A shows representative images of HAEC movement. The cells indicated by the arrows in Fig. 1A started to migrate following 5-HT stimulation. Ketanserin, a selective 5-HT₂ antagonist, did not affect 5-HT-induced migration of HAECs.

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Apparent migration was not observed in the unstimulated cells or 5-HT-stimulated cells in the presence of GR 55562, a selective 5-HT₁ antagonist, and fluoxetine, a 5-HT transporter inhibitor. Mean migration velocity of HAECs is shown in Fig. 1B. 5-HT at concentrations from 1 to 100 nM augmented migration velocity in a concentration-dependent manner. The augmenting effect of 5-HT (100 nM) on migration of HAECs was completely inhibited by GR 55562 and fluoxetine but was not affected by ketanserin. Thus, 5-HT has an enhancing action on migration of HAECs, which is mediated through stimulation of 5-HT₁ receptors. The present study is the first study that demonstrated 5-HT-induced migration of vascular endothelial cells. High concentrations (almost mM order) of 5-HT have been reported to increase proliferation of canine and bovine aortic endothelial cells via 5-HT₂ receptor stimulation [7,8]. In the present study, 5-HT at lower concentrations (nM order) increased migration of HAECs through 5HT₁ receptor stimulation. Thus, 5-HT is a potent enhancer of vascular endothelial cell migration. On the other hand, 5-HT at concentrations from 0.1 nM to 1 μM has been reported to inhibit migration of bovine aortic endothelial cells through activation of 5-HT₂ receptors, although this 5-HT action did not show a concentration-dependent manner [6]. These results suggest that the effect of 5-HT on vascular endothelial cell migra-

![Fig. 1. (A) Time-lapse images of migration of human aortic endothelial cells (HAECs). Migration of vehicle-treated HAECs, 5-hydroxytryptamine (5-HT, 100 nM)-stimulated HAECs and 5-HT (100 nM)-stimulated HAECs in the presence of GR 55562 (100 nM), a selective 5-HT₁ antagonist, ketanserin (100 nM), a selective 5-HT₂ antagonist, or fluoxetine (1 μM), a 5-HT transporter inhibitor, was recorded at the indicated times. (B) Mean migration velocity of HAECs stimulated with 5-HT (1–100 nM) alone or with 5-HT (100 nM) in the presence of GR 55562 (100 nM), ketanserin (100 nM) or fluoxetine (1 μM) is shown. Asterisks denote significant differences (P < 0.05) from the control without 5-HT stimulation. n = 20.](image-url)
tion differs depending on the kind of vessel and species of animals.
5-HT-associated signal transduction in vascular endothelial cells has not been clarified in detail. We therefore investigated the cellular signals involved in 5-HT-induced migration of HAECs. 5-HT stimulation resulted in a significant increase in RhoA activity at 2.5 min after stimulation with 5-HT (Fig. 2A). RhoA/Rho kinase signaling is known to play pivotal roles in endothelial cell movement in response to a variety of stimuli [11–15]: Activation of Rho increases actin-myosin contractility followed by formation of stress fibers and focal adhesion through Rho-associated kinase (ROCK). Fig. 2B shows the effects of inhibition of RhoA on 5-HT-induced migration of HAECs. 5-HT-induced increase in migration velocity of HAECs was abolished in the presence of Y-27632 and H-1152, inhibitors of RhoA (Fig. 2B). Similarly, 5-HT-induced tail retraction and subsequent rear release, which initiated cell migration, were abolished by Y-27632. (Fig. 2C). Moreover, we analyzed the integrity of the F-actin cytoskeleton of HAECs stimulated with 5-HT (Fig. 2D). HAECs stimulated with 5-HT displayed an array of thick bundled actin stress fibers. In contrast, the actin filaments in HAECs in the presence of Y27632 were notably less bundled and had an indistinct and less organized appearance (Fig. 2D). Inhibition of migration is attributed to the absence of dense actin bundles and failure in reorganization into actin stress fibers that are essential for cell migration. Therefore, the RhoA-dependent pathway is involved in 5HT-induced migration of HAECs.
ERK has been reported to be involved in migration of vascular endothelial cells stimulated with EGF-2, angiogenin and GP 1b [16–18]. Association of the Rho and ERK pathways for regulation of actin filaments has also been shown in a variety of cells, including vascular cells [19–23]. In the present study, expression of phosphorylated ERK (p44/p42) was increased from 2.5 min to 15 min after stimulation with 5-HT in a time-dependent manner (Fig. 3A). 5-HT-induced migration of HAECs was abolished in the presence of U0126 and PD98059, inhibitors of ERK (Fig. 3B). 5-HT-induced ERK activation was abolished in the presence of GR 55562 and fluoxetine but not affected in the presence of ketanserin (Fig. 3C). These results indicate that ERK is activated by stimulation of 5-HT1 receptors and involved in 5-HT-induced migration of

![Fig. 2](image-url)
HAECs. Y-27632 abolished 5-HT-induced activation of ERK (Fig. 3D), indicating that ERK is a downstream signal of Rho kinase. Therefore, 5-HT augments migration of HAECs through the RhoA-ERK pathway. Since therapies designed to control angiogenesis offer promise for the treatment of important pathologies, such as tumor growth and metastasis, modulators of the RhoA-ERK pathway as well as 5-HT1-receptor antagonists are proposed to be useful tools for inhibition of angiogenesis by decreasing vascular endothelial cell migration.

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References


