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Irsogladine is a potent inhibitor of angiogenesis

Yasufumi Sato[a,b], Akio Morimoto[b], Akira Kiue[b], Kazuki Okamura[b], Ryoji Hamanaka[b], Kimitoshi Kohno[b], Michihiko Kuwano[b] and Toshiie Sakata[a]

Department of [a]Internal Medicine 1 and *[b]Biochemistry, Oita Medical University, Idaigaoka 1-1, Hasama-machi, Oita, Japan*

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We describe a novel inhibitor of angiogenesis, Irsogladine, an anti-ulcer drug. Irsogladine inhibited plasminogen activator synthesis of, and tube formation by, human microvascular endothelial cells in type I collagen gel treated with an angiogenic growth factor, EGF. Furthermore, Irsogladine administered orally significantly inhibited in vivo angiogenesis in mice. Irsogladine may be useful in the treatment of diseases associated with angiogenesis.

Angiogenesis; Plasminogen activator; Irsogladine

1. INTRODUCTION

Angiogenesis participates in many pathological states such as growth of various tumors, diabetic retinopathy, and arthritis. Therefore, inhibitors of angiogenesis may be useful for the treatment of such diseases. To obtain an effective agent for the treatment of angiogenesis, it is inevitable to understand the cellular and molecular mechanisms of angiogenesis. Growth factors are thought to play an important role in angiogenesis (reviewed in [1]). We previously observed that the induction of tissue-type plasminogen activator (tPA) in human microvascular endothelial cells is indispensable for growth factor-dependent angiogenesis in vitro [2]. Therefore, we examined the ability of substances to inhibit the induction of PA synthesis in growth factor-treated endothelial cells. Here, we describe that Irsogladine, an anti-ulcer drug, inhibited the induction of PA synthesis in endothelial cells as well as in vitro and in vivo angiogenesis.

2. MATERIALS AND METHODS

2.1. *Cells*

Human omental microvascular endothelial (HOME) cells were isolated from omental adipose tissue and were grown in Medium 199 (M-199) containing 10% fetal bovine serum (FBS), as described previously [3].

2.2. *Materials*

Epidermal growth factor (EGF) was purchased from Toyobo Co. (Osaka, Japan). Irsogladine was obtained from the Research Laboratories of Nippon Shinyaku Co. (Kyoto, Japan). Human tPA cDNA was obtained from Dr. W.-D. Schleuning (Schering Akiengesellschaft Pharma Forschung, Berlin, Germany) [4]. Plasminogen activator inhibitor 1 (PAI-1) cDNA was obtained from Dr. D.J. Loskutoff (Research Institute of Scripps Clinic, La Jolla, CA) [5].

Correspondence address: Y. Sato, Department of Internal Medicine 1, Oita Medical University, Idaigaoka 1-1, Hasama-machi, Oita 879-55, Japan. Fax: (81) (975) 494 480.

2.3. *Northern blot analysis*

Northern blot analysis was undertaken as described previously [2,6]. HOME cells were incubated in M-199 containing 1% FBS with or without EGF (10 ng/ml) and/or Irsogladine (10^{-4} M) for 12 h. Total RNA was extracted, fractionated on 1% agarose gels containing 2.2 M formaldehyde, and transferred to a Nytran filter (Schleicher & Schuell). Northern blots were probed with tPA and PAI-1 cDNAs.

2.4. *Tube formation by HOME cells*

Tube formation by HOME cells was examined as previously described [2]. Briefly, HOME cells were grown on the surface of type I collagen gels (Nitta Gelatin, Osaka, Japan). When the cells became confluent, the medium was replaced by M-199 containing 1% FBS with or without EGF (10 ng/ml) and/or Irsogladine (10^{-4} M). After a 3 day incubation, tube-like structures in the gels were quantified by a Cosmozone 1S Image Analyzer (Nikon, Tokyo, Japan). Eight random fields were measured and the total tube length per field ($\times 200$ magnification) was determined.

2.5. *A mouse dorsal air sac method*

In vivo angiogenesis was observed by using a mouse dorsal air sac method as described elsewhere [7]. Briefly, angiogenesis-inducing cells were suspended in phosphate-buffered saline (PBS) at a concentration of 5×10^7 cells/ml. The cell suspension (0.2 ml) was injected into a chamber consisting of a Millipore ring (Millipore Co., MA) and micropore filters (0.22 μm pore size, Millipore, Co., MA) on both sides of the ring. The chamber containing angiogenesis-inducing cells or 10 mg/ml of bovine serum albumin (BSA) was implanted into a dorsal air sac of a 5–7-week-old male ICR mouse. Irsogladine (30, 100, and 300 mg/kg) was given orally twice a day. Mice were killed and carefully skinned on the third day after the implantation.

3. RESULTS AND DISCUSSION

The structure of Irsogladine is shown in Fig. 1. The molecular weight of Irsogladine is 372.17 Da. We examined the effect of Irsogladine on the induction of tPA mRNA in growth factor-treated HOME cells. As shown

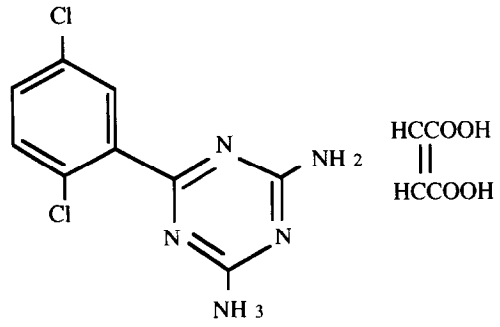

Fig. 1. The structure of Irsogladine.

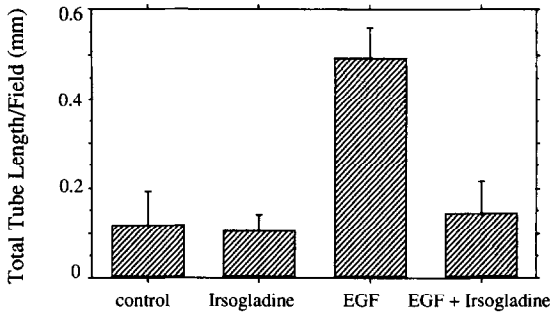

Fig. 3. Effect of Irsogladine on tube formation by EGF-treated HOME cells in type 1 collagen gel. HOME cells on type 1 collagen gel were treated with 10 ng/ml of EGF and/or 10^{-4} M or Irsogladine. After a 3-day incubation, the total length of tube-like structures per field in the gel was determined, using a Cosmozone 1S Image Analyzer, as described in section 2. Values represent mean ± S.D.

in Fig. 2, EGF (10 ng/ml) induced the expression of both tPA and PAI-1 mRNA in HOME cells at a level similar to that previously reported [2,8]. Irsogladine (10^{-4} M) alone did not affect the basal expression of tPA or PAI-1 mRNA in HOME cells, however, simultaneous addition of Irsogladine markedly inhibited the EGF-induced tPA expression but not PAI-1 expression in HOME cells. Thus, Irsogladine was nominated as a candidate for the inhibitor of angiogenesis. Next, we examined whether Irsogladine blocked tube formation by EGF-treated HOME cells in type 1 collagen gels. As shown in Fig. 3, EGF (10 ng/ml) induced tube formation by HOME cells in the gel after a 3-day incubation as was reported previously [2,3]. While Irsogladine (10^{-4} M) alone has no effect on tube formation by HOME cells, simultaneous addition of Irsogladine almost completely blocked the stimulatory effect of EGF on tube formation in the gel. We further examined whether the anti-angiogenic effect of Irsogladine could be reproduced on the in vivo system. We used a mouse dorsal air sac method as an in vivo model of angiogenesis. Various cells types, including Sarcoma 180, B16 melanoma cell lines, as well as HOME cells, were applied to this system as angiogenesis-inducing cells, and HOME cells were found to be stable and have the highest angiogenic potential. Therefore, we chose a HOME cell as an angiogenesis inducer. As shown in Fig. 4, the implantation of the chamber containing BSA did not cause any angiogenesis, indicating that the mechanical maneuver itself did not cause angiogenesis. The chamber containing HOME cells induced marked angiogenesis on the third day after the implantation, however, Irsogladine given orally twice daily inhibited the induction of angiogenesis by HOME cells. The inhibitory effect of Irsogladine was dose dependent, and 300 mg/kg of Irsogladine twice daily significantly inhibited angiogenesis. The mice looked healthy during the experiment, and did not show any signs of toxic effects or reduction of body weight by the treatment with Irsogladine (data not shown).

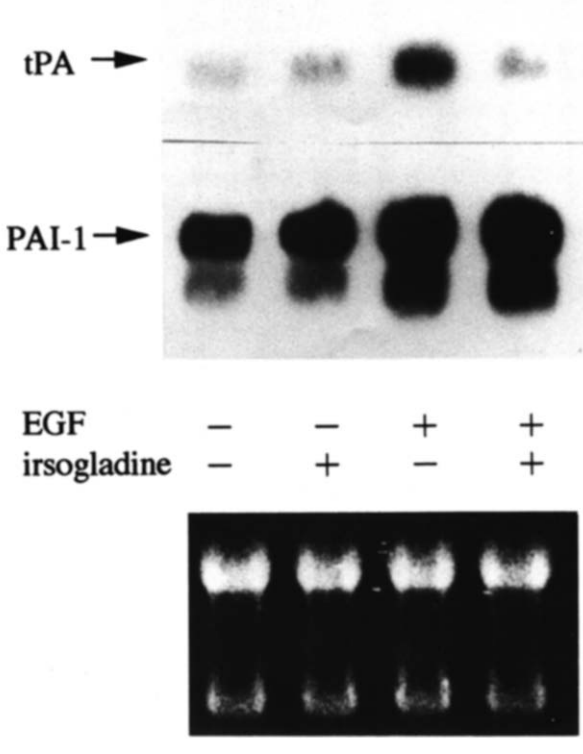

Fig. 2. Northern blot analysis of tPA and PAI-1 mRNA in HOME cells. HOME cells were incubated with 10 ng/ml of EGF and/or 10^{-4} M Irsogladine for 12 h. After the incubation, total RNA was extracted and Northern blot analysis of tPA and PAI-1 was carried out, as described in section 2.

Angiogenesis is a complex phenomenon which includes at least four sequential steps: degradation of vascular basement membrane and interstitial matrices by endothelial cells; migration of endothelial cells; proliferation of endothelial cells; and formation of capillary loops by endothelial cells (reviewed in [9]). A cascade of proteinases is required for the degradation of extracellular matrices. PAs convert plasminogen to plasmin. While plasmin itself is reponsible for the degradation of extracellular matrices, plasmin also converts pro-metalloproteinase to an active form. Endothelial cells have the ability to produce PAs and pro-metalloproteinase,

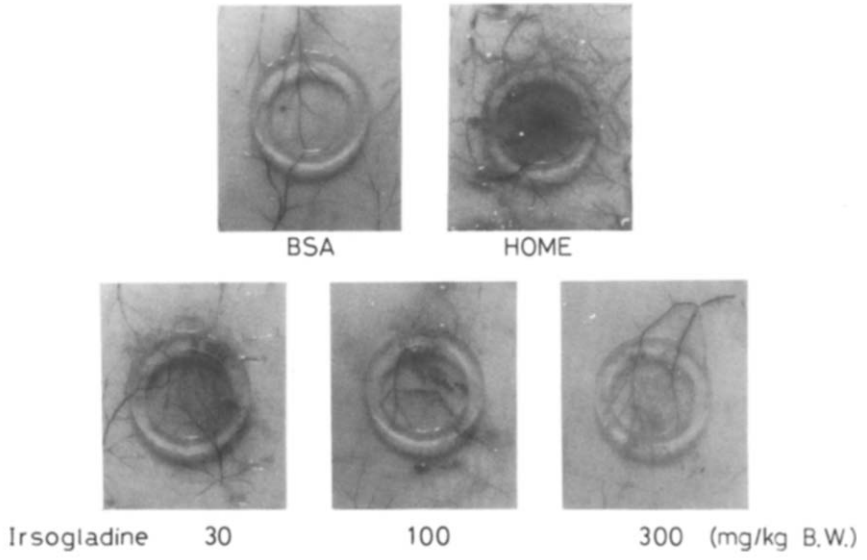

Fig. 4. Effect of Irsogladine on angiogenesis in mice. The Millipore chamber containing HOME cells or BSA was implanted into a mouse dorsal air sac. Irsogladine at a dose of 30, 100, and 300 mg/kg was administered orally to mice twice daily. Mice were killed and skinned 3 days after the implantation as described in section 2.

and thus the induction of PAs in endothelial cells is proposed as a key event for the initiation of angiogenesis (reviewed in [10,11]. We have previously observed that the induction of tissue-type PA (tPA) is indispensable for growth factor-dependent angiogenesis in vitro [2]. A growth factor-like EGF or transforming growth factor α (TGFα) stimulates tPA synthesis, as well as migration and proliferation of HOME cells [12,13], and induces tube formation in type 1 collagen gel [2,8]. The elimination of tPA activity by anti-tPA antibody or serine protease inhibitors inhibited the growth factor-induced tube formation in vitro [2], however, the systemic administration of serine protease inhibitors was not effective for the inhibition of angiogenesis in vivo (data not shown). Therefore, we screened several substances and found that Irsogladine, an anti-ulcer drug, could block the induction of tPA mRNA in, and tube formation by, EGF-treated HOME cells. We further confirmed the anti-angiogenic effect of Irsogladine by an in vivo assay system using a mouse dorsal air sac method. We chose a HOME cell as an angiogenesis-inducing cell for our in vivo system. Although the responsible factor(s) for angiogenesis induced by HOME cells was obscure, we observed that HOME cells produced basic fibroblast growth factor (bFGF), a potent angiogenic growth factor, but not EGF nor TGFα [6]. Therefore, the angiogenic potential of HOME cells is not related to either EGF or TGFα, and an anti-angiogenic effect of Irsogladine may be general and not restricted to the effect of EGF.

The mechanism by which Irsogladine eliminates the induction of tPA in growth factor-treated HOME cells is currently obscure. Migrating endothelial cells express PA activity [14,15], while Gap-junctional communication increases during the migration of endothelial cells [16,17]. These findings suggest a possible relationship between Gap-junctional communication and PA activity of endothelial cells. Irsogladine has been recently shown to modulate Gap-junctional intercellular communication [18]. Thus, Irsogladine may abrogate the induction of PA by modulating Gap-junctional communication of endothelial cells. Further study is currently underway to clarify the mode of action of Irsogladine on endothelial cells. Finally, since Irsogladine is innocuous and has been in clinical use in Japan, it can be a potential candidate for the treatment of diseases associated with angiogenesis.

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