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Clotrimazole, an Imidazole Antimycotic, Is a Potent Inhibitor of Angiogenesis

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Clotrimazole, an imidazole antimycotic, interferes with the rise in cytosolic Ca^{2+} and inhibits cell proliferation in a reversible manner. Here we describe the effect of clotrimazole on vascular endothelial cells (ECs). Clotrimazole inhibited the proliferation of ECs stimulated with typical angiogenic growth factors; vascular endothelial growth factor and basic fibroblast growth factor (bFGF). This inhibitory effect of clotrimazole was dose-dependent and the maximal inhibition was observed at a concentration of 10 m*M*. We did not observe any increase in ^{51}Cr release from ECs during treatment with 10 μM clotrimazole. Moreover, clotrimazole inhibited the basal and bFGF-stimulated migration of ECs. As clotrimazole inhibited two principle components of angiogenesis; the proliferation and migration of ECs, we examined whether clotrimazole inhibited angiogenesis. Tube formation by ECs in type 1 collagen gel was investigated, and clotrimazole was found to be significantly inhibitory. The inhibitory effect of clotrimazole on angiogenesis was further confirmed in an *in vivo* angiogenesis model of murine Matrigel plug assay. These results demonstrate that clotrimazole is a potent inhibitor of angiogenesis.

Key words: Clotrimazole — Angiogenesis — Angiogenesis inhibition — Basic fibroblast growth factor — Vascular endothelial growth factor

Angiogenesis is a process by which new vessels are formed from a pre-existing one. It is thought to be regulated by the balance of angiogenic factors and angiogenesis inhibitors. Various angiogenic factors and angiogenesis inhibitors have been reported so far. Among them, certain growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are important angiogenic factors. Upon stimulation with these factors, endothelial cells express proteases to degrade basement membrane, migrate, and proliferate, culminating in vascular morphogenesis.[1)]

Angiogenesis is a fundamental process in reproduction and development. In adults, physiological angiogenesis can be observed only in restricted sites such as endometrium and ovarian follicle, and in wound repair, and it is normally transient. Persistent angiogenesis, on the other hand, plays a crucial role in several pathological states including solid tumors, diabetic retinopathy and rheumatoid arthritis. In particular, the growth of solid tumors is dependent on tumor angiogenesis, and metastasis is also closely related to angiogenesis. Thus, the inhibition of angiogensis has become a strategy for cancer treatment.[2)]

Clotrimazole is an antimycotic imidazole derivative, and has been in clinical use for more than 20 years. The antimycotic effect of imidazole arises from the inhibition of sterol 14α-demethylase, a microsomal cytochrome P-450-dependent enzyme. Clotrimazole, as well as some other imidazole compounds, also inhibits voltage- and ligand-stimulated Ca^{2+} influx in nucleated cells,[3,4)] and Ca^{2+}-activated K^+ channels in red blood cells.[5)] More recently, clotrimazole has been shown to inhibit the proliferation of normal and cancer cells, including endothelial cells.[6)] Here we present an investigation of the effect of clotrimazole on endothelial cells, showing that clotrimazole is a potent inhibitor of angiogenesis.

MATERIALS AND METHODS

Materials Clotrimazole, miconazole, econazole, imidazole, ronidazole and metronidazole were purchased from Sigma (St. Louis, MO); recombinant human bFGF was from Bachem, Inc. (Torrance, CA); type 1 collagen was from Nitta Gelatin (Osaka); growth factor-reduced Matrigel was from Collaborative Research, Inc. (Bedford, MA); [^{51}Cr]sodium chromate (1 mCi/ml in saline) was from NEN (Boston, MA). VEGF was isolated from the conditioned medium of insect cells infected with a baculovirus-based expression vector as described.[7)] Protein A-purified rabbit anti-human recombinant bFGF antibody was described previously.[8)]

Cells and animals Bovine capillary endothelial (BCE) cells, bovine aortic endothelial (BAE) cells and porcine aortic endothelial (PAE) cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) as described elsewhere.[8–10)] Human umbilical vein endothelial (HUVE) cells were isolated and grown in MCDB-131 supplemented with 5% FCS, 1 μg/

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ml hydrocortisone, 10 ng/ml epidermal growth factor (EGF), and 12 μg/ml bovine brain extract. C57B16 mice were obtained from Japan SLC, Inc. (Hamamatsu).

Cell proliferation Endothelial cells (3×10^4) were plated in a 35 mm plastic dish with DMEM containing 0.5% FCS. After a 6 h incubation, bFGF (1 n*M*) or VEGF (1 n*M*) and/or indicated concentrations of imidazole compounds were added to the medium. After a 3-day incubation, cell numbers were determined.

^{51}Cr-release assay ^{51}Cr-release assay was conducted as described previously.[11] Briefly, confluent BCE cells in a 24-well plate were incubated in DMEM containing 10% FCS and 2 μCi/ml of ^{51}Cr. After an 18-h incubation, each well was washed with phosphate-buffered saline (PBS). Cells were then incubated with DMEM containing 10% FCS plus or minus clotrimazole (10 μM) for 6 h, and the ^{51}Cr content in aliquots of the supernatant was measured in a gamma counter.

Cell migration Cell migration was examined by wound assay as described previously.[12] Briefly, a confluent monolayer of BCE cells was wounded with a razor blade, washed with PBS and further incubated for 18 h in DMEM containing 0.1% bovine serum albumin (BSA). bFGF (1 n*M*) plus or minus clotrimazole (10 μM) was added immediately after the wounding. Cells that migrated across the edge of the wound were counted.

Tube formation by BCE cells Tube formation by BCE cells in type 1 collagen gel was examined as described.[13] Briefly, BCE cells were grown on the surface of type 1 collagen gel. After they reached confluence, the medium was changed to DMEM containing 1% FCS, and bFGF (1 n*M*) and/or clotrimazole (10 μM) were added. After a 3-day incubation, tube-like structures formed in the gel were measured with Cosmozone 1S Image Analyzer (Nikon, Tokyo).

Murine angiogenesis model Matrigel plug assay as originally described by Passaniti *et al*.[14] was performed. Male C57B16 mice were used at 4 weeks of age. Angiogenesis

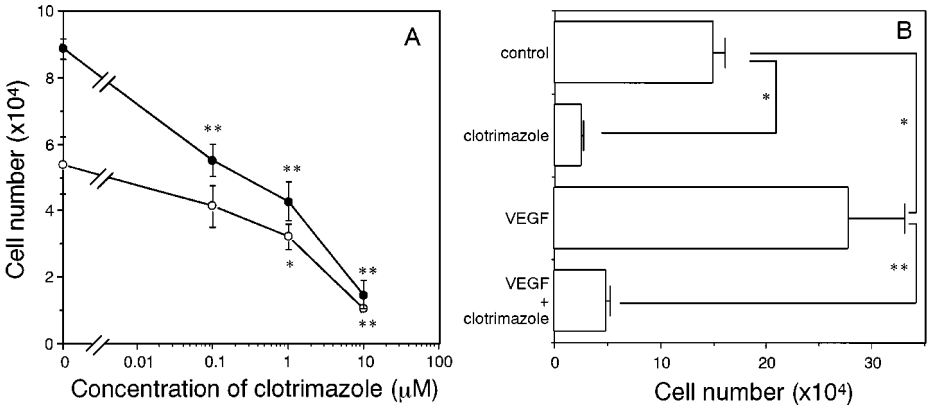

Fig. 1. Effect of clotrimazole on the proliferation of BCE cells. A, BCE cells (3×10^4) were plated in 35 mm plastic dishes in DMEM containing 0.5% FCS. After a 6-h incubation, bFGF (1 n*M*) and/or indicated concentrations of clotrimazole were added to the medium. After a 3-day incubation, cell numbers were determined. Values represent means and SDs of triplicate samples. ○ clotrimazole, ● bFGF plus clotrimazole. * $P<0.05$, ** $P<0.01$ vs. corresponding control. B, BCE cells (3×10^4) were plated in 35 mm plastic dishes in DMEM containing 0.5% FCS. After a 6-h incubation, VEGF (1 n*M*) and/or clotrimazole (10 μM) were added to the medium. After a 3-day incubation, cell numbers were determined. Values represent means and SDs of triplicate samples. * $P<0.05$, ** $P<0.01$ for the indicated comparisons.

Table I. Effect of Clotrimazole on the Proliferation of Various Endothelial Cells

Cell type	Basal without CLT vs. with CLT[a] mean±SD (10^4)		bFGF without CLT vs. with CLT mean±SD (10^4)		VEGF without CLT vs. with CLT mean±SD (10^4)	
BAE	3.22±0.59	1.66±0.39[b]	6.81±0.39	3.38±0.23[b]	5.51±0.88	3.45±0.26[b]
PAE	2.41±0.39	1.83±0.09[b]	ND	ND	5.39±1.22	2.37±0.23[b]
HUVE	10.69±3.54	3.21±0.06[b]	ND	ND	ND	ND

a) CLT, clotrimazole.
b) $P<0.05$ (compared with the corresponding group without CLT).

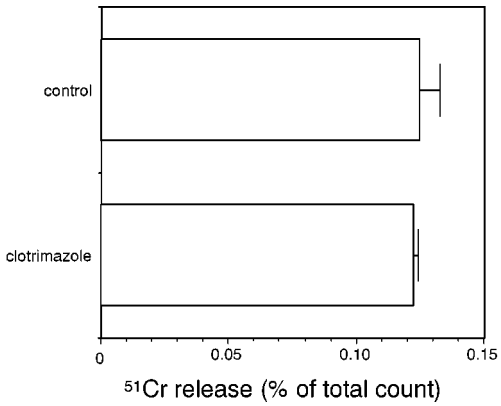

Fig. 2. Effect of clotrimazole on the release of ^{51}Cr from BCE cells. Confluent BCE cells in a 24-well plate were incubated with 2 μCi/ml of ^{51}Cr for 18 h. After an 18-h incubation, each well was washed with PBS, and then incubated with DMEM containing 10% FCS plus or minus clotrimazole (10 μM) for 6 h. The ^{51}Cr contents in aliquots of the supernatant were measured in a gamma counter. Values represent means and SDs of triplicate samples.

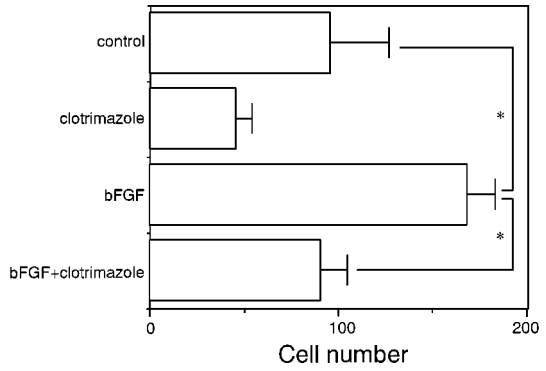

Fig. 3. Effect of clotrimazole on the migration of BCE cells. Confluent monolayers of BCE cells were wounded with a razor blade, washed with PBS, and further incubated for 18 h in DMEM containing 0.1% BSA. bFGF (1 nM) and/or clotrimazole (10 μM) were added immediately after the wounding. Cells that had migrated across the edge of the wound were counted from 5 fields (×200 magnification). Values represent means and SDs. $*$ $P<0.05$ for the indicated comparisons.

was determined as growth of neo-vessels from subcutaneous tissue into solid Matrigel as follows. Growth factor-reduced Matrigel (0.5 ml) supplemented with 8.3 nM bFGF in liquid form at 4°C was injected into the abdominal subcutaneous tissue of mice at the mid-peritoneal area. Clotrimazole (600 mg/kg body weight/day) was orally administered once a day. At the 5-day point, the mice were killed, and the gels were recovered, fixed in 4% paraformaldehyde in PBS, and embedded in paraffin. Five-micrometer sections of gel were stained with Masson's trichrome, and the ingrowth of blood vessels was observed. For quantification of angiogenesis, the number of cells that had invaded the Matrigel was determined. Each group contained 5 animals, and one section of the center of the Matrigel gel from each individual animal was observed at 200 fold magnification. Cells that had invaded the Matrigel were counted from 3 to 5 different fields from one section, and the mean cell number per field of each section was calculated.

Calculations and statistical analysis The statistical significance of differences in the results was evaluated by use of unpaired ANOVA, and P values were calculated by Scheffe's method. A value of $P<0.05$ was taken as the criterion of statistical significance.

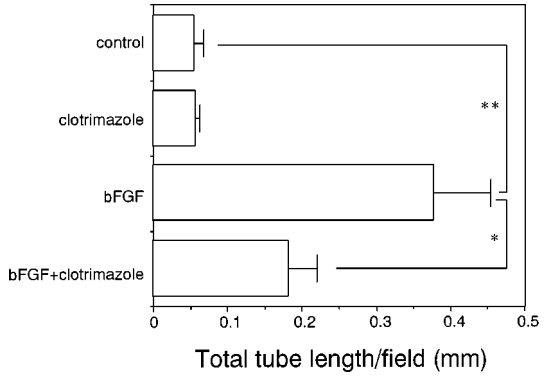

Fig. 4. Effect of clotrimazole on angiogenesis *in vitro*. BCE cells were grown on the surface of type 1 collagen gels. After they had reached confluence, the medium was changed to DMEM containing 1% FCS, and bFGF (1 nM) and/or clotrimazole (10 μM) was then added. After a 3-day incubation, the lengths of the tube-like structures formed in the gel were measured. Total tube length per field (×200 magnification) was calculated from 8 fields. Values represent means and SDs. $*$ $P<0.05$, $**$ $P<0.01$ for the indicated comparisons.

RESULTS

We first examined the effect of clotrimazole on the proliferation of endothelial cells (ECs). We observed that clotrimazole inhibited the basal and bFGF-stimulated proliferation of BCE cells in a dose-dependent manner, and the inhibitory effect of clotrimazole reached maximum at 10 μM (Fig. 1A). The inhibitory effect of clotrimazole on cell proliferation was further characterized by using VEGF, a specific mitogen for ECs. As shown in Fig. 1B, clotrimazole (10 μM) also inhibited the growth of BCE cells stimulated with 1 nM VEGF. The inhibitory

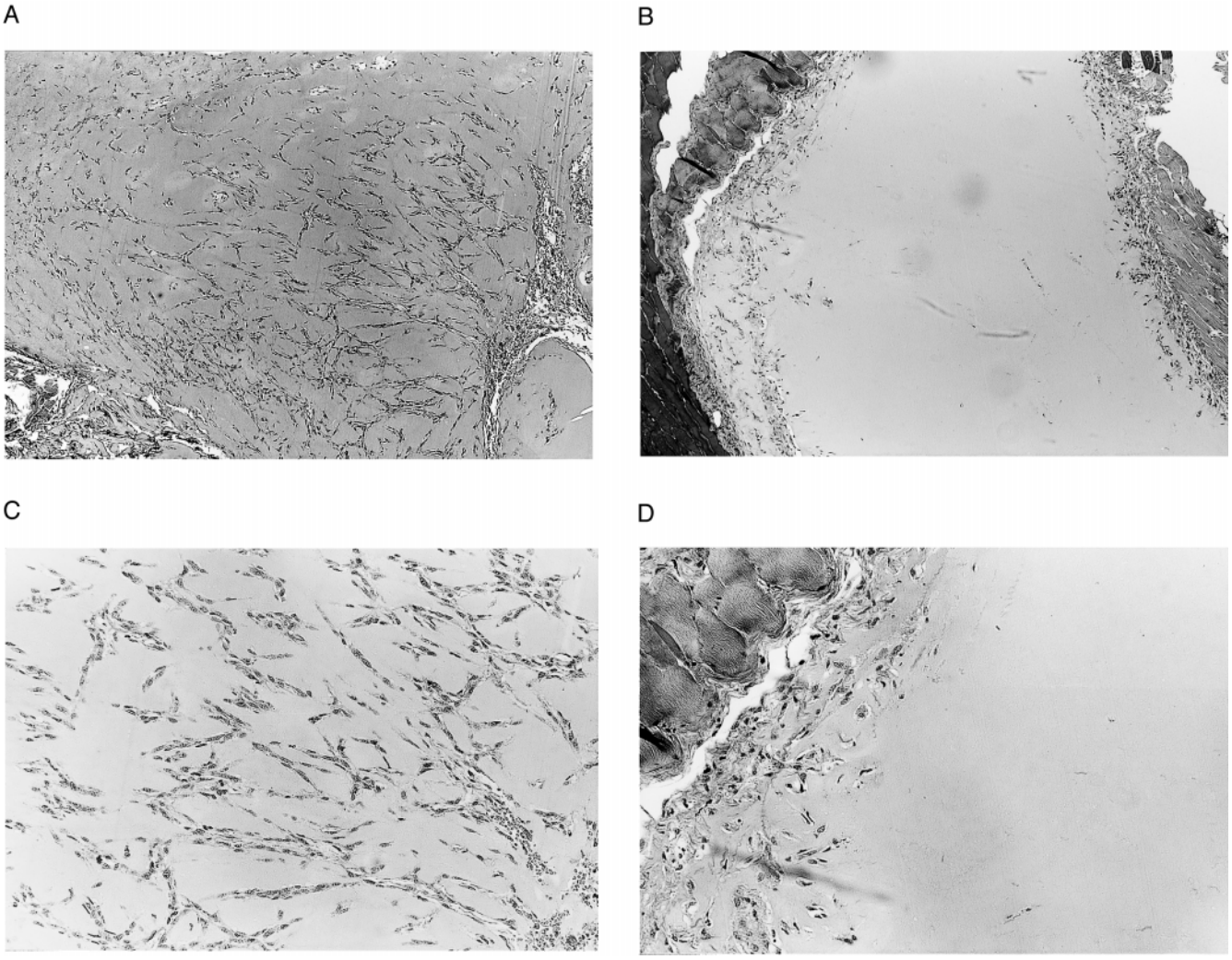

Fig. 5. Murine angiogenesis model. Growth factor-reduced Matrigel (0.5 ml) in liquid form at 4°C supplemented with 8.3 n*M* bFGF was injected into the abdominal subcutaneous tissue of mice at the mid-peritoneal area. Clotrimazole (600 mg/kg body weight) was orally administered once a day. At the 5-day point, the mice were killed, and the gels were recovered, fixed in 4% paraformaldehyde in PBS, and embedded in paraffin. Five micrometer sections of each gel were stained with Masson's trichrome, and the degree of ingrowth of blood vessels was observed. A, control animal, ×100 magnification; B, clotrimazole-treated animal, ×100 magnification; C, control animal, ×200 magnification; D, clotrimazole-treated animal, ×200 magnification.

effect of clotrimazole on the proliferation of ECs was further confirmed by using ECs isolated from other species and sources such as BAE cells, PAE cells and HUVE cells. As shown in Table I, clotrimazole (10 μM) significantly inhibited the bFGF- or VEGF-stimulated proliferation of BAE cells, the VEGF-stimulated proliferation of PAE cells, and the proliferation of HUVE cells in the presence of bovine brain extract and EGF. It was possible that this inhibitory effect of clotrimazole was due to cytotoxicity. Therefore, we examined the cytotoxic effect of clotrimazole by ^{51}Cr-release assay as described in "Materials and Methods." Clotrimazole at 10 μM did not enhance ^{51}Cr release (Fig. 2), indicating that this concentration of clotrimazole was not cytotoxic to BCE cells.

We next examined whether clotrimazole inhibits the migration of ECs, another property of ECs important in angiogenesis. When a monolayer of BCE cells was wounded with a razor blade and further incubated in a serum-free medium, remaining cells migrated into the denuded area during a 24-h incubation. Exogenous bFGF

(1 n*M*) enhanced this process. As shown in Fig. 3, clotrimazole (10 μM) inhibited the basal and bFGF-stimulated migration of BCE cells.

Since clotrimazole inhibited the two principle properties of ECs required for angiogenesis, we tested whether clotrimazole inhibited angiogenesis itself. Tube formation by BCE cells in type 1 collagen gel was investigated as an *in vitro* model of angiogenesis. Tube length was quantified and the effect of clotrimazole was examined as described in "Materials and Methods." When a monolayer of BCE cells cultured on the surface of type 1 collagen gel was stimulated with 1 n*M* bFGF, BCE cells invaded the gel and formed tube-like structures in it. Coadministration of clotrimazole (10 μM) significantly inhibited this effect of bFGF (Fig. 4).

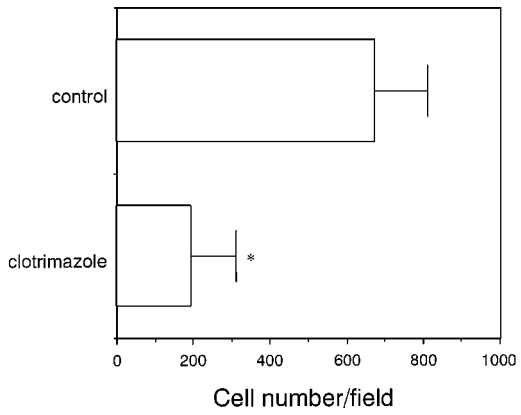

Fig. 6. Effect of clotrimazole on angiogenesis *in vivo*. The number of cells that had invaded the Matrigel was determined as described in "Materials and Methods." Each group contained 5 animals, and the mean cell number per field (×200 magnification) was determined from 5 fields for each animal. Values represent means and SDs of 5 animals. * $P<0.05$ vs. control.

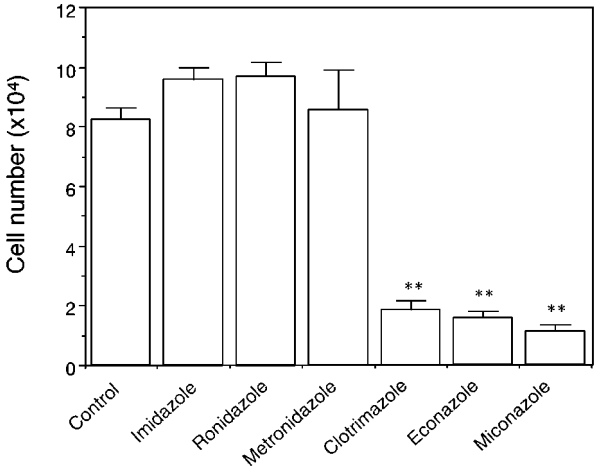

Fig. 8. Effects of imidazole compounds on the proliferation of BCE cells. BCE cells (3×10^4) were plated in 35 mm plastic dishes in DMEM containing 0.5% FCS. After a 6-h incubation, various imidazole compounds (10 μM) were added to the medium, respectively. After a 3-day incubation, cell numbers were determined. Values represent means and SDs of quadruple samples. ** $P<0.01$ vs. control.

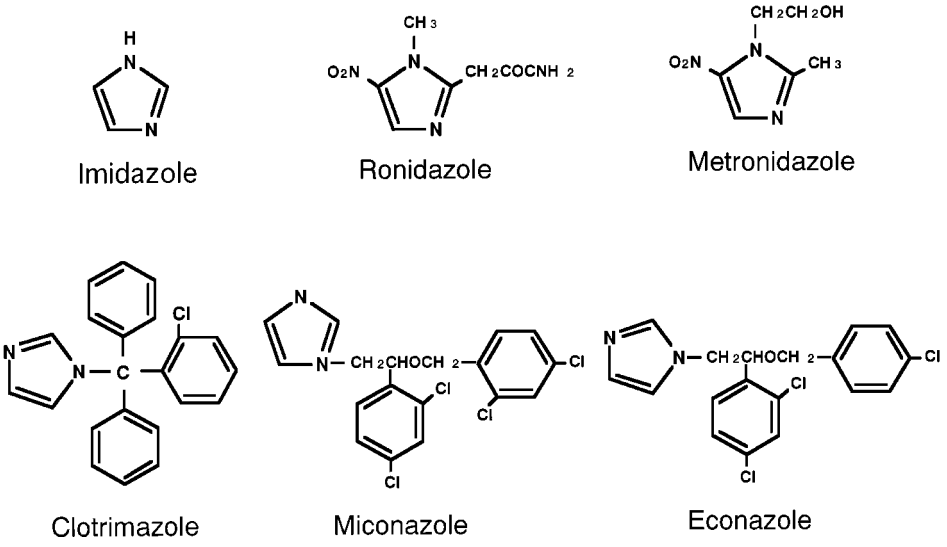

Fig. 7. Chemical structures of various imidazole compounds.

We further confirmed the effect of clotrimazole on angiogenesis *in vivo*. Matrigel plug assay was performed as described in "Materials and Methods." Almost all the cells that invaded the Matrigel were immunohistochemically positive for factor VIII antigen, a marker of endothelial cells (data not shown). A marked cellular invasion and tube structures were observed in growth factor-reduced Matrigel supplemented with 8.3 n*M* bFGF (Fig. 5, A and C). Oral administration of clotrimazole (600 mg/kg/day) inhibited it (Fig. 5, B and D). The extent of angiogenesis was quantified by counting cells that had invaded the gel. As shown in Fig. 6, the clotrimazole treatment caused a significant inhibition of angiogenesis.

Finally, we compared the effect of clotrimazole on the proliferation of BCE cells with those of other imidazole compounds (Fig. 7). In addition to clotrimazole, miconazole and econazole inhibited the proliferation of BCE cells, while imidazole, ronidazole and metronidazole had no inhibitory effect (Fig. 8).

DISCUSSION

Here we have demonstrated that clotrimazole, an imidazole antimycotic agent, inhibited the proliferation and the migration of ECs stimulated with two representative angiogenic factors, bFGF and VEGF. As a result, clotrimazole inhibited tube formation by ECs in type 1 collagen gel. This inhibitory effect of clotrimazole was not due to cytotoxicity, since there was no increase in ^{51}Cr release when the cells were treated with clotrimazole. The anti-angiogenic effect of clotrimazole was further confirmed in an *in vivo* experimental system. Therefore, clotrimazole was considered to be as a potent inhibitor of angiogenesis.

Benzaquen and colleagues[6] recently reported that clotrimazole reversibly inhibited the proliferation of various cancer cells and normal cells including endothelial cells. They also suggested that the inhibitory effect of clotrimazole on the proliferation is correlated with prevention of the rise in cytosolic Ca^{2+} which normally follows mitogenic stimulation. We confirmed that clotrimazole inhibited the rise in cytosolic Ca^{2+} in our endothelial cells using Quin II, a cytosolic calcium indicator, and confocal microscopy (data not shown). It is known that some imidazole compounds, including clotrimazole, miconazole and econazole, are potent inhibitors of voltage-gated Ca^{2+} entry into nucleated cells.[3, 4] Here, we observed that miconazole and econazole also inhibited the proliferation of ECs. Therefore, the anti-angiogenic effect of clotrimazole is thought to be mediated through the inhibition of Ca^{2+} entry into ECs in response to angiogenic growth factors. The importance of Ca^{2+}-mediated signal transduction in angiogenesis was previously described. Presta and co-workers[15] studied the effect of bFGF on GM 7373 cells (chemically transformed bovine ECs), and showed that bFGF acts through two distinct signaling pathways. A protein kinase C-dependent pathway was shown to be involved in the mitogenic activity and a calcium-dependent but protein kinase C-independent pathway was shown to trigger the plasminogen activator cascade. Kohn and co-workers[16] showed that carboxyamidotriazole, a triazole compound which inhibits Ca^{2+} channels of mammalian cells, exerts anti-angiogenic activity by inhibiting the proliferation and the migration of ECs. However, the precise mechanism by which Ca^{2+} modulates the proliferation and the migration of ECs is obscure, and further study is required.

Attempts have been made to find agents having an anti-angiogenic effect among drugs which are already in clinical use for other purposes. Minocycline (a tetracycline antimicrobial),[17] irsogladine (an anti-gastric ulcer drug),[18, 19] captopril (an angiotensin converting enzyme inhibitor),[20] and spironolactone (a renal aldosterone antagonist)[21] are examples. The advantage of this approach is that these drugs have been in clinical use for years, and their side-effects are well characterized. We can now add clotrimazole to the list of angiogenesis inhibitors. Although clotrimazole is in clinical use for external application at present, systemic administration of clotrimazole has been reported without major side-effects.[22] Therefore, we suggest that clotrimazole, and possibly some other imidazole compounds, may be useful for the treatment of angiogenesis-related diseases.

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