

Inhibition of Tumor Growth and Neovascularization by an Anti-Gastric Ulcer Agent, Irsogladine¹

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Abstract

Irsogladine used clinically as an anti-gastric ulcer agent, at 10^{-6} – 10^{-4} M, inhibited cell proliferation and tubular morphogenesis of vascular endothelial cells, but the proliferation of human epidermoid cancer or glioma cells was not inhibited by this drug, even at 10^{-4} M. *In vivo* studies demonstrated that p.o. administration of irsogladine significantly inhibited tumor growth of human glioma cells in mice, and histological analysis showed a dramatic decrease of the neovascularization in the tumors. In mice transplanted with chambers containing human glioma cells or hepatic cancer cells, irsogladine also inhibited angiogenesis. These *in vivo* and *in vitro* assays demonstrate that irsogladine may be a unique and potent inhibitor of tumor angiogenesis.

Introduction

Neovascularization is involved in tumor growth as well as rheumatoid arthritis, diabetic retinopathy, and psoriasis, all of which are termed "neovascularization diseases" (1, 2). The transition from limited to rapid tumor growth often accompanies angiogenesis. The development of blood vessels within tumor tissues is also closely correlated with invasion and metastases of cancer cells in breast cancer, melanoma, lung cancer, prostatic cancer, and other cancers (3, 4). Endogenous positive and negative angiogenic factors released by tumors and stroma components are thought to balance tumor angiogenesis (4, 5). The positive factors include fibroblast growth factors, vascular endothelial growth factor/vascular permeable factor, interleukin 8, platelet-derived growth factor, and epidermal growth factor/transforming growth factor α , whereas negative factors include thrombospondin, platelet factor 4, and angiostatin (4, 5). On the other hand, antiangiogenic agents targeting the tumor vasculature are expected to induce blocking in the blood supply to tumors and to prevent metastasis processes (1). Various antiangiogenic agents have been developed in *in vitro* and *in vivo* models. These include platelet factor 4-related peptide (6), IFN- α (7), steroids (8), fumagillin and its derivative, AGM-1470³ (TNP-470; Refs. 9 and 10), and polysaccharide-peptidoglycan complex (11). Some of these agents are currently in clinical tests (1).

In our laboratory, we have established tumor angiogenesis models *in vitro* with vascular endothelial cells, and *in vivo* by transplanting tumor cells into dorsal air sacs in mice (12). We have previously

reported that increased expression of tissue-type plasminogen activator is required for the angiogenesis process *in vitro* (13). Irsogladine inhibits tubular morphogenesis and tissue-type plasminogen activator synthesis in vascular endothelial cells (14). Irsogladine has been used clinically as an anti-gastric ulcer agent, which strengthens intercellular gap junction communication (15, 16). In this study, we further examined whether irsogladine could modulate tumor growth and tumor angiogenesis. The antitumor effect of irsogladine is discussed in relation to its inhibitory effect on tumor vasculature.

Materials and Methods

Cell Lines, Cell Culture, and Drugs. Human umbilical endothelial cell kit was obtained from Dainippon Seiyaku Co. (Osaka, Japan). Human microvascular endothelial cells, prepared as described previously (13, 14), were cultured in medium 199 supplemented with 10% FBS. Human epidermoid carcinoma KB cells, human hepatic cancer HepG2 cells (purchased from American Type Culture Collection, Rockville, MD), and human glioma U251 cells (12) were cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 60 μ g/ml kanamycin. Endothelial cells and cancer cells (1×10^4 /dish) were plated on 35-mm dishes on day 0, and the next day, various concentrations of drugs were added to the medium. Culture supplements were exchanged every other day. On the indicated day, cells were detached from the culture dishes, and the number of viable cells was counted by a Coulter counter (Coulter Electronics, Hialeah, FL). Irsogladine was obtained from Research Laboratories (Nippon Shinyaku Co., Kyoto, Japan), and its M_r is 372 (14). Fumagillin was obtained from Chinoin Pharmaceutical Chemical Works Co. (Budapest, Hungary).

Tubular Morphogenesis in the Coculture System. We previously established a tumor angiogenesis model system in which vascular endothelial cells underwent tubular morphogenesis when cocultured with tumor cells in type I collagen gels (12, 17). In this study, we specifically assayed tubular morphogenesis by human microvascular endothelial cells. Tumor cells were cultured in the outer chambers of six-well plates (each well, 38×7 mm; Iwaki Glass Co., Chiba, Japan), in 2 ml DMEM containing 1% FBS. Human microvascular endothelial cells were seeded separately in 2 ml medium 199 containing 10% FBS in type I collagen gels (1 ml) in culture plates with 0.4- μ m filters (Millicell-CM; Millipore Corp., Laboratory Products, Bedford, MA) in the inner chamber (30×7 mm). When the endothelial cells reached confluence, the serum content of the medium was reduced from 10 to 1%, and the inner chamber was transferred to the outer chamber. In this system, tubulogenesis by endothelial cells occurs in the collagen gel in the inner chamber, when tumor cells cocultured in the outer chamber secrete angiogenic factors that pass through the filter of the inner chamber. On the third day, phase contrast microscopic pictures of each dish were recorded by a still video camera recorder (R5000H; Fuji, Tokyo, Japan), and the total length of tubular structures per field was measured using a Cosmozone image analyzer (Nikon, Tokyo, Japan; Refs. 12 and 17). Eight random fields per dish were measured, and the total length per field was calculated.

Tumor Angiogenesis Assay in Mice. A dorsal air sac was created in 5–7-week-old male mice according to a previously published method (12). Tumor cells were suspended in PBS at a concentration of 5×10^7 cells/ml, and 0.2 ml of this suspension was injected into a chamber consisting of a ring (Millipore Corp.) covered with Millipore filters (0.2- μ m pore size) on both

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³ The abbreviations used are: FBS, fetal bovine serum; AGM-1470, 6-(chloroacetyl-carbamoyl) fumagillol.

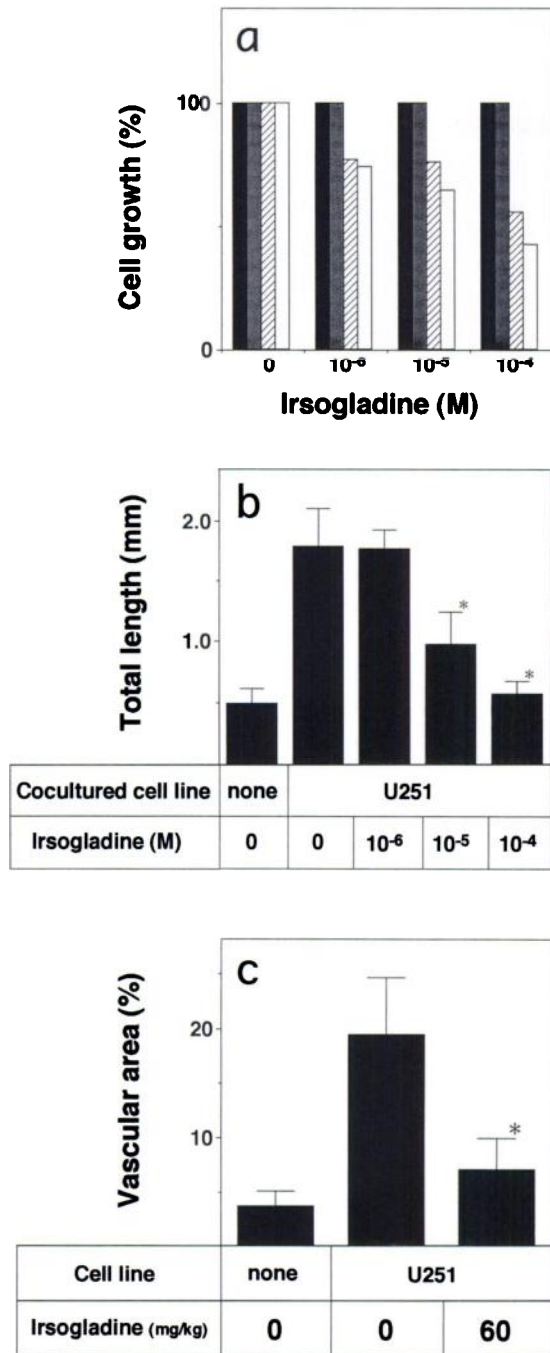


Fig. 1. *a*, differential effects of irsogladine on the proliferation of human vascular endothelial cells and human cancer cells. Exponentially growing human epidermoid cancer KB cells, human glioma U251 cells, human microvascular endothelial cells, and human umbilical endothelial cells were incubated in 35-mm plastic dishes for 5 days with various doses of irsogladine. The number of viable cells was counted by a Coulter counter; 100% cell growth indicates the number of each cell type in the absence of the drug. Columns, means of three dishes. Each value represents the average of triplicate dishes; the data represent the average values of triplicate determinations. ■, KB; □, U251; ▨, human microvascular endothelial cells; □, human umbilical endothelial cells. *b*, quantitative analysis of the effect of irsogladine on tubular morphogenesis of human microvascular endothelial cells cocultured with human glioma U251 cells. The development of tubular structures by vascular endothelial cells cocultured with glioma cells was examined in the absence or presence of 10⁻⁶, 10⁻⁵, and 10⁻⁴ M irsogladine. On the third day, phase contrast microscopic pictures were taken with a still video camera recorder. Eight random fields per dish were measured, and the total tubular length per field was calculated. Columns, mean of five dishes; bars, SD; *, significantly different from untreated controls ($P < 0.01$). *c*, development of capillary networks in a mouse implanted with the human glioma cell line U251 and the effect of p.o. administration of irsogladine. U251 cells at 10⁷ were injected into a chamber covered with a filter, and the chamber was implanted into a dorsal air sac produced in a mouse (25). Mice were killed and carefully skinned on day 5. Irsogladine was administered p.o. every day at 60 mg/kg/day. After the implanted chamber was removed from the s.c. space, a new ring without filters was placed

sides. This chamber containing tumor cells was implanted into a dorsal air sac produced in the mouse by injecting 10 ml air. Five or six mice in each group were killed and carefully skinned on day 5 or 7. After the implanted chamber was removed from the s.c. air fascia, a ring without filters was placed on the same site and photographed. The area of the air sac fascia with a dense capillary network was quantitated with an image processor (Nexus Inc., Tokyo, Japan) through the photograph (12).

Tumor Growth in Nude Mice and Determination of Microvessels. Male BALB/c *nu/nu* mice, 5 weeks old and weighing ~20 g, were used. Mice received s.c. implantations of 1×10^7 human glioma U251 cells. Irsogladine suspended in 5% sodium carboxymethylcellulose was given p.o., and fumagillin in sodium carboxymethylcellulose was given s.c. every day for 4 weeks. Tumor diameters were measured every week, and tumor volume was calculated as (width²) × (length)/2. The tumor was removed and fixed in 10% formalin and embedded in paraffin wax. After the fixation, the tumor was sectioned at a thickness of 8 μm. After the deparaffinization procedure, immunoperoxidase staining with a monoclonal antibody against factor VIII (Nichirei Co., Tokyo, Japan) was performed using standard immunoperoxidase procedures. The number of vessels in each tumor was determined by counting all factor VIII-positive microvessels per ×200 field. We measured the total vessel number in three serial cryostat sections per mouse of five or six mice under various conditions.

Results

Fumagillin and its potent antiangiogenic derivative AGM-1470 were first reported to show their preferential inhibition on proliferation of vascular endothelial cells and antitumor activities against various transplanted tumor types in animals (9, 10). We examined the effect of irsogladine on the proliferation of vascular endothelial and human cancer cells. Irsogladine at 10⁻⁶–10⁻⁴ M inhibited the proliferation of human microvascular endothelial cells and human umbilical endothelial cells in the presence of 10% serum by >30% of the control but did not affect human glioma U251 or human epidermoid cancer KB cells (Fig. 1*a*). Irsogladine thus seemed to specifically inhibit the proliferation of vascular endothelial cells.

Human microvascular endothelial cells undergo tubular morphogenesis when stimulated by epidermal growth factor and transforming growth factor α (13, 14) and vascular endothelial growth factor or interleukin 8 (17). In our coculture model of angiogenesis, human microvascular endothelial cells and bovine endothelial cells show branching, vessel-like structures within collagen gels when cocultured with human glioma cells, as described previously (12, 17). In this model system, human microvascular endothelial cells develop capillary-like structures when cocultured with human glioma U251 cells. Irsogladine at 10⁻⁵ and 10⁻⁴ M inhibited the U251-dependent tubular morphogenesis by 50 and 100%, respectively (Fig. 1*b*).

We used an *in vivo* model of tumor angiogenesis to examine further the effect of irsogladine. In this *in vivo* model, a diffusion chamber containing tumor cells was transplanted into a mouse dorsal air sac (12). Extensive capillary networks developed in the dorsal subcutis in contact with the implanted chamber when the chamber contained U251 cells. Computer image analysis of the area occupied by the capillary networks developed in the chamber ring demonstrated that p.o. administration of irsogladine at 60 mg/kg/day inhibited the development of capillary networks (Fig. 1*c*). p.o. administration of irsogladine at 30 mg/kg/day induced a slight, but significant, inhibition of the development of capillary networks (data not shown).

Another model of tumor angiogenesis was used to examine the

in the same site and photographed (see Ref. 25). Quantitative analysis of the capillary networks in mice was performed using an image processor and photographs, and the results are presented as the mean area ($1 \times \text{mm}^2$) of the capillary networks within the ring of the air sac. Each value represents the mean results obtained from five mice. Columns, means; bars, SD; *, significantly different from untreated controls ($P < 0.01$).

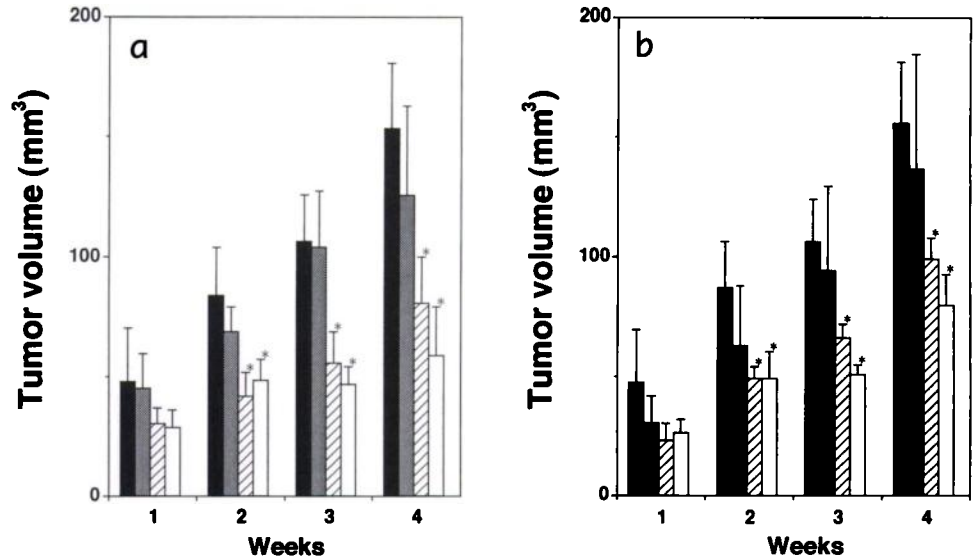


Fig. 2. Antitumor activity of irsogladine (a) and fumagillin (b). Nude mice received s.c. implants of human glioma cells (1×10^7) on day 0 and, beginning the next day, were treated daily with irsogladine (30, 60, and 120 mg/kg/day p.o.) or fumagillin (30, 60, and 120 mg/kg/day s.c.). Tumor volumes (mm^3) were determined at each week after the implantation. ■, control (six mice); □, 30 mg/kg/day irsogladine (five mice); ▨, 60 mg/kg/day irsogladine (six mice); □, 120 mg/kg/day irsogladine (five mice); □, 30 mg/kg/day fumagillin (five mice); ▨, 60 mg/kg/day fumagillin (five mice); □, 120 mg/kg/day fumagillin (six mice). Columns, means; bars, SD; *, significant difference from untreated controls ($P < 0.01$).

effect of irsogladine. s.c. inoculation of 10^7 glioma cells induced tumor growth in nude mice, with average tumor volumes of $150 \pm 30 \text{ mm}^3$ 4 weeks after inoculation. The average tumor volume at 4 weeks was found to be about one-half that of untreated groups when 60 mg/kg/day fumagillin or irsogladine was administered s.c. or p.o., respectively (Fig. 2). The antitumor effects of irsogladine and fumagillin were greater when their doses were increased to 120 mg/kg/day. Daily administration of 30–120 mg/kg/day irsogladine and 30 mg/kg/day fumagillin over a 4-week period did not affect the body weight of the mice. However, there was a loss of body weight in one-half of the mice treated with 60 mg/kg/day fumagillin and in all mice treated with 120 mg/kg/day fumagillin. Histological analysis demonstrated that untreated tumors contained numerous microvessels, which were stained with factor VIII, an endothelial cell-specific marker (data not shown). By contrast, there seemed to be a decrease in the number and diameter of microvessels when treated with 60 mg/kg/day irsogladine or fumagillin. Quantitative analysis demonstrated a dramatic decrease in the number of factor VIII-positive microvessels in mice treated with 60–120 mg/kg/day irsogladine or fumagillin (Fig. 3).

We also examined whether angiogenesis occurring with other types of cancer cells could be inhibited by irsogladine. In the dorsal air sac assay in mice, human hepatic cancer HepG2 cells were transplanted into a chamber, and the effect of p.o.-administered irsogladine was tested (Fig. 4). The presence of HepG2 cells in the chamber promoted the development of capillary networks in the dorsal subcutis in contact with the implanted chamber (Fig. 4, a and b). New development of capillary networks, presumably tumor neovasculatures, was observed in addition to the preexisting microvessels (Fig. 4, a, a', b, and b'). We observed the development of microvessels with curled structures and many tiny bleeding spots in these mice (Fig. 4c). We could not observe the development of such curled microvessels when a chamber without HepG2 cells was transplanted (data not shown). p.o. administration of irsogladine at 30 mg/kg/day (Fig. 4, d and d') and 60 mg/kg/day (Fig. 4, e and e') reduced the development of such tumor microvasculatures. We consistently observed almost similar inhibitory action of irsogladine on hepatic cancer cell-dependent neovascularization in all four mice tested, as seen in Fig. 4. There seemed to be apparent development of neovasculatures when a chamber without tumor cells was placed (Fig. 4, f and f'). Treatment with irsogladine at 60 mg/kg/day did not affect any preexisting vessels (Fig. 4, g and g').

Discussion

Our previous study indicates that irsogladine inhibits the synthesis of tissue-type plasminogen activator and angiogenesis in both *in vitro* and *in vivo* systems (14). In the present study, we have demonstrated that: (a) irsogladine specifically inhibited the proliferation of vascular endothelial cells as opposed to cancer cells; (b) the ability of microvascular endothelial cells to form capillary networks in type I collagen gels *in vitro* and of transplanted glioma cells to form capillary networks in dorsal air sacs *in vivo* were inhibited by irsogladine; (c) irsogladine inhibited tumor growth and neovascularization when human glioma cells were transplanted into dorsal air sacs; and (d) irsogladine also inhibited tumor neovascularization when human hepatic cancer cells were transplanted into dorsal air sacs.

Irsogladine has been found to inhibit the induction of plasminogen activator synthesis in growth factor-treated endothelial cells (14). Migrat-

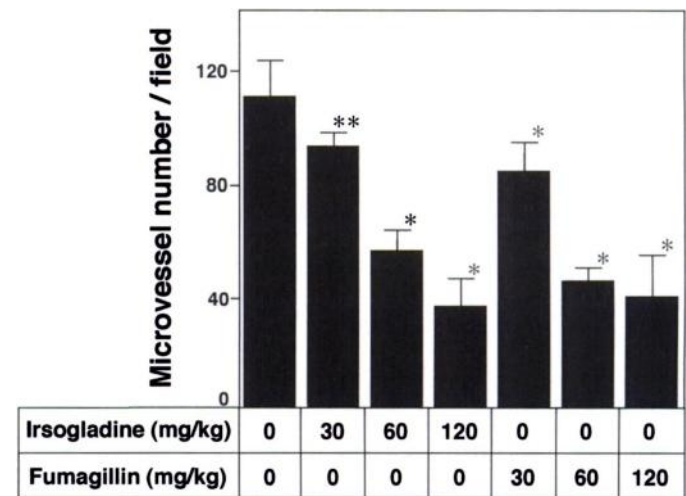


Fig. 3. Tumor neovascularization in animals treated with irsogladine. Immunostaining with antibodies against factor VIII (see "Materials and Methods") showed many microvessels in untreated tumors and a decrease in the number and width of microvessels when treated with drugs. The number of factor VIII-positive microvessels developed in a transplanted tumor was determined by measuring the number of microvessels in each field ($\times 100$) of three serial cryostat sections from each mouse, and an average of five mice is presented. Bar, SD; asterisks, significantly different from untreated control (*, $P < 0.01$; **, $P < 0.05$).

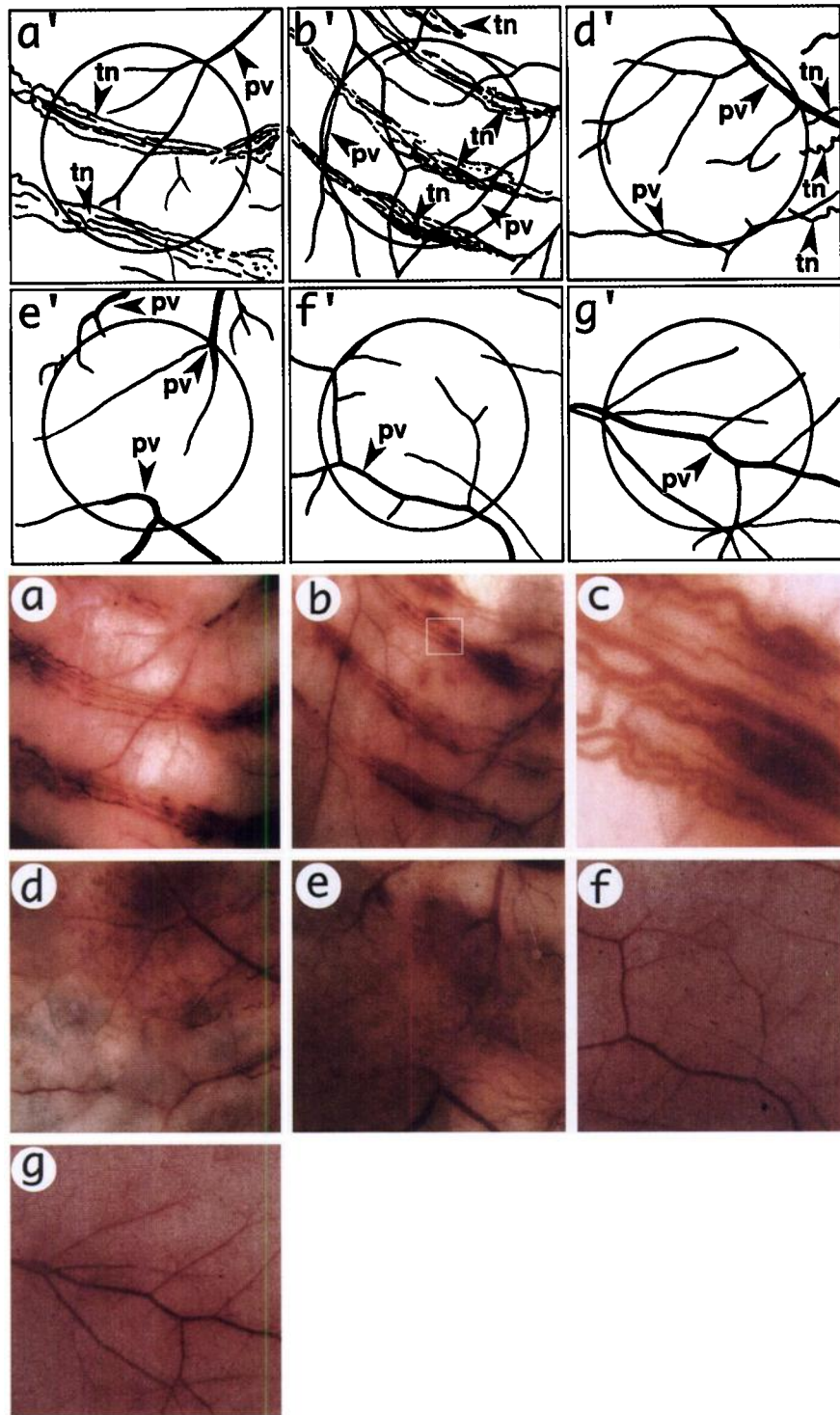


Fig. 4. Formation of capillary networks induced by human hepatic cancer cells in mice and the effect of p.o. administration of irsogladine. Human hepatoma HepG2 cells prepared at a density of 3×10^6 were injected into a diffusion chamber and implanted into a mouse dorsal air space as described in the legend to Fig. 2. Irsogladine was administered p.o. every day for 1 week at 30 and 60 mg/kg daily. Mice were killed and carefully skinned on day 7. After the resection of the mouse dorsal skin, the chamber was carefully removed from the s.c. tissue. A new ring without filters on both sides was placed in the same site to mark the position of the chamber filled with HepG2 cells (*a'*, *b'*, *d'*, and *e'*; circles). Photographs of the capillary networks developed inside the rings in mice implanted with: *a*, chamber containing HepG2 cells in one mouse; *b*, chamber containing HepG2 cells in another mouse; *c*, magnification of the area denoted by the square in *b*; *d*, HepG2 cells implanted and treated with 30 mg/kg/day irsogladine; *e*, HepG2 cells implanted and treated with 60 mg/kg/day irsogladine; *f*, chamber containing HepG2 cells untreated with irsogladine; *g*, chamber without HepG2 cells treated with irsogladine 60 mg/kg/day. *a'*–*g'*, illustrations of *a*–*g*, respectively. *pv*, preexisting vessels; *tn*, tumor neovasculature. In *d* and *d'*, development of short neovasculatures was still observed when treated with 30 mg/kg/day irsogladine. By contrast, there seemed to be no appearance of such short tumor neovasculature when treated with 60 mg/kg/day irsogladine (*e* and *e'*).

ing endothelial cells express plasminogen activator activity (18), and angiogenic factors have been found to enhance the expression and activity of plasminogen activator in endothelial cells (13). Treatment with serine protease inhibitors and anti-plasminogen activator antibodies has been found to inhibit the migration and formation of tubular arrays of vascular endothelial cells (13). These studies indicate the existence of an obligatory coupling between plasminogen activator and angiogenesis. One can expect that inhibition of the protease activity associated with angiogenesis would provide effective antitumor therapy. However, it remains unknown whether the antiangiogenic activity of irsogladine is correlated directly with its inhibitory effect on plasminogen activator

synthesis. On the other hand, irsogladine is also known to modulate the gap junctional intercellular communication in gastric epithelial cells (15). Pepper *et al.* (19) have reported previously that junctional communication is induced in migrating vascular endothelial cells. Intercellular contact between migrating endothelial cells has been found to be essential for the induction of tubular morphogenesis (20). The reinforcement of gap junctional intercellular communication by irsogladine might interfere with cell migration and tubular morphogenesis during neovascularization.

Various strategies have been developed to target tumor neovascularization (1). Among these fungus-producing fumagillin derivatives, AGM-1470 has been used most often. AGM-1470 suppresses tumor

growth of transplanted Lewis lung carcinoma and the B16 melanoma (9, 10), but the underlying mechanism is not known. Here, we describe that irsogladine, which is in clinical use in Japan, also showed specific inhibition of endothelial cell proliferation and inhibited tumor growth *in vivo*. The advantage of irsogladine is that it is a p.o. drug and is less toxic than fumagillin. s.c. administration of fumagillin at 60 and 120 mg/kg/day showed a loss of body weight, and two of six mice died when treated for 12 days with 120 mg/kg/day fumagillin. Irsogladine is p.o. administered to patients with gastric ulcers at 60–80 µg/kg/day, suggesting that ~1000-fold higher doses of the drug could have been used in the mice in our present therapeutic study. p.o. toxicity experiments demonstrate that the LD₅₀s are 5700–6000 mg/kg/day (mouse), 2900–3900 mg/kg/day (rat), and 1000–1200 mg/kg/day (rabbit) when administered daily for up to 2 weeks (21). The p.o. administration of irsogladine at 5000 mg/kg/day for 2 weeks shows no loss of body weight at all in mice (21). Sumi *et al.* (22) have also reported that myelography shows no abnormalities related to irsogladine treatment in rats at 1200 mg/kg/day for 13 weeks.

Development of irsogladine derivatives that have more potent antiangiogenic activity might be useful for further clinical application. A goal in this field is to find an agent that could discriminate between tumor-associated, proliferating vascular cells and normal blood vessels. Further study is required to understand the underlying mechanism by which irsogladine inhibits the tumor-associated vascularization and to determine whether irsogladine will be useful in the treatment of other types of tumors rich in vascularization.

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