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INHIBITOR OF BREAST CANCER REGROWTH AND METASTASIS

(Original report)

Inhibition of breast cancer regrowth and pulmonary metastasis in nude mice by anti-gastric ulcer agent, irsogladine

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Abstract

Irsogladine is a commonly used anti-gastric ulcer agent in Japan, and recent *in vivo* studies have shown it to have anti-angiogenic properties. The exact role of irsogladine as an inhibitor of angiogenesis remains uncertain. In this study, we show that irsogladine inhibited breast cancer regrowth and pulmonary metastasis but had no anti-angiogenic function against HUVEC cells. Irsogladine failed to inhibit proliferation, tubular formation, and the uPA/MMP-1 mRNA expression of HUVEC cells. We also examined the effect of irsogladine in an orthotopic transplant model of human breast cancer metastasis in athymic mice. Human MDA-MB-435 cells were injected into the mammary fat pads. After nine weeks, the tumors were resected under general anesthesia. Irsogladine or vehicle was given p.o. daily thereafter. Daily administration of irsogladine at 120 mg/kg/day over a five-week period had no effect on the body weight of the mice. Tumor regrowth, average volume of pulmonary metastases, and the number of metastases were inhibited by 40, 48 and 64%, respectively. These results suggest that irsogladine may be useful in the breast cancer adjuvant setting.

Key words: angiogenesis, anti-tumor activity, matrix metalloproteinase-1, orthotopic transplant model, urokinase plasminogen activator

Introduction

New blood vessel formation (angiogenesis) is mandatory for growth of breast cancer, and the development within tumor tissues has been implicated in invasion and metastasis in breast cancer, melanoma, prostatic cancer, and other cancers [1, 2]. An effective vessel-targeting therapy should be useful for many types of cancer because delivering the drug to the vessels should be much easier than getting it into all the cells of a solid tumor. Several anti-angiogenic agents are currently undergoing clinical trials [3, 4]. These include interferons [5], tetracycline [6], tamoxifen [7] and thalidomide [8].

Irsogladine has been clinically used as an anti-gastric ulcer agent in Japan, where it acts to strengthen intercellular gap junctions [9]. Irsogladine has been found to inhibit the induction of tissue-type plasminogen activator (tPA) synthesis in endothelial cells and angiogenesis in both in vitro and in vivo models [10]. Recently, irsogladine has also been shown to have anti-tumor activity in murine xenograft models of epidermoid cancer and glioma [11]. To date no work has been performed evaluating irsogladine in breast cancer. In the present study, we investigated the effect of irsogladine in a human breast cancer-athymic nude mouse system [12], specifically looking at local-regional tumor regrowth and the formation of pulmonary metastases after primary tumor resection.

Materials and methods

Cell culture and drugs

Human MDA-MB-435 breast cancer cells (a gift from Dr. Janet Price, The University of Texas M. D. Anderson Cancer Center, Houston) were cultured in minimum essential medium (Life Technologies [GIBCO BRL], Gaithersburg, MD) supplemented with multi-vitamins, sodium pyrophosphate (100 mM), penicillin (10^5 U/100 ml), streptomycin (10 µg/100 ml) and fetal calf serum (5%) (supplements from Sigma Chemical Co., St. Louis, MO) in a 5% CO_2-air atmosphere at 37°C. The HUVEC umbilical vein cell kit and recombinant human vascular endothelial cell growth factor (VEGF) were obtained from Clonetics (San Diego, CA) and Genentech Inc. (South San Francisco), respectively. Irsogladine and VEGF were freshly prepared for each experiment.

Cell proliferation assay

Cell proliferation was determined using the CellTilter Aqueous nonradioactive cell proliferation assay (Promega Corp., Madison, WI). Cultured cells (5 x 10^3 cells/well) were plated with various doses of irsogladine into 96-well tissue culture plates (Costar, Cambridge, MA). After 48 h, 20 µl/well of combined tetrazolium/phenazine methosulfate solution was added. After 60 min at 37°C in a 5% CO_2-air atmosphere, an absorbance reading at 450 nm was measured using Elx 800 (Bio-Tek Instruments, Inc., Winooski, VT). The results are reported as a percentage of untreated control cells. Each group has eight wells.

Tubular formation assay

HUVEC cells (4×10^4 cells/well) were seeded on gels of growth factor reduced Matrigel matrix (Becton Dickinson, Bedford, MA) with or without VEGF (10 ng/ml) and/or irsogladine (100 µM) into 24-well tissue culture plates (Costar). After 24 h, microscopic pictures of each well were taken and the total length of tubular formation per field was measured using a road map pen (American Map, Maspeth, NY). Eight random fields were measured and the total length per field was calculated.

Northern blot analysis

HUVEC cells were incubated with or without VEGF (10 ng/ml) and/or irsogladine (100 µM) for 24 h. Total RNA was extracted and subjected to northern blotting as described previously [13, 14]. Briefly, the RNA was fractionated on 1% agarose gel containing 2.2 M formaldehyde, and transferred to an Optitran membrane (Schleicher and Schuell, Inc., Keene, NH). The membranes were baked in a vacuum oven at 80°C, prehybridized, hybridized, washed, and exposed to an X-ray film with an intensifying screen at -80°C. The probes specific for human urokinase-type plasminogen activator (uPA), matrix metalloproteinase-1 (MMP-1) and ß-actin were an 1.5-kb Pst I-Pst I fragment of pEMBL8, a 2.0-kb EcoRI-Pst I fragment of pSP64 and an 1.4-kb EcoRI-EcoRI fragment of pBluescript SK-, respectively. All plasmids were obtained from the American Type Culture Collection, Rockville, MD. After exposure to X-ray film and development, expression was measured densitometrically using Adobe Photoshop 4.0 (Adobe System Inc., Seattle, WA). Relative levels of specific uPA and MMP-1 mRNA were compared

after normalization by dividing each expression densitometrically by that of ß-actin on the same filter.

Human breast cancer xenograft-nude mouse model

The nude mouse xenograft model was performed as described previously by Sledge et al. [12]. Briefly, female BALB/c nude mice (Harlan Sprague Dawley, Indianapolis, IN), six to eight weeks old, were used. MDA-MB-435 cells (5×10^5 cells/mouse) were injected into the mammary fat pads of 40 nude mice. After nine weeks, the tumors were resected under general anesthesia. Irsogladine, suspended in 0.5% sodium carboxymethylcellulose (vehicle), was given p.o. daily thereafter. The control mice were given only vehicle. The administrations were continued through week 14. The lungs were removed and fixed in Bouin's solution. Macroscopically visualized tumors were enumerated and measured. Tumor volume was calculated as (length) x $(\text{width})^2$ /2. Animal experiments were performed according to NIH guide lines and approved from institutional animal care and use committee.

Statistical analysis

Statistical analysis was performed using Statview (version 4.02; Abacus Concepts, Berkeley, CA) software. Data were evaluated by Fisher's exact probability test or Student's *t*-test.

Results

Irsogladine has been shown to specifically inhibit proliferation of vascular endothelial cells [11]. We examined the effect of irsogladine on the proliferation of HUVEC and MDA-MB-435. Irsogladine at 100 μM inhibited the proliferation of HUVEC and MDA-MB-435 by 15% but had no effect at one and 10 μM (data not shown). There was no difference between the two cell lines. As shown in Figure 1, VEGF induced tubular formation by HUVEC cells, but irsogladine did not block the stimulatory effect of VEGF. Although irsogladine has been found to inhibit the induction of tPA mRNA in epidermal growth factor (EGF) treated endothelial cells [10], irsogladine has no effect on the uPA/ MMP-1 cascade in HUVEC cells incubated without growth factors for 24 h in a serum free medium (Table 1).

We have previously used an athymic mouse model of human breast cancer to evaluate the effect of anti-angiogenic agents on tumor regrowth and metastasis [12]. Eight of 40 primary tumor-bearing mice died during tumor resection and 34 survival mice were used for the following study. Daily administration of irsogladine at 120 mg/kg/day over a 5-week period did not affect the body weight of the mice, though one of 18 control mice died before the study was completed. Primary tumor regrowth occurred in 14 of 16 irsogladine-treated mice and all of vehicle-treated controls. As shown in Table 2, orally delivered irsogladine inhibited tumor regrowth by 40% of control.

As shown in Table 2, daily treatment with irsogladine at 120 mg/kg/day decreased the incidence, the volume, and the number of pulmonary metastases. Only one of 17 control mice failed to develop pulmonary metastases, compared with five of 16 irsogladine-treated mice. Similarly, the volume and the number of metastases were inhibited by 48 and 64%, respectively (Table 2).

Discussion

In our laboratory, we have used a human breast cancer-athymic nude mouse model system to evaluate the effect of adjuvant anti-angiogenic therapy after primary tumor resection [12]. In this system, tumor regrowth and spontaneous pulmonary metastasis were inhibited by irsogladine orally administered after primary tumor resection.

Irsogladine, which has been clinically used as an anti-gastric ulcer agent in Japan, has been reported to have efficacy in metastatic [15] and inoperable gastric cancer [16] when combined with UFT (a mixture of tegafur and uracil in a molar ratio of 1:4). Experimentally, irsogladine has been shown to inhibit angiogenesis [10, 11] and carcinogenesis [17, 18].

Irsogladine, a potent inhibitor of angiogenesis, has been found to inhibit the induction of tPA mRNA in EGF-treated endothelial cells [10]. In human breast cancer, uPA rather than tPA has been shown to play a more important role both in tumor-related angiogenesis and as a prognostic agent [19], and MMPs have been shown to be involved in endothelial cells during the angiogenic process [20, 21]. We examined the hypothesis that irsogladine might affect the uPA/MMP cascade in vascular endothelial cells. Irsogladine failed to inhibit proliferation, tubular formation, and the uPA/MMP-1 mRNA expression of human vascular endothelial cells. These results suggest that HUVEC cells might be not responsive to irsogladine.

Sato et al. [10] have suggested that an anti-angiogenic effect of irsogladine may be general and not restricted to the effect of EGF. The reason was that the angiogenic potential of human omental microvascular endothelial cells, which had not produced EGF [22], was not related to EGF. Ren et al. [23] have suggested that the mechanism may be independent of plasminogen activation in vivo because the agent inhibited bFGF-induced angiogenesis in tPA-knockout and uPA-knockout mice. In addition, irsogladine has been reported to upregulate

intercellular communication via gap junctions between cultured rabbit gastric endothelial cells [9], and the junctional communication has been reported to be induced in migrating vascular endothelial cells [24]. Irsogladine upregulated expressions of connexin26 and 32, gap junction proteins, in the rat liver [25]. These findings suggest that the modulation of gap junctional intercellular communication by irsogladine might correlate to its anti-angiogenic effect, but the exact role of irsogladine as an inhibitor of angiogenesis remains uncertain.

Irsogladine modestly inhibited not only tumor regrowth of MDA-MB-435 mammary fat pad tumors but pulmonary metastases and might be a unique and useful drug in treatment of breast cancer adjunctive to surgery. Further study is required to clarify the mechanism by which irsogladine inhibits the tumor regrowth and metastasis.

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References

1. Weidner N, Semple JP, Welch WR, Folkman J: Tumor angiogenesis and metastasis correlation in invasive breast carcinoma. N Engl J Med 324: 1-8, 1991

2. Fidler LJ, Ellis LM: The implications of angiogenesis for the biology and therapy of cancer metastasis. Cell 79: 85-88, 1994

3. Barinaga M: Designing therapies that target tumor blood vessels. Science 275: 482-484, 1997.

4. Twardowski P, Gradishar WJ: Clinical trial of antiangiogenic agents. Curr Opin Oncol 9: 584-589, 1997

5. Tsuruoka N, Sugiyama M, Tawaragi Y, Tsujimoto M, Nishihara T, Goto T, Sato N: Inhibition of in vitro angiogenesis by lymphotaxin and interferon-gamma. Biochem Biophys Res Commun 155: 429-435, 1988

6. Tamargo RJ, Bok RA, Brem H: Angiogenesis inhibition by minocycline. Cancer Res 51: 672-675, 1991

7. Gagliardi A, Collins DC: Inhibition of angiogenesis by antiestrogens. Cancer Res 53: 533-535, 1993

8. D'Amato RJ, Loughnan MS, Flynn E, Folkman J: Thalidomide is an inhibitor of angiogenesis. Proc Natl Acad Sci USA 91: 4082-4085, 1994

9. Ueda F, Kyoi T, Mimura K, Kimura K, Yamamoto M: Intercellular communication in cultured rabbit gastric epithelial cells. Jpn J Pharmacol 57: 321-328, 1991

10. Sato Y, Morimoto A, Kiue A, Okamura K, Hamanaka R, Kohno K, Kuwano M, Sakata, T: Irsogladine is a potent inhibitor of angiogenesis. FEBS Lett 322: 155-158, 1993

11. Ono M, Kawahara N, Goto D, Wakabayashi Y, Ushiro S, Yoshida S, Izumi H, Kuwano M, Sato Y: Inhibition of tumor growth and neovascularization by an anti-gastric ulcer agent, irsogladine. Cancer Res 56: 1512-1516, 1996

11. Sledge GW Jr., Qulali M, Goulet R, Bone EA, Fife R: Effect of matrix metalloproteinase inhibitor batimastat on breast cancer regrowth and metastasis in athymic mice. J Natl Cancer Inst 87: 1546-1550, 1995

13. Nozaki S, Sledge GW Jr, Nakshatri H: Cancer cell-derived interleukin 1alpha contributes to autocrine and paracrine induction of pro-metastatic genes in breast cancer. Biochem Biophys Res Commun 275: 60-62, 2000

14. Patel NM, Nozaki S, Shortle NH, Bhat-Nakshatri P, Newton TR, Rice S, Gelfanov V, Boswell SH, Goulet RJ Jr, Sledge GW Jr, Nakshatri H: Paclitaxel sensitivity of breast

cancer cells with constitutively active NF-kappaB is enhanced by IkappaBalpha super-repressor and parthenolide. Oncogene 19: 4159-4169, 2000

15. Hosokawa T, Otani Y, Ogawa K, Kajiwara T: Complete disappearance of metastatic abdominal tumors from gastric cancer after treatment with irsogladine maleate. J Cancer Res Clin Oncol 118: 565-566, 1992

16. Hosokawa T, Ogawa K, Otani Y, Kajiwara T: Two cases of gastric cancer remarkably reduced with a combined dosage of irsogladine maleate preparation and UFT. Oncol Rep 1: 93-95, 1994

17. Sugie S, Okamoto K, Ueda F, Watanabe T, Tanaka T, Mori H: Suppressive effect of irsogladine maleate on diethylnitrosamine-initiated and phenobarbital-prompted hepatocarcinogenesis in male F344 rats. Jpn J Cancer Res 89: 371-376, 1998

18. Hirose Y, Tanaka T, Makita H, Yang M, Satoh K, Hara A, Maeda M, Toriyama HB, Mori H, Tsuda H: Suppressing effects of 6-(2,5-dichlorophenyl)-2,4-diamino-1,3,5-triazine and related synthetic compounds on azoxymethane-induced aberrant crypt foci in rat colon. Jpn J Cancer Res 87: 549-554, 1996

19. Janicke F, Schmitt M and Graeff H: Clinical relevance of the urokinase-type and tissue-type plasminogen activators and of their type 1 inhibitor in breast cancer. Semin Thromb Hemostas 17: 303-312, 1991

20. Hanemaaijer R, Koolwijk P, le Clercq L, de Vree WJ, van Hinsbergh VW: Regulation of matrix metalloproteinase expression in human vein and microvascular endothelial cells. Effects of tumour necrosis factor alpha, interleukin 1 and phorbol ester. Biochem J 296: 803-809, 1993

21. Fisher C: Gilbertson-Beadling S, Powers EA, Petzold G, Poorman R, Mitchell MA: Interstitial collagenase is required for angiogenesis in vitro. Dev Biol 162: 499-510, 1994

22. Okamura K, Sato Y, Matsuda T, Hamanaka R, Ono M, Kohno K, Kuwano M: Endogenous basic fibroblast growth factor-dependent induction of collagenase and interleukin-6 in tumor necrosis factor-treated human microvascular endothelial cells. J Biol Chem 266: 19162-19165, 1991

23. Ren CJ, Ueda F, Roses DF, Harris MN, Mignatti P, Rifkin DB, Shapiro RL: Irsogladine maleate inhibits angiogenesis in wild-type and plasminogen activator-deficient mice. J Surg Res 77: 126-131, 1998

24. Pepper MS, Spray DC, Chanson M, Montesano R, Orci L, Meda P: Junctional communication is induced in migrating capillary endothelial cells. J Cell Biol 109: 3027-3038, 1989

Table 1. Level of uPA and MMP-1 mRNA in HUVEC cells

	Control	Irsogladine	VEGF	VEGF + Irsogladine
uPA/ß-actin[a]	0.51	0.57	0.52	0.63
MMP-1/ß-actin[a]	1.36	1.24	0.99	0.97

[a]Relative levels of specific uPA and MMP-1 mRNA were compared after normalization by dividing each expression densitometrically by that of ß-actin on the same filter.

Table 2. Effect of irsogladine on human breast cancer xenograft-nude mouse model

	Control (n = 17)	Irsogladine (n = 16)	IR[a] (%)	*p* value[b]
Primary tumor regrowth	17/17 (100%)	14/16 (88%)	-	.23
Volume of regrowth tumor (mm^3)	7389 ± 1346 [c]	4466 ± 832	39.6	.08
Without pulmonary metastases	1/17 (5.9%)	5/16 (31%)	-	.04
Number of metastases	13.3 ± 5.2	4.8 ± 1.3	63.9	.14
Volume of metastases (mm^3)	37.8 ± 11.4	19.5 ± 5.9	48.4	.17

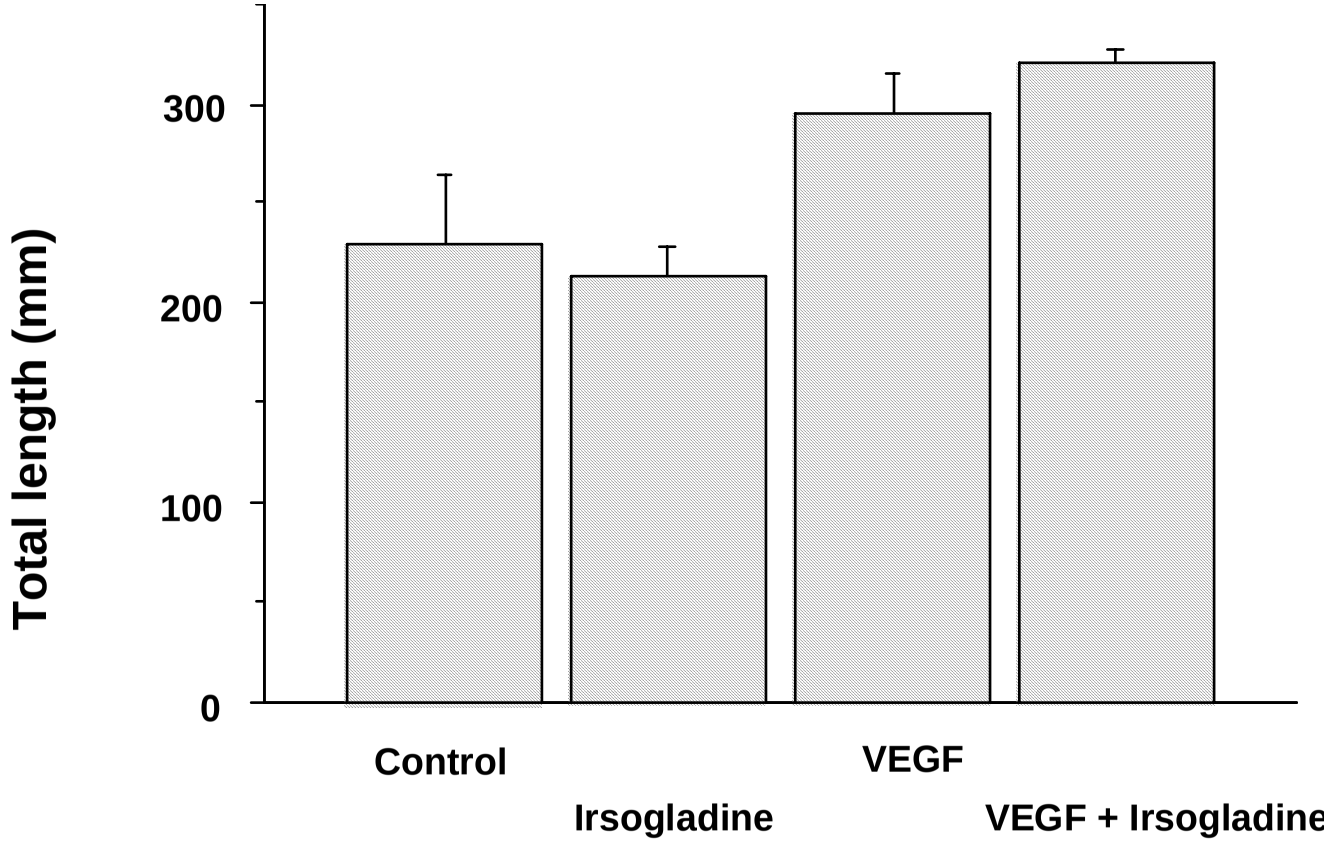
[a]IR (inhibition ratio) = {1-(irsogladine treated group/control group)} x 100

[b]by Fisher's exact probability or Student's t test

[c]mean ± SE per mouse

LEGENDS

Figure 1. Effect of irsogladine on tubular formation by VEGF-treated HUVEC cells. HUVEC cells were seeded on gels of growth factor reduced Matrigel matrix with or without VEGF (10 ng/ml) and/or irsogladine (100 μM) into 24-well tissue culture plates. After 24 h, microscopic pictures of each well were taken and the total length of tubular formation per field was measured. Eight random fields were measured and the total length per field was calculate. Total length is represented as mean length ± SE.


Total length (mm)
300
200
100
0
Control
Irsogladine
VEGF
VEGF + Irsogladine