Inhibitory Effect of TNP-470 on Lymph Node Metastasis of Human Gastric Carcinoma Line Established by Orthotopic Implantation

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ABSTRACT

We assessed the anti-tumor and anti-metastatic effects of angiogenic inhibitor TNP-470, 6-O-(N-chloroacetyl-carbamoyl)-fumagillol, semisynthtic analogue of fumagillin, on lymph node metastasis of human gastric carcinoma using our established orthotopic implantation model of AZL5G. This line was derived from a parental human gastric cancer cell line, AZ521, which had low capacity for metastasis. 5×10^6 cells were implanted orthotopically into the stomachs of nude mice, which had been randomly divided into 3 groups: a control group given saline solution, a group receiving 15 mg/kg TNP-470, a group receiving 30 mg/kg TNP-470. TNP-470 (15 or 30 mg/kg) was given s.c. on alternate days for 6 weeks from day 1 after orthotopic implantation. In the control group, lymph node metastasis developed in 5 of 6 mice (83.3%). Lymph node metastasis developed in 3 of 4 mice (75.0%) receiving of 15mg/kg TNP-470, and in 3 of 5 mice (60.0%) receiving of 30mg/kg TNP-470. TNP-470 was revealed to have no inhibitory effect on the incidence of lymph node metastasis. However, it was also observed that administration of 30mg/kg TNP-470 reduced the number of lymph node metastases statistically. The inhibitory effect on the production of vascular endothelial growth factor (VEGF) by AZL5G cells were not altered by TNP-470 administration at the concentration ranging from 10^{-1} to 10^3 ng/ml. These results indicate that administration of TNP-470 in co-administration cytotoxic agents may be effective on keeping micrometastases dormant in the clinical therapeutic strategy for gastric cancer patients.

Key words : Gastric cancer cells, Lymph node metastasis, Orthotopic implantation, TNP-470

INTRODUCTION

Lymph node metastasis, a frequent occurrence in several human carcinomas, is one of the most predominant prognostic factors in the cancer treatment[1]. Although lymph nodes are by far the most common site of metastasis for human carcinomas, the actual mechanisms by which carcinoma cells metastasize to lymph

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nodes are still unclear. These mechanisms need to be characterized in order to develop new therapeutic modalities, and there is a need to develop better relevant animal metastatic models for this purpose. We have developed an orthotopic implantation model of human gastric carcinoma line AZ521 and describe a cell line, designated AZL5G, with a highly metastatic potential to lymph nodes[2].

Angiogenesis is essential for the growth of solid tumors[3], especially in the early phase following several steps of the complex metastatic process. Malignant tumors are always dependent on the development of blood supply through angiogenesis for growth, and inhibition of angiogenesis may be an effective treatment for metastasis of solid tumors. TNP-470 is a potent angiogenesis inhibitor and an analogue of fumagillin, which is a natural antibiotic secreted by Aspergillus fumigatus fresenius[4]. At present, correlation between angiogenesis and lymph node metastasis and efficacy of this agent for lymph node metastasis are not clear. In this paper, we examined the inhibitory effect of TNP-470 on lymph node metastasis of human gastric carcinoma cells established orthotopic implantation in nude mice.

MATERIALS AND METHODS

Anti-angiogenic agent

TNP-470 was the kind gift of Takeda Chemical Industries, Ltd. (Osaka, Japan). Its structure, disposition and metabolism have already been reported[4,5]. In *in vivo* experiment, TNP-470 was suspended in a vehicle composed of 0.5% ethanol plus 5% gum arabic in saline. In *in vitro* experiment, TNP-470 was dissolved in dimethylsulfoxide and RPMI-1640 (GIBCO, Grand Island, NY, USA).

Animals

Athymic female BALB/c nu/nu mice, 6-7 weeks old, which originated from the Central Institute for Experimental Animals (Kawasaki, Japan), were purchased from CLEA Japan, Inc. (Tokyo, Japan). Nude mice weighing 18-22g were used.

Cell lines

Human gastric carcinoma cell line, AZL5G with highly lymph node metastatic capability[2], were used in this study. This cell line showed poorly differentiated adenocarcinoma line with medullary type, that were established by *in vivo* stepwise selections according to the method described by Morikawa *et al*.[6], and maintained in our laboratory. Briefly, a human gastric carcinoma cell line, AZ521 (5×10^6/0.1ml) was implanted orthotopically under the serosal membrane in the greater curvature of the antrum in nude mice. After 6 weeks, the mice were killed and autopsied. The regional lymph nodes were resected to evaluate the metastatic potential of cell lines, and cells from lymph node metastatic foci were expanded *in vitro* and subsequently implanted orthotopically. By repeating these procedures four times we obtained AZL5G. As shown in Table 1, AZL5G cells had an enhanced potential for lymphogenous metastasis. It was also observed that none of the lines exhibited pulmonary metastasis within the experimental interval.

Table 1 Metastasis After Ortothopic Implantation of Gastric Carcinoma Lines

Gastric carcinoma lines	Total number of mice	No. of local tumor growth	No. of mice with lymph node metastasis
AZ521	14	14	2(14.3%)
AZL5G	15	15	14(93.3%)*

Metastases were assessed 6 weeks after implantation.
*$p<0.05$ vs AZ521 were considered significant.

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords by means of 0.25% trypsin digestion (DIA-IATRON, Tokyo, Japan).

Experimental metastasis assay

We performed experimental metastasis assay using lymph node metastasis model, to in-

vestigate the inhibitory effect of TNP-470. AZL5 G cells (5×10^6/0.1 ml in PBS) were implanted orthotopically in nude mice using a 1ml tuberculin syringe fitted with a 30-gauge needle. Mice were divided into 3 groups; a control group given saline solution (n=6), a group receiving 15 mg/kg TNP-470 (n=4), a group receiving 30 mg /kg TNP-470 (n=5). The TNP-470 (15 or 30 mg/ kg) was given s.c. on alternate days for 6 weeks from day 1 after orthotopic implantation. After 6 weeks, the mice were killed and autopsied. Then, the frequency of lymph node metastases was evaluated macroscopically and histopathologically. In addition, tumors growing on the stomach were removed and weighed in usual manner.

Cell proliferation assay *in vitro*

Human umbilical vein endothelial cells (HUVEC) (1×10^4/well) were suspended in RPMI-1640 with 10% FBS, and cultured in a 96-well plate. Culture supernatants were replaced the next day (day 1) with fresh medium, containing TNP-470 at the concentrations of 10^{-1}, 1, 10^1, 10^2 and 10^3 ng/ml. On day 4, the number of viable cells was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; Sigma, Milwaukee, WI, USA] assay. The MTT assay was previously described. Briefly, 10μl of 5 mg/ml MTT was added and cells were incubated another 4 hour to allow cleavage of MTT to occur. A purple formazan product was then formed by the action of mitochondrial enzymes in living cells. Cells were solubilized with 150 μl of isopropanol with 0.04 N HCl_2 and at the end of the assay the number of viable cells was estimated from the absorbance at 570 nm and using an MTP-120 microplate reader (Corona Electric Co., Ibaragi, Japan). Growth rate was calculated for each concentration as absorption of the experimental well on day 4/ absorption of the control well on day 4.

Vascular endothelial growth factor (VEGF) production

AZL5G cells were cultured in 96-well plates at 1×10^4 cells/well in RPMI-1640 medium with 10% FBS. The culture media were changed to RPMI-1640 containing no FBS but various concentration of TNP-470 (10^{-1}, 1, 10^1, 10^2 and 10^3 ng /ml) the next day (day1). Supernatants were collected after 2 days, and concentration of vascular endothelial growth factor (VEGF) were measured using VEGF enzyme-linked immunosorbent assay kits (IBL, Fujioka, Japan).

Statistical analysis

The χ^2 test was used to compare the number of mice with metastasis in each group. Other data were analyzed statistically using the unpaired ***t***-test. A ***P***-value of less than 0.05 was considered statistically significant.

RESULTS

Primary tumor growth

Six weeks after orthotopic implantation with AZL5G, the primary gastric tumor showed extensive local growth in every mouse (Fig. 1).

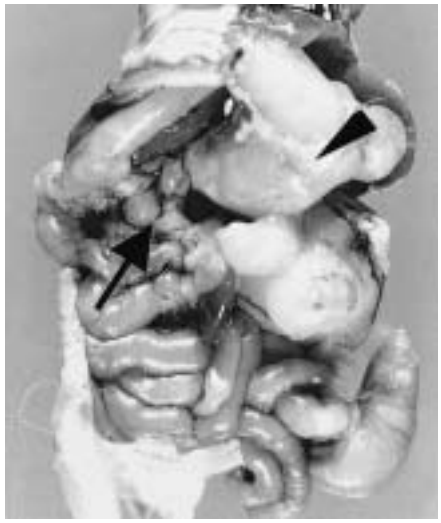

Fig. 1 Macroscopic views of primary tumor growth and metastasis. Gastric tumor (AZL5 G) shows an extensive local growth 6 weeks after orthotopic implantation in every mouse (arrowhead). An arrow shows regional lymph node metastasis.

It was also observed that the implanted AZL5G tumors resulted in regional lymph node metastasis in nude mice. Table 2 shows the primary gastric tumor weight in the control group and the TNP-470 groups at killing. TNP-470 had no inhibitory effect on primary tumor growth.

Table 2 Inhibitory Effect of TNP-470 on Primary Tumor Growth

Group	Total number of mice	Primary Tumor Weight
Control	6	2.51 ± 0.27
TNP-470 15 mg／kg	4	2.07 ± 0.23
TNP-470 30 mg／kg	5	2.10 ± 0.33

Primary tumor weight were evaluated 6 weeks after implantation.

Histopathological characteristics

Histopathological findings of the primary and metastatic lesions are presented in Fig. 2, all of which show poorly differentiated adenocarcinoma of the medullary type, and histopathological appearance of them strongly resembled that of primary gastric tumor.

Lymph node metastasis

The frequency of lymph node metastases is summarized in Table 3. The lymph node metastatic rate was 83.3% in the control group. Although the incidence of lymph node metastasis was not reduced in the TNP-470 groups statistically, the number of lymph node metastases was reduced in the TNP-470 groups. The actual number of lymph node metastases was 3.8 ± 2.3 in the control group, 1.8 ± 1.3 in the 15mg/kg TNP-470 group and 1.2 ± 1.3 in the 30mg/kg TNP-470 group. TNP-470 reduced the number of lymph node metastases statistically ($p<0.05$).

Cell proliferation

We also examined the effect of TNP-470 on cell proliferation of HUVEC. As shown in Fig. 3, the growth of cultured cells with TNP-470 was inhibited in an almost dose-dependent manner.

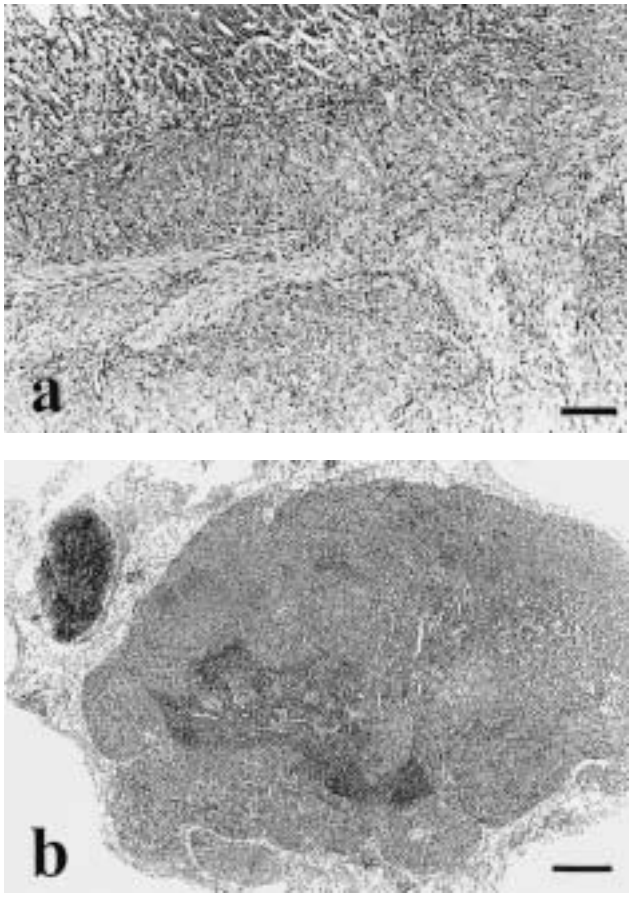

Fig. 2 Microscopic views of the tumors in nude mouse after orthotopic implantation. (a) primary gastric tumor of AZL5G shows poorly differentiated adenocarcinoma of the medullary type. (b) Regional lymph node involved with AZL5G cells. Tissues were fixed, embedded, sectioned, and stained with H&E using standard procedures. For (a) Bar = 100μm. For (b) Bar = 300μm.

Table 3 Inhibitory Effect of TNP-470 on Lymph Node Metastasis

Group	Total number of mice	Incidence of lymph node metastasis	Mean No. of lymph node metastases ± SD
untreated (saline solution)	6	5 (83.3%)	3.8 ± 2.3
TNP-470 (15 mg／kg)	4	3 (75.0%)	1.8 ± 1.3
TNP-470 (30 mg／kg)	5	3 (60.0%)	1.2 ± 1.3*

Metastases were assessed 6 weeks after orthotopic implantation.
*$p<0.05$ vs control group was considered significant.

VEGF production

To investigate the inhibitory effect, we determined on the amount of VEGF produced by

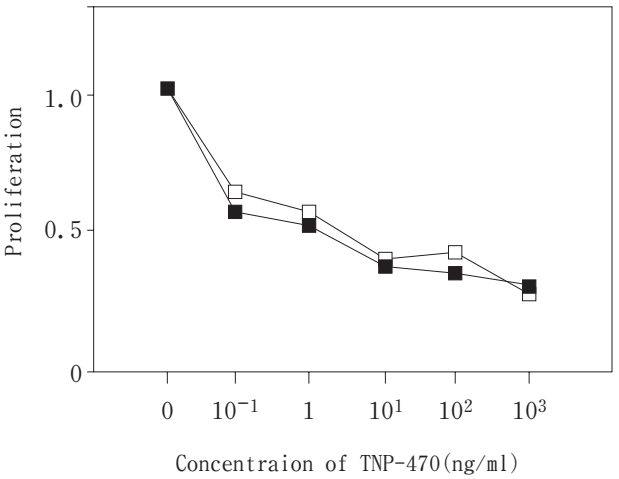

Fig. 3 Effects of TNP-470 on *in vitro* proliferation of HUVEC. The open squares denote first experiment. The closed squares denote second experiment. The proliferation is shown as the ratio of the growth for each concentration with/without TNP-470.

AZL5G cells with TNP-470 treatment. As shown in Fig. 4, the production of VEGF by AZL5G

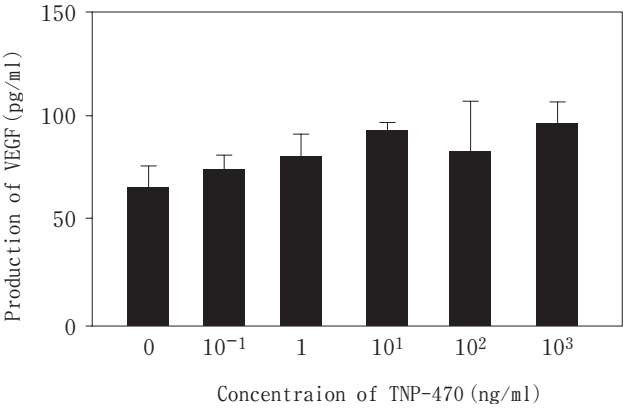

Fig. 4 Effects of TNP-470 on production of VEGF by AZL5G. Bars represent the mean ± standard error.

cells were not altered by TNP-470 administration at the concentration ranging from 10^{-1} to 10^{3} ng/ml.

DISCUSSION

Growth of neoplastic cells is a fundamental component of the complex multistep process leading to the formation of metastases, that has been shown to be dependent on angiogenesis especially in solid tumors[3], and recent researches concerned metastasis have focused on the anti-angiogenic therapy[7,8]. Angiogenic inhibitor TNP-470, 6-O-(N-chloroacetyl-carbamoyl)-fumagillol, semisynthtic analogue of fumagillin, has strong inhibitory activities against both tumor growth and metastasis[8,9]. It is thought that this agent exerts its anti-tumor effect by preventing tumor neovascularization and endothelial cell proliferation[5]. Although it was also reported that TNP-470 inhibited liver metastasis in human colon cancer[7,8], its inhibitory effect has not been examined for lymph node metastasizing gastric carcinoma line established by orthotopic implantation using cell suspension technique in nude mice. In the present study, an orthotopic implantation model of human gastric carcinoma cell lines designated as AZL5G, with a highly metastatic potential to lymph nodes, were used.

We have previously reported the inhibitory effect of liver metastasis by using an intrasplenic injection model with a highly metastatic human gastric carcinoma cell line, AZH5c[10], and liver metastasis could be completely inhibited by administration of 30 mg/kg TNP-470. In the early models demonstrating hematogenous metastasis, tumor cells were injected into the spleens of animals[6,10,11]. These models do not permit study of several steps of the complex metastatic process such as the local tumor growth, local invasion and intravasation of tumor cells, which are important components of the metastatic process leading to the formation of metastatic foci. Recently, animal models implanting tumor cells or tissues orthotopically into the original organ could reflect the entire scope of hematogenous liver metastasis, that have been considered to be more suitable to evaluate the interactions between tumor cells and the microenvironment in the organ[12,13]. Furthermore, we have reported human gastric carcinoma cell line, AZH5G with a highly liver metastatic capability, which was established by orthotopic tumor cell implantation[14], and liver metastasis could be completely inhibited by administration

of 30 mg/kg TNP-470 (date not shown). Thus, orthotopic implantation models should be useful for investigation of the metastatic mechanisms and for establishment of a new therapeutic approach for human gastric cancer.

In this study, TNP-470 had no inhibitory effect on primary tumor growth in lymph node metastatic experiments. Some reports have indicated that this agent has no significant effect on primary tumors transplanted orthotopically[15,16]. However, administration of 30mg/kg TNP-470 reduced the number of lymph node metastases statistically. These findings suggested that a rapidly proliferating tumor is angiogenesis-dependent such as primary tumor in orthotopic implantation models, and angiogenesis may be more dominant in the metastatic site. On the other hand, anti-metastatic effects by TNP-470 were weak in lymphogenous metastasis as to the incidence of lymph node metastasis. In this regard, it is assumed that there may be other factors in lymphogenous metastatic capability of AZL5G cells *in vivo* such as the interactions of cell-adhesion receptors with ECM components. Although the action of angiogenic factors on lymphatic vessels is not still clear, there is a possibility that TNP-470 also have an inhibitory effect on the growth of lymphatic endothelial cells as to reduced number of lymph node metastases.

VEGF production by AZL5G cells were not inhibited by administration of TNP-470. Consequently, this cytostatic agent seems to be important in anti-angiogenic activity for lymph node metastasis influenced by the growth of vascular endothelial cell.

In conclusion, angiogenic inhibitor TNP-470 had less inhibitory effect on incidence of lymph node metastasis, but reduced the number of lymph node metastases. Angiogenic inhibitor TNP-470 in co-administration of cytotoxic agents may be effective on keeping micrometastases dormant. Thus, this therapeutic strategy should be useful for gastric cancer patients with curative resection of primary neoplastic lesions.

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(Accepted for publication, Dec. 26, 2001)