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The inhibitory effects of TNP470 on tumour growth of head and neck carcinoma cell producing interleukin-8

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Abstract

The anti-tumour effect of the angiogenic inhibitor TNP470, σ-(chloro-acetyl-carbamoyl) fumagillol, a synthetic analogue of fumagillin, was studied *in vitro* and *in vivo* using KB cells, one of the human head and neck carcinoma cell lines that produce interleukin(IL)-8. In the *in vitro* study, the combination treatment of TNP470 and anti-IL-8 antibody significantly reduced the proliferation of KB cells. In the *in vivo* studies, TNP470 administration by any route (intratumoral: it, intraperitoneal: ip, intravenous: iv) reduced the tumour volume significantly, compared to the control group. Among the groups administered TNP470, the anti-tumour effect was strongest in the it group. Furthermore, the concurrent treatment of anti-IL-8 antibody and TNP470 also maximally reduced the tumour volume. The combination therapy of TNP470 and anti-IL-8 antibody was very effective. These results suggest that combination therapy of TNP470 and anti-IL-8 antibody could be beneficial for solid tumours, such as head and neck cancer.

Key words: Angiogenesis Inhibitors; Head and Neck Neoplasms; Carcinoma, Squamous Cell; Interleukin 8

Introduction

Angiogenesis is essential for the growth of solid tumours, especially in the early phase of tumour growth. This fact suggests that anti-angiogenesis agents may have an anti-tumour effect on solid tumours. TNP470 (AGM-1470), a synthetic analogue of fumagillin, has been reported to be an angiogenic inhibitor. There are several reports of *in vivo* experiments indicating the anti-tumour angiogenic effects of TNP470 on human tumour cells and animal tumours such as: B16 melanoma,[1,2] M5076 reticulum cell sarcoma,[1,2] Walker 256 carcinoma,[2] GCH-1 NUC-1, human cell lines of ovarian cancer,[3] and Nakajima cells of uterine endometrial cancer,[3] Lewis lung carcinoma,[1,2] DMBA-induced mammary tumours,[4] and VX-2 carcinoma.[5,6] TNP470 inhibited the growth of endothelial cells and solid tumours with relatively few side effects.[7,8]

We investigated the anti-tumour activity of TNP470 using KB cells. It is known that KB cells, one of the human head and neck squamous carcinoma cell lines, produce interleukin-8 (IL-8), which is a chemotactic cytokine for T lymphocytes and neutrophils,[9–12] and induces an angiogenic response *in vitro* and *in vivo*. Apart from this, IL-8 is a multifunction cytokine that can stimulate the division of endothelial cells.[13–15]

It has been reported that IL-8 is related to many diseases, such as rheumatoid arthritis,[16] psoriasis,[17] wound repair,[13] diabetes retinopathy,[18–19] malignant melanoma,[20] bronchogenic carcinoma,[21,22] and human cancers,[23–29] including human head and neck cancer.[30,31] Therefore, we expected that anti-IL-8 antibody may be effective in inhibiting cancer cell proliferation and tumour growth. We hypothesized that not only TNP470 but also anti-IL-8 antibody would have an anti-tumour effect by inhibiting angiogensis, resulting in growth reduction of tumour cells producing IL-8.

Subjects and methods

Subjects

TNP470 was a kind gift from Takeda Chemical Industries (Osaka, Japan). Anti-IL-8 antibody was purchased from Biogenesis Inc. (USA). Male BALB/c (nu/nu) mice, aged five weeks, purchased from Nippon SCL Inc. (Tokyo, Japan), were used in these experiments. They were kept in a room at 24°C±2°C and 40–70 per cent humidity with a 12-hour light/dark cycle. They were supplied with sterile water and food.

Cell line

KB cells, a human oral floor squamous cell carcinoma cell line, were grown in RPMI-1640

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(Life Technologies, Inc., Gaithersburg, MD, USA) supplemented with 10 foetal calf serum (FCS), 2-mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (complete medium) at 37°C in a five per cent CO_2 atmosphere.

Production of angiogenic factors

The detection of IL-8 in the supernatants of KB cells treated or untreated with TNP470 for 24, 48 and 72 hours, was examined using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Inc., Minneapolis, MN, USA). Immunoassay was performed according to the manufacturer's instructions. The minimum level detectable by ELISA is 5.0 pg/ml of IL-8.

Effects of TNP470 and anti-IL-8 antibody on KB cell proliferation

KB cells were seeded on a ϕ 10-cm plastic dish (Falcon,; Becton Dickinson Labware, Lincoln Park, NJ) at 1×10^6 cells/well in complete medium. After 24-, 48- and 72 hour-treatments with anti-IL-8 antibody and/or TNP470, KB cell proliferation was determined by cell counting after washing with cold phosphate-buffered saline (PBS), trypsinizing and staining with trypan saline.

Cell cycle analysis

KB cells treated or untreated with TNP470 were seeded on a ϕ 10-cm plastic dish at 1×10^6 cells/well. After 48- and 72-hour treatments, KB cells were trypsinized, and cell pellets were collected. After being suspended in 500 μl of PBS, KB cells were digested with 20 μg/mlRNase at 37°C for 30 minutes and chilled on ice for 10 minutes, and then cellular DNA was stained with 50 μg/mol propidium iodide by incubation for one hour at room temperature in the dark. Cell cycle distribution was analyzed by flow cytometry using the Becton Dickinson FACS system.

Tumour growth

KB cells (1×10^7/mouse) were transplanted subcutaneously into the lumbar portion of nude mice. Mice were divided into four groups: a control group given no TNP470, a group receiving 10 mg/kg of TNP470 (n = 5) intratumorally, a group receiving 10 mg/kg of TNP470 (n = 5) intraperitoneally and a group receiving 10 mg/kg of TNP470 (n = 5) intravenously. TNP470 (10 mg/kg) was given once a week for five weeks. Also, mice injected with KB cells were divided into three groups: a control group given no TNP470 nor anti-IL-8 antibody, a group receiving anti-IL-8 antibody (10 mg/kg) intraperitoneally alone and a group receiving both anti-IL-8 antibody (10 mg/kg) intraperitoneally and TNP470 (10 mg/kg) intratumorally (n = 5). The longest diameter (mm) and the shortest diameter (mm) in all animals were measured every week for five weeks. Tumour volumes were calculated by the following formula: tumour volume (mm^3) = longest diameter (mm) × (shortest diameter)^2 (mm^2) × ½. At five weeks, mice were sacrificed under deep anaesthesia with pentobarbitone at the end of the experiment. The tumours were weighed, and immediately small tissue samples were taken from the tumours and used for morphological microscopic studies with haematoxylin-eosin and immunohistochemical studies.

Immunohistochemical analysis of microvessels

After deparaffinization, sections were stained for factors viii (Dako, Glostrup, Denmark) by the ABC technique using an ABC kit (Vector Laboratories, Burlingame, CA, USA) After counterstaining with methyl green solution, light microscopic observation was performed.

Apoptosis

Tumours were minced in cold Tris-buffered saline and homogenized. They were filtered and washed with cold Tris-buffered saline followed by resuspension in 500 μl of lysis buffer containing 500 mM Tris-HCl (pH 9.0), 2 mM EDTA, 10 mM NaCl, one per cent sodium dodecyl sulfate (SDS) and 1 mg/ml proteinase K (Wako Chemical). Deoxynucleotidyl transferase-mediated cUDP nick end labelling (TUNNEL) was performed using a commercial kit, Apop Taq Plus (Oncor, Gaithersburg, MD, USA) for deparaffinized paraffin-embedded tumour sections. Counting of immunoreactive cells was based on the distribution of apoptotic tumour cells in three different fields within the same section; the apoptotic index was expressed as the percentage of TUNNEL-positive cells over the total number of the cells.

Statistical analysis

Data were analysed using the two-sample *t*-test with significance set at $p<0.05$.

Results

Effects of TNP470 and anti-IL-8 antibody in vitro

The quantity of IL-8 produced by KB cells was related to KB cell proliferation (Figure 1). As IL-8 production from KB cells increased, KB cell proliferation increased. The addition of IL-8 to the culture of KB cells didn't increase the KB cell proliferation, whereas the proliferation was significantly inhibited by the addition of anti-IL-8 antibody ($p<0.01$), as shown in Figure 2. TNP470 showed inhibiting activity on KB cell proliferation, and the production of IL-8 was suppressed (Figure 3). The simultaneous addition of TNP470 (10 ng/kg) and anti-IL-8 antibody (10 μg/kg) strongly reduced the proliferation of KB cells ($p<0.01$) (Figure 4). Next, the action point of TNP470 in the cell cycle phase was examined. The KB cells migrated from the G_1 to S phase due to TNP470. Also, TNP470 promoted the KB cells into the S phase of the cell cycle (Figure 5).

Effects of TNP470 and anti-IL-8 antibody in vivo

TNP470 (10 mg/kg) was administered every week into nude mouse transplanted tumours (1×10^7 KB cells) intratumorally, intraperitoneally and intrave-

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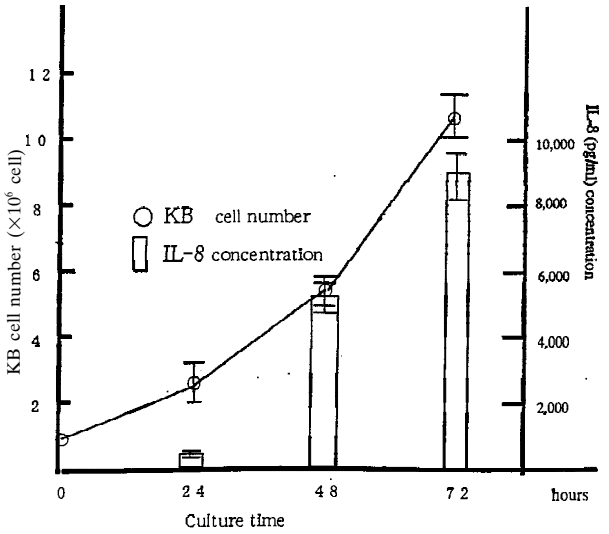

FIG. 1

The relationship between KB cell proliferation and IL-8 concentration *in vitro*. With increasing of KB cell proliferation, IL-8 concentration also increased.

nously. The grafted tumours increased in size and weight. The tumours in the control group grew most in size. In contrast, the tumour growth in the intratumorally administered group was most significantly inhibited in size and weight five weeks after starting TNP470 administration ($p<0.05$). The intraperitoneal and the intravenous groups also showed significantly reduced tumours by treatment with TNP470 ($p<0.05$), compared to the control (Figure 6). Both anti-IL-8 antibody and TNP470 were simultaneously injected into tumour-bearing

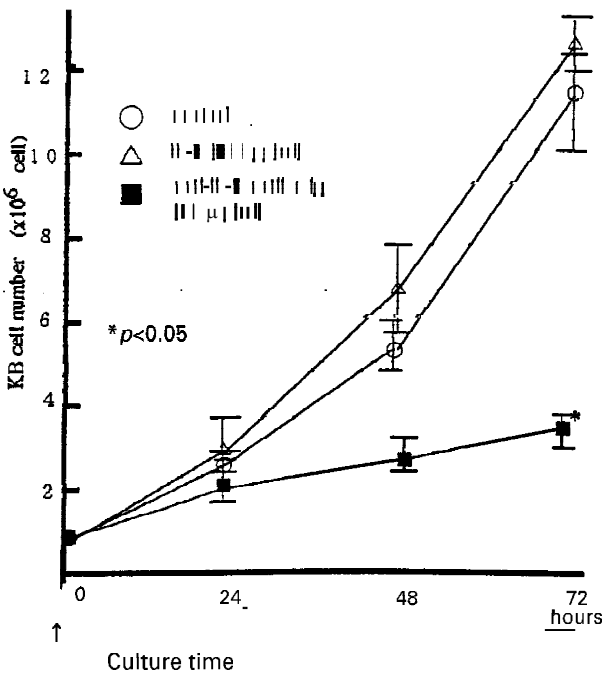

FIG. 2

Effects of IL-8 and anti-IL-8 antibody *in vitro* on KB cell proliferation. The difference in inhibitory effect on tumour proliferation between the group treated with anti-IL-8 antibody and the control group was significant at $p<0.05$. v Control, △ IL-8, m Antibody.

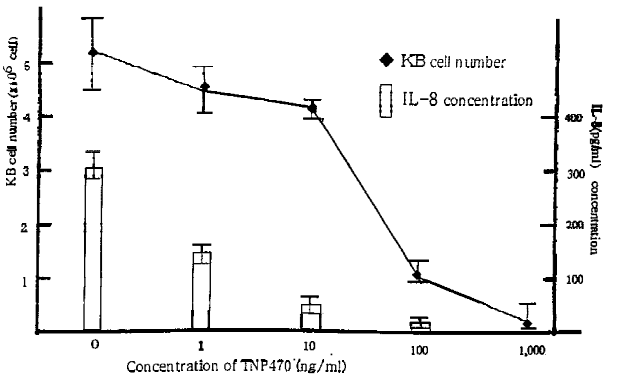

FIG. 3

The inhibitory effect of TNP470 *in vitro* on proliferation of KB cells and IL-8 concentration. With increasing TNP470 concentration, the proliferation of KB cells and IL-8 concentration was inhibited significantly.

nude mice at two to seven weeks after transplantation. Anti-IL-8 antibody alone had a significant inhibitory effect on tumour growth. Furthermore, the group injected with combined TNP470 (10 mg/kg, intratumoral) and anti-IL8 antibody (10 μg/kg, intraperitoneal) showed significant tumour shrinkage (Figure 7). There was no difference in the density of microvessels evaluated by anti-factor viii staining between the control group and the TNP470 intratumoral group (data not shown). Also, there was no difference in the apoptotic index between the two groups (data not shown).

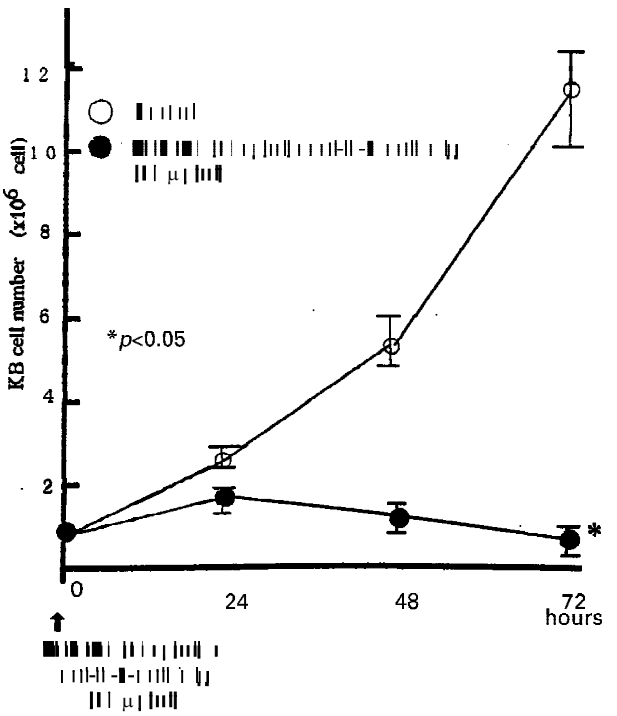

FIG. 4

The effect of TNP470 and anti-IL-8 antibody *in vitro* on the proliferation of KB cells. The group treated with TNP470 and anti-IL-8 antibody showed significant inhibition of tumour proliferation, compared to the control group. The difference between the treated group and the control group was significant at $p<0.05$. v Control, v TNP470 + anti-IL-8 antibody.

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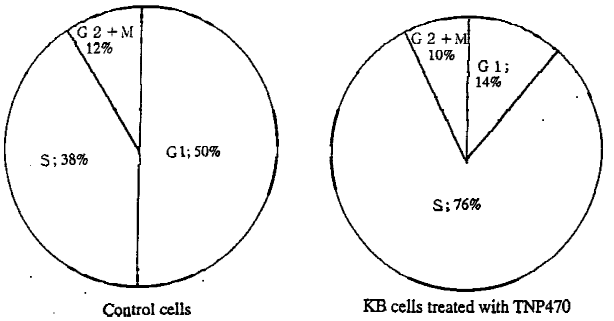

FIG. 5

Cell cycle analysis of KB cells treated with TNP470.

Side-effects of TNP470

During the treatment period, the TNP470-treated mice did not show significant side-effects, such as hair loss, intestinal disturbance or infection. Mice treated with TNP470 showed some weight loss, but it was not statistically significant ($p>0.05$).

Discussion

Growth of solid tumours is reported generally to depend on angiogenesis for oxygen and nutriments.[32] Therefore, angiogenetic inhibitors could be candidates for a new treatment modality against cancer. It has been reported that TNP470, which is an angiogenetic trap inhibitor, has an inhibitory effect on tumour growth of a variety of tumours.[33–38] However, as far as we are aware, there are few reports concerning its inhibitory activity on head and neck squamous carcinoma.[39] In the present study, we investigated the inhibitory effects of TNP470 on KB cells. It has been reported that IL-8 is produced from some head and neck carcinoma, including KB cells,[30,31] as in human breast cancer,[23] ovarian carcinoma,[24] human liver and pancreatic carcinoma,[25] human gastric carcinoma,[26] non-small cell lung carcinoma[27,28] and colorectal carcinoma.[29] It has been also reported that IL-8 receptors are expressed on cancer cells in the head and neck region.[31] IL-8 is a multifunction cytokine that can stimulate the division of endothelial cells, and IL-8 was found to be a mediator of angiogenesis.[31] It was expected that IL-8 might play an important role in tumour growth or cell proliferation through angiogenesis.

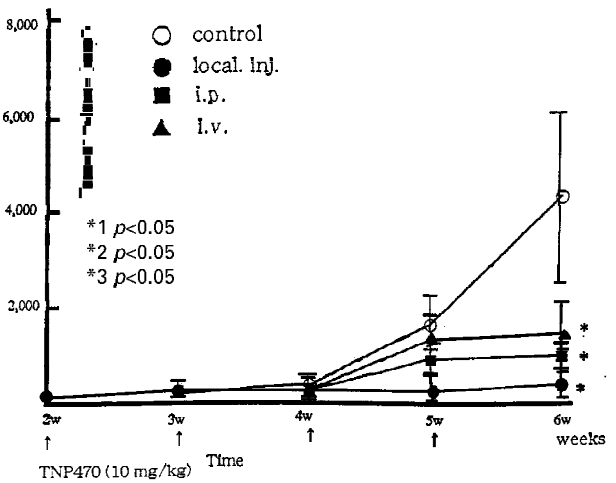

FIG. 6

The effects of TNP470 on established human epithelial carcinoma of BALB/c nude mice. Treatment was performed intratumorally (1), intraperitoneally (2), or intravenously (3). The experiments were performed two weeks after transplantation of the tumours. The differences between the three groups (intratumoral, intraperitoneal, and intravenous) and the control group were significant at $p<0.05$.

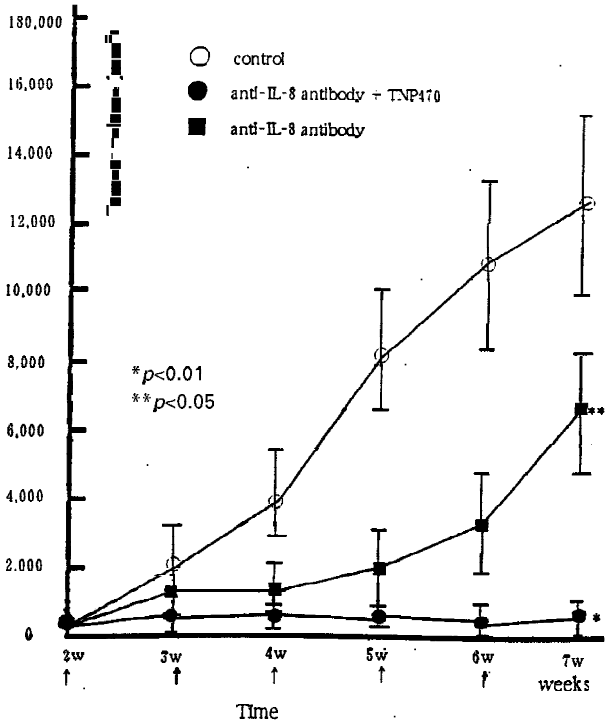

FIG. 7

The inhibitory effects of TNP470 and anti-IL-8 antibody. TNP470 was administered intratumorally, and anti-IL-8 antibody was administered intraperitoneally. The experiments were performed two weeks after tranplantation of the tumours. The difference between the group treated with anti-IL-8 antibody + TNP470 and the control group was significant at $p<0.01$.

In this study, we added TNP470 and anti-IL-8 antibody to the culture of KB cells. The proliferation was strongly inhibited *in vivo* by both agents. This result indicates that IL-8 plays an important role on tumour cell proliferation. The inhibitory effect of a combination of TNP470 and anti-IL-8 antibody was stronger than the addictive effect of TNP470 and anti-IL-8 antibody. This phenomenon suggests that TNP470 may suppress the productive of IL-8 by KB cells. There are no reports on the combined treatment of TNP470 and anti-IL-8 antibody against human head and neck carcinoma. In our study, TNP470 inhibited KB cell proliferation by promoting the KB cells into the S phase of the cell cycle. However, it has been reported that TNP470 inhibits endothelial cell proliferation by preventing the entry of the cell into the G_1 phase of the cell cycle.[40] On the other hand, the number of apoptotic cells was not increased by TNP470. Therefore, the mechanisms of the inhibitory effect of TNP470 on tumour

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growth was not clarified by our study. Also, in this study, TNP470 had the greatest effect of cancer cells by intratumoral injection. TNP470 has an inhibitory effect on cancer cells with relatively few side-effects. The combination of TNP470 and anti-IL-8 antibody showed very high effectiveness on tumour proliferation and growth. These results suggested that the topical administration of TNP470 combined with anti-IL-8 antibody would be more effective on human head and neck cancers, including advanced cases.

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