Synthetic Terrein Inhibits Progression of Head and Neck Cancer by Suppressing Angiogenin Production

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Abstract. *Background/Aim: Head and neck cancers are the fifth most common cancer type worldwide, affecting more than half a million patients annually. Development of effective therapeutic drugs is, therefore, required for this type of disease. This study assessed the effects of synthetic terrein on head and neck cancer. Materials and Methods: Synthetic terrein was prepared by using the modified Altenhach's procedure. The effect of synthetic terrein on cell proliferation of head and neck cancer cells and HUVECs was assessed. Angiogenin secretion and ribosome biogenesis were examined by ELISA and silver staining of the nucleolar organizer region. A mouse xenograft model was prepared by inoculating mice with suspensions of cells of the human head and neck cancer cell line OSC-19 subcutaneously into the dorsal region of each mouse. Ki-67, CD31 and angiogenin expression in xenografted tumors was examined by immunohistochemistry. Results: Synthetic terrein inhibited the growth of various head and neck cancer cells. In addition, an in vivo experiment revealed that synthetic terrein inhibited a xenograft tumor growth in athymic mice. Immunohistochemical analysis revealed that expression of Ki-67, CD31 and ANG was down-regulated in synthetic terrein-treated tumors, compared to controls. Synthetic terrein suppressed the ANG secretion and ribosome biogenesis in cancer cells, and cell proliferation in vascular endothelial cells. Conclusion: The mechanism underlying the anti-tumor effects of synthetic terrein against head and neck cancer consists of the inhibition of both tumor cell proliferation and angiogenesis via the suppression of ANG production.*

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Key Words: Synthetic terrein, head and neck cancer, squamous cell carcinoma, cell proliferation, angiogenesis.

Head and neck cancers arise from the oral cavity, pharynx, larynx, nasal cavity or paranasal sinuses (1), and are the fifth most common cancer type worldwide, affecting more than half a million patients annually (2). At the time of diagnosis, 60% of patients have advanced locoregional disease (3). A major challenge in treating locally advanced head and neck cancer is obtaining a high cure rate while preserving organ structures and function. The majority of these patients require a combination of surgery, radiation or chemotherapy, with radiation and chemotherapy playing a particularly important role in organ preservation (1).

Although conventional chemotherapy using cisplatin, fluorouracil or docetaxel continues to provide a gradual improvement in outcome, it also has considerable toxicity. In search for more tolerable and efficacious anticancer agents, cetuximab has emerged as the first molecular targeting drug to confer a survival advantage in head and neck cancer (4). However, conventional chemotherapy with cisplatin still appears more efficacious than cetuximab when used in combination with radiation (3).

For further enhancement of positive outcomes, new therapeutic agents need to be developed, particularly compounds targeting specific molecular pathways that are crucial for cancer biology. Among them, drugs targeting both cancer proliferation and angiogenesis are unique and of great interest (5, 6).

Terrein ($C_8H_{10}O_3$) was first isolated from *Aspergillus terreus* as a fungal metabolite (7) and was later found to be produced by other species of *Aspergillus* and *Penicillium* (8). In general, the availability of terrein has been completely dependent on the identification of natural sources. However, we recently reported the synthesis of terrein by using a modified Altenbach's procedure (9). Terrein exhibits various biological activities, including antibacterial (10), plant growth inhibition (11), melanogenesis inhibition (8, 12, 13), an anti-inflammatory effect in dental pulp (14), and keratinocyte proliferation inhibition (15). Terrein also inhibits cell proliferation in cervical cancer (16), breast cancer (17), lung cancer (18), hepatic cancer (19), ovarian cancer (20) and prostate cancer and suppresses vascular endothelial cell tube formation, one of the critical steps in angiogenesis (21). Accordingly, terrein is a very attractive agent for cancer therapy due to its targeting of both cancer cell proliferation and tumor angiogenesis. To date, however, terrein has not been tested in *in vivo* experiments in any cancer model, and its antitumor effects in head and neck cancer are still unknown.

In the present study, we investigated the antitumor activity of synthetic terrein toward head and neck cancer both *in vitro* and *in vivo* and evaluated its potential as a lead compound for head and neck cancer therapy.

Materials and Methods

Cell culture and reagents. The human head and neck cancer cell lines OSC-19 and OSC-20 were obtained from JCRB Cell Bank (Tokyo, Japan). HSC-3 and HSC-4 were obtained from the Cell Engineering Division of RIKEN BioResource Center (Tsukuba, Ibaraki, Japan). Normal human umbilical vein endothelial cells (HUVECs) were purchased from LONZA Japan (Tokyo, Japan). All cancer cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture (DME/F-12) supplemented with 10% fetal bovine serum (FBS). HUVECs were cultured in EGM™ -2 (LONZA Japan, Japan). Synthetic (+)-terrein was prepared from dimethyl L-tartrate and the structure of (+)-synthetic terrein was confirmed to be good agreement with natural terrein as previously reported (9).

Cell proliferation. Cancer cells and HUVECs were seeded at a density of 10^5 cells and 3×10^5 cells per 60-mm dish, respectively, and cultured for 24 h. They were then cultured in the presence of synthetic terrein for an additional 24, 48, 72, 96 or 120 h. Thereafter, the cells were detached by trypsinization and counted.

Growth of OSC-19 xenograft tumors in athymic mice. Five-week-old male athymic mice (nu/nu) were obtained from CLEA Japan Inc. (Tokyo, Japan). OSC-19 cells, 8×10^5 per mouse, were inoculated subcutaneously into the dorsal region of each mouse. Seven days after inoculation, the animals were treated with local subcutaneous injections of PBS or synthetic terrein (30 mg/kg) twice weekly. Eight mice per group were used. Tumor sizes and body weights were measured weekly, and the former were recorded in cubic millimeters (length × width^2/2). The animals were sacrificed at day 63 and the tumor tissues were removed and weighed. All animal experiments were approved by the institutional animal care and use committee of Okayama University.

Immunohistochemistry for OSC-19 xenograft tumor specimens. Paraffin blocks of specimens were cut at a 4-μm thickness. Immunohistochemistry (IHC) was performed with anti-ANG monoclonal antibody used at a 1:100 dilution (ab10600; Abcam, Cambridge, UK) and anti-Ki67 polyclonal antibody at a 1:50 dilution (M7240; Dako, Carpinteria, CA, USA). Blood vessels were stained with anti-CD31 polyclonal antibody at a 1:50 dilution (ab28364; Abcam, Cambridge, UK). Sections were incubated with the primary antibodies at 4˚C for 16 h, and visualized with a VECTASTAIN ABC kit (Vector Laboratories, Cambridgeshire, UK) for anti-CD31 antibody or the Envision system (Dako, Carpinteria, CA, USA) for anti-ANG antibody and anti-Ki67 antibody. The sections were counterstained with Mayer's hematoxylin. The numbers of Ki67-positive cancer cells and the areas occupied by CD31-positive blood vessels in three visual fields under a microscope (×100) were quantified and averaged using the image analysis software package ImageJ (version 1.43r; NIH, Bethesda, MD, USA), as reported previously (22).

Enzyme-linked immunosorbent assay (ELISA) detection of human ANG. A total of 10^5 cells per 60-mm dish were cultured for 24 h in DME/F12 supplemented with 10% FBS. The medium was exchanged and the cells were cultured in the presence of synthetic terrein. The culture media were collected at various time points, and the secretion levels of ANG protein were determined by ELISA (R&D Systems) and normalized to the cell numbers.

Silver staining of the nucleolar organizer region (NOR). Tissue sections (4 μm thickness) were de-paraffinized with xylene and rehydrated in graded alcohols. Then, the sections were autoclaved in 10 mmol/L citrate buffer (pH 6.0) at 120˚C for 20 min for protein retrieval. Silver-staining was performed by immersing sections in a freshly-prepared solution containing 1 part by volume of 2% gelatin in 1% formic acid and 2 parts of aqueous silver nitrate (Sigma-Aldrich, St. Louis, MO, USA) solution for 25 min at room temperature, as previously described (23). After thorough rinsing in distilled water, the sections were dehydrated and mounted. Silver-stained NOR dots were counted in 60 randomly selected nuclei at ×1,000 magnification.

Statistical analysis. Data were analyzed by the unpaired Student's *t*-test for analysis of the 2 groups, and Fisher's protected least significant difference (Fisher's PLSD) for multiple group comparisons. Results were expressed as the mean±S.D. Values of $p<0.05$ were considered statistically significant.

Results

Synthetic terrein inhibited the proliferation of head and neck cancer cells in vitro. Because terrein is known to inhibit the proliferation of breast (17), prostate (21), hepatic (19), ovarian (20) and cervical cancer cells (16), we examined whether synthetic terrein would decrease proliferation of cells of the head and neck cancer lines OSC-19, OSC-20, HSC-3 and HSC-4 *in vitro*. The results showed that

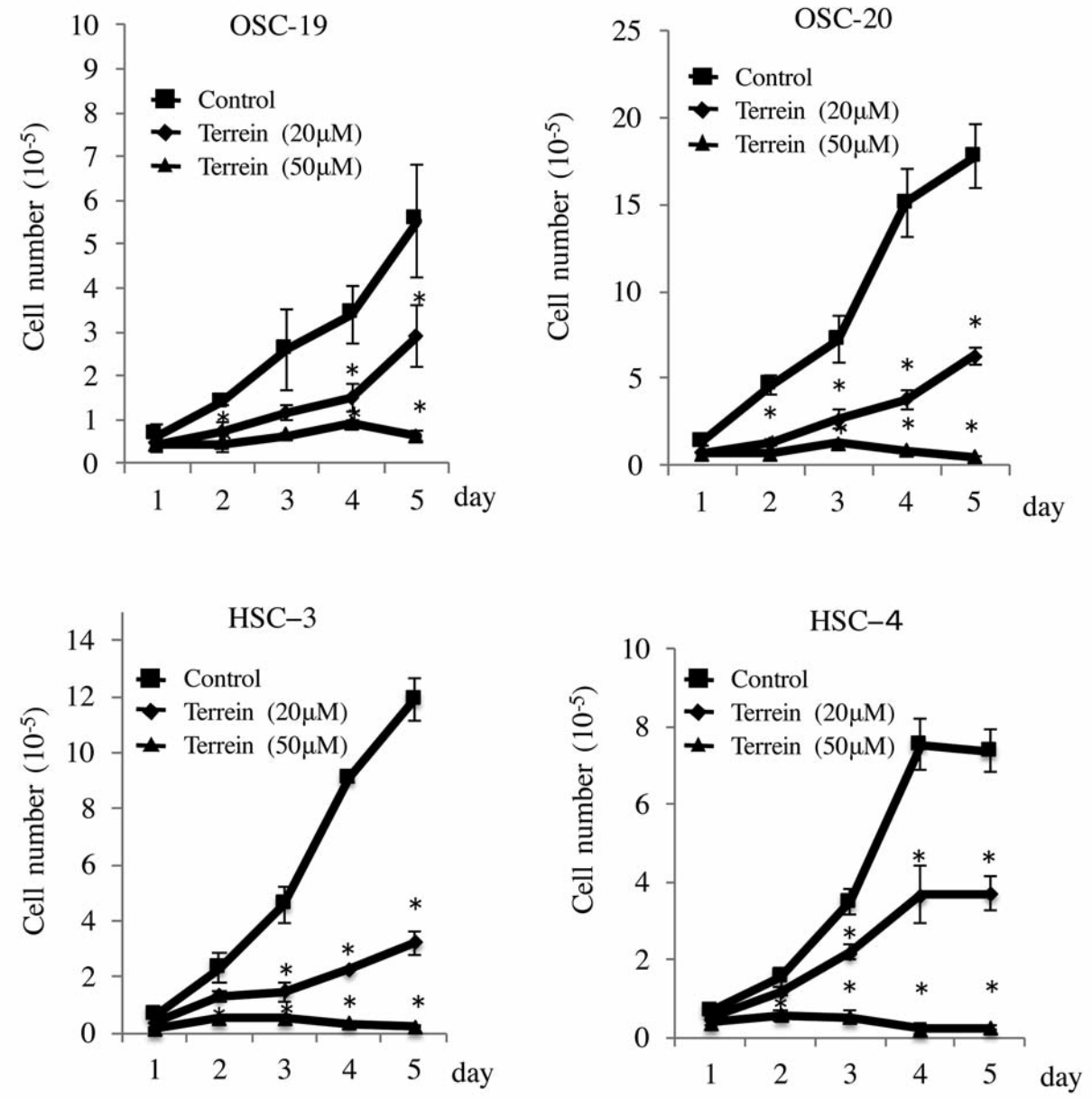

Figure 1. *Inhibition of proliferation of OSC-19, OSC-20, HSC-3 and HSC-4 cells by synthetic terrein in vitro. Cells were seeded at a density of 10^5 cells per 60-mm dish and cultured for 24 h. They were then cultured in the presence of synthetic terrein for an additional 24, 48, 72, 96 or 120 h. The cells were subsequently detached by trypsinization and counted. All experiments were repeated 3 times. Data are presented as the mean±SD of triplicates from a typical experiment. *p<0.05.*

20 μmol/L synthetic terrein inhibited the proliferation of all four cell lines (Figure 1). These findings suggested that synthetic terrein may be an attractive candidate as an anti-tumor agent in head and neck cancer.

Synthetic terrein inhibited the growth of OSC-19 xenografted cells in athymic mice. During the xenograft experiment, there were no significant differences in body weight, grooming behavior or food and fluid intakes between the PBS- (control) and synthetic terrein-treated groups. Treatment with synthetic terrein significantly decreased the growth rate of OSC-19 xenograft tumors compared to the rate in the PBS-treated group (Figure 2). The end tumor volumes at the end of the experiment (day 63) for the groups of animals treated with PBS and synthetic terrein were 3,054.7±1,785.2 and 717.8±291.5 mm^3, respectively, indicating an approximately 76.5% decrease in

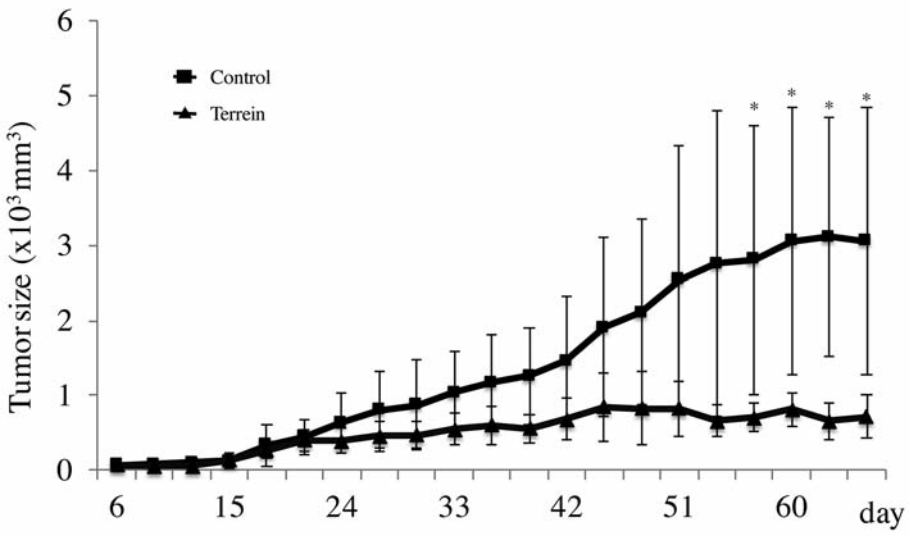

Figure 2. *Inhibition by synthetic terrein of xenograft growth of OSC-19 xenografts in athymic mice. OSC-19 cells, 8×10^5 per mouse, were inoculated subcutaneously into the dorsal region of each mouse. The animals were treated with local subcutaneous injections of PBS or synthetic terrein (30 mg/kg) twice weekly. Eight mice per group were used. Tumor sizes and body weights were measured weekly, and the former were recorded in cubic millimeters (length × width^2/2). The animals were sacrificed at day 63 and the tumor tissues were removed and weighed. *p<0.05.*

tumor growth rate for the synthetic terrein-treated animals (Figure 2). And the end tumor weights at day 63 for the groups of animals treated with PBS and synthetic terrein were 2,000±930 and 975±345 mg, respectively, indicating an approximately 51.3% decrease in tumor growth rate for the synthetic terrein-treated animals. These results suggest that synthetic terrein effectively inhibited the xenograft tumor growth of head and neck cancer cells in athymic mice.

Synthetic terrein inhibited tumor proliferation and angiogenesis. As described above, synthetic terrein inhibited tumor growth of human head and neck cancer *in vivo*. To quantify these effects, IHC staining with the anti-Ki67 antibody was performed. The percentage of Ki67-positive cells decreased from 157.7±18.0 in the control OSC-19 tumor group to 111.6±7.9 in the synthetic terrein-treated OSC-19 tumor group, representing a 29.2% decrease in cell proliferation (Figure 3). The CD31-positive areas in the control and synthetic terrein-treated OSC-19 tumor groups were 35,817±12,017 and 21,709±8,163 μm^2, respectively. This represents a 39.4% decrease in tumor angiogenesis (Figure 3). IHC staining with an ANG-specific antibody showed strong ANG expression in the nucleus and cytoplasm of OSC-19 tumor cells from control (PBS-treated) animals. ANG expression in the nucleus and cytoplasm was decreased in OSC-19 tumor cells from synthetic terrein-treated animals (Figure 3), indicating that synthetic terrein suppressed the production of ANG in OSC-19 cells *in vivo*. These results suggest that the antitumor activity of synthetic terrein against OSC-19 xenograft tumors was exerted by the inhibition of both tumor cell proliferation and angiogenesis *via* the suppression of ANG production.

Synthetic terrein reduced ANG production and ribosome biogenesis in OSC-19 cells in vitro. To quantify the changes in angiogenin and ribosome biogenesis, we examined the effects of synthetic terrein on angiogenin secretion and the nucleolar organizer region (NOR) of the OSC-19 cells *in vitro*. Treatment of OSC-19 cells with synthetic terrein suppressed angiogenin secretion from 2.7±1.0 (pg/10^{3}cells/day) in the control to 1.0±0.3 and 0.5±0.1 in the 20 μM and 50 μM terrein-treated groups, respectively. Synthetic terrein also decreased the average number of NOR dots per cell from 22.6 ± 4.0 in the control to 15.0 ± 2.9 in the synthetic terrein-treated group, indicating a significant decrease in ribosome biogenesis in the latter (24) (Figure 4).

Synthetic terrein suppressed the proliferation of HUVECs in vitro. Finally, to elucidate the direct effects of synthetic terrein on angiogenesis, we examined whether synthetic terrein would decrease the proliferation of HUVECs *in vitro*. The results showed that 20 μM synthetic terrein inhibited the proliferation of HUVECs (Figure 5), suggesting that synthetic terrein directly inhibits angiogenesis.

Discussion

Terrein was isolated as a fungal metabolite in 1935 (7). Recently, we succeeded in producing terrein by a novel synthesis method that would allow for large-scale terrein

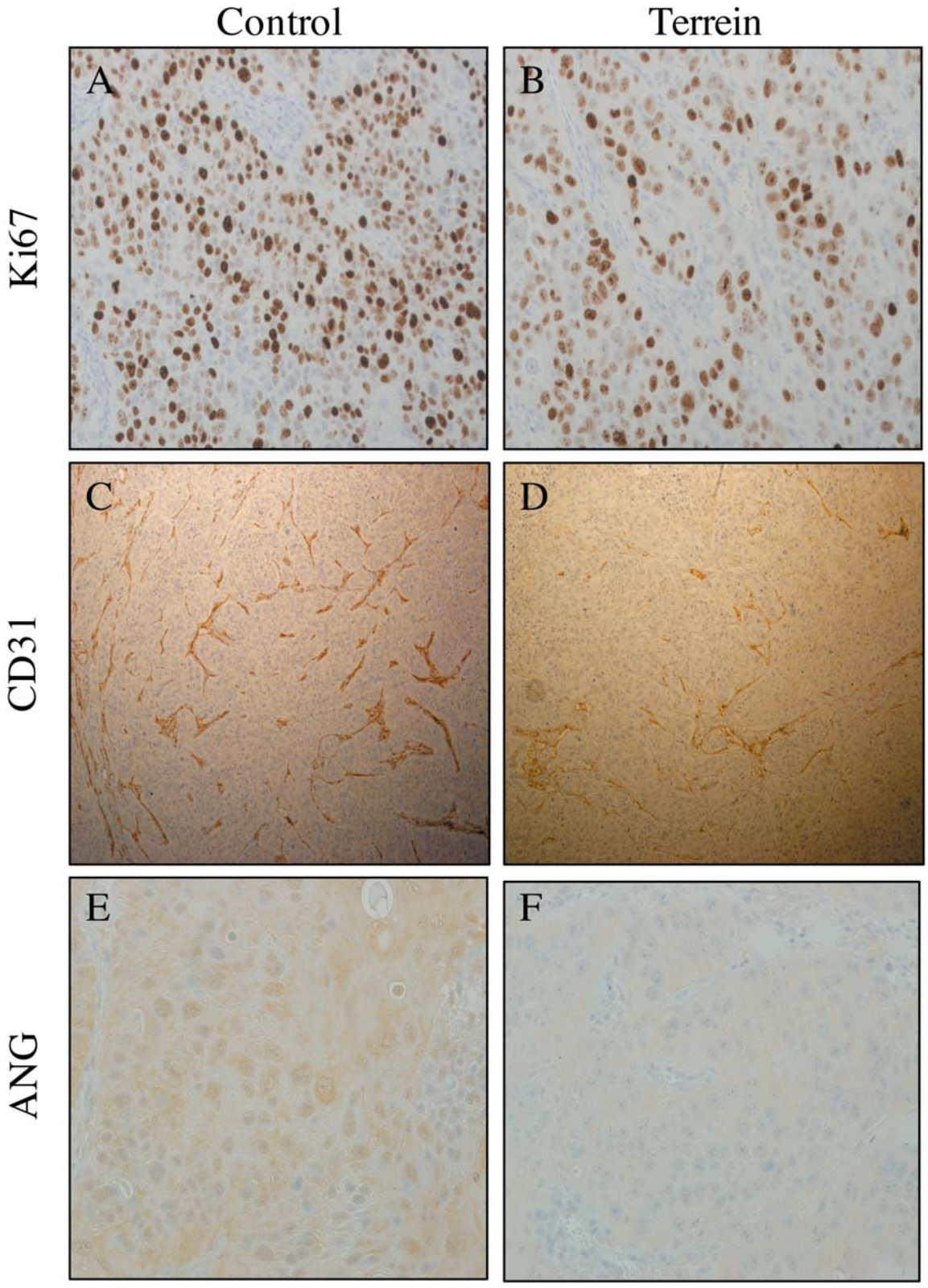

Figure 3. *IHC staining of OSC-19 xenograft tumor specimens. A-F, IHC staining for Ki67, CD31 and ANG in tumors from the control (PBS-treated) and synthetic terrein-treated groups. Ki67-positive cancer cells and CD31-positive blood vessel areas in three visual fields under a microscope (×100) were quantified and averaged. A, B: ×100 magnification; C, D: ×40 magnification; E, F: ×100 magnification.*

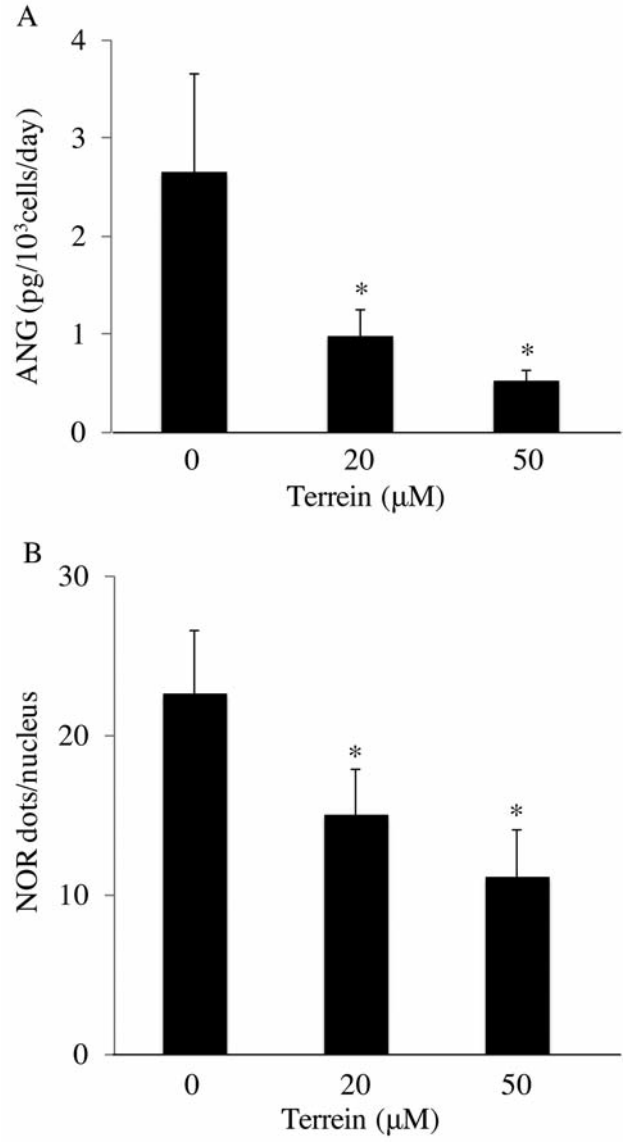

Figure 4. *Effect of synthetic terrein on angiogenin secretion and ribosome biogenesis of OSC-19 cells in vitro. A: OSC-19 cells were cultured in the presence of synthetic terrein. The culture media were collected at various time points, and secretion levels of ANG protein were determined by ELISA and normalized to cell numbers. The data shown were collected at 120 h. B: NOR dots in cancer cells were examined to obtain a quantitative assessment of the changes in ribosome biogenesis caused by synthetic terrein. Silver-stained NOR dots were counted in 10 randomly selected nuclei, and the numbers were averaged. Results are shown as the mean±SD for each group. *p<0.05.*

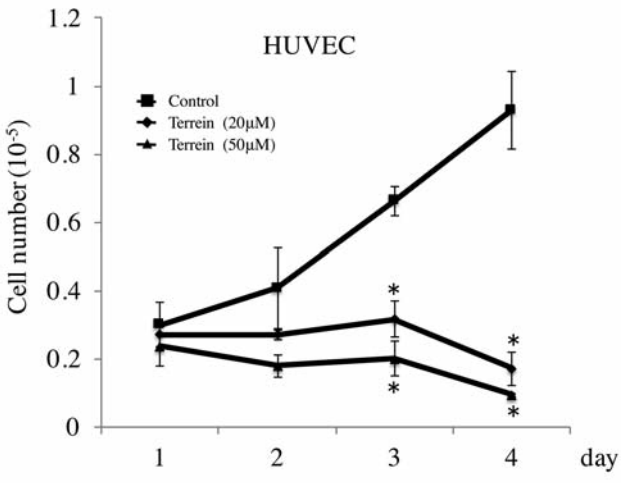

Figure 5. *Effects of terrein on cell proliferation of HUVECs in vitro. HUVECs were seeded at a density of 3×10^5 cells per 60-mm dish and cultured in the presence of synthetic terrein for an additional 24, 48, 72 or 96 h. The cells were subsequently detached by trypsinization and counted. All experiments were repeated three times. Data are presented as the mean±SD of triplicates from a typical experiment. *p<0.05.*

production (9). This is the first study to demonstrate that our synthetic terrein exerts anti-tumor effects in head and neck cancers in *in vivo* experiments *via* suppression of angiogenin production and cancer cell proliferation.

Previous studies have shown that terrein exhibits various biological activities. In mouse melanocytes (Mel-Ab), terrein functions as a melanogenesis inhibitor at a concentration from 5 to 100 μM by reducing the tyrosinase production *via* mitogen-activated protein kinase (MAPK) activation (13). Terrein also functions as an anti-inflammatory agent (14). At a concentration of 10 μM, terrein has been shown to reduce intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression in dental pulp cells by blocking nuclear factor-kappa B (NF-κB) and amino kinase terminal (AKT) activation. As we reported previously, our synthetic terrein suppresses vascular endothelial growth factor (VEGF) expression in gingival fibroblasts by blocking signal transducer and activator of transcription-3 (STAT-3) and MAPK activation at the concentration of 10 μM (9). In osteoblast-like cells (MC-3T3 E1) grown on a titanium surface, 10 μM terrein were found to promote osteoblast differentiation by blocking NF-κB nuclear translocation. Moreover, terrein shows anti-oxidative activity through up-regulation of MAPK and focal adhesion kinase (FAK) activity (25). In epidermal keratinocytes, 50 μM terrein were also shown to suppress MAPK activation and cell proliferation without cytotoxicity (15).

The inhibitory effects of terrein on growth of various cancer cells have also been reported. In cervical cancer cells (HeLa), terrein induces apoptosis through p53 and MAPK regulation with an IC_{50} of 0.29 mM (16). In breast cancer cells (MCF-7), terrein also induces apoptosis by blocking AKT activation and activating caspase-7 with an IC_{50} of 1.1 nM (17). In lung cancer cells (NCI-H292), terrein inhibits proteasome activity with an IC_{50} of 0.3 mM

and can induce apoptosis at a concentration of 0.3 mM (18). In hepatoma cells (Bel-7402), terrein inhibits cell proliferation with cell-cycle arrest and mesenchymal epithelial transition (MET) with an IC_{50} of 11.6 μM, without inducing apoptosis (19). In ovarian cancer cells (SKOV3), terrein inhibits cell proliferation through cell-cycle arrest and suppression of the expression of LIN28, which is a marker of stemness in ovarian cancer stem cells (20). In prostate cancer cells (LNCaP-CR) and vascular endothelial cells (HUVEC), terrein works as a suppresser of ANG secretion with IC_{50}s of 13 μM and 20 μM, respectively. Terrein also inhibits tube formation of vascular endothelial cells (21).

In this study, the anticancer activity of our new synthetic terrein and its mechanism of action in head and neck cancer were investigated. Synthetic terrein was shown to inhibit cell proliferation of head and neck cancer cells (OSC-19) with an IC_{50} of 21 μM. Treatment of OSC-19 cells with terrein for different time periods from 5 to 60 min did not change the phosphorylation of AKT and MAPK (data not shown). However, synthetic terrein suppressed ANG secretion with an IC_{50} of 11 μM, and inhibited ribosome biogenesis. This effective dose was almost the same as that in prostate cancer cells and was much lower than that observed in cervical cancer cells, lung cancer cells and normal epidermal keratinocytes. These data suggest that the mechanism of action and effective dose of terrein required to induce cancer cell death are highly cell type-dependent, and terrein does not exhibit any severe cytotoxic action on normal epithelial cells at this dose.

We also found that synthetic terrein inhibited tumor xenograft growth *in vivo*. IHC staining showed that treatment with synthetic terrein decreased Ki67-positive cancer cells, CD31-positive vessels in the tumors and ANG expression in cancer cells. ANG is up-regulated in various types of human cancers, including cervical, breast, lung, liver, ovarian, prostate, head and neck, colon, colorectal, endometrial, gastric, kidney, pancreatic, and urothelial cancers, as well as astrocytoma, leukemia, lymphoma, melanoma, and osteosarcoma (26). ANG has the dual effect of inducing cancer cell proliferation and angiogenesis by stimulating ribosomal RNA transcription in both cancer cells (24, 27) and vascular endothelial cells (23). In addition, ANG has an anti-apoptotic effect by targeting p53, and this effect leads to cancer progression (28). Our *in vivo* experiment showed that terrein exerted a direct anti-proliferation effect in addition to its anti-angiogenesis effect by suppressing ANG expression in head and neck cancer.

In conclusion, our results demonstrated that synthetic terrein effectively suppressed head and neck cancer progression through inhibition of tumor proliferation and angiogenesis. Thus, terrein is a potential candidate for an anticancer agent in head and neck cancer therapy.

Conflicts of Interest

None declared.

Acknowledgements

This work was supported by Grants-in-Aid for Young Scientists (B) (Nos. 22791978, 24792213) to Soichiro Ibaragi from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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Received March 1, 2016
Revised April 11, 2016
Accepted April 12, 2016