Tranilast, an Anti-Allergic Drug, Down-Regulates the Growth of Cultured Neurofibroma Cells Derived from Neurofibromatosis Type 1

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Neurofibromas are benign tumors that comprise primarily of Schwann cells and fibroblasts. Mast cells have been found scattered in the tumor tissue, and their role in promoting the proliferation of neurofibroma has been suggested. Tranilast (N-[3,4-dimethoxycinnamoyl]anthranilic acid) is an anti-allergic drug that inhibits release of the chemical mediators from mast cells and it used for the treatment of keloids and hypertrophic scars by its inhibition of growth-promoting transforming growth factor (TGF)-β, from fibroblasts. We assumed that tranilast would suppress neurofibroma cell growth. In order to prove this hypothesis, we investigated the effectiveness of tranilast in inhibiting the tumor growth using a new cell culture system obtained from patients with neurofibromas. We called this culture system with the mixture of Schwann cells and fibroblasts “NF1 cells culture”. Mast cells were differentiated from CD34+ peripheral blood mononuclear cells of normal healthy subjects, and were co-cultured with NF1 cells. Three days after tranilast (10 ~ 100 μM) added to the culture dishes, we counted viable cell numbers and measured the concentrations of TGF-β1, stem cell factor (SCF) and tryptase, which exists in the histamine granule, in the culture medium. Tranilast significantly suppressed proliferation of the NF1 cells and lowered the levels of TGF-β1, SCF and tryptase. These results suggest that tranilast retards tumor proliferation through not only suppression of cell growth factor, but also the inhibition of a chemical mediator released from mast cells. Thus, tranilast can be a potent therapeutic agent to inhibit the growth of neurofibromas. ——— tranilast; von Recklinghausen disease; neurofibroma; mast cell; co-culture.

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animal experiments to investigate its inhibitory effects in other diseases. In particular, Jones et al. (2004) reported that tranilast reduces mesenteric vascular collagen deposition and mast cells in experimental diabetic rats.

Histologically, neurofibromas mainly consist of Schwann cells and fibroblasts. However, mast cells have been found scattered in the tumor tissue. Their role in promoting the proliferation of neurofibroma has been strongly suspected. Affected patients were found to have a mutation in the NF1 gene with a phenotype of NF1<sup>+/−</sup> in cells throughout the body, including Schwann cells. It is postulated that a second hit against the mutated gene within Schwann cells, leading to the NF1<sup>−/−</sup> phenotype, triggers a series of events including chemotactic attraction of mast cells to the tumor, and allows mast cells to release cytokines which promote the proliferation of neurofibromas (Riccardi 1981; Zhu et al. 2002; Viskochil 2003; Yang et al. 2003).

With the above background information, we postulated that the drug suppresses the growth of neurofibromas with its dual action on both fibroblasts and mast cells. However, these findings were obtained from experiments either using murine neurofibroma cell lines or using cells from human malignant schwannoma. Therefore, the experimental results may not have relevance to the pathogenesis of human neurofibromas.

In order to obtain more appropriate experimental conditions, we developed special culture systems for the disease using surgically resected tissues from patients with neurofibromas. We succeeded in establishing a natural “mixed culture” consisting of fibroblasts and Schwann cells, and called it NF1 cells. Furthermore, we established a coculture of mast cells with the NF1 cells so that we could closely examine the mechanism of tumor promotion by mast cells. We used mast cells from cytokine-induced stem cells of healthy subjects.

With these novel culture systems closely resembling human neurofibroma, we tested the effectiveness of tranilast in inhibiting the tumor growth and in suppressing cytokine mediators released from mast cells in culture. Our results are the first experimental report of an anti-allergic agent being added to a culture established directly from human neurofibroma cells.

**MATERIAL AND METHODS**

This study was approved by the Ethical Review Committee of Yamaguchi University, and four patients provided informed consent for use of their tumor samples in the study. NF1 cells, a mixture of fibroblast cells and Schwann cells, were taken from neurofibroma and cultivated by the explant culture method. Mast cells for the cultures were prepared according to the technique of Rottem et al. (1994). Then we co-cultured the cells from the two culture systems, as described below, and used the combined culture to test the growth inhibitory effect of tranilast.

**Preparation of fibroblasts and Schwann cells derived from NF1 tumors**

Tumor tissues for the cultures were prepared from neurofibromas obtained in plastic surgery. They were sterilized with calcium and magnesium-free phosphate buffered saline [PBS(−)] supplemented with antibiotics (Penicillin-G, Streptomycin, and Amphotericin B, all from Invitrogen Corp, Carlsbad, CA, USA) and cut into fine pieces with scissors. The cultured cells were established according to the explant culture technique. In short, fine pieces of neurofibroma tissues were treated with 0.25% trypsin (Trypsin 250, Becton Dickinson, Franklin Lakes, NJ, USA)/DMEM (Dulbecco’s Modified Eagles Medium, Invitrogen Corp, Carlsbad, CA, USA) and kept at 4°C for one night. The trypsin treatment was stopped by the addition of DMEM containing 10% fetal calf serum (FCS, Invitrogen Corp, Carlsbad, CA, USA) as a trypsin inhibitor, and the pieces were then gently shaken for 30 min. As the primary culture, this cell suspension (2×10<sup>6</sup> cells) was poured into 75 cm<sup>2</sup> flasks (Becton Dickinson, Franklin Lakes, NJ, USA). For the culture, DMEM containing 5% FCS was used. In two weeks, the fibroblasts detached from the tissue pieces and migrated and proliferated on the surface of the culture flask (Fig. 1A). The medium was changed to Neuro Basal Medium (NBM, Invitrogen Corp, Carlsbad, CA, USA) containing 5% FCS, N2-supplement (Invitrogen Corp, Carlsbad, CA, USA). In another two to four weeks, morphologically characterized neural cells started to appear spread over the confluent layer of the proliferated fibroblasts (Fig. 1B). These neural cells were identified as Schwann cells by immunohistochemical analysis with anti-human S-100 antibody (diluted 1 : 250; DAko, Tokyo, Japan) (Fig. 1C) and type VI collagen antibody (diluted 1 : 200; LSL, Tokyo, Japan, data not shown). Although we found that Schwann cells were inferior in growth potency to fibroblasts, we succeeded in growing Schwann cells in up to four passages of culture. We called this culture formation with the mixture of Schwann cells and fibroblasts “NF1 cells”. NF1 cells were cultured at 37°C in a 5% CO<sub>2</sub> incubator. The present study used cells of the second to third passages.

**Preparation of mast cells derived from PBMC**

Mast cells were grown and differentiated from Granulocyte Colony Stimulating factor (G-CSF) mobilized CD34<sup>+</sup> peripheral blood mononuclear cells (PBMC; Camblex, Walkersville, MD, USA) in the culture with AIM-V medium (Invitrogen Corp, Carlsbad, CA, USA) containing 20 ng/ml SCF and 10 ng/ml Interleukin-3 (IL-3) for six weeks (both were from R&D systems, Minneapolis, MN, USA) (Fig. 2A). The degree of differentiation of the mast cells obtained from the G-CSF-mobilized CD34<sup>+</sup> PBMC was evaluated by metachromasia with toluidine blue staining and immunohistochemical analysis with anti-human tryptase antibody (diluted 1 : 1000; Calbiochem, San Diego, CA, USA). We injected rabbits with anti-human recombinant rabbit IgG (Miltenyi Biotec, Bergisch Gladbach, Germany) as a microbeads-coating second antibody (Fig. 2B).

**Preparation of mast cell / NF1 cell co-culture**

For the co-culture experiment, we set up a direct culture system (Fig. 3). The suspension of mast cells was poured onto the layer of NF1 cell culture, and the mast cells thus gained direct contact with the NF1 cells. First, the NF1 cells were seeded on 1.8 cm<sup>2</sup> wells, and incubated for a while after the NF1 cells had attached. Then, mast
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The number of cells placed in the containers was set strictly to $1 \times 10^4$ for the NF1 cells and to $5 \times 10^3$ for the mast cells. As control and monoculture systems, a monoculture containing either NF1 cells or mast cells was also produced. In all of the cultures, DMEM containing 1% FCS was used.

Preparation of tranilast solution

For preparation of the stock solution of tranilast (Kissei Pharmaceutical Co., Matsumoto, Japan), 32.7 mg of powdered tranilast was dissolved into 10 ml of 1% NaHCO$_3$ at 80°C to make a $1 \times 10^{-2}$M solution. It was further diluted with DMEM containing 1% FCS to the final concentration of 10 $\mu$M ($1 \times 10^{-3}$M), 50 $\mu$M ($5 \times 10^{-3}$M), and 100 $\mu$M ($1 \times 10^{-2}$M) before applying it to the co-culture and monoculture (control).

Analysis of proliferation of cells in monoculture and co-culture

In the monoculture system, the magnitude of proliferation of NF1 cells was analyzed 1, 3, 5 and 7 days after the tranilast treatment using the following procedures. Before collecting the cells, the culture supernatant was first taken out for measurement of cytokines and tryptase. The NF1 cells remaining in the culture wells were washed once with PBS(−), and then soaked in PBS(−) containing 0.1% trypsin and 0.02% ethylene-diamine-tetra-acetic acid (EDTA) for 10 min until the cells scattered. The cell suspension was transferred to 15-ml conical tubes and centrifuged at 1,000 rpm for 5 min. After the supernatant was removed, 1.0 ml of DMEM was added to the tube in order to resuspend the cells.
In the co-culture system, NF1 cell removal and suspension were obtained by the same procedure as described above. The culture medium containing mast cells was centrifuged to separate the supernatant for the measurements. In the mast cell co-culture system as control, the mast cells in the culture medium were first pipetted out to the conical tube. The cell suspension was then centrifuged to obtain the supernatant for the measurement later on.

The NF1 cells attached to the wells were treated similarly as in the other systems. The number of cells in each suspension was counted as follows: 100 μl of the cell suspension was poured into a 96-well microplate, and 10 μl of water-soluble tetrazolium salt-1 (WST-1) coloring agent (Dojindo Laboratories, Kumamoto, Japan) was added to each well. This assay is based on WST-1 cleaved to formazan by mitochondrial enzymes. The reaction continued for 4 hours at 37ºC in a 10% CO₂ incubator. Absorbance was measured using a microplate reader (Model 550, Bio-Rad Laboratories, Hercules, CA, USA) by setting the test wavelength at 450 nm and the reference wavelength at 650 nm, and the obtained values were converted into cell numbers based on the absorbance of standards with known cell numbers.

**Flow cytometry analysis of the cell cycle**

DNA staining was performed using propidium iodide (PI, Sigma-Aldrich, St. Louis, MO, USA) to measure the cell cycle rate. Details of the flow cytometric analysis and staining procedure were the same as those previously described (Krishan 1975; Herzenberg et al. 1976; Xu et al. 2005). NF1 cells that had been cultured with tranilast for three days were rinsed thoroughly in PBS(−), then incubated in 0.02% EDTA/PBS(−) and 0.1% trypsin for 15 min each to disperse the cells. The action of the trypsin was stopped by adding FCS containing trypsin inhibitor, and the dispersed cells were centrifuged for 5 min at 1,000 rpm. The supernatant was discarded and the cells in the precipitate were fixed in 75% ethanol dissolved in H₂O. Fixed cells were counted using flow cytometry, and the DNA content of each cell was analyzed using CXP Software (Cytomics FC500, Beckman Coulter, Fullerton, CA, USA).

**Cytokine levels in the culture media**

The TGF-β1 and SCF released into the culture media were measured with human enzyme-linked immunosorbent assay (ELISA) kits for the respective analyses (R&D Systems, Minneapolis, MN, USA). The concentrations obtained were converted to pg per 10⁴ cells.

**Tryptase levels**

Tryptase, a tetrameric serine proteinase, is a specific component of mast cell degranulation and a target for therapeutic intervention against type I allergy. To evaluate mast cell activity from the co-cultured media, we measured the tryptase level using a Mast Cell Degranulation Assay Kit (Chemicon International, Temecula, CA, USA). The concentrations obtained were converted to pg per 10⁴ cells.

**Statistical analyses**

Each set of experiments was done in six to ten replicate measurements. The Mann-Whitney U test was used to test for differences in cell numbers in the presence of tranilast. The Kruskal-Wallis test, a nonparametric version of one-way analysis of variance, was used to judge the dose-dependency of responses to tranilast. P values less than 0.05 were considered statistically significant. The dependency of the cell cycles (G₀/G₁/S phases) on the dosage of tranilast was tested by the chi-square test for independence (L × M contingency table).

**RESULTS**

**Tranilast-treated NF1 cells in the monoculture system Cell growth**

In the monoculture system, the number of NF1 cells per well with or without tranilast treatment was counted on the 1st, 3rd, 5th and 7th days (Fig. 4). Within a couple days, the cell proliferation was suppressed proportionately with
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The tranilast concentrations. There was no significant difference in the cell numbers on the 1st day, but by the 3rd day, the cells of the tranilast treatment group were significantly suppressed compared to those of the controls ($p < 0.005$ for the 3rd, 5th and 7th days).

**Cell cycle**

Three days after the tranilast treatment, the cultured NF1 cells were stained with PI and their intensity was measured by flow cytometry for evaluation of their cell cycle. As shown in Fig. 5, the percentage of cells in the G2/M phases was decreased while that in G0/G1 phases was increased in a dose-dependent manner ($p < 0.05$ by chi-square test or L × M contingency table).

**Tranilast-treated NF1 cells in the co-culture system**

**NF1 cell growth**

Three days after adding 50 μM of tranilast to the NF1 cells and PBMC-derived mast cells in the co-culture, the number of NF1 cells had decreased significantly compared with the control without the addition of tranilast ($p < 0.05$). With cell counts of NF1 cells without tranilast treatment set as 100, the ratio of cell counts for the monoculture of NF1 cells, measured in six replicates, was computed as 83.1, and that for the co-culture system was 78.0. The ratio for the monoculture of mast cells was 83.9. There was no significant difference in these ratios (Fig. 6). In addition, we did not observe any significant difference in the mast cell counts between the two culture systems (data not shown).

**Cytokine release**

Measurement of the cytokine levels in the culture
medium revealed that TGF-β₁ and SCF levels were significantly decreased on the 3rd day after the addition of tranilast ($p < 0.01$). Setting cytokine levels for the control without tranilast as 100, TGF-β₁ levels were computed as 85.3 for the monoculture of NF1 cells, 87.0 for the co-culture system, and 83.9 for the monoculture of mast cells. (Figs. 7 and 8) The same ratios for the SCF levels were 47.1 and 39.1, respectively. In the conditioned medium in the mast cell culture, there was no detection of SCF levels. There was no significant difference among culture systems in either cytokine ratio.

**DISCUSSION**

The pathogenetic mechanism of neurofibroma in von Recklinghausen disease is very complicated. Viskochil (2003) summarized the following mechanisms for the

**Tryptase levels**

The concentration of tryptase in the culture medium measured on the 3rd day was suppressed conspicuously for the co-culture system and monoculture of mast cells. Their relative ratios to the control were computed as 45.8 and 25.7, respectively and were significantly different from each other ($p < 0.05$). In the conditioned medium in the NF1 cell culture, there was no detection of tryptase level (Fig. 9).
pathogenesis of neurofibroma: (1) The presence of NF1<sup>−/−</sup> Schwann cells is essential for the tumorigenesis, but the co-presence of NF1<sup>+/−</sup> in the vicinity of the NF1<sup>−/−</sup> cells is also required (Zhu et al. 2002). (2) Although NF1 patients possess hemi-allele mutation, development into neurofibromatosis is allegedly dependent on mast cells which respond to the inflammation occurring in neurofibroma tissue (Viskochil 2003; Yang et al. 2003). Yang et al., in particular, hypothesized that mast cells act as an epigenetic contributor for the Schwann cell and thus promote tumorgenesis.

In recent years, the role of mast cells within several tumor tissues, such as gastric cancer, has been investigated. Yano et al. (1999) reported that mast cell infiltration around gastric cancer cells correlates with tumor angiogenesis and metastasis. Normal mast cells have also been found to exist during the development of other soft tissue tumors such as malignant lymphoma and Hodgkin’s lymphoma (Fukushima et al. 2006; Hedstrom et al. 2007; Rygol et al. 2007). Chemical mediators released from mast cells were thought to be responsible for the enhanced proliferation of tumor cells.

Tranilast, developed originally as an anti-allergy agent, was empirically found to be effective in suppressing the formation of hypertrophic scars and keloids, and thus was approved in Japan as the sole oral therapeutic agent against those conditions. It suppresses chemical mediators released from mast cells, and also inhibits TGF-β, and collagen bio-
synthesis from hypertrophic scars and keloid fibroblasts. Only tranilast possesses this property of dual suppression. From the analogy of cellular composition to the keloid tissue, we postulated that tranilast can be a potent suppressor of neurofibroma cell growth as well.

In order to clarify how mast cells work for the promotion of tumorigenesis, it is essential to have an experimental model to elucidate the pathogenic mechanism for the neurofibroma. The experimental models that have been reported to date have been based on tumor cells derived from either NF1-transgenic mice or malignant human schwannoma. Thus, they do not truly represent human neurofibroma. The culture system established in the present study is the first successful “mixed culture” of fibroblast and Schwann cells, obtained from surgical specimens of neurofibroma. We called the cell mixture NF1 cells. We established a co-culture of the mast cells with NF1 cells to enable us to closely examine the mechanism of mast cells for promotion of the tumor. This is the first report to prove cell-cell interaction affect on neurofibroma cell growth using special culture system closely resembling human neurofibroma.

From the series of experiments, we found the NF1 cells of the tranilast treatment group were significantly suppressed in number compared to those of the control group after the 3rd day. The NF1 cells and PBMC-derived mast cells in the co-culture, the number of NF1 cells, and the TGF-β1 and SCF levels in the culture medium also decreased significantly compared with the control. Furthermore, the concentration of tryptase in the culture medium measured was conspicuously suppressed. TGF-β1 is well known as a potent cell growth factor for fibroblasts. The effects of TGF-β1 on the proliferation of fibroblasts involve not only autocrine but also paracrine regulation. When tranilast was added to culture systems, tranilast suppressed the release of cytokines in all mono/co-culture systems; hence, we consider that tranilast can influence the release of TGF-β1 from both fibroblasts and mast cells.

SCF is a mast-cell growth factor released from fibroblasts (Valent et al. 1992; Linenberger et al. 1995; Broudy 1997). Actually, mast cells have well-developed receptors for SCF called c-kits. Furthermore, mast cells have c-kits, but never release SCF. Although it has not been clearly documented as a proliferation mechanism for neurofibroma, we postulated that the fibroblast releases SCF stimulated by chemical mediators from the mast cells in the neurofibroma. This in turn leads to increased release of SCF from the fibroblasts with further activation of the mast cells, thus forming a positive feedback chain, enhancing tumor growth and accumulation of collagen fibers in the tumor tissue. The results of our experiment revealed that a co-culture system inhibited SCF release from the fibroblasts with or without tranilast, but tranilast could inhibit SCF release more. As far as we know, there has been no report demonstrating the suppression of SCF by tranilast. We measured the concentration of SCF in culture medium after the addition of tranilast. SCF was decreased although the number of mast cells was not affected. However, mast cells take a long time to grow; thus, a three-day period of exposure to tranilast was too short to reduce the number of mast cells.

Secretory granules from mast cells contain many pro-inflammatory mediators including heparin, cytokines, chemokines, and many proteases (Hallgren et al. 2001). Tryptase is a tetrameric serine protease specifically found in mast cells as well as in chymase. It has emerged as the major component of mast cell granules, comprising up to 20% of the total protein of mast cells derived from lung, colon and skin tissue (Schwartz et al. 1985; Harvima et al. 1999; He et al. 2004, 2005). Their overproduction in tissues rich with collagen matrix was thought to be responsible for the localized accumulation of collagen. Therefore, we measured them in the culture medium with and without tranilast treatment. As expected, tranilast was found to be a potent suppressor of the synthesis of tryptase, which is produced specifically by mast cells associated with the tumor. However, we could not clarify whether tranilast suppresses tryptase directly or suppresses it indirectly by inhibiting the release of chemical mediators from the mast cells.

The primary mode of therapy for von Recklinghausen disease-associated neurofibroma is surgical resection of the tumors; pharmacological treatment has not yet been established. Therefore, patients with systematic disease, without surgical indication suffer both emotionally and physically from intense itching and pain accompanying the tumor. The first event for tumor genesis is deletion of a normal allele in Schwann cells; on this account, tranilast cannot prevent the occurrence of the tumor. However, since the tumor generally takes a benign course, the drug is expected to retard its growth. Furthermore, its action against the release of chemical mediators will have a beneficial effect on the common manifestations of pain or itching originating from the tumor.

The results of this study suggest that tranilast can be a suitable anti-tumor agent because of its potent suppressive effect on both mast cells and fibroblasts through its inhibition of their release of growth-stimulating cytokine.

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