In-vivo activation of Raf-1 inhibits tumor growth and development in a xenograft model of human medullary thyroid cancer

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Apart from surgical resection, there are no effective therapies for medullary thyroid cancer, a neuroendocrine tumor derived from parafollicular C cells. We have previously shown that activation of raf-1 in TT-raf cells by estradiol suppresses tumor cell growth and calcitonin secretion *in vitro*. TT-raf cells are a human medullary thyroid cancer cell line that contains an estrogen-inducible raf-1 construct. The in-vivo effects of raf-1 activation in this cell line, however, have not been characterized. Therefore, we utilized TT or TT-raf cells in a murine subcutaneous xenograft model to study tumor development and growth. Activation of raf-1, in mice with TT-raf tumors, led to a significant decrease in medullary thyroid cancer tumor formation. Control groups, however, had a high rate of medullary thyroid cancer tumor development. These data indicate that raf-1 activation by estradiol treatment in this TT-raf xenograft model inhibited tumor development. Furthermore, to determine whether raf-1 activation could also inhibit the growth of established tumors, estradiol and control pellets were implanted after tumor development. The TT-raf group that received estradiol pellets showed an 8-fold decrease in tumor volume compared with the TT-raf control group. Taken together, these results suggest that in-vivo activation of raf-1 in a murine model of medullary thyroid cancer not only led to a reduction in tumor development, but also inhibited the growth of established tumors. These results suggest that strategies to activate the raf-1/MEK/ERK1/2 signaling pathway may be a viable approach to treat patients with metastatic medullary thyroid cancer. *Anti-Cancer Drugs* 17:849–853

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Introduction

Medullary thyroid cancer (MTC) is a neuroendocrine (NE) tumor originating from the calcitonin-producing thyroid C cells and is the third most common form of thyroid cancer accounting for 3–5% of all thyroid cancers [1,2]. Currently, surgical resection, including total thyroidectomy and appropriate lymph node dissection, is the only potential curative therapy for MTC. Fifty percent of patients with MTC, however, will have recurrent disease after surgery. Therefore, it is necessary to develop alternative therapies to control tumor growth, possibly through the manipulation of various cellular signaling pathways [1]. Although MTC is rare, there has been much interest in the molecular pathways that regulate the growth and metastatic phenotype of these tumors. It has been previously shown that ras-activating mutations, common in other forms of carcinomas, are extremely rare in MTC [1,3–5]. This suggests that the activation of downstream ras-activated pathways, such as the raf-1/MEK/ERK1/2, may not be advantageous to MTC tumor development. Activation of the raf-1/MEK/ERK1/2 pathway has been shown to have effects on both cell cycle progression and protection against apoptosis. We and others, however, have previously shown that activation of the raf-1/MEK/ERK1/2 pathway, *in vitro*, inhibits growth and proliferation of MTC, and reduces levels of NE hormones such as calcitonin and chromogranin A (CgA) [1,4,6–8]. On the basis of these in-vitro data, it is possible that the manipulation of this signaling pathway could be used as a potential therapeutic strategy in the treatment and management of MTC. Interestingly and perhaps surprisingly, to our knowledge, there is no published report on in-vivo activation of raf-1 and its effect on MTC tumor growth. On the basis of our reports and of others, however, we speculate that raf-1 activation in an in-vivo animal MTC model should reduce the tumor development and growth. With the aim of understanding better the role of raf-1 in MTC, we developed a xenograft model of human MTC utilizing TT and TT-raf cells. In this study, we show the tumor development within

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4 weeks in male nude *nu/nu* mice using human MTC cells. Furthermore, activation of the raf-1 pathway in these tumors led to a significant reduction in tumor growth and development. Importantly, our results suggest that raf-1 activation could be a therapeutic strategy to control MTC tumor growth.

Methods

Cell culture

Human medullary thyroid carcinoma cells (TT) and TT-raf cells were a kind gift from B. Nelkin (Johns Hopkins). The TT and TT-raf cells were maintained in RPMI 1640 (Invitrogen, Carlsbad, California, USA) with and without phenol red, respectively, supplemented with 18% fetal bovine serum (Sigma, St Louis, Missouri, USA), 100 IU/ml penicillin and 100 μg/ml streptomycin (Invitrogen). TT-raf cells were kept under selection with 200 μg/ml G418 (Invitrogen). Cells were maintained in a humidified atmosphere of 5% CO_2 in air at 37°C [7].

Raf-1 activation

TT-raf cells were previously created by transfecting TT cells with a retroviral construct containing the raf-1 catalytic domain fused to the hormone-binding domain of the human estrogen receptor (ΔRaf-1:ER) [9]. To activate raf-1, in TT-raf cells, 1 μmol/l of β-estradiol (E2) (Sigma) was added to the media. For control treatment, an equivalent volume of ethanol (C), the solvent for E2, was added to the media. After 48 h, cells were harvested, and total cellular protein was isolated and subjected to Western blot analysis as described previously [5,10,11].

Animal surgery and qualitative tumor analysis

All animal care and surgery was performed in compliance with our animal care protocol approved by the University of Wisconsin–Madison animal care and use committee.

Experiment 1

Fifty-six male nude athymic, *nu/nu* mice (Charles Rivers, Wilmington, Maryland, USA) received subcutaneous injections into the right flank, of either TT or TT-raf cells (10^6). Mice were divided into four groups of 14 animals per group. In this study, both groups (TT and TT-raf) received C or E2 60-day sustained release pellets (Innovative Research of America, Sarasota, Florida, USA) at the time of tumor cell injection. Pellets used to activate the Raf-1:ER construct are of 3 mm diameter containing either 2.5 mg of E2 or control. Pellets are implanted in the cervical interscapular space by using a commercially available trochar (Innovative Research of America). Each pellet is designed to slowly release E2 by matrix-driven delivery, through controlled erosion and diffusion of the pellet matrix (Innovative Research of America). Mice were observed for tumor development. The number of mice within each group that developed tumors and the subsequent tumor volume were recorded. Tumor volume was determined by measuring the length (l) and width (w) of each tumor with vernier calipers, and calculated using the formula $w^2 \times l \times 0.52$.

Experiment 2

In the second study, mice received C or E2 pellets after the development of a visible tumor (tumor size around 2–4 mm). At 20 days after receiving treatment, tumors were measured, harvested and total tumor cell lysates were prepared as described below for Western blot analysis.

Western blot analysis

Tumors were removed from the mice after killing them and were flash frozen in liquid nitrogen. A 1-mm^3 piece of tissue was taken from the tumor and then pulverized in liquid nitrogen. The tissue powder was then placed in 500 μl of lysis buffer (50 mmol/l Tris, pH 7.5, 150 mmol/l NaCl, 1% Igepal CA-630, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 0.1 μmol/l phenylmethylsulfonyl fluoride, 5 mmol/l ethylene diaminetetraacetic acid, 12 μl/ml Protease Inhibitor Cocktail; Sigma) and then incubated in ice for 30 min. The tumor lysates were centrifuged at 18 000 g at 4°C for 30 min and the supernatant was collected. The concentrations of total cellular proteins were quantified by the bicinchoninic acid protein assay kit (Pierce, Rockford, Illinois, USA). The denatured proteins (30 μg) from each sample were subjected to electrophoresis on 10% Bis-Tris precast polyacrylamide gels (Invitrogen) and transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, New Hampshire, USA). Membranes were then blocked for 1 h in milk solution (1 × Tris buffered saline, 5% nonfat dry milk, 0.05% Tween 20) and incubated at 4°C overnight with the appropriate primary antibodies. Primary antibody dilutions were as follows: phospho-ERK1/2 (1:1000; Cell Signaling Technology, Beverly, Massachusetts), CgA (1:1000; Zymed Laboratories, San Diego, California USA) and G3PDH (1:10 000; Trevigen, Gaithersburg, Maryland, USA). Following primary antibody incubation, membranes were washed either 3 × 5 min (phospho-ERK1/2) or 3 × 10 min (CgA, G3PDH) with 1 × TBS-T (1 × TBS with 0.05% Tween 20). The membranes were then incubated with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:2000; Cell Signaling Technology) for 1 h at room temperature. Then, the membranes were washed either 3 × 5 min (phospho-ERK1/2) or 3 × 10 min (CgA, G3PDH) with 1 × TBS-T, and developed with Immunstar (Bio-Rad Laboratories, Hercules, California, USA) in accordance with the manufacturer's instructions.

Statistical analysis

Analysis of variance with Bonferroni post-hoc testing (SPSS software 10.0, SPSS, Chicago, Illinois, USA) was utilized for statistical comparisons. < 0.05 was considered significant. Unless noted, data are represented as means ± SE.

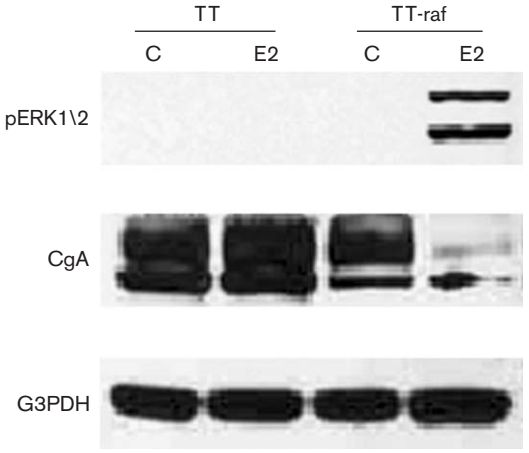
Results

Raf-1 pathway activation in TT-raf cells reduces neuroendocrine hormone, chromogranin A, in tissue culture

It has been shown by our group and others that treatment of TT-raf cells with E2 led to the activation of the raf-1 pathway, which then reduces NE hormone production and also cellular growth [4,7,8,12,13]. To ensure that the TT-raf cells respond to E2, we carried out an experiment before injecting the mice with TT and TT-raf cells. Both TT and TT-raf cells were treated with C and/or E2 as described in Methods. To determine the raf-1 pathway activation in TT-raf cells, we measured the levels of active, phosphorylated mitogen-activated protein kinase (pERK1/2) by Western blot analysis. Cell lysates from TT-raf cells treated with E2 showed a marked increase in pERK1/2 as shown in Fig. 1. The TT and TT-raf cells treated with C, as well as the TT cells treated with E2, however, showed no detectable levels of pERK1/2. The increase in pERK1/2 in the TT-raf cells, treated with E2, indicates activation of the raf-1 pathway.

Having activated the raf-1 pathway in MTC in cell culture, the effects of raf-1 on the production of the bioactive hormone CgA were assayed using Western blot analysis. It has been previously shown that activation of the raf-1/MEK/ERK1/2 pathway caused a reduction in NE hormone production [4,8]. No observable change was seen in the baseline level of CgA production in TT cells treated with either C or E2, or in TT-raf cells treated with control. Activation of raf-1 in TT-raf cells treated with E2, however, caused a marked reduction in the level of CgA, as compared with control treatments. This result is consistent with previous observations [4,8].

Fig. 1



Raf-1 activation in medullary thyroid cancer (TT-raf cells) caused the induction of phosphorylated mitogen-activated protein kinase (pERK1/2) and a reduction in neuroendocrine hormone, chromogranin A (CgA). Western blot analysis of 30 μg total cell lysates from TT and TT-raf cells treated *in vitro* with control (C) or β-estradiol (E2).

Table 1 Total number of mice that grew tumors per treatment group after receiving control or E2 pellets at the time of tumor cell injection

Group	Cells	Pellet implanted	No. of mice with medullary thyroid cancer tumors
1	TT	Control	11/14
2	TT	E2	12/14
3	TT-raf	Control	12/14
4	TT-raf	E2	2/14
P-value	–	–	<0.05

In the group with TT-raf tumors that received E2, fewer mice developed tumors. E2, β-estradiol.

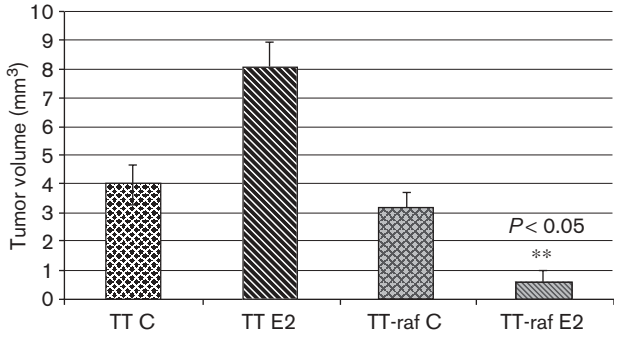
Activation of raf-1 in TT-raf cells inhibits xenograft tumor development

Raf-1 activation inhibits growth and hormone production in MTC *in vitro*, but does raf-1 activation inhibit tumor development and hormone production in an in-vivo model? On the basis of our in-vitro data, we hypothesized that the results would be translatable to an in-vivo animal model of human MTC. Mice in Experiment 1 received TT or TT-raf tumor cell injections and C or E2 pellets simultaneously and were observed for xenograft tumor development. Of the 14 mice that received tumor cell injections of TT-raf cells treated with E2 pellets, only two (14%) of the mice developed tumors (Table 1). Twelve (86%) of the mice injected with TT-raf cells in the control group, and 11 (79%) and 12 (86%) of the mice that received TT cell injections in the control and E2-treated groups, however, developed tumors, respectively (Table 1). The results of this experiment suggest that the activation of raf-1 in TT-raf cells prevents tumor formation.

Raf-1 activation in TT-raf xenograft tumor model reduces tumor growth

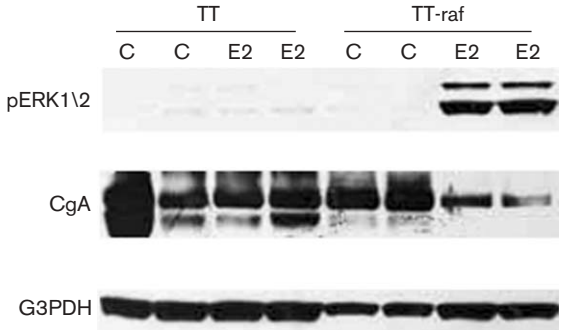
We observed that simultaneous activation of raf-1 in TT-raf cells, at the time of subcutaneous tumor cell injection, led to inhibition of tumor formation in mice. In order to determine the effect of raf-1 activation on tumor growth, however, we allowed the mice to develop visible tumors (around 2–4 mm) before the raf-1 pathway was activated (Experiment 2). In this study, mice developed measurable tumors and then the pellets (C or E2 pellets) were implanted. As shown in Fig. 2, for the TT-raf group receiving E2 pellets, the average tumor volume at the time of killing the mice was $0.6 \pm 0.4\,mm^3$. Interestingly, this average tumor volume was markedly less than that in the TT-raf control group that had an average tumor volume of $3.2 \pm 0.5\,mm^3$. Mice that received TT cells and C and E2 treatments, however, had much larger tumors with volumes of 4.0 ± 0.7 and $8.1 \pm 0.8\,mm^3$, respectively (Fig. 2). Interestingly, as shown in Fig. 2, TT xenograft tumors that received E2 developed larger tumor size than that in its control groups. The increase in tumor size with E2 treatment in TT xenograft tumor is interesting and, perhaps, might be due to the action of E2. In contrast,

Fig. 2



Tumor volume for the xenograft tumors after 20 days of treatment. TT and TT-raf cells were injected subcutaneously into the flank of nude mice. Established tumors were treated with either control (C) or β-estradiol (E2) pellets for 20 days. Then, the sizes of the tumors were measured as described in Methods. Treatment of TT-raf xenografts resulted in at least 8-fold reduction in tumor volume compared with the control groups.

Fig. 3



Raf-1 activation in TT-raf tumors caused the induction of phosphorylated mitogen-activated protein kinase (pERK1/2) and a reduction in neuroendocrine hormone, chromogranin A (CgA). Western blot analysis of 30 μg total cell lysates from tumors treated *in vivo* with either control (C) or β-estradiol (E2). Samples shown are representative of each treatment group.

E2-treated TT-raf xenograft tumors (raf-1-activated tumor) showed a reduction in tumor size of 8-fold in volume as compared with the C group. We, however, speculate that the actual reduction of tumor size would be more than 8-fold if one accounts for the E2 activity. Statistical analysis showed that the differences between the control groups (TT C, TT E2, TT-raf C) and the TT-raf E2 group are statistically significant with $P < 0.05$.

Confirmation of raf-1 activation

Next, we wanted to determine whether the tumor size reduction was actually due to the activation of the raf-1 pathway. Tumors from all four groups of mice were removed after the animals were killed. Proteins were isolated from the tumors as described in Methods and Western blot analysis was performed. Here, we show representative examples of the tumors from each treatment group. As shown in Fig. 3, pERK1/2 was detected only in E2-treated TT-raf tumors as compared with the control TT-raf tumors. As expected, there is no detectable pERK1/2 in TT tumors treated either with control or E2. This result is in agreement with Fig. 1 and with previously published in-vitro data [8]. These data support our conclusion that the tumor reduction seen in the TT-raf xenograft tumors is indeed due to the activation of raf-1.

The effect of raf-1 activation on neuroendocrine markers

It has been previously shown that the levels of CgA produced by MTC correlates with the levels of other NE hormones such as calcitonin [8]. Therefore, to assess the effect of raf-1 activation on NE hormone production, we used Western blot analysis to detect the levels of CgA in the tumors from each treatment group. The samples from the TT-raf tumors that received E2 treatment showed a distinct reduction in CgA levels in comparison with control groups (Fig. 3).

Discussion

We have been interested in the role of various signaling pathways in the modulation of growth and hormone production of NE tumor cells *in vitro*. Earlier, we and others reported that the activation of raf-1 in MTC cells led to the reduction in hormone production and to growth inhibition. Although the MTC cell line (TT) has been utilized previously in in-vitro studies for sometime, it has only recently been used for the development of a xenograft model to study the tumor development and progression [14,15]. Here, we showed that the activation of raf-1 in a TT-raf xenograft model not only prevented tumor formation but also reduced tumor growth.

We have previously reported the development of a mouse model of carcinoid syndrome by using a gastrointestinal carcinoid cell line (BON) [16]. In this study, utilizing similar techniques, we have successfully developed a xenograft model for raf-1 activation in human MTC cells. Utilizing an established nude mouse model of xenograft tumor growth, we developed TT and TT-raf xenograft tumors, and studied the effects of raf-1 pathway activation in MTC tumors both at the time of tumor challenge and after the development of an established tumor. Activating raf-1 at the time of tumor challenge resulted in no tumor growth. Only 14% of the mice (two mice) developed measurable tumors at this time point as compared with control groups. TT-raf tumors given C treatment and TT tumors given either C or E2 treatment, however, developed tumors with a frequency of 86, 79 and 86%, respectively. At this point in the study, the reason that the two mice developed tumors in

E2-treated TT-raf cells is not known. It is possible that these two tumors did not receive an adequate amount of E2 to activate the raf-1 construct in the TT-raf cells. On the basis of the results of this experiment, we believe that raf-1 activation inhibits tumor formation in the mice model. To further confirm this observation and the effect of raf-1 activation on tumor progression, TT and TT-raf tumors were allowed to develop to a measurable size. This second experiment was performed to study the effects of raf-1 activation on established tumors and verify that we had actually activated the raf-1 pathway. The TT-raf tumors that received E2 had an average volume of $0.6 \pm 0.4\,mm^3$, whereas the TT-raf tumors given C treatment had an average volume of $3.2 \pm 0.5\,mm^3$. TT tumors that received C or E2 pellets had an average volume of 4.0 ± 0.7 and $8.1 \pm 0.8\,mm^3$, respectively. Another point of interest is the increased size of the TT tumors that were treated with E2. The size difference in this group is statistically significant with $P < 0.05$. The increase in the tumor size by E2 could be the result of the inhibition of natural killer cells in *nu*/*nu* mice [17].

Furthermore, in this study, in-vivo activation of the raf-1 pathway also resulted in a reduction of the NE hormone CgA. The mice with established TT-raf tumors that received E2 showed a significant decrease in CgA after 20 days of treatment as illustrated by Western blot analysis. Treatment of TT-raf tumors with E2 also showed an induction of pERK1/2. The presence of pERK1/2 in the TT-raf tumors treated with E2 indicates that the raf-1 pathway is activated in these tumors. Raf-1 activation in an in-vivo model of MTC leads to a slower onset of tumor and the reduction of hormone present in an established tumor, as evident by the low amount of CgA. On the basis of both previous in-vitro and current in-vivo data, raf-1 activation appears to be a possible therapeutic target for treatment of MTC. Activation of raf-1 would best function in conjunction with surgical resection or as a palliative treatment for patients with highly metastatic disease.

The xenograft mouse model described here may therefore provide a suitable model system in which to identify and elucidate the effects and activation of raf-1 and other genes in MTC tumors. The challenge, however, is to deliver activated raf-1 to tumor cells. Alternatively, it is worthwhile to explore and identify raf-1-activating pharmacological compounds. Recently, we have reported that ZM336372 is an raf-1-activating compound and treatment of carcinoid cells with ZM336372 leads to growth inhibition and a reduction in NE hormone production [5]. In summary, effective prevention of NE tumors such as MTC is clearly the ultimate goal for eradicating the disease. The xenograft model will certainly help shed more light on the process of tumor development and progression, and help researchers to identify more targets on the raf-1 pathway components to inhibit tumor growth.

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