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ORIGINAL PAPER

Lung Tumor Development in the Presence of Sphingosine 1-phosphate Agonist FTY720

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Abstract Urethane is a chemical carcinogen which causes lung tumorigenesis in mice with similarities to human adenocarcinoma (AC). The sphingosine 1-phosphate agonist FTY720 administered to mice in doses above 5 mg/kg/day has been able to prevent hepatocellular carcinoma and bladder cancer. We used BALB/c mice in urethane-induced lung cancer model to investigate the effects of a lower dose of FTY720 (1 mg/kg/day). The benefits of FTY720 were associated with the time point of the compound administration. FTY720 30 Group presented lower incidence and smaller area of lung nodules, decreased PCNA and increased Caspase-3 expressions. The findings in FTY720 0 Group (nodule multiplicity and area, PCNA expression) were similar to Urethane Group suggesting that the administration of the compound at early time point did not affect lung tumor development. FTY720 90 Group presented the biggest nodule area which was associated with increased PCNA and decreased Caspase-3 expressions. FTY720 (30 days and 90 days) administration decreased CD4+splenocytes and blood lymphocytes which caused opposite effects in lung tumor development - impairment and improvement respectively.

In conclusion, FTY720 in low dose did not provide lung tumor inhibition in mice but its administration 30 days after the chemical carcinogen (Urethane) injection was associated with impaired tumor development

Keywords Lung cancer · Urethane · FTY720 · Apoptosis · Proliferation · CD4 + T cell

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Introduction

Administration of chemical carcinogens to mice has been used to model multistage human lung carcinogenesis. In mouse, lung tumors arise from nonciliated airway (Clara) or type II alveolar epithelial cells and the gene mutations observed are similar to those identified in human lung cancers. Moreover, mouse lung tumors induced by chemical carcinogens and human adenocarcinoma (AC) present similarities in gene expression profiles supporting the utility of mice models [1–4].

Urethane (ethyl carbamate) is a chemical carcinogen which induces mice lung tumorigenesis and has been used to classify inbred strains into sensitive, intermediate, and resistant categories of susceptibility to lung AC. By 20 weeks after urethane administration, all sensitive A/J mice develop an average of 30 tumors/mouse, most intermediate BALB mice develop tumors but with a low multiplicity of 2 tumors/mouse, and less than half of resistant C57BL/6 mice develop tumors with a mean multiplicity <1 tumor/mouse [1]. O'Donnell et al. (2006) showed that in BALB mice the earliest microadenomas did not appear until 9 weeks after urethane exposure (1 mg/g of

body weight) and the mean number of microadenomas remained slightly above one tumor per mouse at all subsequent timepoints with an 80% incidence at 20 weeks [5].

BALB/c intermediate susceptibility has been associated with the presence of both sensitive *Kras* polymorphism and a unique *Pulmonary adenoma resistance* (*Par2*) allele that down-modulates their neoplastic predilection [6]. Another finding in BALB/c mice at early time point (1 week) after urethane administration was the increase of circulating neutrophils and inflammatory cells in bronchoalveolar lavage (BAL) fluid besides of increased TNF-α and IL-12p70 levels. These results suggest that the early appearance of local and peripheral inflammation after urethane administration play a role in BALB/c adenoma development [7].

FTY720 is a new compound produced by modification of a metabolite from *Isaria sinclairii* (myriocin) which impairs tumor growth and metastasis in animal models. FTY720 phosphate (FTY720P) bear structural similarity to sphingosine-1 phosphate (S1P) and binds to four of five S1P receptors ($S1P_1$, $S1P_3$, $S1P_4$, $S1P_5$). In a mice model of melanoma FTY720 not only inhibited both S1P- and VEGF-induced angiogenesis but also impaired primary and metastatic tumor growth. These data suggest that although FTY720 has been shown to have $S1P_1$ agonist activity its anti-tumor effect is mediated by functional antagonism of vascular S1P receptors [8].

Mouse breast cancer development in mice after the injection of JygMC(A) cells was markedly suppressed by FTY720 (5 mg/kg). In addition, tumor metastasis was significantly prevented even at a low dose (2 mg/kg/day), resulting in a significant prolongation of animal survival [9]. The anticancer effect of FTY720 (5 mg/kg and 10 mg/kg) was also confirmed by significantly decreased human bladder cancer cells growth in mice [10]. Lewis Lung Carcinoma (LLC1) cells injected in C57BL/6 mice were inhibited by FTY720 (10 mg/kg) with the tumor size presenting less than half the size of tumors in control animals [11]. An androgen-independent human prostate tumor (CWR22R) inoculated into castrated nude mice treated with FTY720 (10 mg/kg) had suppressed growth associated with reduced proliferation rate and suppression of angiogenic factors [12]. Hepatocellular carcinoma cells lines MHCC-97 L and MHCC-97H (lower and higher metastatic potential respectively) were inoculated in nude mice liver and FTY720 was administered (5 mg/kg or 10 mg/kg) to evaluate tumor development. Tumor volume and metastases were significantly suppressed in both doses of FTY720 in association with VEGF diminished expression [13]. As described above all models for the evaluation of FTY720 efficacy in cancer were performed using tumor cells and high doses of the drug administration. In a previous study our group showed that FTY720 did not inhibit but limited the number of lung nodules development after Urethane injection. This beneficial effect occurred when FTY720 was administered 30 days after the carcinogen injection and was associated with decreased expression of PCNA in lung tissue [14]. In order to further investigate the mechanisms associated with Urethane-induced lung cancer and FTY720 it was our aim in the present study to measure lung nodule numbers and size, tumor associated events such as proliferation (PCNA), apoptosis (active Caspase-3) and changes in the immune system.

Materials and Methods

Animals and Experimental Design

Eight to 10-week-old male BALB/c mice (bred in a local colony CEDEME-UNIFESP) were placed in collective cages and cared for in accordance with the Principles of Laboratory Animal Care (NIH publication 86-23, revised 1985) and the regulations of the Brazilian Committee on Animal Experimentation. Urethane dissolved in 0.9% NaCl (1.5 g/kg) was administered in two doses i.p. with the interval of 48 h (Sigma Chemical Company, St Louis, MO). FTY720 (Novartis, Basel, Switzerland) diluted in sterile distilled water (1 mg/kg/day) was administered by gavage. Mice receiving urethane were treated with distilled water (Urethane n=6), FTY720 1 mg/kg/day during five days starting the day after the last dose of urethane (FTY720 0 n=8), FTY720 1 mg/kg/day during five days starting 30 days after the last dose of urethane (FTY720 30 n=8), or FTY720 1 mg/kg/day during five days starting 90 days after the last dose of urethane (FTY720 90 n=8). Four months after urethane administration all mice were anesthetized with Xylazine (Agribrands, Brazil) and Ketamine (Vetbrands, Brazil) diluted in 10 mL of sterile PBS (phosphate buffered solution-OXOID LTD Hampshire England), the abdomen and thorax were opened for the harvesting of blood, spleen and lungs.

Peripheral Blood Cell Counting

Blood (10 μL) collected from the vena cava was dropped toward one end of a slide and with a cover slip it was smeared back over the slide. A Panotico kit (Laborclin, Paraná, Brazil) was used for staining and leukocytes were identified and counted at microscopy.

Flow Cytometry (CD4 + T cells)

Spleen single cell suspension was prepared by pressing it through a 400 μm sterile nylon mesh and submitted to 1 min of distilled water with the aim of causing hemolysis.

1×106 cells from spleen single cell suspensions were incubated with rat anti-mouse (BD Biosciences Pharmingen) CD4 Gam FITC for surface marker staining. Cells were washed with FACS buffer (PBS/2% FCS) and evaluated in FACScalibur Flow Cytometer (BD Biosciences) using Cell Quest software. At least 10,000 cells were evaluated.

Histological analysis

Lungs were fixed in 4% buffered formalin followed by paraffin embedded, cut into 4 μm sections that were placed on glass slides, stained with hematoxylin and eosin (H&E), and these section were reviewed by a pathologist (blinded to the treatment arm).

Measurement of Nodule Area

Areas of lung adenomas (H&E staining slides) were measured with the help of an image analysis system. For this purpose, each lesion was photographed, saved in the software Image Pro Plus 3.0, drawn round with an electronic pen and its area was immediately calculated by UHTSCSA Image Tool 3.0.

Immunohistochemistry

Serial longitudinal lung sections of 4 μm were deparaffinized in xylene and rehydrated in graded ethanol, then pretreated in a microwave (Eletrolux, SP, Brazil) with 10 mM citric acid buffer (pH=6) for 3 cycles of 5 min each at 850 W for antigen retrieval. They were pre-incubated with 0.3% hydrogen peroxide in PBS for 5 min for inactivation of endogenous peroxidase, and then blocked with 5% normal goat serum in PBS for 10 min. The specimens were then incubated with anti-PCNA polyclonal antibody (PC 10, Dako Corporation, Denmark) at a concentration of 1:400 or caspase-3 (1:200) (Asp175 Cell Signaling Technology). Incubation was carried out overnight at 4°C within the refrigerator. This was followed by two washes in PBS for 10 min. The sections were then incubated with biotin-conjugated secondary antibody (Vector Laboratories, Burlingame, CA, USA or Dako Corporation, Denmark) at a concentration of 1:200 in PBS for 1 h. After that, the sections were washed twice with PBS followed by the application of preformed avidin biotin complex conjugated to peroxidase (Vector Laboratories, Burlingame, CA, USA or Dako Corporation, Denmark for Caspase-3) for 45 min. The bound complexes were visualized by the application of a 0.05% solution of 3-3'-diaminobenzidine solution, and counterstained with hematoxylin. For control studies of the antibodies, the serial sections were treated with mouse IgG (Vector Laboratories, Burlingame, CA, USA) at a concentration of 1:200 or rabbit IgG (Vector Laboratories, Burlingame, CA, USA) at a concentration of 1:200 in place of the primary antibody. Additionally, internal positive controls were performed with each staining batch.

Quantification of Immunohistochemistry

Counts of PCNA and Caspase-3 immunoreactivity are expressed as the percentage of positive tumor cells. For this purpose, 500 tumor cells were counted in each nodule using a microscope magnification of 400×. The percentage of positive cells was determined as number of positive cells/total number of cells ×100.

Statistical Analysis

Data from immunohistochemistry, cells in blood and spleen are expressed as mean and standard deviation and the statistical analysis was performed by ANOVA followed by Tukey's pairwise comparisons. Mood Median test was used for all other evaluations. The level of statistical significance was defined as p-value <0.05.

Results

Lung nodules occurred more often in Urethane Group (100%) whereas in all groups treated with FTY720 there was a decrease in this incidence. In the FTY720 30 Group, 25% of mice (2 from 8) did not develop lung nodules. The multiplicity mean/variation of lung nodules was slightly decreased in FTY720 30 Group. When all nodule area in each group were summed, FTY720 treatment after 90 days of Urethane injection was associated with the biggest area and highest range. On the other hand, FTY720 administered 30 days after Urethane injection was associated with the smallest total area of nodules and the lowest range (Table 1). Urethane and FTY720 0 presented similar multiplicity mean for number of nodules and variation of nodule area.

In comparison to Control Group, Urethane administration caused no change in leukocytes and CD4 + T splenocytes when investigated 120 days after the carcinogen injection. CD4 + T cell population decreased in mice treated with FTY720 30 days and 90 days after urethane injection. CD4 + T cells did not return to normal values in spleen when mice were evaluated 90 (FTY720 30) or 30 (FTY720 90) days after FTY720 administration. It is possible that Group FTY720 0 had also had a decrease in CD4 + T cells after the drug administration and recovered normal values of CD4+120 days later. Blood lymphocytes decreased in all groups treated with FTY720 whereas neutrophils increased in the same groups. Blood monocytes were higher in FTY720 30 (Table 2).

Table 1 Incidence, multiplicities and area of lung nodules in BALB/c mice evaluated four months after Urethane injection

Lung nodule				
Groups	Incidence	Multiplicity mean/ variation (μm^2)	Area mean (μm^2)	Total Nodule area/ variation (μm^2)
Control	0/8 (0%)	0	0	0
Urethane	6/6 (100%)	2.5/(2-4)	5,009	34,118.86 (413.5 – 9,091.3)
FTY720 0	7/8 (87.5%)	2.5/(0-4)	5,875	42,843.38 (275.3 – 10,860.1)
FTY720 30	6/8 (75.0%)	1.5/(0-4)	3,086	21,241.12 (513.1 – 3,167,4)
FTY720 90	7/8 (87.5%)	1.5/(0-5)	7,298	125,696.75 (535.4 – 87,924.8)

PCNA (proliferation cell nuclear antigen) expression in lung nodules was higher in FTY720 90, FTY720 0, and Urethane Groups when compared with FTY720 30 (Table 3, Fig. 1). This finding is probably the reason why we observed a smaller nodule area in FTY720 30 when compared with the other Groups (Table 1). Active Caspase-3 expression was significantly higher in FTY720 30 than in other groups. The PCNA and active Caspase-3 findings suggest that the diminished incidence and area of nodules observed in FTY720 30 Group could be due to decreased proliferation and increased apoptosis. On the other hand, the increased area of nodules observed in FTY720 90 Group was associated with increased PCNA and significantly decreased active Caspase-3 expressions.

Discussion

It has been described that Urethane administration in mice causes lung nodules with size range from 15 μm^2 to 200 μm^2 and positive staining for PCNA [15]. In our model Urethane injection was associated with nodules from 413.5 to 9091.3 μm^2 and positive expression for PCNA. Moreover, Cha et al. [16] evaluated BALB/c female mice 7 days after Urethane injection and observed a significant decrease in spleen cell number, expression of CD4, CD8, B cells and macrophages suggesting that this carcinogen promotes immunosuppression. In opposite, Stathopoulos et al. [7] found in BALB/c mice after 1 week of Urethane administration the increase of circulating neutrophils and inflammatory cells in bronchoalveolar lavage (BAL) fluid besides of increased TNF-α and IL-12p70 levels. These results suggest that in different sites it is possible to have inflammatory and suppressive response after Urethane injection. In our model it was not possible to show that Urethane alone causes decrease in spleen CD4 + T cell population probably because mice were evaluated 120 days after urethane injection which allowed the return to normal percentage of this cell population. Nevertheless if there was an immunosuppression associated with the carcinogen it might have caused an impaired immune response in the early period after Urethane injection promoting tumor development. In agreement we observed that the Urethane Group was similar to FTY720 0 Group for nodule size and PCNA expression. These results suggest that FTY720 administered immediately after Urethane injection could not provide protection against tumor proliferation and growth which could be associated with the immunosuppression caused by the carcinogen. When additional immunosuppression (Cyclosporin – CsA) was associated with Urethane (1 mg/g) in Swiss mice, Yokota et al. [17] obtained even more dramatic results. In both groups (mice receiving Urethane only and mice receiving Urethane + CsA) similar number of lung adenomas was found but the average size of the adenomas was bigger in mice receiving CsA (1555±104 μm) than in Urethane only (1012±61 μm). Therefore, CsA enhanced the growth of lung adenomas induced by Urethane.

In our previous study [14] it was observed that Urethane caused in BALB/c mice the development of a higher

Table 2 Leukocytes in blood and CD4 + T cells in spleen of BALB/c mice evaluated four months after Urethane injection

		Measurements in blood		
Groups	Spleen CD4+(%)	Lymphocytes	Neutrophils	Monocytes
Control	62.8 ± 17.4^{ab}	70.5 ± 8.3^{a}	27.0 ± 8.0^{a}	3.0±0.8
Urethane	62.2 ± 11.5^{a}	67.0 ± 3.8^{a}	30.0 ± 1.5^{a}	2.5±3.0
FTY720 0	68.4 ± 9.6^{a}	46.5 ± 8.3^{b}	51.0 ± 7.3^{b}	2.5±1.8
FTY720 30	49.4 ± 14.0^{b}	44.0 ± 10.0^{b}	51.0 ± 5.0^{b}	4.0±3.0
FTY720 90	43.2 ± 7.5^{b}	45.5 ± 5.3^{b}	52.5 ± 5.5^{b}	2.5±1.0
p-value	$^{a\ versus\ b}<0.005$	$^{a\ versus\ b}<0.005$	$^{a\ versus\ b}<0.005$	0.152

Table 3 Proliferation (PCNA) and apoptosis (Caspase-3) tumor associated markers in lungs of BALB/c mice evaluated four months after Urethane injection

	% of positive cells	
Groups	PCNA	Caspase-3
Urethane	13.1±8.7	2.5±0.8[b]
FTY720 0	15.1±8.9	10.2±4.5[a]
FTY720 30	10.8±5.5	13.8±7.7[a]
FTY720 90	13.9±5.7	1.8±1.0[b]
p-value	0.6	[a versus b] 0.017

number of lung nodules when compared with FTY720-treated mice. Moreover, FTY720 treatment 30 days after Urethane injection was associated with an important decrease in lung nodule numbers but no difference in PCNA expression was observed. In the present study FTY720 30 Group when compared with Urethane and FTY720 0 Groups presented lower incidence and smaller area of lung nodules in association with a slight decrease in PCNA expression. The impairment in the lung nodule development in this Group could be due to FTY720 administration during a hyperplasia phase (from 25 to 75 days after Urethane injection as shown by Monobe & Manabe [15]) when neoplastic changes had not occurred yet. Moreover, the decrease in CD4 + T splenocytes and blood lymphocytes observed in FTY720 30 Group was not associated with the improvement of lung nodule development confirming that the lymphopenia caused by this compound does not impair the function of immune system cells. On the other hand, FTY720 90 Group in which the drug administration occurred during the malignant neoplastic period (from day 100 to 250 after Urethane administration, Monobe & Manabe [15]) presented a more vigorous development of lung nodules associated with higher PCNA and lower active Caspase-3 expression. In this group it was also observed a decrease in CD4 + T splenocytes and blood lymphocytes. The decrease in spleen and blood lymphocytes caused by FTY720 in our model was associated with either impairment (FTY720 30) or improvement (FTY720 90) of tumor development suggesting that besides of the well established role played by lymphocytes in lung cancer there were other mechanisms involved. FTY720 causing cancer growth inhibition or improvement was mainly related to the time point of the drug administration instead of changes in percentage of immune system cells.

A possible mechanism is FTY720 promoting decrease or increase in tumor cells proliferation. PCNA is a 36 kDa nuclear protein which is strongly expressed in the late G1 through S-phases of the cell cycle. It is frequently used as a marker of cell proliferation and is known to correlate well with grades of malignancy [18, 19]. Monobe & Manabe [15] showed that the increase of PCNA staining was associated with malignancy in mice injected with Urethane. Therefore, our findings that FTY720 30 promoted a decrease in the size of lung nodules which was associated with a diminished expression of PCNA have significant clinical meaning. In patients with non-small cell lung cancer (NSCLC), Hung et al. [20] found a positive significant correlation among the cancer diameter, overall survival (61.4% in 5 years and 40% in 10 years) and

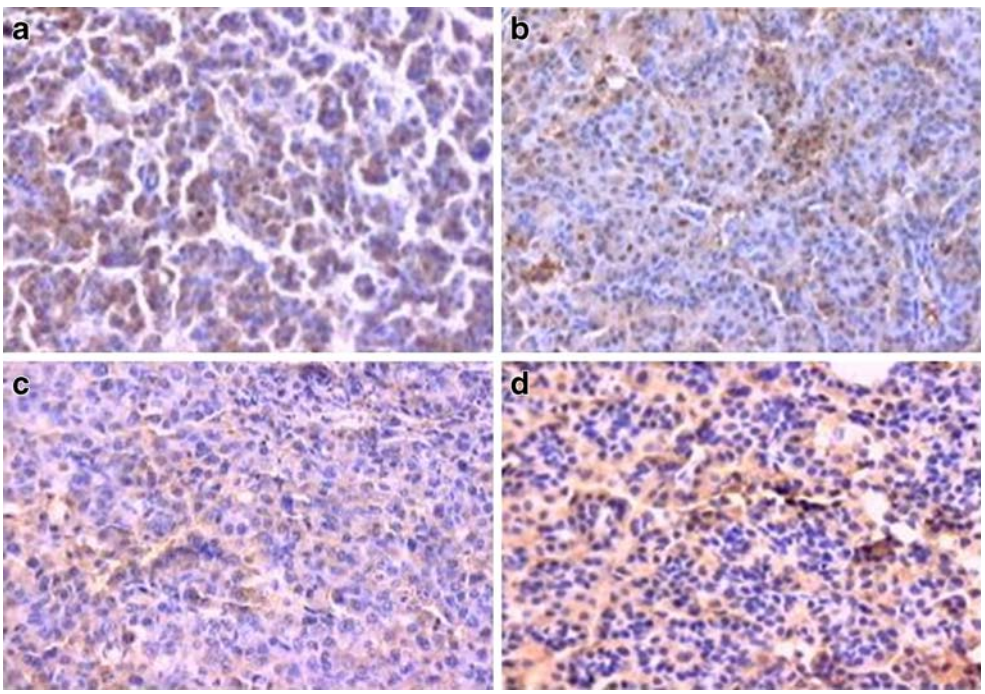

Fig. 1 Immunohistochemistry (PCNA – proliferation cell nuclear antigen and Caspase-3) performed in lung of BALB/c mice four months after Urethane injection. A representative case of Urethane and FTY720 group is shown for PCNA (Urethane – A, FTY720 – B) and Caspase-3 (Urethane – C, FTY720 – D)

disease-free survival (around 73% for both 5 and 10 years). Also, decreased proliferation index (Ki-67) has been associated with decrease of the tumor size [21]. FTY720 administered 30 days after Urethane induction prevented high levels of tumor proliferation and caused the decrease in nodule sizes in comparison with the other groups.

Another possible FTY720 mechanism of action in tumor development is increasing apoptosis since this event plays an important role in neoplasia. Caspase activation (cleavage of procaspase to active caspase) is the most specific indicator of this mechanism. Several cell lines have been shown to be susceptible to FTY720-induced apoptosis [9, 10, 22]. Hung et al. [20] showed that FTY720 antitumor effects in hepatocellular carcinoma (HCC) cells was mediated by caspase-3-dependent apoptosis. In agreement we found that FTY720 30 Group presented the highest level of caspase-3 expression in lung nodules whereas FTY720 90 presented the lowest one.

Considering that recent therapeutic strategies in cancer are based on the direct destruction of proliferating tumor cells as these cells can stimulate non-neoplastic cell to alter their transcriptional program to aid neoplastic growth [23] FTY720 could be an important drug in preventing cancer development. Moreover, the advantage of the anticancer function of FTY720 has been pointed by its ability to selectively induce apoptosis of cancer cells but not normal somatic cells [9, 10, 24].

Our treatment with FTY720 did not cause a dramatic decrease in lung nodule development as we used a non-high dose of the drug (1 mg/kg/day) and it is possible that both the prevention of tumor cell proliferation and the level of apoptosis needed to prevent tumor growth were not reached. Nevertheless, we obtained reduction in tumor growth when FTY720 was administered 30 days after urethane injection and the events associated were increased apoptosis and decreased proliferation of tumor cells. In conclusion our data showed that the benefits of FTY720 lower dose in lung tumor development is dependent on the time point of the compound administration.

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