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ARTICLE *in* INTERNATIONAL JOURNAL OF CANCER · MARCH 2006

Impact Factor: 5.09 · DOI: 10.1002/ijc.22468 · Source: PubMed

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Int. J. Cancer: **120,** 1150–1153 (2006)

SHORT REPORT

Modulation by budesonide of DNA methylation and mRNA expression in mouse lung tumors

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Biomarkers are being developed that can aid in the evaluation of cancer therapeutic and chemopreventive drugs. Two suggested biomarkers found in mouse lung tumors are DNA hypomethylation and alterations in mRNA expression of genes, such as 18S RNA, caspase 3, cyclin B2, cyclin E1, iNOS and survivin. Budesonide is very efficacious in preventing lung tumors in mice, so that its ability to modulate biomarkers in lung tumors was determined. Lung tumors were induced by vinyl carbamate in female strain A/J mice. Budesonide (2.0 mg/kg diet) was administered for 2, 7 and 21 days or for 14 days followed by a 7-days' holding period prior to the killing of the mice at week 27. After 2 days of budesonide treatment, the size of the lung tumors was reduced. Tumor size continued to decrease during the 21 days of treatment. In the tumors, 2 days of treatment resulted in (*i*) increased methylation of DNA, reversing DNA hypomethylation, (*ii*) increased expression of 18S RNA and (*iii*) decreased mRNA expression of caspase 3, cyclin B2, cyclin E1, iNOS and survivin. Termination of budesonide treatment at 7 days prior to killing did not affect the size of the tumors, but did result in increased mRNA expression of the 5 genes, approaching the expression level in tumors from control mice. Hence, budesonide rapidly decreased the size of lung tumors, reversed DNA hypomethylation and modulated mRNA expression of genes; with the molecular alterations requiring continued treatment with the drug for maintenance.

Key words: budesonide; biomarkers; chemoprevention; DNA hypomethylation; lung cancer; mRNA expression; mouse lung tumors

The development of cancer chemopreventive and therapeutic agents would be aided by the use of biomarkers. Biomarkers have the advantage of requiring a shorter duration of treatment than either the prevention or regression of tumors. In clinical trials, the greatest utility of biomarkers appears to be in studies that determine their modulation by relatively short-term treatment with therapeutic and chemopreventive agents. Recent advances in the molecular biology of cancer have suggested several biological and molecular endpoints that might be useful as biomarkers, including DNA hypomethylation and alteration in mRNA expression of cancer-related genes.

Budesonide, a synthetic anti-inflammatory glucocorticoid used to control mild-to-moderate persistent asthma,[1] has been shown to prevent the formation of lung adenomas and adenocarcinomas in mice when administered either by inhalation or in the diet.[2–7] As a chemopreventive agent, budesonide appears to decrease both the growth rate of tumors and the progression of adenomas to adenocarcinomas.[4] Lung tumors from budesonide-treated mice were smaller and contained fewer carcinomas when compared with tumors from mice that were not administered the drug. Budesonide has also been demonstrated to prevent the occurrence of DNA hypomethylation in mouse lung tumors[4,5] and to modulate the mRNA expression of cancer-related genes.[4,8] Another drug that is effective in preventing mouse lung tumors, R115777 (a farnesyl transferase inhibitor), has also been shown to prevent DNA hypomethylation in lung tumors.[9] We report here the ability of budesonide to modulate tumor size, DNA hypomethylation, and mRNA expression of genes when administered to mice with established lung tumors.

Material and methods

Chemicals

Vinyl carbamate (purity >99%) was obtained from TorontoResearch Chemicals (North York, ON, Canada); AIN-76A diet (casein 20%, DL-methionine 0.3%, cornstarch 15%, sucrose 50%, corn oil 5%, cellulose 5%, AIN mineral mixture 3.5%, AIN vitamin mixture 1.0% and choline bitartrate 0.2%) from Dyets (Bethlehem, PA); and budesonide from Sigma (St. Louis, MO).

Treatment of mice

Female strain A/J mice from Jackson Laboratory (Bar Harbor, ME) were housed in the AAALAC accredited laboratory animal facility at the Medical University of Ohio. At 8 weeks of age, the mice were administered 16 mg/kg vinyl carbamate by intraperitoneal injection once a week for 3 consecutive weeks. At 2, 7 and 21 days prior to killing, the mice were administered 2.0 mg/kg budesonide in their diet. Other mice received budesonide for 14 days, followed by a 7-day holding period prior to killing. Each treatment group contained 10 mice, except for the control group that had 18 animals. All the mice were killed at 27 weeks after the first dose of vinyl carbamate.

At necropsy, the lung was excised. The right lung was frozen in liquid nitrogen and stored at −70°C, until used for the analysis of DNA methylation and mRNA expression of genes. The left lung was fixed in formalin. The size of the tumors in the left lung was measured independently by 2 individuals, who were blind to the treatment of the mice. A dissecting microscope was used to determine the size of the tumors in 0.2-mm increments. Tumor size was determined for a total of 378 tumors from the control group and 160–188 tumors from each of the treatment groups that received budesonide. The lung was then embedded in paraffin, sectioned and stained with hematoxylin and eosin for histopathologic evaluation of tumors.

DNA hypomethylation: Dot blot analysis

DNA was isolated from normal lung tissue and tumors by digestion with RNase A and proteinase K followed by organic extraction with phenol and chloroform. Purified DNA (1 μg) was denatured in 100 μl of 0.4 M NaOH/10 mM EDTA at 100°C for 10 min, neutralized with 2 M ammonium acetate and dotted onto a Hybond™ nitrocellulose membrane. The membranes were dried, incubated with 7% milk in Tris-buffered saline + Tween-20 (pH 7.6) for 1 h, and then incubated with a 1:10,000 dilution of rabbit polyclonal primary antibody specific against 5-methylcytosine in DNA (Megabase Research Products, Lincoln, NE) for 2–3 hr. They were then

Grant sponsor: U.S. National Cancer Institute; Grant numbers: N01-CN-15125, 25126.
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Received 20 June 2006; Accepted after revision 11 October 2006
DOI 10.1002/ijc.22468
Published online 12 December 2006 in Wiley InterScience (www.interscience.wiley.com).

TABLE I – ASSAY AND PROBES USED FOR REAL TIME RT-PCR

Gene	Applied Biosystems assay ID	Probe sequence	Exon	NCBI gene reference
18S	Hs99999901_s1	TGGAGGGCAAGTCTGGTGCCAGCAG		
Caspase 3	Mm00438045_m1	CGGCGGGGAGCTTGGAACGCTAAGA	1	NM_009810
Cyclin B2	Mm00432351_m1	AGGCGCTAGCTCCCAAGGATCGTCC	3	NM_007630
Cyclin E1	Mm00432367_m1	GATAGCAGTCAGCCCTGGGATGATA	3	NM_007633
iNOS	Mm00440485_m1	GCCACATCGGATTTCACTTGCAAGT	4	NM_010927
Survivin	Mm00599749_m1	CCCCAGAGCGAATGGCGGAGGCTGG	1	NM_009689

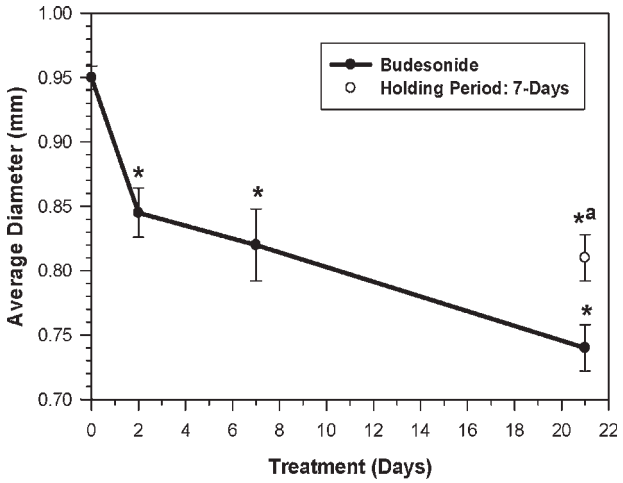

FIGURE 1 – Effect of chemopreventive agents on the diameter of mouse lung tumors. Results are means ± SE for 378 tumors from the control group and 160–188 tumors from each of the treatment groups that received budesonide. The asterisk indicates significant difference from the control group, p-value <0.01 and the "a" indicates significant difference from mice administered budesonide for 21 Days, p-value < 0.05.

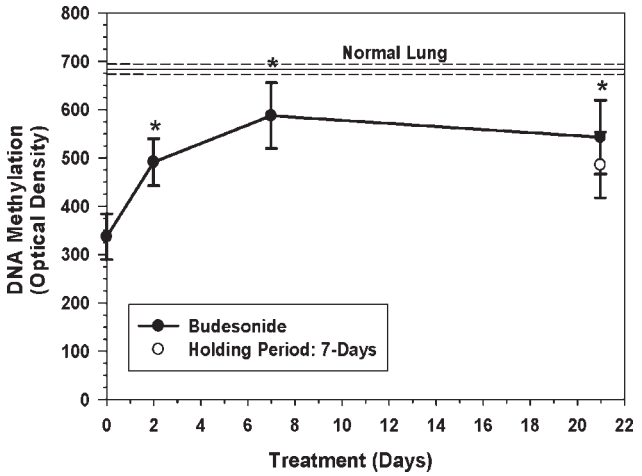

FIGURE 2 – Effect of budesonide on DNA methylation in mouse lung tumors. The optical density for DNA methylation in normal lung tissue from 6 mice was 684 ± 21.8 and is indicated by horizontal solid and dashed lines. Results are means ± SE for 6 lung tumors from different mice. The asterisk (*) indicates statistical difference from the control group, p-value <0.05.

washed and incubated with a 1:1,000 dilution of horseradish peroxidase-conjugated secondary anti-rabbit-IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 hr. After washing again, the membranes were treated with enhanced-chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech, Arlington Heights, IL), and exposed to Kodak autoradiograph films. Optical density of the dots was quantified with a Scion Image Analysis System (Scion Corp., Frederick, MD). Equal DNA loading of the membrane was demonstrated by methylene blue staining.

mRNA expression: Real time RT-PCR

Total RNA was isolated and cDNA-synthesized by reverse transcription with random hexamers and the High Capacity cDNA Achieve Kit (Applied Biosystems, Foster City, CA). The cDNA was PCR-amplified with primers specific for the gene of interest and for the reference gene, β-actin, using a 7500 Real-Time PCR System (Applied Biosystems). The PCR amplification mixture contained 20-ng template, TaqMan universal PCR master mix and assays-on-demand gene expression primers and probes in a total volume of 30 μl. The assays and probes are listed in Table I. The incubations were performed at 50°C for 2 min, followed by 95°C for 10 min, and then by 40 cycles consisting of 95°C for 15 sec and 60°C for 1 min. Gene expression relative to the β-actin reference gene was determined by the $2^{-\Delta\Delta CT}$ method.[10] The relative gene expression was then used to calculate the mRNA expression of a gene relative to its expression in normal lung tissue. Each treatment group contained either 10 lung tumors or 10 lung tissue specimens.

Statistical analysis

The results were analyzed for statistical significance by ANOVA followed by the Dunnett's test. Statistical significance was indicated by a p-value <0.05.

Results

Body weight and toxicity

There were no deaths or any observed sign of toxicity in any of the treatment groups. The average terminal body weight of the mice in the different treatment groups varied between 21.8 and 24.8, with none of the groups being significantly different from the control group. The liver-to-body weight ratio also did not significantly differ among the treatment groups, being between 4.1 and 4.5 g/100 g body weight.

Tumor yield and size

The lung tumors induced by vinyl carbamate were solid adenomas. Tumor multiplicity did not differ among the 5 different treatment groups having a range of 21.7–25.1 lung tumors/mouse. Budesonide reduced the size of the tumors within 2-days of treatment, which continued to decrease during the 21-days evaluated (Fig. 1). When treatment with budesonide ceased 7 days prior to killing, tumor size was stable, neither decreasing further nor increasing.

Modulation of DNA hypomethylation

The DNA in lung tumors was hypomethylated relative to normal lung tissue (Fig. 2). Two days of treatment with budesonide increased the methylation of DNA, *i.e.*, significantly reversed the DNA hypomethylation in the tumors. The level DNA methylation after 7 days of treatment approached that found in normal lung tissue and remained elevated for 21 days. Termination of treatment with budesonide at 7 days prior to killing decreased the level of DNA methylation in the tumors, so that it no longer differed from the level found in tumors of mice that did not receive the drug. Thus, continued treatment with budesonide was required to maintain the reversal of DNA hypomethylation in the tumors.

TABLE II – EFFECT OF BUDESONIDE ON mRNA EXPRESSION OF GENES

Gene	Relative mRNA expression (relative to control lung)				
	Day 0	Day 2	Day 7	Day 21	Holding for 7 days[1]
18S	1.41 ± 0.17[2]	13.4 ± 1.08*	12.2 ± 1.18*	11.7 ± 1.18*	9.05 ± 1.31*
Caspase 3	1.64 ± 0.12	1.06 ± 0.071*	0.90 ± 0.049*	0.74 ± 0.033*	1.33 ± 0.19
Cyclin B2	1.61 ± 0.064	0.628 ± 0.033*	0.662 ± 0.060*	0.331 ± 0.065*	1.83 ± 0.081
Cyclin E1	0.989 ± 0.055	0.487 ± 0.017*	0.461 ± 0.015*	0.695 ± 0.045*	1.06 ± 0.101
iNOS	0.933 ± 0.072	0.521 ± 0.059*	0.654 ± 0.071*	0.391 ± 0.062*	0.739 ± 0.070
Survivin	1.79 ± 0.102	0.645 ± 0.099*	0.614 ± 0.043*	0.330 ± 0.073*	2.01 ± 0.135

[1]Fourteen days of treatment followed by a 7 days' holding period.–[2]Results are means ± SE for 10 lung tissues and tumors.
*The asterisk indicates significant difference from the control group (Day 0).

Modulation of mRNA expression

The expression of 18S RNA in control tumors was not different from that in normal lung (Table II). Two days of treatment with budesonide increased the expression of 18S RNA by about 13-fold. The expression of 18S remained elevated for the 21 days of budesonide treatment. Termination of budesonide treatment at 7 days prior to killing did not significantly alter the large increase in 18S RNA expression.

The effect of budesonide on the expression of caspase 3, cyclin B2, cyclin E1, iNOS and survivin is presented also in Table II. These genes were chosen since their mRNA expression in vinyl carbamate-induced mouse lung tumors was modulated by 14-days of treatment with targretin.[11] The mRNA expression of caspase 3, cyclin B2 and survivin were increased in lung tumors by 1.6–1.8-fold, while the expression of cyclin E1 and iNOS was not altered in the tumors, relative to control lung tissue. Within 2 days of treatment, budesonide decreased the mRNA expression of caspase 3, cyclin B2, cyclin E1, iNOS and survivin that remained decreased for the 21 days of evaluation. Termination of budesonide treatment at 7 days prior to killing increased the expression of caspase 3, cyclin B2 and survivin, so that their expression in tumors was no longer different from tumors of mice that did not receive the drug. In fact, the mRNA expression of the 3 genes was greater than their expression in normal lung. The expression of cyclin E1 and iNOS was also increased when budesonide treatment was terminated for 7 days. In the case of these 2 genes, their mRNA expression increased, so that it was no longer different from their expression in control lung.

Discussion

Budesonide administered to mice with lung tumors was shown to modulate the size of the tumors as well as 2 potential biomarkers in the tumors, *i.e.*, DNA hypomethylation and mRNA expression of genes. In mice with lung tumors, 2 days of treatment with budesonide was sufficient to increase the methylation of DNA, reversing DNA hypomethylation. This short-term treatment also increased the mRNA expression of 18S and decreased the expression of caspase 3, cyclin B2, cyclin E1, iNOS and survivin in the tumors. The rapid modulation of the 2 molecular alterations by budesonide was accompanied by a decrease in tumor size. When treatment with budesonide ceased for 7 days before the mice were killed, there was an increase in both the methylation of DNA and the mRNA expression of caspase 3, cyclin B2, cyclin E1, iNOS and survivin, approaching the level of methylation and expression found in tumors from mice that were not administered the drug. Hence, modulation of the 2 biomarkers by budesonide required only a very short duration of treatment, as well as continued treatment to maintain the modulation.

The effect of budesonide on the mRNA expression of caspase 3, cyclin B2, cyclin E1, iNOS and survivin was determined, because targretin, another effective chemopreventive agent, was reported to decrease the expression of the 5 genes in mouse lung tumors.[11] In that study, targretin was administered for 14 days to mice with vinyl carbamate-induced lung tumors. The mice were then killed. Targretin decreased the mRNA expression of caspase 3, cyclin B2, cyclin E1, iNOS and survivin in the tumors. Targretin also increased the methylation of DNA in lung tumors, reversing the DNA hypomethylation in the tumors. Thus when administered to mice with lung tumors, 2 drugs with very different structural characteristics and pharmacologic activity, budesonide (a glucocorticoid) and targretin (an RXR agonist) reversed DNA hypomethylation and decreased the mRNA expression of genes in the tumors.

Consequences of the remethylation of DNA in tumors could include (*i*) an increased DNA binding of methylated DNA-binding proteins, including MeCP2 and MBD 1–4, (*ii*) an increased DNA binding of histone deacetylase resulting in decreased histone acetylation, condensation of the chromatin and decreased mRNA transcription, including protooncogenes, and (*iii*) a restored imprinting of genes.[12–17] Hence remethylation of DNA by condensing the chromatin could prevent or decrease the accessibility of transcription machinery to the DNA, resulting in a decrease in mRNA expression. In support of this conjecture, targretin has been reported to decrease the expression of many more genes than it increased in lung tumors.[11] Hence, the ability to remethylate DNA could in part be responsible for the downregulation of many genes. This suggestive relationship between remethylation of DNA and decreased expression of genes warrants further investigation. However, the results do support modulation of DNA hypomethylation and mRNA expression of genes as biomarkers in lung tumors.

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