BASIC RESEARCH

Curcumin, but not Prima-1, decreased tumor cell proliferation in the syngeneic murine orthotopic bladder tumor model

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OBJECTIVE: Cigarette smoking is the main risk factor for bladder cancer development. Among the mediators of this effect of smoking is nuclear factor-kappa B. Curcumin suppresses cellular transformation by downregulating the activity of nuclear factor-kappa B. Prima-1 is a compound that induces apoptosis in human tumor cells, restoring the function of mutant p53. Our study aimed to evaluate the effects of curcumin and prima-1 in an animal model of bladder cancer.

METHODS: Tumor implantation was achieved in six- to eight-week-old female C57BL/6 mice by introducing MB49 bladder cancer cells into the bladder. Intravesical treatment with curcumin and Prima-1 was performed on days 2, 6, 10, and 14. On day 15, the animals were sacrificed. Immunohistochemistry was used to determine the expression of cyclin D1, Cox-2, and p21. Cell proliferation was examined using PCNA.

RESULTS: Animals treated with curcumin exhibited a higher degree of necrosis than animals in other groups. Immunohistochemistry showed reduced expression of cyclin D1 in the curcumin-treated group. All of the cells in mice treated with curcumin were p21 positive, suggesting that the p53 pathway is induced by this compound. Prima-1 did not induce any change in tumor size, necrosis, cell proliferation, or the expression of proteins related to the p53 pathway in this animal model.

CONCLUSION: Curcumin showed activity in this animal bladder cancer model and probably acted via the regulation of nuclear factor-kappa B and p53. Therefore, curcumin is a good choice for the use in clinical trials to treat superficial bladder cancer as an alternative to bacillus Calmette-Guerin. In contrast, Prima-1 does not seem to have an effect on bladder cancer.

KEYWORDS: Bladder cancer; Treatment; Curcumin, Prima-1; Apoptosis.

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INTRODUCTION

Cancer of the urinary bladder is diagnosed in approximately 71,500 people in the United States, and approximately 14,500 individuals die of the disease each year.[1] The condition affects individuals 60 to 70 years of age, and racial and ethnic variations in its incidence have been observed.[2-4] Current treatment for superficial urothelial carcinoma includes endoscopic tumor resection, followed by intravesicular administration of bacillus Calmette-Guerin (BCG), a treatment that has well-known side effects.[5] Topical chemotherapy with mitomycin, thiotepa, and epirubicin has been used as an alternative, but this treatment has no impact on long-term survival or disease progression. The toxicity and inefficacy of these intravesicular agents prompted us to explore new treatments for superficial urothelial carcinoma of the bladder.

The use of tobacco is one of the main causes of bladder cancer development, which is due to the presence of certain compounds present in cigarette smoke (CS). Exposure to some of these compounds is associated with the induction of nuclear factor-kappa B (NF-kB). We previously studied the effects of curcumin in an orthotopic murine bladder tumor model, demonstrating that curcumin has an inhibitory effect on bladder urothelial cancer, possibly by downregulating NF-kB-related genes,[6] Therefore, curcumin could be an option for the treatment of urothelial neoplasms.[7] The tumor suppressor protein p53 inhibits tumor growth primarily through its ability to induce apoptosis. Mutations in p53 occur in at least 50% of human tumors, and p53 is characteristically mutated in high-grade, invasive urothelial carcinoma. Prima-1 is a low-molecular-weight compound able to induce apoptosis in

No potential conflict of interest was reported.

human tumor cells by restoring the transcriptional transactivation function of mutant p53. In the presence of Prima-1, mutant p53 proteins regain their sequence-specific DNA binding ability and their active conformations *in vitro* and in living cells. *In vivo* studies in mice revealed that Prima-1 has an anti-tumor effect with no apparent toxicity.[8]

Now, using the same animal model,[9] we assessed whether curcumin function is enhanced by Prima-1 or if there is a specific role of curcumin that could be used to treat bladder cancer.

MATERIALS AND METHODS

Orthotopic Tumor Implantation

Six- to- eight-week-old female C57BL/6 mice were put under general anesthesia with an intraperitoneal injection of 150 μL of a mixture of xylazine-ketamine-PBS (1:1:8). Then, a 24-gauge Teflon intravenous IV catheter was inserted through the urethra into the bladder using xylocaine gel. To prepare the bladder for tumor implantation, a chemical lesion on the bladder wall was made by intravesicular administration of 0.15 M $AgNO_3$ (10 μL). This promoted an adequate and controlled diffuse cauterization of the bladder wall. After 10 seconds, the contents were washed out by transurethral infusion of PBS. Then, a suspension of 5×10^5 viable tumor cells of the murine transitional cell carcinoma cell line MB49 (MB49) was introduced into the bladder. The day of the administration of tumor cells was considered to be Day 1.

Treatment

Treatment was performed by administering the tested drugs four times. Twenty-four hours following tumor cell introduction (Day 2), therapy with 100 μL intravesicular curcumin (100 μM - Sigma) or 100 μL Prima-1 (100 μM) was initiated. The ''curcumin+Prima-1'' treatment group received 100 μL of a 1:1 mixture of curcumin (200 μM) and Prima-1 (200 μM). Mice were assigned to 1) the control group, which received 100 μL saline (n = 10); 2) the ''only tumor'' group (n = 10), which received tumor cells and were not handled again until sacrifice; and 3) treatment groups receiving curcumin (n = 10), Prima-1 (n = 10) or a mixture of both (n = 10). The other three drug applications occurred on days 6, 10, and 14.

Assessment of the tumors

The mice were evaluated on a daily basis for viability and gross hematuria. At Day 15, they were sacrificed by CO_2 inhalation. The bladder, lungs, kidneys, and liver were resected for histological examination.

Histopathology analysis

The specimens were fixed in 10% buffered formalin and sectioned for histological examination. For the bladder slides, tumors were measured, and the degree of necrosis was recorded. Sections of the lungs, kidney, and liver were also analyzed to identify the presence of metastases.

Immunohistochemistry

Three-micrometer sections from the paraffin block were placed on adhesive-coated slides. For antigen retrieval, the slides were placed in citrate buffer (1 mM, pH 6.0) and heated for 30 min in the steamer. The slides were then incubated overnight at 4℃ with monoclonal antibodies targeting cyclin D1 (Spring, 1:100 dilution), Cox-2 (Spring, 1:100), p21 (Millipore, 1:50), and PCNA (Dako, 1:200). The LSAB system was used for immunostaining. The color was developed using a 3,3′-diaminobenzidine substrate-chromogen solution, followed by counterstaining with Harris hematoxylin. Samples were then dehydrated, preserved with a cover slip and reviewed using light microscopy.

Statistical analysis

Statistical analyses were performed using ANOVA and chi-squared tests for the evaluation of histological characteristics and immunohistochemistry findings between different groups of animals using the GraphPad Prism 5.0 software.

RESULTS

Initially, the study included 50 animals distributed among the groups. Five animals in the control (saline) group were excluded from the study due to a possible bladder infection observed by microscopy. Two animals died prematurely, probably as the result of the anesthesia. Five mice died later, possibly due to the neoplasm. In four animals, the tumor implantation was not successful. These animals were also excluded from the study. After these exclusions, the study included 34 mice.

The results are shown in Table 1. We found that animals treated with curcumin showed a higher degree of necrosis than the mice in all other groups. The mean percentage of tumor necrosis in mice treated with curcumin was 67%, whereas in the other groups, the mean was only 27% ($p<0.001$).

The mean tumor sizes at the end of the study in the untreated and saline-injected groups were 4.2 mm and 6.2 mm, respectively. Although these sizes seem much larger than that in the curcumin-treated group (mean size 2.9 mm), there were no statistically significant differences.

We next compared the expression of genes regulated by NF-κB. Cyclin D1 was expressed in fewer cells in the group treated with curcumin, with a mean percentage of 23% compared with 34%, 53%, 50%, and 60% in the Prima-1, curcumin and Prima-1, saline control, and untreated control groups, respectively ($p<0.001$). There was no statistically significant difference in Cox-2 expression between groups ($p = 0.064$).

We also analyzed the expression of p21, which is regulated by p53, a frequently mutated gene in invasive bladder cancer. Because Prima-1 restores the activity of the mutated p53 protein, we expected to find some difference in the expression of p21 among these groups of animals. However, there were no differences in the expression of p21 between the different groups of mice ($p = 0.55$).

In addition, proliferative activity, as measured using PCNA, was similar among the treatment groups.

DISCUSSION

Our results confirm our previous finding that intravesicular injection of curcumin was able to promote bladder tumor necrosis. Although there was no statistically significant difference in tumor size between the different groups, tumors treated with curcumin were smaller, especially when compared with those exposed to saline alone (mean 2.85 mm vs. 6.23 mm).

Recently, we showed that curcumin promotes apoptosis in MB49 mouse bladder tumor cells *in vitro* and causes a

Table 1 - Summary of the findings for the different groups of animals treated with curcumin, Prima-1, or curcumin+Prima-1 and the control animals.

	Morphological aspects		Immunohistochemistry			
Group	Percentage of necrosis (Mean)	Size (mm)	PCNA (Mean)	Cyclin D1 %(Mean)	Cox-2 %(Mean)	p21 %(Mean)
Curcumin (N = 6)	67%	2.88	66%	23%	33%	100%
Prima-1 (N = 8)	28%	3.46	47%	34%	25%	43%
Curcumin+Prima-1 (N = 8)	26%	3.10	69%	53%	75%	63%
Control (saline) (N = 4)	38%	6.23	66%	50%	0%	67%
Control (N = 8)	23%	4.94	71%	60%	28%	67%
	$p = 0.001$	$p > 0.05$	$p > 0.05$	$p < 0.001$	$p = 0.064$	$p = 0.55$

decrease in tumor size *in vivo* in the syngeneic orthotopic murine bladder cancer model.[7,8] Garg and Aggarwal[10] previously discovered that curcumin functions by modulating TNF-induced NF-kB activation. NF-kB activates the transcription of genes related to cell proliferation, including cyclin D1 and Cox-2. We confirmed that curcumin affects NF-kB function in our animal model, reducing the expression of cyclin D1 in mice treated with this substance compared to untreated animals, in agreement with our previous results. The mean percentage of cyclin D1-positive tumor cells was 23% in animals treated with curcumin and 49% in untreated mice ($p<0.01$). However, we did not find differences in the regulation of other genes related to the NF-kB pathway among the groups of animals. The Cox-2 expression profile varied widely in animals treated with curcumin, Prima-1, or both. In addition, there was no difference in terms of cell proliferation.

Chen et al.[11] were able to show that BCG activates multiple intracellular signaling pathways, including NF-kB, in MB49 cultures. BCG is the standard of care for the treatment of low-grade pTa and pT1 urothelial carcinomas and reduces tumor recurrence without affecting specific cancer survival rates. However, one-third of patients fail to respond to BCG, and BCG can cause many side effects. In this *in vivo* mouse model, we demonstrated that curcumin affects tumor behavior in a manner similar to that of BCG without side effects. Curcumin, a compound derived from *Curcuma longa Linn*, is not easily absorbed, and the possibility of using it as an intravesicular anti-cancer drug is very attractive.

The rationale for testing Prima-1 in this animal model was that Prima-1 can restore p53 transcriptional activity. We expected to observe an effect of Prima-1 on tumor size, apoptotic index or p21 protein expression either when Prima-1 was used alone or when it was used together with curcumin. The transcription of p21/WAF1 is controlled by p53, and the loss of its expression has been related to poor prognosis in bladder cancer.[12] However, we saw no differences in these studies between animals treated with or without Prima-1. We can speculate that p53 plays no role in this tumor model.

However, 100% of tumor cells in mice treated with curcumin were p21 positive, suggesting that p53 can be induced by this compound, as previously observed in in vitro and in vivo models of bladder cancer[13] and in other tumors, such as neuroblastomas[14] and adenocarcinomas of the prostate[15] and colon.[16]

We conclude that curcumin is a good choice for use in clinical trials as an alternative to BCG to treat superficial bladder cancer because curcumin controls the genetic pathways important in the carcinogenesis of this neoplasia. In contrast, Prima-1 does not seem to have an effect on this tumor.

AUTHOR CONTRIBUTIONS

Watanabe FT conceived and designed the study and was also responsible for the draft of the manuscript. Chade DC was responsible for the data collection. Reis ST conceived and designed the study and was also responsible for the statistical analysis. Piantino C was responsible for the administrative support. Dall'Oglio MF was responsible for the data collection, critical revision and important intellectual content of the manuscript. Leite KRM conceived and designed the study and was responsible for the critical revision and important intellectual content of the manuscript. Srougi M supervised the study.

REFERENCES

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics. 2010. CA Cancer J Clin. 2010;60:277-300, Epub 2010 Jul 7, doi: 10.3322/caac.20073
2. Lynch CF, Cohen MB. Urinary system. Cancer. 1995;75:316-29, doi: 10.1002/1097-0142(19950101)75:1+<316::AID-CNCR2820751314>3.0.CO;2-T.
3. Scosyrev E, Noyes K, Feng C, Messing E. Sex and racial differences in bladder cancer presentation and mortality in the US. Cancer. 2009;115:68-74, doi: 10.1002/cncr.23986.
4. Howe HL, Wu X, Ries LA, Cokkinides V, Ahmed F, Jemal A, et al. Annual report to the nation on the status of cancer, 1975-2003, featuring cancer among U.S. Hispanic/Latino populations. Cancer. 2006;107:1711-42, doi: 10.1002/cncr.22193.
5. Herr HW, Wartinger DD, Fair WR, Oettgen HF. Bacillus Calmette-Guerin therapy for superficial bladder cancer: a 10-year followup. J Urol. 1992;147:1020-3.
6. Leite KR, Chade DC, Sanudo A, Sakiyama BY, Batocchio G, Srougi M. Effects of Curcumin in an Orthotopic Murine Bladder Tumor Model. Int Braz J Urol. 2009;35:599-607, doi: 10.1590/S1677-55382009000500012.
7. Chade DC, Andrade PM, Borra RC, Leite KR, Andrade E, Villanova FE, et al. Histopathological characterization of a syngeneic orthotopic murine bladder cancer model. Int Braz J Urol. 2008;34:220-6; discussion 226-9, doi: 10.1590/S1677-55382008000200013
8. Bykov VJ, Issaeva N, Shilov A, Hultcrantz M, Pugacheva E, Chumakov P, et al. Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound. Nat Med 2002;8:282-8, doi: 10.1038/nm0302-282.
9. Günther JH, Jurczok A, Wulf T, Brandau S, Deinert I, Jocham D, et al. Optimizing syngenic orthotopic murine bladder cancer (MB49). Cancer Res. 1999;59:2834-7.
10. Garg A, Aggarwal BB. Nuclear transcription factor kB as a target for cancer drug development. Leukemia. 2002;16:1053-68, doi: 10.1038/sj.leu.2402482.
11. Chen F, Zhang G, Cao Y, Hessner MJ, See WA. MB49 Murine Urothelial Carcinoma: Molecular and Phenotypic Comparison to Human Cell Lines as a Model of the Direct Tumor Response to Bacillus Calmette-Guerin. J Urol. 2009;182:2932, doi: 10.1016/j.juro.2009.08.018.
12. Korkolopoulou P, Konstantinidou AE, Thomas - Tsagli E, Christodoulou P, Kapralos P, Davaris P. WAF1/p21 protein expression is an independent prognostic indicator in superficial and invasive bladder cancer. Appl. Immunohistochem. Mol. Morphol. 2000;8:285-92, doi: 10.1097/00022744-200012000-00005.
13. Tian B, Wang Z, Zhao Y, Wang D, Li Y, Ma L, et al. Effects of curcumin on bladder cancer cells and development of urothelial tumors in a rat bladder carcinogenesis model. Cancer Lett. 2000;264:299–308, doi: 10.1016/j.canlet.2008.01.041.

14. Liontas A, Yeger H. Curcumin and resveratrol induce apoptosis and nuclear translocation and activation of p53 in human neuroblastoma. Anticancer Res. 2004;24:987–98.
15. Kawamori T, Lubet R, Steele VE, Kelloff GJ, Kaskey RB, Rao CV, et al. Chemopreventive effect of curcumin, a naturally occurring anti-inflammatory agent, during the promotion/progression stages of colon cancer. Cancer Res. 1999;59:597–601.
16. Chauhan DP. Chemotherapeutic potential of curcumin for colorectal cancer. Curr Pharm Des. 2002;8:1695-706, doi: 10.2174/1381612023394016.