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Topical CTC-96 accelerates wart growth in rabbits infected with cottontail rabbit papillomavirus

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Abstract

CTC-96, a cobalt containing complex, was tested as a putative topical therapeutic agent for the treatment of papillomavirus-induced tumors in our cottontail rabbit papillomavirus (CRPV)-rabbit model system. Following experimental infection of domestic rabbits with CRPV, CTC-96 was applied to infection sites twice daily, 5 days a week for a total of 8 weeks. Two levels of concentrations of aqueous CTC-96 were compared to placebo control-treated animals. With increasing dose of CTC-96 we observed tumors earlier, larger, and more often across eight infected sites on each animal.

Key words: Papillomavirus; Rabbit; CTC-96; Topical application

1. Introduction

Our laboratory and others have used cottontail rabbit papillomavirus (CRPV) to

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experimentally induce papillomas in domestic rabbits for the testing of putative antiviral agents (Ostrow et al., 1992; Kreider et al., 1990; Shikowitz et al., 1986). For the last 5 years we have utilized this animal model system in relatively rapid in vivo screening of putative anti-papillomavirus agents under contract to the National Institutes of Allergy and Infectious Diseases.

There are both clinical and molecular foundations for using the CRPV-rabbit model for testing the therapeutic effects of drugs which have a potential for the treatment of human papillomavirus-induced disease. Progression of cutaneous papillomas to invasive carcinomas is observed in about 25–70% of rabbits with experimentally induced lesions (Rous and Beard 1935; Syverton 1952). The rate of growth of papillomas and their conversion to malignancy is enhanced by various chemical treatments (Friedewald, 1942). This model shares some similarity to the human cutaneous disease known as epidermodysplasia verruciformis (EV) which is associated with human papillomavirus (HPV) type 5. Both EV and CRPV cause benign and malignant cutaneous lesions, and their growth and progression can be stimulated by environmental cofactors such as mutagens or ultraviolet light, respectively (Orth et al., 1980; Ostrow et al., 1982; Pfister et al., 1983). Rabbit and human papillomaviruses also contain analogous transforming genes which function in similar fashions (Meyers et al., 1992b; Defeo-Jones et al., 1993).

We have sought to develop new treatment modalities that might prove more effective and less toxic in the treatment of HPV-induced tumors. Current treatment of severe or neoplastic HPV-induced disease include surgery (laser or operative) and to a lesser extent drug treatments (podophyllin, 5-FU, interferons). These drug treatments have systemic side effects, incomplete resolutions, and frequent recurrences (Chamberlain et al., 1972; Senff et al., 1988; Benjamin et al., 1988, Weck et al., 1988; Stellato, 1992). Abramson et al., 1992, reports that laryngeal papillomatosis was reduced by about 50% following phototherapy, but it produced a generalized skin photosensitivity for at least 6 weeks, as well as other minor reactions. Latent viral DNA was maintained in the surrounding tissues following this therapy.

It has been demonstrated that the CTC class of compounds have therapeutic effects in the treatment of various DNA viruses. CTC-96 (patent pending) is a derivative of CTC-23 (patent #4866054), which is a member of a class of organometallic complexes (Wooley and Whalen, 1992). The latter cobalt-containing complex had been found to have in vitro activity against herpes simplex virus type 1 (HSV 1) in plaque reduction assays on human foreskin fibroblast monolayer cultures with little cytotoxicity up to 1 mg/ml (Dunkel et al., 1991). Efficacy was also observed in vivo in topical administration of CTC-23 at 1 mg/ml for rabbit ocular HSV 1 infection (Dunkel et al., 1991). Both CTC-23 and CTC-96 demonstrated moderate in vitro activity against HSV 1, HSV 2, varicella zoster virus (VZV) and Epstein-Barr virus (EBV).

In topical treatments begun shortly after infection, both CTC-23 and CTC-96 demonstrated in vivo activity against HSV 2 in mice. No protection from death was observed by Vogt et al., 1992 in murine models using systemic HSV 1 or cytomegalovirus (CMV). Topical use of CTC-23 and CTC-96 was shown by Devlin et al., 1993 to be effective in treating HSV 1-induced epithelial and stromal keratitis in

rabbits. Wooley and Whalen, 1992 demonstrated that CTC-23 is an immune modulator effective in the treatment of murine type II collagen-induced arthritis.

In this report we summarize our tests of the most potent and least toxic compound now in the series (CTC-96) (Claudia Stuart, Redox Pharmaceuticals, personal communication) in our CRPV-rabbit model as a topical therapeutic agent for the treatment of papillomavirus-induced tumors. We demonstrated a dose-dependent increase in the size of tumors treated with prolonged topical applications of CTC-96. This is an important finding because these compounds are being considered as possible therapeutics for other viral infections. Our results show that use of these compounds may be contraindicated in patients with papillomavirus infections.

2. Materials and methods

2.1. Preparation of virus

CRPV was prepared by standard methods (Watts et al., 1983) which produce a 10% w/v homogenate of cottontail rabbit warts cleared of cellular debris. The virus was titred by serial dilution and injection on domestic female dutch belt rabbits, producing warts in about 3 to 4 weeks.

2.2. Experimental protocol

Our study was designed to test the effectiveness of CTC-96 (provided by Redox Pharmaceutical Corp., a division of Chai-Tech Corp., Greenvale, NY) in our rabbit papillomavirus system. This treatment protocol has proven useful in other trials (Kreider, 1990; Ostrow, 1993). Seven rabbits were randomly placed into each of three groups and infected with CRPV. Each rabbit was infected at 8 sites on the back with 100 μl/site of a 1:4 dilution of the stock virus (approx. 32 ID50 units) by injection with a Ped-O-Jet injector (Brandsma et al., 1991). Two hours later topical treatments began which consisted of twice daily topical applications of 100 μl/site with the aid of a rubber policeman twice a day, 5 days a week for 8 weeks.

Group 1 inoculation sites were painted with water. Group 2 inoculation sites were painted with 1 mg/ml aqueous CTC-96, and Group 3 inoculation sites with 10 mg/ml of CTC-96. The stock solutions were prepared weekly and stored at 4°C. One animal in Group 2 died the first day due to unrelated causes. All animals were monitored weekly for body weight and daily for first appearance of tumors by observation or palpation. Tumor size was measured for all animals in the fifth and eighth week of the study. Red cell, white cell, and differential cell counts were determined for all animals prior to the start of the study at in the eighth week of the study. Tumor volumes were obtained from the product of the greatest length, width and height at each site determined using a calipers. All animals were kept in a single room, lighted by timed fluorescent lights and were caged individually. As CTC-96 has a distinctive color, it was not possible to observe the growth of tumors in a blind fashion. The presence of the tumors and measurements of their size were determined by two of the authors.

2.3. Statistical analysis

The statistical significance of the presence or absence of warts in the individual sample groups compared to the control groups was measured by a chi-square test. Dose-response results were based upon a linear and logistic regression analysis. Analysis of variance (ANOVA) was used to ensure that statistically significant differences between treatments was greater than differences observed between the animals nested within the three treatments.

3. Results

3.1. Clinical observations

Three groups of rabbits were infected with cottontail rabbit papillomavirus using an inoculation gun (Brandsma et al., 1991). We have used this method successfully to produce warts in rabbits and the results are similar in nature to those derived from scarification of the skin. This new method permits less trauma to the animals, and more reproducibility in delivering the live virus. It makes early detection and measurements of tumor size more reliable. Within 2 h following viral infection at eight dorsal sites, the injection sites of each animal in the first group received a topical dose of water (100 μl/site), each animal in the second group received a topical dose of 1 mg/ml aqueous CTC-96, and the third group received a topical dose of 10 mg/ml aqueous CTC-96. Animals were dosed twice per day, 5 days a week for 2 months.

In the fifth week of the study, all animals had developed at least one tumor and 87.5%, 95.8% and 100% of the infection sites produced tumors in the control, 1 mg/ml and 10 mg/ml groups, respectively (Table 1). We noted that tumors appeared earlier in animals receiving the CTC-96 and grew larger. The average latent period (time to first detection of tumors) and standard error to detection of a tumor was 26.0 $\pm$ 0.8, 23.9 $\pm$ 0.6, and 22.3 $\pm$ 0.5 days, respectively (Table 1). A regression analysis across the three groups produced a coefficient of correlation of -0.34 ($P<0.001$). The average size of the tumors at 5 weeks were 10.8 $\pm$ 1.8, 17.7 $\pm$ 2.8 and 40.6 $\pm$ 4.1 mm^3, respectively, which appeared to show a dose-response.

Papillomavirus infections are not systemic and each infection site could be considered as an experimental unit. It is necessary to segregate the animal-to-animal variability from the within-animal variability. Wart size at 5 weeks is analyzed in the analysis of variance in Table 2. The differences of the three levels of CTC-96 was greater than the animal-to-animal variability ($F=7.30$, $df=(2,17)$, $P=.005$). There was a large amount of variability within the 8 sites of each rabbit but the 20 animals were not significantly different from each other ($F < 1$, N.S.). The analysis for the other measures in Table 1 is similar to this one.

Similar findings were observed in the eighth week of the study when 91.1%, 97.9% ($P>0.05$) and 98.2% of the infection sites still contained tumors in the control, 1 mg/ml and 10 mg/ml groups, respectively. One previously observed additional tumor had regressed in the control group and one had regressed in the third group. The average sizes of the tumors were 82.6 $\pm$ 12.3, 191.3 $\pm$ 22, 300.6 $\pm$ 40.4 mm^3,

Table 1
Summary measures of CRPV-induced warts by dose concentrations of CTC-96

	Time post-infection	Group 1	Group 2	Group 3
Dose CTC-96 (μg/ml)		0	1	10
Number of animals at each dose		7	6*	7
Proportion of tumors n/N (%)**	5 weeks	49/56 (87.5%)	46/48 (95.8%)	56/56 (100%)
	8 weeks	51/56 (91.1%)	47/48 (97.9%)	55/56 (98.2%)
Time to first tumor (days)				
Mean (S.E.M.)		26.0 (0.8)	23.9 (0.6)	22.3 (0.5)
Median (range)		26 (19–42)	23 (19–37)	21 (19–30)
Average tumor size for all infection sites within group	5 weeks	10.8 (1.8)	17.7 (2.8)	40.6 (4.1)
Mean (S.E.M.) (mm^3)	8 weeks	82.6 (12.3)	191.3 (22)	300.6 (40.4)

*One animal in this group died of unrelated causes.
**There are 8 infection sites per animal. All animals developed and maintained most tumors by 5 weeks. n = number of tumors; N = number of infection sites.

respectively. The differences between each of the two sample groups and the control tumor sizes were highly significant ($P < 0.001$) and again demonstrated a positive dose-response (correlation = 0.41).

3.2. Toxicity

Two sample biopsies of tumors from each of three animals in groups 1 and 3 were submitted for histological analysis in addition to normal, untreated tissues from the same animals in group 3. No significant differences were observed in mitoses, atypia

Table 2
Analysis of variance for wart size at 5 weeks*

Source	Degrees of freedom	Sum of squares	Mean of square	F	P-value
Levels of CTC-96, nested in animals	2	3576.5	1788.3	7.3	$P = .005$
Treatment error	17	4164.4	245		
Between rabbits	19	7740.9	407.4	.58	N.S.
Sites within rabbits	140	98,196	701.4		
Total	159	105,937			

Each of 20 rabbits had 8 infections sites. All sites within each rabbit were treated with one of three levels of CTC-96. The 3 levels of CTC-96 (treatments) are compared to the variability between the 20 animals.
*The analysis for other measures is similar.

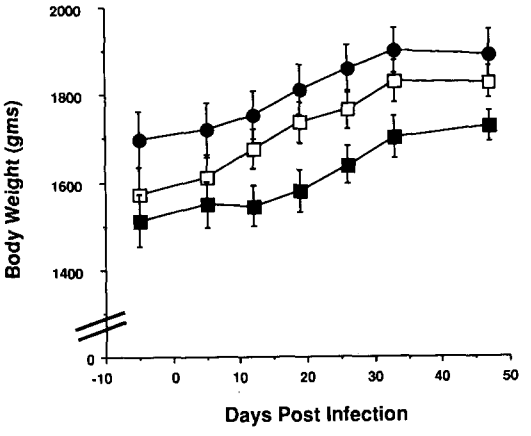

Fig. 1. Body weights of animals during the course of the study. CRPV infected rabbits were treated with topical water or CTC-96 at the concentrations indicated. Shown is the average body weight for each group of animals. -●-, Control; -□-, 1 mg/ml; -■-, 10 mg/ml.

or degree of differentiation between treated and control animals. We also treated one site distal from the infection sites of each of three rabbits with the highest dose of CTC-96 for 2 weeks. No histological differences were observed compared to untreated normal skin. No major pathological side effects of the treatment were observed and hematologic parameters measured were not different between the groups. At the highest dose of CTC-96, mean white cell counts and standard errors of the mean for all animals prior to the start of treatment and just before termination of treatment were $1.0 \pm 0.1 \times 10^9$/ml and $0.9 \pm 0.1 \times 10^9$/ml, respectively, and red cell counts were $4.5 \pm 0.2 \times 10^9$/ml and $5.0 \pm 0.3 \times 10^9$/ml, respectively. Average body weights of the animals of all groups of animals increased at approximately the same rate (11–16%) over the course of the study, although group 3 lagged somewhat in the middle third of the study (Fig. 1).

4. Discussion

We have been involved in a program of screening reagents which may be useful for the treatment of papillomavirus-induced disease. Our program has detected two agents thus far which have been efficacious in reducing the number and size of papillomas. The first was the use of systemic Ribavirin (Ostrow et al., 1992) which has since been shown to be effective in preliminary clinical trials of patients with laryngeal papillomatosis (McGlennen et al., 1993). This work has demonstrated that results in our rabbit system can directly reflect potential results of putative anti-papillomaviral agents in humans. The second agent we found involved a crude extract of *Aspergillus niger* which was applied topically to infection sites (Ostrow et

al., 1993). The positive results of that study in rabbits paralleled anecdotal reports of similar positive results to human and animal warts exposed to this extract.

In the current study, we observed that tumors appeared slightly sooner and grew significantly faster in animals which were treated with CTC-96. The reduction in the length of the latent period and the increase in size of the tumors was directly related to the dose of CTC-96 used. No reduction was observed in the number of experimental tumors treated with topical CTC-96.

The mechanism by which CTC-96 promotes wart growth is not clear. A closely related variant of this compound was previously found to promote epithelial growth and differentiation in burn wound healing studies (Claudia Stuart, unpublished data). Such promotion of growth and differentiation have been found in many classic studies to promote papillomatous growth (Friedewald, 1942), enhance amplification of HPV 1 genomes (Bossens et al., 1992), and enable the complete productive formation of HPV 11 virions (Dollard et al., 1992) or HPV 31 virions (Meyers et al., 1992a). If CTC-96 is capable of producing hyperproliferation and enhancing differentiation of the epidermis, it is likely that those aspects could enhance the growth of CRPV-induced tumors which we observed here. We did not, however, observe any histological differences in our study between treated animals and controls. The increased tumor growth may be the result of a quantitative rather than a qualitative difference in growth or differentiation. CTC-23 can also scavenge superoxides thus producing an anti-inflammatory response and immune modulation (Wooley and Whalen, 1992). Local immune modulatory effects of these compounds may also enhance papillomatous growth in a manner not yet understood.

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