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Effect of topically applied resveratrol on cutaneous herpes simplex virus infections in hairless mice

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Abstract

Resveratrol (3,5,4′-trihydroxystilbene) is a natural component of certain foods, such as grapes, that has been shown to have anti-herpes simplex virus (HSV) activity in vitro. To determine if it is active in vivo, the abraded epidermis of SKH1 mice were infected with HSV-1 and topically treated with 12.5 or 25% resveratrol cream or cream only. Initial studies demonstrated that: (1) 25% resveratrol cream topically applied two, three, or five times a day effectively suppressed lesion development whereas 12.5% resveratrol cream effectively suppressed lesion formation when applied five times a day starting 1 h after infection; (2) when treatment was begun 1, 6, or 12 h after infection, both 12.5 and 25% resveratrol were effective at 1 and 6 h after infection, but not if applied 12 h after infection. Comparative studies between resveratrol cream, 10% docosanol cream (Abreva™) and 5% acyclovir ointment (Zovirax™) were also carried out. When treatment was begun 1 h after infection and repeated every 3 h five times a day for 5 days, 12.5 and 25% resveratrol significantly ($P = 0.0001$) inhibited the development of HSV-1 induced skin lesions. Acyclovir was as effective ($P = 0.0001$) as resveratrol. Animals that were topically treated with docosanol were not protected and developed lesions in a manner indistinguishable from cream only controls. These studies were repeated with an HSV-1 acyclovir-resistant virus. As before, 12.5 and 25% resveratrol cream effectively suppressed lesion formation. The skin of resveratrol-treated animals showed no apparent dermal toxicity such as erythema, scaling, crusting, lichenification, or excoriation. These studies demonstrate that topically applied resveratrol inhibits HSV lesion formation in the skin of mice.

Keywords: HSV; Resveratrol; In vivo

1. Introduction

Resveratrol (3,5,4′-trihydroxystilbene) is a non-flavanoid phenol compound produced naturally by some spermatophytes, such as grapes, in response to injury or fungal attack. It is present in grape skins, but not flesh, and therefore is found in red wine where it has been identified as the major active compound of stilbene phytoalexins. It has been reported that resveratrol has anti-cancer properties (Jang et al., 1997), antimycotic properties (Jeandet et al., 1995), and beneficial cardiac effects (Goldberg et al., 1995). Along with its antimycotic properties, it has also been shown to have selective antibacterial properties (Docherty et al., 2001) and anti-herpes simplex virus (HSV) activity in vitro (Docherty et al., 1999).

HSV is a common human virus that affects the majority of the population (Whitley and Gnann, 1993). Infection with HSV normally results in limited benign lesions, but it is capable of causing life-threatening disease or blindness. The discovery of acyclovir over 30 years ago (Elion et al., 1977) was a major development in the management of HSV infections and its use or the use of its analogues has relieved considerable human suffering.

However, the possibility of the emergence of drug-resistant virus mutants has stimulated the continuing development of anti-HSV drugs with an emphasis on mechanisms of action other than inhibition of DNA synthesis by nucleoside analogues. This has led to studies, with varying degrees of success, on anti-HSV agents that act through an anti-sense mechanism (Flores-Aguilar et al., 1997), inhibition of HSV ribonucleotide reductase (Bourne et al., 1992), inhibition of viral proteases (Waxman and Darke, 2000), and inhibitors that target HSV helicase/primase (Crute et al., 2002; Kleymann et al., 2002). One group of inhibitors that is par-

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ticularly interesting are agents that inhibit cell cycle factors required for HSV replication (Schang et al., 1998).

Because resveratrol effectively inhibits HSV in vitro and because it is reported to inhibit the cell cycle (Ragione et al., 1998), we examined its effectiveness against HSV in vivo in the hairless mouse model of cutaneous HSV-1 infection. In assessing the effectiveness of resveratrol in vivo, it was compared with two approved topical treatments, acyclovir ointment and docosanol cream (Sacks et al., 2001).

2. Materials and methods

2.1. Virus

Two different HSV-1 strains were used in this study. HSV-1 (84–373) was isolated from an oral lesion while HSV-1 (0–1116) was isolated from an adult brain. HSV-1 (0–1116) was kindly provided by Dr. D. Parris and is acyclovir resistant (Parris and Harrington, 1982). HSV-1 (84–373) pools were prepared in Vero cells in Media 199 supplemented with 5% fetal calf serum, 0.075% $NaHCO_3$, and 50 μg/ml gentamycin sulfate. HSV-1 (0–1116) pools were prepared in an identical manner except that the tissue culture media also contained 50 μM acyclovir.

Viral stocks were quantified by the plaque assay in Vero cells.

2.2. In vivo studies

All animal studies were reviewed and approved by the IACUC. Five-week-old SKH1 mice (Charles River, MA), which are hairless, euthymic, and immunocompetent, were used in all studies. Each group of animals, controls or treated, contained 8–9 mice with an average weight of 20.6 ± 0.6 gm/mouse at the beginning of the study.

Mice were infected by abrading the epidermis of the dorsal aspect of the neck with a pedicure/manicure device (Medisana®, USA, Inc., Charlotte, NC) equipped with a sapphire needle burr rotating at 5000 rpm. The needle burr effectively abraded a section of epidermis approximately 1 mm in diameter exposing the underlying dermis. Cells in the dermal layer were infected by placing 10 μl of fluid containing 10^6 pfu of HSV-1 on the abraded site and lightly rubbing for 3–5 s. Drug treatment of animals began 1, 6, or 12 h after infection.

Beginning 1 day after infection, the abraded site was evaluated and scored according to the following schedule: 0, no visible change in the abrasion or surrounding tissue; 0.5, the earliest lesion characterized by slight swelling at the abrasion and mild erythema; 1, papule(s) at or around the abrasion; 2, ulcerated papules at or around the abrasion with eschar formation; 3, fusion of ulcerated lesions into large eschar; 4, large open ulcerated lesion; 5, death or sacrifice.

The animals were evaluated daily for 11 days beginning 1 day after infection. In control animals, it was noted that peak acute disease occurred at about 7–8 days after infection.

2.3. Drugs

Resveratrol was obtained from Royalmount Pharma, Inc., Montreal, Que., Canada. The chemical was assayed by HPLC and found to be greater than 99% pure. A cream, consisting of a polyethylene glycol base, was formulated by the same company to contain resveratrol at concentrations of 12.5 or 25%. The cream without resveratrol was used as the control.

Acyclovir (Zovirax™) was purchased as a 5% ointment. Docosanol (Abreva™) was purchased as a 10% cream.

Acyclovir ointment, which is approved for primary genital herpes and herpes labialis in immunocompromised persons, but not normal subjects, was applied according to the manufacturer's instructions every 2.5 h six times a day for 7 days. The ointment was spread using a sterile plastic applicator stick for each mouse. The ointment was liberally applied approximately 1 cm in diameter on, and well beyond, the margins of the abrasion site. Docosanol is an over-the-counter drug for herpes labialis, and according to the manufacturer's instructions, should be applied five times a day until healing. In our studies, it was applied five times a day for 7 days because lesion formation was greatest at that time and further treatment provided no benefit in the mouse model. Docosanol was applied to the lesion site in the same manner as acyclovir. Resveratrol cream was applied in an identical manner as acyclovir or docosanol two, three, or five times a day, depending on the experiment, for 5 days. If applied twice a day, the applications were 8 h apart; if applied three times a day, the applications were 4 h apart; and if applied five times a day, the applications were 3 h apart.

2.4. Data analysis

Statistical significance was tested by the unpaired *T* test using the Bonferroni correction for multiple comparisons. A *P*-value of less than 0.006 was considered statistically significant.

3. Results

3.1. Number of applications per day and resveratrol concentration

Initial studies were designed to determine if resveratrol was effective in vivo and to establish the number of applications per day, the concentration and the time of application after initial infection that would most effectively limit HSV-induced lesion formation. Mice infected with HSV-1 were treated with 12.5 or 25% resveratrol two, three, or five

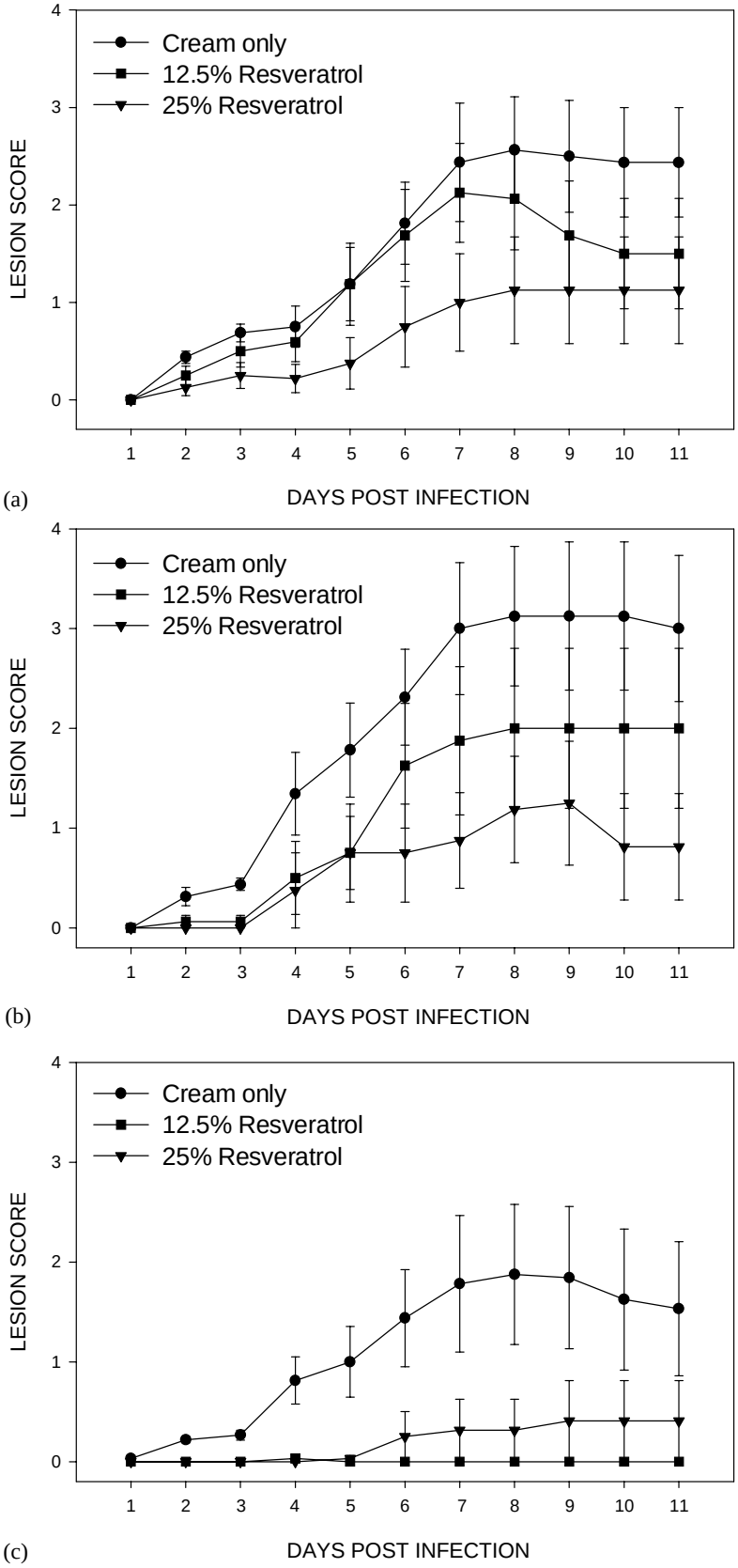

Fig. 1. Varying numbers of applications per day of resveratrol to HSV infected mouse skin. The skin of SKH1 mice were infected with HSV-1 and topical treatment with 12.5 or 25% resveratrol cream or cream only was applied twice a day 8 h apart (a); three times a day 4 h apart (b); or five times a day 3 h apart (c) beginning 1 h after infection. Data points represent the average value of 8/9 mice per group ± standard error.

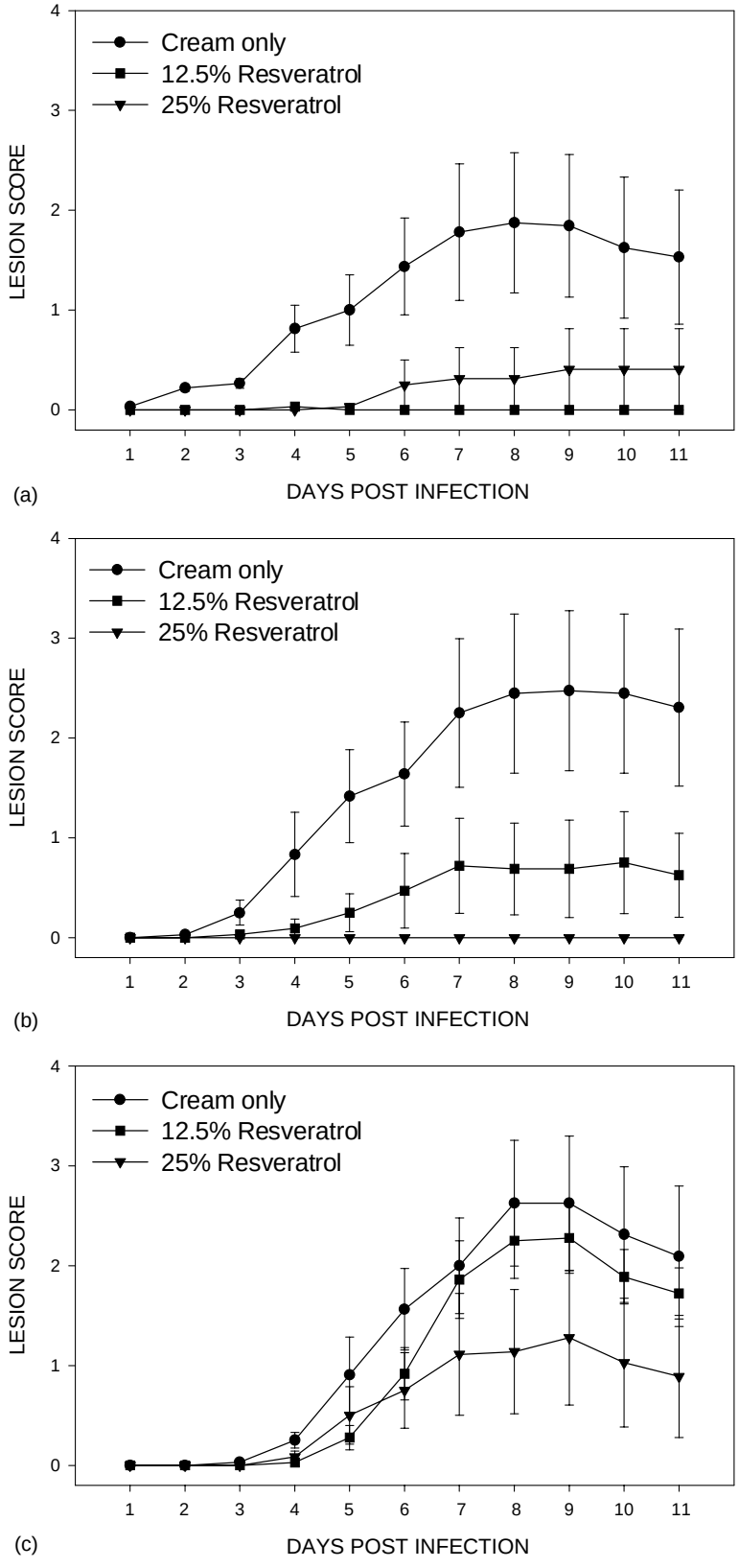

Fig. 2. Resveratrol treatment of HSV infected mouse skin starting at various times after infection. The skin of SKH1 mice were infected with HSV-1 and topical treatment with 12.5 or 25% resveratrol cream or cream only was begun at 1 h (a); 6 h (b); or 12 h (c) after infection. Mice were treated every 3 h five times a day. Data points represent the average value of 8/9 mice per group ± standard error.

times a day beginning 1 h after infection for 5 days. Results in Fig. 1a reveal that two treatments per day, 8 h apart, did not significantly decrease lesion formation in mice treated with 12.5% resveratrol compared to the cream control. However, mice treated with 25% resveratrol had reduced lesion formation when compared to the cream control. Similarly, Fig. 1b shows a decrease in lesion formation in animals treated three times a day, 4 h apart, with 25% resveratrol, but not 12.5% resveratrol when compared to the cream control. However, both 12.5 and 25% resveratrol significantly decreased lesion formation when applied every 3 h five times a day for 5 days when compared to the cream control (Fig. 1c).

3.2. Time of application and resveratrol concentration

After infection of the abraded skin of SKH1 mice with HSV-1, treatment was started at 1, 6, and 12 h after infection using resveratrol cream applied every 3 h five times a day at concentrations of 12.5 and 25% or cream only. Results in Fig. 2a and b demonstrate that resveratrol treatment was most effective when started 1–6 h after infection. When treatment was started 1 h after infection (Fig. 2a), lesion formation was profoundly limited by 12.5 and 25% resveratrol when compared to the cream only control. Similar results were obtained when treatment was delayed for 6 h (Fig. 2b). If treatment was started 12 h after infection, the difference between the cream control and 12.5% resveratrol-treated mice was not significant (Fig. 2c), but 25% resveratrol remained efficacious.

3.3. Effectiveness of resveratrol treatment compared to other anti-HSV drugs

The studies described previously established that resveratrol was most effective when treatment was started within 6 h of infection and when drug was applied five times a day. Once these parameters of treatment with resveratrol were established, the efficacy of the drug was compared to other known, topically applied anti-HSV drugs. For this purpose, two anti-HSV drugs with completely different modes of action that are currently available to the public were selected for this comparative study. Docosanol is sold under the trade name Abreva™ and inhibits HSV by interfering with the fusion of the virus envelope with the plasma membrane (Pope et al., 1998) while acyclovir which is marketed as Zovirax™ disrupts viral DNA synthesis (Elion et al., 1977).

Animals were abraded and infected in the usual manner and treatment with 12.5% resveratrol, 25% resveratrol, docosanol, acyclovir, or cream only was begun 1 h after infection. Results demonstrate that 12.5 and 25% resveratrol and 5% acyclovir significantly inhibited the development of HSV-1 induced skin lesions compared to the cream only control (Fig. 3; $P = 0.0001$). Animals that were treated with cream only or 10% docosanol developed comparable lesions (Fig. 3).

3.4. Effectiveness of resveratrol on acyclovir-resistant HSV-1

Studies were conducted on the ability of resveratrol to inhibit acyclovir-resistant HSV-1 in vivo. As be-

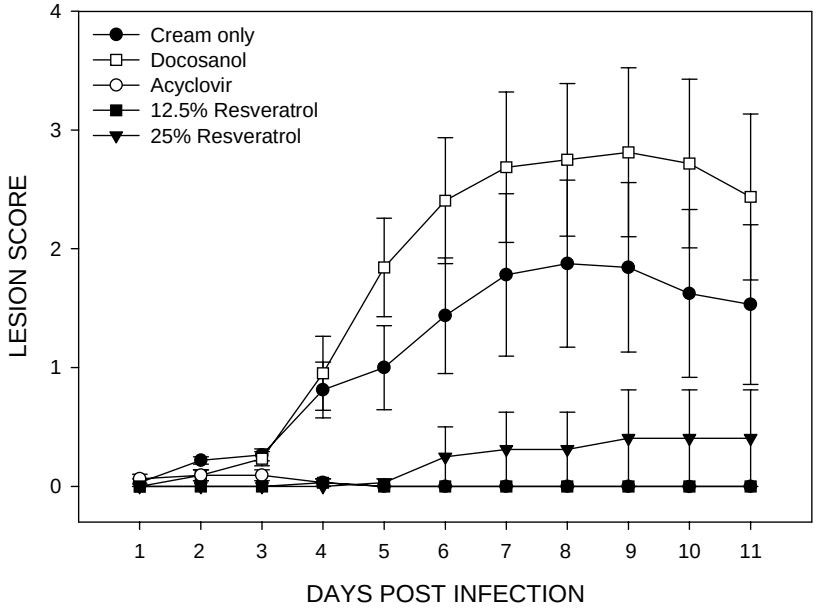

Fig. 3. Treatment of HSV infected mouse skin with resveratrol, docosanol, or acyclovir. The skin of SKH1 mice were infected with HSV-1. Beginning 1 h after infection, the infected site was topically treated with 12.5 or 25% resveratrol cream or cream only every 3 h five times a day for 5 days. Animals receiving docosanol were treated every 3 h five times a day for 7 days while animals receiving acyclovir were treated every 2.5 h six times a day for 7 days. Data points represent the average value of 8/9 mice per group ± standard error.

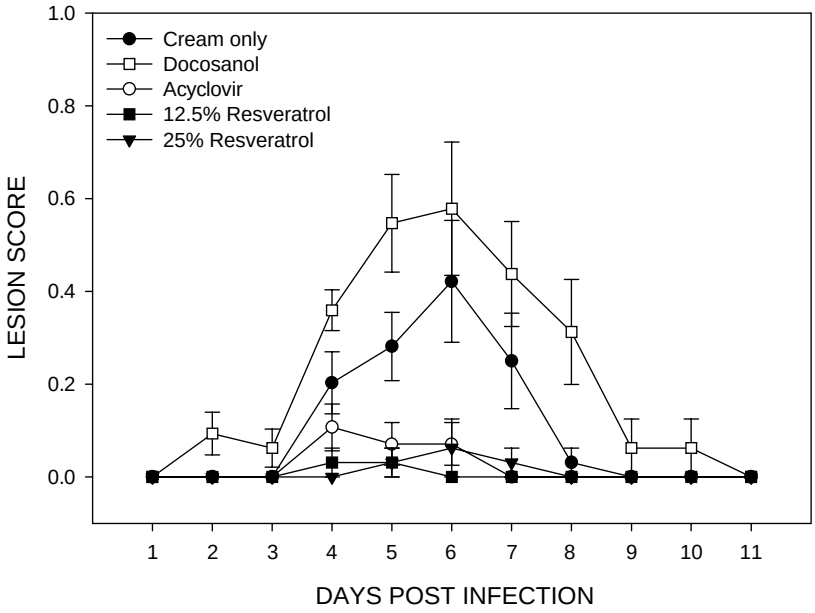

Fig. 4. Treatment of acyclovir-resistant HSV infected mouse skin with resveratrol, docosanol, or acyclovir. The skin of SKH1 mice were infected with acyclovir-resistant HSV-1. Beginning 1 h after infection, the infected site was topically treated with 12.5 or 25% resveratrol cream or cream only every 3 h five times a day for 5 days. Animals receiving docosanol were treated every 3 h five times a day for 7 days while animals receiving acyclovir were treated every 2.5 h six times a day for 7 days. Data points represent the average value of 8/9 mice per group ± standard error.

fore, docosanol and acyclovir were included as control drugs.

Abraded mice were infected with acyclovir-resistant HSV-1 (0–1116) and treatment was begun 1 h after infection with 12.5% resveratrol, 25% resveratrol, docosanol, acyclovir, or cream only in a manner identical to the previous study. Lesion development in the mice was scored daily for 11 days.

It should be noted that HSV-1 (0–1116) induced only mild lesions in the skin of SKH1 mice when compared to HSV-1 (84–373), the other virus used in this study. A maximum lesion score for HSV-1 (0–1116) was two while a maximum lesion score for HSV-1 (84–373) was five.

The results in Fig. 4 demonstrate that 12.5 and 25% resveratrol suppressed lesion formation when compared to the cream only control. Surprisingly acyclovir also suppressed lesion development in the skin of mice infected with HSV-1 (0–1116). However, the cream control and docosanol were both ineffective in preventing lesion formation (Fig. 4).

3.5. *Toxicity*

Throughout these studies, the skins of animals treated with cream only or resveratrol cream were visually examined on a daily basis for signs of toxicity. None of the animals showed any apparent signs of dermal toxicity such as erythema, scaling, crusting, lichenification, or excoriation.

4. Discussion

The results of this study demonstrate that resveratrol is able to significantly reduce HSV-induced lesion formation in the skin of SKH1 mice. The in vivo effectiveness of resveratrol is influenced by drug concentration, number of applications per day and the amount of time between initial infection and the start of treatment. When compared to two commercially available drugs, resveratrol was found to be as effective as acyclovir in lesion suppression and superior to docosanol, which did not suppress lesion formation in this animal model.

Like acyclovir, resveratrol was most effective the earlier it was applied after infection. Because most patients self medicate when they first feel a herpes outbreak beginning, early application is most likely to occur. As such, the activities of resveratrol shown here could translate into efficacy in humans.

Resveratrol is a drug that has garnered significant attention primarily because of its alleged beneficial cardiac properties (Fremont, 2000; Soleas et al., 1997) and its anti-cancer activity (Jang et al., 1997). However, its natural function appears to be, at least in part, its' antimicrobial activity that protect certain foods such as grapes from fungal attack (Jeandet et al., 1995).

Because of this wide range of activities, resveratrol has been extensively studied and numerous properties attributed to it (Belguendouz et al., 1998; Fukuhara and Miyata, 1999; Fremont et al., 1999; Naderali et al., 2000; Narayanan et al.,

2003; Cai et al., 2003). It has been reported that resveratrol inhibits cell DNA polymerase (Sun et al., 1998), ribonucleotide reductase (Fontecave et al., 1998), and is capable of disrupting the cell cycle (Ragione et al., 1998; Holian and Walter, 2001).

Attempts to assign a specific property of the drug with anti-HSV activity may be premature. Nevertheless, it is interesting to note that HSV encodes ribonucleotide reductase (Goldstein and Weller, 1988), DNA polymerase (Knopf, 1979), and requires cell cycle factors such as cdk-1 and cdk-2 to replicate (Hossain et al., 1997; Schang et al., 1998). All are potential targets for resveratrol. However, it has been shown that HSV DNA polymerase is refractory to the inhibitory effects of resveratrol (Stivala et al., 2001) and would therefore not be a likely target for the drug. On the other hand, HSV ribonucleotide reductase cannot be eliminated as a possible target for resveratrol. Because resveratrol has been shown in vitro to target immediate early events in HSV replication (Docherty et al., 1999), the large subunit of ribonucleotide reductase is of interest (Smith et al., 1998). However, it was reported that a ribonucleotide reductase HSV-1 mutant reduced virus yield only four- to five-fold and is dispensable for virus growth and DNA synthesis (Goldstein and Weller, 1988) while resveratrol has been shown to inhibit HSV by greater than 99% in vitro (Docherty et al., 1999). Thus, while resveratrol could affect HSV by targeting the large subunit of ribonucleotide reductase, it is unlikely that this would be the sole source of inhibitory properties on HSV replication.

Recent studies have indicated that HSV requires cellular functions associated with cell cycle progression in order to replicate (Hossain et al., 1997). More specifically, it has been shown that drugs that inhibit cdk-1 and cdk-2 also inhibit HSV replication (Schang et al., 1998). Because resveratrol has been shown to disrupt the cell cycle, it may be this affect that contributes to the inhibitory effects on HSV replication (Ragione et al., 1998; Holian and Walter, 2001; Delmas et al., 2002). The precise method by which resveratrol disrupts the cell cycle is unknown, but it is known that resveratrol inhibits phosphorylation (Jayatilake et al., 1993; Stewart et al., 1999) and that phosphorylation/dephosphorylation play a significant regulatory role in the cell cycle (Norbury and Nurse, 1992). As such, disruption in phosphorylation mechanisms of the cell cycle as well as for herpes essential proteins could potentially account for the observed effects of resveratrol in vivo.

In Fig. 4 of this study, resveratrol, docosanol, and acyclovir were examined for their ability to control lesion formation in vivo using an acyclovir-resistant virus. Resveratrol, but not docosanol, inhibited lesion development; but surprisingly, acyclovir also inhibited this virus even though it was acyclovir resistant. Acyclovir-resistant strains of HSV have been defined as those with an ED5O (ED50: percentage plaque number plotted against the log 10 of the drug concentration) of greater than 2 μg/ml (Field, 2001). The acyclovir-resistant strain used in these studies qualifies as resistant since virus stocks were prepared in the presence of 50 μM acyclovir (i.e. 11.26 μg/ml). However, the acyclovir ointment used contained 5% acyclovir which is more than four thousand times the concentration used in the preparation of the virus and apparently sufficient to suppress this strain of acyclovir-resistant virus in vivo under the circumstances presented by this model. Additionally, abrogation of the barrier function of the stratum corneum of the skin by the needle burr used in this study may have made all treatments more effective compared to the same treatments in cutaneous models where the stratum corneum is intact. For example, acyclovir ointment was almost 100% effective in the present study, but only 20–30% efficacious in the dorsal cutaneous guinea pig model of HSV-1 infection (McKeough and Spruance, 2001).

In these studies, a chemical found in common foods such as grapes inhibited HSV-induced lesion formation in vivo. The level of inhibition was similar to that of the commonly used and effective drug, acyclovir. Currently, studies are ongoing in an attempt to understand the mechanism of action by which resveratrol suppresses HSV infection.

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