Transforming Growth Factor-α (TGF-α) in a Semisolid Dosage Form: Preservative and Vehicle Selection

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The selection of an ideal semisolid vehicle for growth factors presents a challenge. Some antimicrobial agents are known to delay wound healing. The objective of this investigation was to identify appropriate preservatives and vehicles for TGF-α. Criteria for acceptance are noninterference with the mitogenic activity of TGF-α as well as adequate product preservation. Vehicles considered were o/w creams, ointments, and a gel. Combinations of six preservatives were tested. Selection was determined using both microbial preservative challenge and TGF-α mitogenic assay. In the former, 10 species of microorganisms were inoculated into formulation samples. At selected time intervals, it was determined whether colonies decreased, increased, or remained constant. In the mitogenic assay, samples of either preservatives or formulation prototypes were introduced to TGF-α-stimulated fibroblast cell cultures. Mitogenesis was determined by measuring ^{3}H-dThd uptake into newly synthesized DNA. As preservatives, sorbic acid and quaternium-15 appear to satisfy both selection criteria. A thermosetting gel appears most promising as vehicle.

KEY WORDS: transforming growth factor-α (TGF-α); semisolid formulation; mitogenic assay; preservative challenge test.

INTRODUCTION

Growth factors are increasingly being investigated for their ability to promote wound-healing (1–3). In past studies, the growth factor has often been used either in solution form (4) or as a powder manually incorporated into a commercially available anti-bacterial cream such as Silvadene (3). The latter technique does not ensure a homogeneous distribution of the active ingredient. In addition, marketed antibacterial creams may contain their own active ingredients, which probably confound effects due to growth factors alone. Presently, there is no growth factor-containing semisolid product available for clinical use.

The selection of an ideal semisolid vehicle for growth factors presents a challenge. In this study, creams were the primary vehicles of interest but other types of semisolid bases were also investigated. Our goal was to identify an appropriate preservative and vehicle for transforming growth factor-α (TGF-α). TGF-α, a 50-amino acid polypeptide, is a potent stimulator of epidermal cell growth (5).

The presence of water in emulsions requires the use of preservatives to curtail bacterial growth. The general mechanism of action of preservatives is to destroy bacterial cell walls (6). Some antimicrobial agents are found to interfere with wound healing (7). On the other hand, an ideal vehicle for TGF-α should not retard the process of wound-healing. Acceptance criteria for TGF-α's preservative and vehicle are, therefore, adequate product preservation and noninterference with the mitogenic activity of TGF-α.

The difficulties associated with performing routine *in vivo* experiments that measure cell growth potential necessitates *in vitro* bioassays. DNA synthesis is synonymous with cell proliferation and is considered evidence of cell growth (8). In order to follow DNA synthesis, laboratories commonly use mitogenic or proliferation assays involving ^{3}H-thymidine incorporation. Since the main cell affected in wound-healing processes appears to be the fibroblast (9), we used the mouse fibroblast as model cell to test the effect preservatives and vehicles have on cellular proliferation.

MATERIALS AND METHODS

Materials

TGF-α was supplied by Oncogen, Seattle, WA. Purity of the supplied material was reported at greater than 99%. Stearyl alcohol (Ashland Chemicals), Pluronic F-127 (BASF), quaternium-15 (Dow), phenoxyethanol (Emery), PEG stearate/glycol stearate and glycol stearate (Gattefosse), cetyl esters and cetostearyl alcohol (Henkel), sorbic acid (Monsanto), methylparaben and propylparaben (Napp Chem.), benzyl alcohol (Stauffer), PEG-8 and PEG-75 (Union Carbide), and petrolatum (Witco) all conformed to USP or NF requirements.

Formulations

(1) Emollient cream. An oil in water (o/w) emulsion containing petrolatum and stearyl alcohol with water, nonionic emulsifiers, solvents/emollients, and preservatives
(2) Hydrophilic cream. An o/w emulsion containing cetyl esters and cetostearyl alcohol with water, nonionic emulsifiers, solvents/emollients, and preservatives.
(3) Stearate cream. A mixture of PEG stearate/glycol stearate with emollients/solvents, water, and preservatives.
(4) Thermosetting gel. A poloxamer (polyoxyethylene–polyoxypropylene block copolymer) gel with water, solvents, and preservatives.
(5) Modified PEG ointment. A variation of polyethylene glycol ointment NF using PEG-8 and PEG-75.

Preservative Challenge

Five microorganisms required by USP XXII were used: two gram-negative bacteria (*Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 9027), a gram-positive bac-

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terium (*Staphylococcus aureus* ATCC 6538), a yeast (*Candida albicans* ATCC 10231), and a mold (*Aspergillus niger* ATCC 16404). In addition, several isolates were included: four gram-negative bacteria (*Enterobacter gergoviae* WW 75, *Pseudomonas aeruginosa* ATCC 15442, *Pseudomonas pickettii* WW10311, and *Pseudomonas cepacia* WW 252) and a yeast (*Candida tropicalis* ATCC 13803). Methods of preparing inoculum mixtures for each type of organism were adapted from McGinnis and Rinaldi (10).

Each test product was aseptically dispensed into 10 125 × 20-mm test tubes (10 g/tube). Every test tube preparation was inoculated with 0.1 mL of the prepared inoculum of interest. The target level for inoculation was 10^5–10^6 colony-forming units (CFU) per g of product. Depending on product viscosity, the inocula were evenly distributed in the tubes using a Vortex-Genie mixer or by stirring with a sterile swab or applicator stick. Within 30 min after inoculation and mixing, a sterile cotton swab was used to remove some of the contaminated material and streak it onto agar-containing petri dishes. The subculture step was repeated 24 hr, 48 hr, 7 days, 14 days, 21 days, and 28 days unless two consecutive subcultures did not yield growth. At that point, sampling was discontinued until the final 28-day subculture.

Inoculated tubes of test products were incubated at ambient room temperature. The subculture plates were incubated at 32°C for 48 hr. Levels of increasing or decreasing microbial growth were observed.

Mitogenic Assay

A detailed description of the fibroblast proliferation assay, including the tissue culture method is described by Leister and Kozick (11). Briefly, cultured C3H-10T$^{1/2}$ cells were treated with TGF-α (15 ng/mL) and placed in a 37°C incubator with a 5% CO_2/95% air atmosphere for 30 min. Vehicles and preservatives were then added at designated concentrations followed by the addition of tritiated thymidine. Subsequently, the cultures were placed in a 37°C incubator with a 5% CO_2/95% air atmosphere for 24 hr. Stimulated DNA was then measured as tritiated thymidine incorporated into the cell material.

RESULTS AND DISCUSSION

The effect of individual preservatives on fibroblast proliferation was evaluated. Fibroblast cells were first treated with TGF-α, followed by varying concentrations of each preservative. Twenty-four hours after treatment, DNA synthesis was measured as tritiated thymidine incorporated into the acid-insoluble cell material. Data obtained are expressed as percentage thymidine incorporation, i.e., the growth exhibited by a plate of cells containing a preservative compared to the growth exhibited by the TGF-α control which has no preservative. ^{3}H-dThd incorporation measures newly synthesized DNA, which is considered a representative bioresponse of increased cell growth (11).

The cell proliferation assay requires sample dilution. Studying a range of preservative concentrations identifies the general cell response trend due to a specific preservative. It also identifies the range of dilution wherein assay interference, i.e., sensitivity to preservatives resulting in cell death, is evident. A steep curve indicates a decrease in expected cell growth due to the presence of a preservative. A relatively flat activity curve is a positive sign that the preservative does not cause death of fibroblast cells.

Figure 1 shows the effects of several preservatives on the ability of TGF-α to stimulate growth of mouse fibroblast cells. With readings close to 100% throughout all the dilutions ranging from 1:200 to 1:25, sorbic acid does not appear to interfere with cell growth. A similar flat curve is observed with a methyl/propyl paraben combination. Thymidine incorporation, however, was attained only at 60–80% throughout the range of dilutions. Conversely, phenoxyethanol and benzyl alcohol markedly inhibited growth as evidenced by the steep slope of the curves. Quaternium-15 interfered with cell growth to a lesser extent.

Propylene glycol was selectively added into some of the formulations for its antimicrobial and cosolvent properties. The solvent has been reported to affect the osmolality of body fluids (12). We had concerns that this might result in the inhibition of cell growth but there was no evidence of inhibition. Throughout the range of dilutions tested, propylene glycol shows approximately 80–90% thymidine incorporation.

The preservatives we have chosen are either commonly found in parenteral products or rated as nonprimary skin irritants. It has been recognized, however, that the often large number of ingredients in an emulsion necessitates evaluation of an entire formulation rather than by components (13). Since semisolid vehicles are more complex systems than the individual preservative solutions which were previously investigated, it was necessary to test the preservatives when they are incorporated into prototype formulations. All formulations were subjected to both the microbial preservative challenge test and the cell proliferation assay.

Preservative Challenge of Formulations

A number of preservatives were incorporated into three o/w emulsions, a modified USP polyethylene glycol ointment, and a thermosetting gel (Table I). All placebo formu-

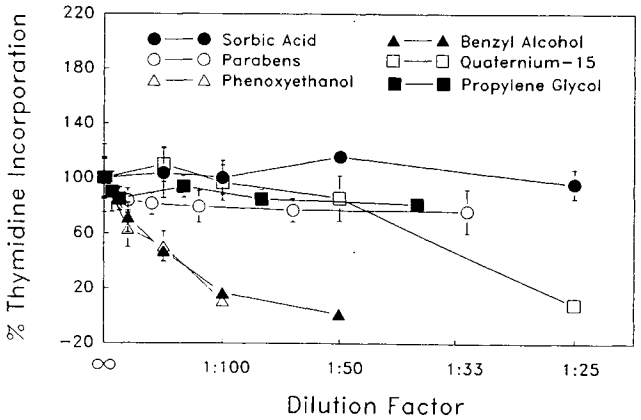

Fig. 1. Effect of preservatives on cell growth where initial concentrations are 0.2% (w/w) for sorbic acid; 0.18% and 0.02% (w/w), respectively, for a combined methylparaben and propylparaben system; 1.0% (w/w) each for both phenoxyethanol and benzyl alcohol; 0.02% (w/w) for quaternium-15; and 7.5% (w/w) for propylene glycol. Dilution factor is defined as the ratio of the volume of the preservative solution to the volume of the cell culture medium.

Table I. Group of Preservative Combinations Used in Five Semisolid Vehicles

Vehicle type	Percentage, w/w						
	Methyl-paraben	Propyl-paraben	Sorbic acid	Quat-15	Benzyl alcohol	Phenoxy-ethanol	Propylene glycol
Emollient cream	0.18	0.02	—	—	—	—	7.5
	0.30	0.20	—	—	—	—	7.5
	0.30	—	0.20	—	—	—	5.5
	—	—	0.20	—	—	1.0	5.5
	—	—	—	0.02	1.0	—	7.5
Hydrophilic cream	—	—	—	0.02	1.0	—	—
	—	—	0.20	—	—	1.0	(4% SD alcohol instead)
Stearate cream	0.30	0.20	—	—	—	—	—
Thermosetting gel	0.18	0.02	—	—	—	—	—
Modified PEG ointment	—	—	—	—	—	—	—

lations exhibited adequate preservation at the end of 28 days. Since the TGF-α formulation would be intended for patients with chronic wounds, we set up an additional criteria that formulations should rapidly start exhibiting antimicrobial activity. For a preservative challenge test, a criterion for acceptability is to achieve a count of less than 10 CFU by the end of the first week (14). Comparisons using data restricted to the first week of preservative testing revealed significant differences (Table II).

Several observations were made. The PEG ointment is inadequately preserved against mold, even at the end of the month-long study (data not shown). The results in Table II show that samples of emollient cream with quaternium 15/benzyl alcohol and phenoxyethanol/sorbic acid are superior to the hydrophilic creams containing the same preservatives. While it may appear that the presence of propylene glycol apparently improves the antimicrobial activity of formulations as a whole, the difference in inactive ingredients may have a vital role as well. Another observation is that a methylparaben/propylparaben combination does not adequately preserve the emollient cream formulation. Replacing a para-hydroxybenzoate ester with sorbic acid, however, significantly improves the preservation of a nonionic emulsion. A similar observation was made by Charles and Carter (13).

Mitogenic Assay of Formulations

The primary goal of the mitogenic assay was to evaluate the mitogenic effect of TGF-α in the presence of all ingredients found in our semisolid formulations. While analytical techniques may characterize proteins with a high precision, potency assays are critical in determining biological activity concentrations. It is important that assay interference, i.e., sensitivity of fibroblast cells to formulation ingredients, did not occur with the prototypes. The mitogenic assay was intended to test the biological activity of TGF-α.

The same set of prototypes listed in Table I was subjected to the mitogenic assay. Figure 2 is a graphical comparison of cell activity after fibroblast cells were treated with vehicles containing the preservatives, following an initial treatment with 15 ng/mL TGF-α. The set of graphs represents only the four vehicles found acceptable by the mitogenic assay.

With the exception of the preservatives, all other vehicle ingredients were first assumed to be inert with respect to any effects on fibroblast cells. We considered a vehicle "acceptable" when the assay showed cell growth stimulation by

Table II. Comparison of Vehicles and Preservative Systems Using First-Week Results of the Microbial Preservative Challenge Test

Vehicle type	Preservatives	No. of species remaining at		
		24 hr	48 hr	7 days
Emollient cream	0.18% methyl- and 0.02% propylparaben, 7.5% propylene glycol	9	9	6
	0.3% methyl- and 0.2% propylparaben, 7.5% propylene glycol	10	7	5
	0.3% methylparaben, 0.2% sorbic acid, 5.5% propylene glycol	3	3	0
	0.02% quat-15, 1.0% benzyl alcohol, 7.5% propylene glycol	1	1	0
	0.2% sorbic acid, 1.0% phenoxyethanol, 5.5% propylene glycol	1	1	0
Hydrophilic cream	0.02% quat-15, 1.0% benzyl alcohol	7	4	1
	0.2% sorbic acid, 1.0% phenoxyethanol, 4.0% SD alcohol	3	3	0
Stearate cream	0.3% methyl- and 0.2% propylparaben	7	5	2
Thermosetting gel	0.18% methyl- and 0.02% propylparaben	10	10	10
Modified PEG ointment	No preservatives	4	3	1

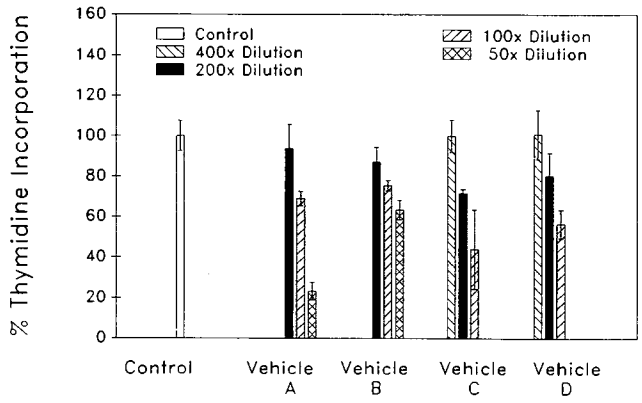

Fig. 2. Effect of semisolid vehicles on cell growth where fibroblast cells were treated with vehicle A, an anhydrous modified PEG ointment without preservatives; vehicle B, a thermosetting gel containing methylparaben/propylparaben (0.18:0.02) as preservative system; vehicle C, a stearate cream containing the same preservatives as vehicle B; vehicle D, a hydrophilic cream containing quaternium-15/benzyl alcohol (0.02:1.0) as preservative system; and control, wherein no vehicle was added.

TGF-α. Evidence of fibroblast activity was taken to imply that the preservatives in a particular formula do not interfere with the assay and can therefore be used for further TGF-α formulation.

A comparison of results from the two methods emphasizes our opposing needs. The preservative challenge measures cell death. On the other hand, the mitogenic assay measures cell growth. The complexity of using both assays as basis of vehicle selection for TGF-α is that a balance is needed with the preservative choice. As noted by Kabara (6), a balance must be achieved between killing microbial organisms in a product and injuring cells in the user (patient).

Formulations susceptible to microbial growth cannot be considered for further investigation even if the vehicles proved noninterfering with the mitogenesis assay. The parabens showed insufficient elimination of microbial organisms in the preservative challenge test (Table II). Further use of benzyl alcohol and phenoxyethanol was discontinued due to the marked inhibition of cell growth in their presence (Fig. 1). Additional evaluation was therefore limited to sorbic acid and quaternium-15 based on the combined results from the two assays.

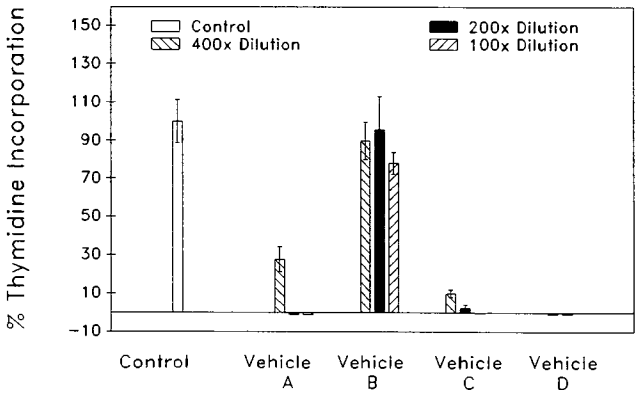

Fig. 3. Effect of semisolid vehicles, containing 7.5% propylene glycol and sorbic acid/quaternium-15 (0.2:0.02), on cell growth where fibroblast cells were treated with vehicle A, a emollient cream; vehicle B, a thermosetting gel; vehicle C, a stearate cream; vehicle D, a hydrophilic cream; and control, wherein no vehicle was added.

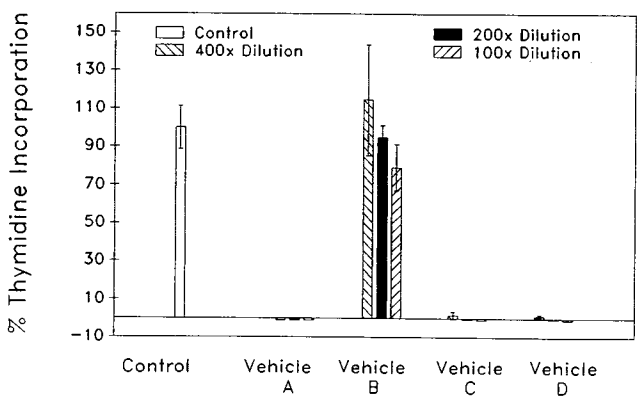

Fig. 4. Effect of semisolid vehicles, containing 7.5% PEG-8 and sorbic acid/quaternium-15 (0.2:0.02) on cell growth where fibroblast cells were treated with vehicle A, an emollient cream; vehicle B, a thermosetting gel; vehicle C, a stearate cream; vehicle D, a hydrophilic cream; and control, wherein no vehicle was added.

Two new sets of vehicles were prepared using only a sorbic acid/quaternium-15 combination as the preservative system. Sorbic acid has poor solubility in the formulations and requires a cosolvent, either propylene glycol or PEG-8. One set consisted of three o/w emulsions and a thermosetting gel, all of which contained propylene glycol. The second set consisted of the same four vehicles but with PEG-8 substituted for propylene glycol.

From the preservative challenge test results, we concluded that all prototypes are adequately preserved. Except for the emollient cream formulations which cleared up in 48 hr, there was no microbial activity in all vehicles by 24 hr. The mitogenic assay, however, reveals that only the thermosetting gel (Figs. 3 and 4) shows mitogenic activity of TGF-α. DiBiase and Rhodes (15) ran a study comparing a number of semisolid formulations for epidermal growth factor product development. They also observed that a poloxamer gel showed favorable results as a vehicle for a growth factor.

The assumption that the vehicle components, excluding preservatives, were inert with respect to cell growth had encouraged us to expect similar results from the proliferation assay in this case. We did not observe this. Since the mitogenic assay did not detect equal TGF-α stimulation among the vehicles that were tested, the earlier assumption that nonpreservative components were insert with respect to cell growth may not be correct. It is possible that interactions between some formulation excipients may have inhibited the mitogenic effect of TGF-α or enhanced the cell-killing ability of the preservative system. In either case, little or no cell growth would be detected. It is interesting that "inert" vehicles (creams, lotions, ointments) have been found to affect the rate of wound-healing in pigs (16).

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REFERENCES

1. S. E. Lynch, R. B. Colvin, and H. N. Antoniades. Growth factors in wound healing. *J. Clin. Invest.* 84:640–646 (1989).
2. M. Chvapil, J. A. Gaines and T. Gilman. Lanolin and epidermal growth factor in healing of partial-thickness pig wounds. *J. BCR* 9:279–284 (1988).
3. T. K. Hunt and F. B. La Van. Enhancement of wound healing by growth factors. *N. Engl. J. Med.* 321:111–112 (1989).
4. L. B. Nanney. Epidermal and dermal effects of epidermal growth factor during wound repair. *Soc. Invest. Derm.* 94:624–629 (1990).
5. R. Derynck. Transforming growth factor α. *Cell* 54:593–595 (1988).
6. J. J. Kabara. Cosmetic preservation—the problems and the solutions. In J. J. Kabara (ed.), *Cosmetic and Drug Preservation: Principles and Practice,* Marcel Dekker, New York, 1984, pp. 3–5.
7. L. Bolton *et al.* Repair and antibacterial effects of topical antiseptic agents in vivo. In H. I. Maibach and N. J. Lowe (eds.), *Models in Dermatology, Vol. 2. Dermatopharmacology and Toxicology,* S. Karger, Basel, 1985, pp. 145–158.
8. R. Baserga. Introduction to cell growth: Growth in size and DNA replication. In R. Baserga (ed.), *Tissue Growth Factors,* Springer-Verlag, New York, 1987, pp. 1–8.
9. W. Van Winkle. The fibroblast in wound healing. *Surg. Gynecol. Obstet.* 124:369–386 (1967).
10. M. R. McGinnis and M. G. Rinaldi. Antifungal drugs: mechanisms of action, drug resistance, susceptibility testing, and assays of activity in biological fluids. In V. Lorian (ed.), *Antibiotics in Laboratory Medicine,* 2nd ed., Williams & Wilkins, Baltimore, 1986, pp. 223–281.
11. K. J. Leister and L. M. Kozick. Development and validation of a cell culture-based bioanalytical assay. In *Pharmaceutical Technology Conference Proceedings,* Aster, Eugene, OR, 1991, pp. 331–347.
12. L. Bekeris *et al.* Propylene glycol as a cause of an elevated serum osmolality. *Am. J. Clin. Pathol.* 72:633–636 (1979).
13. R. D. Charles and P. J. Carter. The effect of sorbic acid and other preservatives on organism growth in typical nonionic emulsified commercial cosmetics. *J. Soc. Cosmet. Chem.* 10:383–394 (1965).
14. T. Parsons. A microbiology primer for the microbiology manager. *Cosmet. Toilet.* 105:73–77 (1990).
15. M. D. DiBiase and C. T. Rhodes. Investigations of epidermal growth factor in semisolid formulations. *Pharm. Acta Helv.* 66:165–169 (1991).
16. W. H. Eaglstein and P. M. Mertz. "Inert" vehicles do affect wound healing. *J. Invest. Dermatol.* 74:90–91 (1980).