Oral Mucosal Permeability and Stability of Transforming Growth Factor Beta-3 *In Vitro*

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Purpose. To investigate the permeability and localization of topically applied ^{125}I-TGF-β3 in porcine floor-of-mouth mucosa as a function of concentration and exposure.

Methods. The ^{125}I-TGF-β3 diluted in three different vehicles was applied to the tissue samples mounted in perfusion cells maintained at 37°C. Flux and K_p values were calculated from the perfusate collected over a 24 hour period. The quantity of ^{125}I-TGF-β3 present in the tissue was determined by horizontal sectioning and subsequent counting. The stability of ^{125}I-TGF-β3 in saliva and in the tissue was analyzed by SDS polyacrylamide gradient gel electrophoresis.

Results. ^{125}I-TGF-β3 was relatively stable in saliva and in the epithelium; approximately 50% of the total counts in the deeper epithelium were resident in the 25kDa TGF-β3 homodimer. A steady-state flux was reached ~6 hours post application and K_p value was $4.0 \pm 0.6 \times 10^{-6}$ (mean ± sem). Penetration of ^{125}I-TGF-β3 to the basal cell layer was concentration dependent but reached nanomolar concentrations even after extensive surface rinsing, representing over one-thousand fold the IC_{50} for epithelial cell cycle arrest.

Conclusions. The data suggest that topical application of TGF-β3 to the oral mucosa in an appropriate vehicle can provide effective therapeutic delivery to the tissue.

KEY WORDS: transforming growth factor; permeability; chemotherapy; oral mucosa; mucositis and pharmacokinetics.

INTRODUCTION

There has recently been increasing interest in delivering drugs both locally within the oral cavity and systemically across the oral mucosa (1–3). The oral mucosa is a well vascularized tissue with extensive areas of non-keratinized epithelium with a permeability greater than that of skin but less than the gastrointestinal lining (4). In both epidermis and oral epithelium it has been clearly demonstrated that the major barrier to substances diffusing across the tissue consists of lipid and glycolipid organized in the intercellular regions of the superficial or keratinized cell layers (4,5). Although several studies have examined factors influencing systemic oral mucosal delivery of small molecular weight compounds and peptides (1,2), there is considerably less data on local administration of such molecules to the oral epithelium. One of the major difficulties in examining oral mucosal delivery has been the choice of suitable models for the human oral mucosa, which differs from that of most small laboratory animals. We have shown that the pig oral mucosa is remarkably similar to human in terms of structure (4,6) and permeability (7).

Among cancer victims, one of the most distressing side-effects of anti-cancer therapy is mucositis and ulceration of the lining epithelium of the oral cavity and upper intestinal tract. This reflects damage to the epithelial cell progenitor compartment as a result of chemotherapy and radiotherapy (8,9). The use of growth factors to modulate the severity of oral and gastrointestinal mucositis, administered by both local and systemic routes, has recently been investigated (10–12). Local delivery minimizes systemic exposure of often pleotrophic growth factors and potentially increases the therapeutic index. Here we focus on the delivery of transforming growth factor beta-3 (TGF-β3), a polypeptide homodimer of molecular mass 25 kDa (13–15) by oral mucosal administration. For studies of drug distribution, the traditional measures of flux and permeability constant alone are not useful as they reflect the rate at which a compound passes across (or is cleared) from the total mucosal thickness whereas it is concentration at sites within the tissue (and, specifically, the progenitor cell compartment) that is relevant to the present studies. In this study we demonstrate that TGF-β3 is capable of penetrating across the full thickness of the oral epithelium and is relatively stable both in saliva and after traversing the epithelium. Further, we show that TGF-β3 can penetrate to the basal epithelial cell compartment in quantities that would be sufficient to elicit a biological response.

MATERIALS AND METHODS

Preparation of TGF-β3 and ^{125}I-TGF-β3

Recombinant TGF-β3 was prepared by the refolding and dimerization of TGF-β3 monomer expressed in E.coli (16,17); this has an IC_{50} in vitro of ~50pg/ml in a standard CCL64 growth inhibition assay (18). ^{125}I-TGF-β3 was labelled by the Bolton-Hunter method (NEN™ Life Science Products, Boston MA) to a specific activity of ~2200Ci/mmole, purified by C4 reverse phase HPLC by acetonitrile/0.1% TFA gradient elution, buffer exchanged into 50mM sodium acetate, 50mM n-acetyl-methionine, 5% sucrose and 0.25% BSA, lyophilized, dissolved in 2mM HCl, 10% ethanol and stored frozen at −70°C.

Tissue Preparation

Porcine floor-of-mouth and buccal mucosa was excised at slaughter and within 2 hours of removal, discs of tissue 8–10 mm in diameter were mounted in continuous flow perfusion chambers, exposing an area of epithelial surface of approximately 20 mm^2 (19). The perfusion chambers were placed in water heated blocks that could be maintained at 37°C and PBS (0.01 M, pH 7.6) pumped through the receiving chambers across the connective tissue side of the biopsies at approximately 1.5 ml/hr. Perfusate was collected in vials at 1 or 2 hr intervals for periods up to 24 hrs using a fraction collector. We have previously shown that penetration of compounds across oral mucosa is a passive diffusion process and not influenced by tissue vitality (20), thus permitting valid flux measurements up to 24 hours.

^{125}I-TGF-β3 was diluted 1:10 in three different vehicles: (i) phosphate buffered saline (PBS), (ii) an acidic viscous solvent

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containing glycine, pH 3.5 and 0.1% gelatin (gel-1) and (iii) a neutral viscous solvent containing 0.1% methocellulose, pH 7 (gel-2). Each formulation was placed on the epithelial surface within 1 hour of preparation; it was subsequently removed after the tissue had been exposed for times varying between 1 and 60 minutes for the gels and up to 24 hours for the PBS. The tissue surface was subsequently rinsed with PBS.

Measurements of Flux and K_p for ^{125}I-TGF-β3

Vials containing perfusate were mixed with scintillation fluid and counted in a Beckman Gamma Counter until a 2σ value of 2% was obtained; quenching was corrected. A minimum of 5 specimens was used for each set of experimental conditions. All values were plotted to show the cumulative penetration of the compounds with time across the oral mucosa and flux was calculated per sq. cm. of tissue per minute. A permeability constant (K_p) was calculated from the relationship: $K_p = J/\Delta C$ where J is the flux at steady state, and ΔC is the average concentration gradient expressed in cpm/cm^3. The units of K_p are cm/min.

Quantitation of ^{125}I-TGF-β in Mucosal Tissue

After removing tissue discs from the chambers, they were mounted on cork disks and snap-frozen in liquid nitrogen for sectioning in a cryostat at −20°C. Sections 10 μm thick were then cut parallel to the epithelial surface (en-face) and collected and pooled so as to represent three tissue strata; outer epithelial layers (150 μm), deeper epithelial layers and basement membrane (150 μm) and residual connective tissue. The average thickness of porcine floor-of-mouth epithelium is 250 μm (4). The exact histological location of each series of horizontal sections was checked by preparing histological cross-sections of the tissue, as described by Cox and Squier (21). The pooled tissue sections were solubilized and counted so as to determine the relative proportion of ^{125}I-TGF-β3 in different tissue layers at different times.

Stability Measurements of ^{125}I-TGF-β3 in Saliva and Oral Mucosa

Specific detection of ^{125}I-TGF-β3 in tissue and biological fluids requires methods which discriminate between intact and proteolyzed or hydrolyzed ^{125}I-TGF-β3. Here we have used SDS-polyacrylamide gel electrophoresis to establish the proportion of TGF-β3 which was still in the biologically active homodimer form in the epithelium or in saliva.

The stability of ^{125}I-TGF-β3 in human saliva was measured *in vitro* at 37°C with respect to time. Human saliva was collected, centrifuged at 13,000 × g for 5 minutes at RT and the supernatant transferred to a 1.5 ml siliconized microfuge tube. One μCi of ^{125}I-TGF-β3 in a one μl volume (containing 2.8 μg TGF-β3 in 20% isopropanol, 20 mM acetic acid) was added to 100 μl of cleared human saliva, to the gel-1 formulation or to phosphate buffered saline, and incubated at 37°C. Twenty microliter samples were collected at 0 minutes, 10 minutes, 60 minutes, 4 hours and 24 hours and analyzed by SDS polyacrylamide gradient gel electrophoresis.

To examine stability in the mucosa, representative fractions of the tissue exposed to the gel-2 formulation were analyzed by SDS-gel electrophoresis to assess whether the ^{125}I-TGF-β3 was intact and the iodine radiolabel retained. Tissue samples were harvested after exposure to ^{125}I-TGF-β3 in gel-2, sectioned into the three strata described above, incubated in SDS sample buffer, sonicated for 20 seconds, boiled, centrifuged for 5 minutes at 10,000 × g, applied to a 5 to 20% gradient SDS-PAGE gel and exposed to X-ray film. A standard preparation of ^{125}I-TGF-β3 and prestained protein molecular weight markers were included as controls. Using the methods described above, the effect of four different parameters were examined as follows:

The Effect of Temperature on Permeability of TGF-β3 in Mucosa

To establish initial kinetics of TGF-β3 penetration across the mucosa and to determine the effect of temperature on the permeability of TGF-β3 in mucosa (Study 1), ^{125}I-TGF-β3 (2.71×10^8 cpm/ml; 100 μg protein/ml) in PBS was applied to the epithelial surface of floor of mouth mucosa. The perfusion chambers were maintained at 25°C or 37°C and the quantity appearing in the receptor chamber determined over 24 hours.

Effect of Vehicle on Permeability of TGF-β3 in Mucosa

The relationship between vehicle characteristics and the permeability of ^{125}I-TGF-β3 in oral mucosa (Study 2) was examined by determining the mean flux (in cpm/cm^2/min) of ^{125}I-TGF-β3 in the three vehicles and the amounts at different depths in the mucosa by counting activity of ^{125}I-TGF-β3 (in cpm). In order to replicate the short exposure times that might be encountered when an agent is placed on the surface of the oral mucosa in vivo, tissue samples were incubated with ^{125}I-TGF-β3 for 1 hour, the ^{125}I-TGF-β3 removed from the epithelial surface, rinsed once with PBS and subsequently a PBS solution was placed on the surface to keep it moist for the remaining time. Samples were collected from the receptor side over a period of 24 hours and counted. Subsequently, specimens were frozen at 24 hours, sectioned horizontally, and the tissue counted to determine the localization of TGF-β3 in four different strata. Seven replicates were used for each treatment condition.

Effect of Exposure Time on Permeability of TGF-β3 in Mucosa

In order to further define the tissue availability of TGF-β3 after relatively short exposure times (Study 3) ^{125}I-TGF-β3 in gel-1 or gel-2 was placed on the epithelial surface of the tissue in the chambers for periods of 1, 2, 5, 10, or 60 minutes, removed and the epithelial surface rinsed once with PBS and subsequently a PBS solution was placed on the surface to keep it moist for the remaining time, up to 5 hours, before the experiment was terminated. The ^{125}I-TGF-β3 was diluted into 100 μg/ml (3.9 μM) cold TGF-β3, and 9 μCi and 3 μCi ^{125}I-TGF-β3 (100 μg protein) were used for the 1–10 and 60 minute time points, respectively. The total amount applied was determined by counting the drug removed at the end of the application period to which was added the counts present in the PBS used to rinse the epithelial surface plus the counts in the PBS solution used to keep the surface moist. The tissue was subsequently frozen and sectioned and the distribution of the TGF-β3 in each of three strata was determined by counting as already described.

In order to quantify the amount of protein present in the tissue, the counts obtained were converted to nM of protein present in a given layer. The specific activity of the ^{125}I-TGF-β3 was 0.95 μCi/μg (0.32 μCi/μg for 60 minute time point) where 5.5 × 104 cpm = 1 pmole TGF-β3 (for 1 to 10 minute time points) and 1.8 × 104 cpm = 1 pmole TGF-β3 (for 60 minute time point).

Effect of Concentration and Tissue Rinsing on Permeability of TGF-β3 in Mucosa

We hypothesized that TGF-β3 was passively diffusing through the mucosal interstitial spaces according to the laws of mass action. If this were true, TGF-β3 permeability should be concentration dependent. The application of 100 μg and 5 μg ^{125}I-TGF-β3 (10 μ Ci) in gel-2 was compared (Study 4) using five replicates for each. For the 100 μ/ml groups, ^{125}I-TGF-β3 was diluted into 100 μg/ml (3.9 μM) cold TGF-β3 and approximately 6 μgCi in 9.5 μg TGF-β3 was applied to each mucosal sample. For the 100 μg/ml groups, the specific activity of the material applied was 1.0 μCi/μg where 5.7 × 10^4 cpm = 1 pmole TGF-β3. For the 5 μ/ml group, ^{125}I-TGF-β3 was diluted into 5 μg/ml (0.2 μM) cold TGF-β3 and approximately 10 μCi in 0.48 μg TGF-β3 was applied to each pig mucosa sample. For the 5 μg/ml groups, the specific activity of the material applied was 20.8 μCi/μg where 1.2 × 10^6 cpm = 1 pmole TGF-β3. After a 2 minute exposure, the surface was rinsed 1 or 5 times and a PBS solution was placed on the surface to keep it moist until the tissue was harvested at 5 hours.

Statistical Comparisons

Comparisons of the flux, K_p and tissue distribution values for the experimental conditions described above were made using ANOVA and Tukey's post-hoc test to identify means that were significantly different among the values obtained for the three formulations.

RESULTS

Stability of ^{125}I-TGF-β3 in Saliva and Oral Mucosa

The stability of ^{125}I-TGF-β3 in human saliva measured *in vitro* at 37°C with respect to time are shown in Fig. 1A. Some inconsistency in the loading of the saliva samples was noted (particularly at the 10 minute time point), which represents the difficulty of accurately dispensing the viscous salivary fluid. The additional bands above and below the ^{125}I-TGF-β3 band at the 10 minute time point were also observed by SDS-gel electrophoresis of the non-iodinated starting material and probably reflect traces of TGF-β3 monomer (13kDa) or TGF-β3 aggregates of higher molecular weight (~52kDa). A decrease in ^{125}I-TGF-β3 concentration was observed by 4 hours, with a further decrease noted by 24 hours. In control incubations, no loss of ^{125}I-TGF-β3 was observed in the gel-1 formulation at 37°C over 24 hours (Fig. 1B). A small reduction in ^{125}I-TGF-β3 concentration with respect to time was observed in the PBS formulation (Fig. 1C), which may simply reflect partitioning to the siliconized wall of the microfuge tube. In contrast to incubation in gastric fluid (data not shown), no proteolytic degradation of ^{125}I-TGF-β3 in human saliva could be detected at any time point tested.

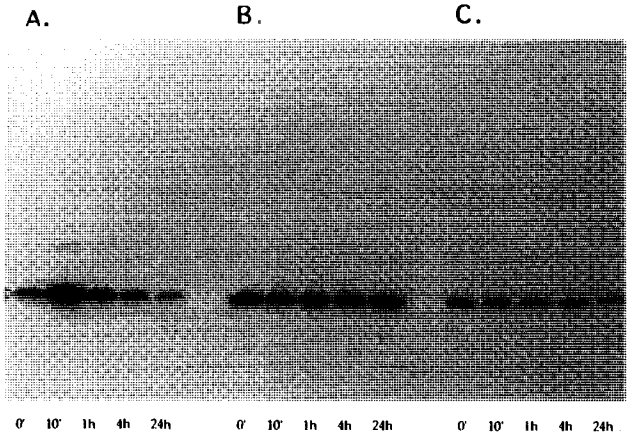

Fig. 1. Effect of human saliva on the electrophoretic mobility of ^{125}I-TGF-β3 with respect to time. ^{125}I-TGF-β3 was incubated with either human saliva (panel A), Gel-1 (panel B) or phosphate buffered saline (PBS; panel C) for the times indicated (0, 10 minutes, 1 hour, 4 hours, or 24 hours) at 37°C. Samples subsequently were subjected to 5–20% gradient SDS-gel electrophoresis and autoradiography. The position of the 25kDa ^{125}I-TGF-β3 homodimer is indicated by the arrow.

The results of the analysis of the representative fractions of the tissue exposed to gel-2 are shown in Fig. 2. In lane 1, 3096 cpm of mucosal extract was applied and 1483 cpm, or 48% of the counts, were contained within the TGF-β3 25kDa homodimer; in lane 2, 487 cpm were applied and 215 cpm, or 44% of the counts, were contained within the TGF-β3 homodimer; in lane 3, 341 cpm were applied and 144 cpm, or 42% of the counts, were contained within the TGF-β3 homodimer. Data obtained from eight such experiments indicated that approximately 50% of the ^{125}I-TGF-β3 extracted from the tissue was intact 25kDa dimer, with some 13kDa monomer also present.

Study 1. Effect of Temperature on Permeability of TGF-β3 in Mucosa

Although ^{125}I-TGF-β3 could be detected in the perfusate at the first sampling time (2 hours) a steady state was not reached until 6 hours at 37°C and approximately 10 hours at 25°C. Increasing chamber temperature from 25°C to 37°C significantly increased K_p approximately two fold, the mean values (± sem) being 2.0 ± 0.3 × 10^{-6} at 25°C and 4.0 ± 0.6 × 10^{-6} at 37°C.

Study 2. Effect of Vehicle on Permeability of TGF-β3 in Mucosa (Table 1)

TGF-β3 in gel-1 reached a flux of approximately 1 cpm/cm^2/min at 1 hour and maintained a value close to this level for 24 hours, whereas TGF-β3 in PBS and gel-2 both reached a similar maximum flux (7 cpm/cm^2/min) at 3 hours and 1 hour respectively (Fig. 3). Thereafter, there was a decline that varied between the two formulations to a lower flux value that was maintained for the subsequent 20–24 hours. Mean values (± sem) for the flux over these latter times were 3.24 ± 0.51,

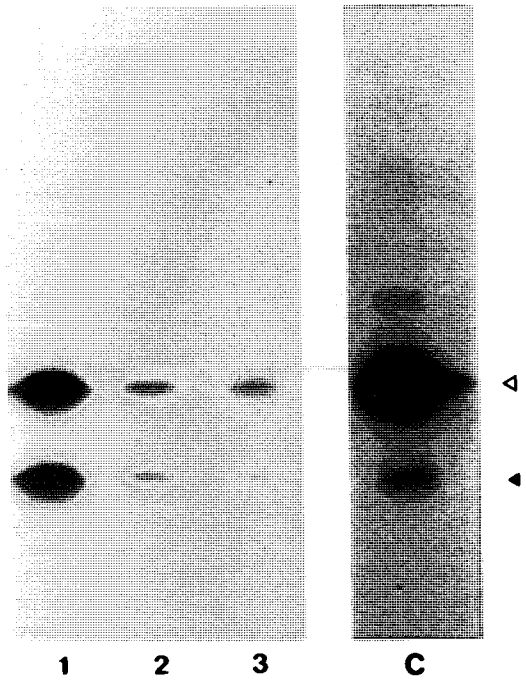

Fig. 2. SDS-gel electrophoresis of porcine floor-of-mouth mucosal sample lysates exposed to ^{125}I-TGF-β3. Tissue samples roughly corresponding to the outer, basal and connective tissue layers were lysed by sonication in SDS sample buffer, followed by autoradiography with X-ray film (Kodak X AR5). Gel-2 formulation was used to apply the ^{125}I-TGF-β3 to the porcine floor-of-mouth mucosa. Lane 1: outer epithelial layer-sample 1 (3096 cpm); lane 2: basal epithelial layer-sample 1 (487 cpm); lane 3: connective tissue layer-sample 1 (341 cpm); lane C: control ^{125}I-TGF-β3. Note that the applied cpm do not reflect the penetration of ^{125}I-TGF-β3 within that tissue layer. The position of the biologically active 25kDa ^{125}I-TGF-β3 protein dimer is indicated by the open arrow; the position of the 13kDa ^{125}I-TGF-β3 monomer (biologically inactive) is indicated by the solid arrow.

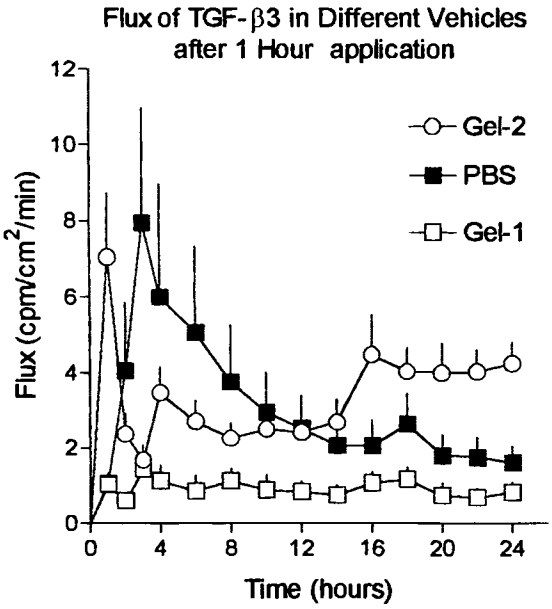

Fig. 3. Flux (cpm/cm^2/min) of TGF-β3 in different vehicles across oral mucosa after one hour application. Bars represent standard error.

0.93 ± .06 and 3.42 ± 0.37 for PBS, gel-1 and gel-2, respectively. Statistical comparisons (ANOVA) indicated that flux from gel-2 and PBS were significantly greater than from gel-1 ($p < 0.001$). There was no significant difference in flux between PBS and gel-2.

Counts representing the quantity of TGF-β3 that was present in the tissue sections (Table 1) reflected the flux values described above. A greater proportion of the TGF-β3 that was applied was detected in the tissue using gel-2 (0.53%) than when using PBS (0.22%) or gel-1 (0.12%). In terms of the concentration in the various tissue layers, it is apparent that a large amount (<50% of the total in the tissue) remains in the outer, epithelial layer after 24 hours.

Table 1. Tissue Distribution of TGF-β3 in Different Vehicles[a]

Treatment (after 1 hr)	Cpm applied to surface	Depth of tissue fraction	Mean cpm ± sem	Percent of total tissue counts	Percent of amount applied
Gel-1 removed	4892562	60 μ	3298 ± 624	54%	0.07%
		90 μ	684 ± 189	11%	0.01%
		90 μ	400 ± 83	7%	0.01%
		CT	1684 ± 280	28%	0.03%
		Total in tissue:	6066 ± 574	100%	0.12%
PBS removed	5440800	60 μ	4469 ± 1122	37%	0.08%
		90 μ	2037 ± 770	17%	0.04%
		90 μ	1266 ± 639	11%	0.02%
		CT	4193 ± 820	35%	0.08%
		Total in tissue:	11966 ± 1729	100%	0.22%
Gel-2 removed	4870689	60 μ	12350 ± 2595	48%	0.25%
		90 μ	8169 ± 2551	32%	0.17%
		90 μ	3118 ± 1330	32%	0.06%
		CT	2017 ± 371	8%	0.04%
		Total in tissue:	25654 ± 4283	100%	0.53%

[a] Relative distribution of TGF-β3 in oral mucosa sectioned horizontally to the epithelial surface after application in different vehicles; tissue samples were harvested after 24 hours in perfusion chambers. Cpm (± standard error) represents the amount of TGF-β3 in tissue fractions at different depths expressed in microns (μ). Connective tissue fraction (CT) includes the entire lamina propria.

Study 3. Effect of Exposure Time of Permeability of TGF-β3 in Mucosa (Table 2)

The value for the TGF-β3 present in the PBS solution was minimal for gel-1, representing only about 2% of the total counts applied, but represented 17% of the total counts for gel-2.

The penetration of TGF-β3 in gel-1 (Table 2) shows a fairly predictable pattern with time, i.e., there is more drug at greater depths in the tissue with increasing exposure. The counts in the tissue after TGF-β3 was applied in gel-2 are strikingly different, particularly at the three shortest exposure times (1, 2, and 5 minutes). The greater amounts in the tissue and the relative similarity of the counts even after different time periods might be explained by drug adhering at the surface after its removal. This interpretation is supported by the greater number of counts in the surface PBS solution removed after 5 hours (see above), which could represent back diffusion of drug that had not diffused into the tissue after the initial heavy loading of the surface layer. This would contribute to a greater flux into the deeper layers.

The tissue region of greatest interest is represented by the second tissue fraction, 150 μm thick, which contains the deepest epithelial layers including the proliferative zone. The volume of this layer in an 8 mm diameter tissue disk was estimated to be approximately 7.5 μl. The results from these experiments (Table 2) show significant quantities (2 minute sample amounting to 44 nM or 20,000X the in vitro IC_{50} activity) of TGF-β3 penetrate to the basal cell layer even after only 1 or 2 minute exposure to the gel-2 formulation. Note that despite surface rinsing, substantial levels of TGF-β3 remained in the tissue for the entire five hour incubation period.

Table 2. Tissue Distribution of TGFβ3 After Application for Different Times[a]

Treatment	Cpm applied to surface	Depth of tissue fraction	Mean Cpm ± sem	Percent of total tissue counts	Percent of amount applied
		150 μ	8567 ± 944	49.36%	0.33%
Gel-1 applied for 60 minutes	2613020	150 μ	4712 ± 984	26.32%	0.18%
		CT	4227 ± 325	24.31%	0.16%
		Total in tissue:	17506 ± 1190	100%	0.67%
		150 μ	32301 ± 17885	43.34%	0.39%
Gel-1 applied for 10 minutes	8348273	150 μ	22752 ± 7842	39.45%	0.27%
		CT	8247 ± 2353	17.21%	0.10%
		Total in tissue:	63299 ± 19186	100%	0.76%
		150 μ	19091 ± 5523	53.33%	0.22%
Gel-1 applied for 5 minutes	8595869	150 μ	12498 ± 4193	32.58%	0.15%
		CT	3941 ± 729	14.00%	0.05%
		Total in tissue:	35530 ± 9400	100%	0.41%
		150 μ	7852 ± 1699	54.99%	0.09%
Gel-1 applied for 2 minutes	8543563	150 μ	4162 ± 1262	28.98%	0.05%
		CT	1953 ± 364	16.03%	0.02%
		Total in tissue:	13967 ± 2019	100%	0.16%
		150 μ	11533 ± 1804	61.11%	0.13%
Gel-1applied for 1 minute	8666859	150 μ	6539 ± 1638	28.52%	0.08%
		CT	2515 ± 847	16.37%	0.03%
		Total in tissue:	20588 ± 3590	100%	0.24%
		150 μ	21162 ± 3401	51.62%	0.56%
Gel-2 applied for 60 minutes	3846092	150 μ	10887 ± 2449	26.00%	0.27%
		CT	10173 ± 2391	22.38%	0.26%
		Total in tissue:	42222 ± 5093	100%	1.09%
		150 μ	34306 ± 5073	73.22%	0.39%
Gel-2 applied for 10 minutes	8945587	150 μ	7692 ± 2913	16.13%	0.10%
		CT	4988 ± 650	10.65%	0.06%
		Total in tissue:	46986 ± 5718	100%	0.54%
		150 μ	34236 ± 5438	45.02%	0.43%
Gel-2 applied for 5 minutes	8097469	150 μ	25835 ± 6081	31.09%	0.34%
		CT	20831 ± 6281	23.72%	0.28%
		Total in tissue:	80902 ± 14750	100%	1.05%
		150 μ	30280 ± 4297	41.31%	0.35%
Gel-2 applied for 2 minutes	8550012	150 μ	18346 ± 5251	23.81%	0.21%
		CT	25692 ± 4107	34.88%	0.31%
		Total in tissue:	74318 ± 7368	100%	0.87%
		150 μ	46420 ± 6000	50.83%	0.54%
Gel-2 applied for 1 minute	8693458	150 μ	22287 ± 2402	26.40%	0.26%
		CT	22667 ± 6710	22.77%	0.26%
		Total in tissue:	91374 ± 11658	100%	1.06%

[a] Relative tissue distribution of TGF-β3 in oral mucosa sectioned horizontally to the epithelial surface; samples were harvested after 5 hours in perfusion chambers. See legend for Table 1.

Study 4. Effect of Concentration and Tissue Rinsing on Permeability of TGF-β3 in Mucosa (Table 3)

Decreasing the concentration of TGF-β3 applied from 100 μg/ml to 5 μg/ml substantially reduced the amount of TGF-β3 penetrating into the basal cell layer from 5 nM to 0.8 nM (Table 3). In addition, the effect of rinsing the ^{125}I-TGF-β3 from the porcine floor-of-mouth mucosal surface either once or five times with PBS for 60 seconds was investigated to mimic the cleansing effects of salivary flow in the oral cavity. Rinsing reduced the amount present in the deeper strata from 56 nM to 5 nM (Table 3).

DISCUSSION

The permeability of porcine floor-of-mouth to ^{125}I-TGF-β3 was investigated by determining flux and K_p and by measuring the amount of ^{125}I-TGF-β3 in different tissue strata. Several important methodological points that relate to the interpretation of the data should be noted here. First, the ^{125}I-TGF-β3 preparation used here exhibited identical biological activity to unlabelled TGF-β3 in a standard epithelial cell proliferation assay (18). Second, TGF-beta was iodinated using conditions for iodination of TGF-beta to high specific activity (>3000Ci/mmol) suitable for receptor binding studies (personal communication-NEN Custom Synthesis Group, Billerica, MA). Third, ^{125}I-TGF-β3 and TGF-β3 showed identical retention times when chromatographed by reverse-phase HPLC (data not shown), suggesting that ^{125}I labeling did not markedly alter the lipophilicity of TGF-β3. Finally, the radiolabel, with a mass of 125 Daltons, would contribute less than 1% to the total mass of ^{125}I-TGF-β3. Thus, we believe that the vast increase in sensitivity of the assays gained by using iodinated TGF-β3 in oral mucosa far outweighs any potential alteration in physical behavior of the labelled compound.

For a therapeutic compound to be successfully delivered to its site of action in the oral mucosa, it must resist degradation in the mouth and during penetration across the oral tissue. Saliva in the oral cavity has very little proteolytic activity and it is likely that a molecule as large as TGF-β3 will traverse the epithelial barrier by a paracellular route (20,21) thus avoiding intra-cellular proteases. No proteolysis of ^{125}I-TGF-β3 was apparent in human saliva and when the deep epithelial layer was extracted and analyzed using SDS gel electrophoresis, approximately 50% of the total counts were resident in the 25kDa dimer (Fig. 2).

Horizontal sectioning of the frozen tissue permits localization and quantification of a labelled compound in the different histological strata. From knowledge of the specific activity of ^{125} I-TGF-β3, it is possible to convert cpm to reflect molar quantities in the tissue. By this method it was apparent that increased exposure of the tissue surface to the compound did not result in a corresponding increase in ^{125}I-TGF-β3 in the deeper epithelial layers. For example, drug content only increased twenty-fold for exposure times that increased by up to sixty-fold. The phenomenon, by which different duration of surface application of TGF-β3 (Table 2) all result in a similar order of tissue loading, can be ascribed to the "reservoir effect" of the epithelium. Although this phenomenon has been inferred from kinetic studies in oral mucosa, (23,24,25) it is poorly understood. The permeability barrier in non-keratinized epithelia consists of groups of lipid lamellae located in the intercellular spaces of the superficial epithelial layer (22,26). These limit the penetration of non-polar compounds, which may become trapped in a non-lipid or fluid lipid intercellular compartment of the barrier layer. Thus the surface layer of the epithelium may take up a compound relatively rapidly (depending on its lipophilicity and the nature of the vehicle). Once saturated, this layer cannot adsorb any more material, regardless of the duration of exposure. Subsequently, the adsorbed material diffuses into the deeper layers of the tissue at a fairly constant rate that is more dependent on the capacity (or loading) of the reservoir than the duration of surface exposure. However, it is apparent that the vehicle also plays a critical role in this process. For example, TGF-β3 was released from the gel-1 vehicle at a rate proportional to the duration of exposure between 1 and 10 minutes, and a reservoir effect was only evident between 10 and 60 minutes (Table 2). On the other hand, the superior permeabilizing properties of gel-2 permit a rapid uptake, so that the reservoir is saturated in as short a time as 1 min. and longer exposures make little difference to tissue loading (Table 2). This phenomenon also explains the similar order of counts found in the tissue exposed to gel-2 (~1%) regardless of the amount of compound and duration of application (Table 2). Contributing to the "reservoir effect," and not distinguishable from it in this study, is the adherence of TGF-β3 at the epithelial surface. Gel-2 contains methocellulose, a muco-adhesive that could contribute to persistence of TGF-β3 in close proximity to the mucosal surface. These data demonstrate that permeability of TGF-β3 in oral mucosa may be significantly modulated by formulations that influence adhesion which, in turn, affects penetration.

Materials adherent to the oral mucosa will be subject to the effect of continual salivary flow in the oral cavity. The effect of rinsing indicated that even after five rinses, the concentration of TGF-β3 in the tissue is more than a thousand-fold the IC_{50} for cell cycle arrest in the deeper epithelial layers. Furthermore, as we have already discussed, ^{125}I-TGF-β3 appears to be stable in human saliva for at least 1 hour at 37°C. Reduction of the TGF-β3 concentration from 100 μg/ml to 5 μg/ml reduced the concentration of TGF-β3 in the basal cell layer from 5 nM to 0.8 nM respectively. However, this latter concentration is still 400 times the biologically effective dose, suggesting that lower applications may be clinically effective in man.

Local delivery of therapeutic compounds to the oral mucosa has the advantage that the oral mucosa is readily accessible and contains extensive regions which are non-keratinized and therefore relatively permeable so that the methodology described here provides a promising approach to treating mucositis, which is a distressing mucosal condition. However, the

Table 3. Effect of Rinsing and Concentration on Basal TGF-β3 Delivery[a]

Treatment (rinse)	TGF-β3 applied	Basal TGF-β3
1X	100 μg ml	56 nM
5X	100 μg ml	5 nM
5X	5 μg ml	0.8 nM

[a] nM concentration of TGF-β3 in basal cell layer after 2 minute exposure to gel-2 and subsequent rinsing. Samples were harvested after 5 hours in perfusion chambers.

high vascularity, absence of first pass hepatic clearance and relatively large surface area of the oral mucosa also suggests that this may be a valuable route for systemic therapy using bioactive peptides and proteins.

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