Topical Tritiated Thymidine for Epidermal Growth Fraction Determination

Jeffrey A. Klein, M.D., Jerry L. McCullough, Ph.D., and Gerald D. Weinstein, M.D.
Department of Dermatology, California College of Medicine, University of California, Irvine, Irvine, California, U.S.A.

Direct autoradiographic identification of the epidermal growth fraction (GF) requires the delivery of tritiated thymidine ([3H]dThd) to the skin during the time interval of an entire cell cycle. The GF in normal human epidermis has not been directly measured using this technique because the systemic infusion of radioactive [3H]dThd in benign skin conditions is precluded by ethical considerations. Studies were undertaken to assess the feasibility of measuring the epidermal GF in vivo by the topical delivery of [3H]dThd. The percutaneous penetration of [3H]dThd in various vehicles was evaluated to select an effective topical delivery system. A vehicle consisting of Azone, isopropanol, and water (2:49:49) was the best of 4 different vehicles tested.

The optimal penetration of [3H]dThd, with respect to the concentration of Azone over a range of 0–4%, was achieved at 2%. During the initial 24 h following a single topical application of [3H]dThd to hairless mice the labeling increased linearly with time. In vivo studies in hairless mice produced a GF of 95% by both continuous systemic [3H]dThd infusion, and by twice daily topical [3H]dThd. Azone vehicles induced epidermal hyperplasia which was minimized by lowering the Azone concentration and by decreasing the frequency of applications from 24 to 48 h. These studies establish the rationale for using topical delivery of [3H]dThd for the in vivo measurement of epidermal GF. J Invest Dermatol 86:406-409, 1986

The growth fraction (GF) is an important kinetic parameter in experimentally defining the rate of epidermal cell reproduction. The GF of a cell population is by definition that fraction of cells actually in the process of proliferation [1]. The traditional method for measuring GFS requires a continuous systemic infusion of tritiated thymidine ([3H]dThd) and sequential biopsies for autoradiographic analysis to detect cells synthesizing DNA during the [3H]dThd infusion. When the percentage of labeled basal cells attains a plateau, essentially all actively proliferating cells have been labeled. The height of this plateau, expressed as the percentage of labeled cells, is an estimate of the GF for the cell population under investigation.

Systemic infusion of [3H]dThd has been used to measure GF in animals [2] and terminal cancer patients [3]. However ethical considerations preclude systemic [3H]dThd infusion in humans with benign skin conditions. Local intradermal injection of [3H]dThd given sequentially over the entire cell cycle greatly reduces the dosage of radioactive labeled drug needed to measure cell cycle time (Tc). The brief Tc in a psoriatic plaque (36 h) [4] permits the use of multiple intradermal [3H]dThd injections in measuring the GF [2]. However, in healthy human skin the repeated trauma of multiple injections into the same site during the normal 13-day cell cycle would more than likely produce a proliferative “wounding” response, and therefore an inaccurate measurement of GF. Topical delivery of [3H]dThd should minimize the systemic exposure of this radioactive compound and also avoid the trauma of repeated injections. The present study was therefore undertaken to assess the feasibility of using topical delivery of [3H]dThd as an alternative in vivo method for measuring epidermal GF. Our experiments were designed so as: (1) to select an effective vehicle for the topical delivery of [3H]dThd using an in vitro percutaneous diffusion model; and (2) to investigate the time course of autoradiographic labeling in hairless mouse epidermis in vivo.

MATERIALS AND METHODS

[methyl-3H]Thymidine, with specific activity (SA) of 25.0 and 47.0 Ci/mmol, was purchased from Amersham (Arlington Heights, Illinois). 1-Dodecylazacycloheptan-2-one (Azone) and N-methyl pyrrolidone were gifts from Nelson Laboratories (Irvine, California). Vehicle N (alcohol 47.5%, water, laureth-4, isopropyl alcohol 4%, propylene glycol) was a gift from Neutrogena Corp. (Los Angeles, California). Glass diffusion chambers with 5-mm inside diameter were purchased from Crown Glass (Somerville, New Jersey). Alzet miniosmotic pumps (model 2001) were purchased from Alza Corp. (Palo Alto, California). Hairless mice were of the Skh:hr-1 strain obtained from Temple University.

In Vitro Studies In vitro studies used full-thickness excised normal human abdominal autopsy skin, stored at −20°C, thawed, and placed on glass diffusion chambers. A topica l dose of 10 μCi of [3H]dThd (SA = 25) in 50 μl of vehicle was applied to each chamber. For each source of cadaveric skin at least 3 diffusion cells were run for each test condition. The reservoir below the dermis contained phosphate-buffered saline. The dermal reservoirs were stirred constantly at 28°C. At each time point the entire contents of the dermal reservoir was emptied completely by as-

Manuscript received April 1, 1985; accepted for publication October 21, 1985.

Supported in part by United States Public Health Service Grant AM 27110 from the National Institutes of Health and by the Southern California Dermatology Foundation.

*This work was presented in part at the Annual Meeting of the Western Regional Section of The Society for Investigative Dermatology, Carmel, California, February 9, 1983.

Reprint requests to: Jerry L. McCullough, Ph.D., Department of Dermatology, California College of Medicine, University of California, Irvine, Irvine, California 92717.

Abbreviations:
GF: growth fraction
LI: labeling index
SA: specific activity
[3H]dThd: tritiated thymidine
Tc: cell cycle time
piration. The radioactivity of this solution was measured using a scintillation counter to obtain the total cpm that had diffused into the reservoir between time points. The amount of residual [³H]dThd in the dermis at the conclusion of a percutaneous absorption experiment was determined by removing the epidermis, digesting the dermal portion of the punch biopsy in NaOH, and measuring the radioactivity.

In Vivo Studies In the in vivo experiments 20 μCi of [³H]dThd in 50 μl of vehicle were applied to the backs of hairless mice. Funnel-shaped collars were placed on the mice to prevent them from ingesting the applied [³H]dThd. Biopsies from the back and abdomen were processed for autoradiography. Autoradiographic analysis involved tissue fixation of biopsies in 10% buffered formalin and embedding in paraffin; 6 μm-thick tissue sections on slides were dipped in Kodak NTB2 emulsion and developed after 8 weeks. The number of labeled basal cells per 1000 basal cells was counted under oil immersion and this fraction reported as the labeling index (LI).

To determine the effects of a single application of [³H]dThd, SA = 25 Ci/mmole, on epidermal LI, groups of 2 mice were sacrificed and biopsied sequentially at times 3, 6, 12, and 24 h. To compare the effect on epidermal LI of varying the SA of the topical [³H]dThd, 2 groups of 15 mice received a single application of [³H]dThd, with SA of either 25 or 47 Ci/mmole, at time (t) = 0. Three mice from each group were sacrificed and biopsied daily on days 1–5.

To compare topical vs systemic delivery of [³H]dThd, SA = 25 Ci/mmole, over a 1-week period, one group of 14 mice was given twice daily (8 AM and 5 PM) topical applications, with 2 groups of 2 mice being sacrificed and biopsied daily (6 PM). A second group of 3 mice was implanted with intraperitoneal osmotic diffusion pumps containing [³H]dThd (SA = 25 Ci/mmole) which diffused continuously into the systemic circulation. Dorsal skin was biopsied on days 1, 2, 4, 5, 6, and 7. On day 1 an additional biopsy was taken from the abdomen of each mouse. Epidermal labeling indices were compared after once-daily and once-every-other-day dose schedules using topical [³H]dThd (SA = 47 Ci/mmole). In one group daily dosages were applied at t = 0, 1, 2, 3, and 4 days; another group was treated every-other-day, with dosages applied at t = 0, 2, and 4 days. Each group contained 15 mice. Subgroups were sacrificed and biopsied at least 24 h after the most recent dose, on days 1–5.

Hyperproliferative effects of the vehicle and its various components were examined with 5 treatment groups each of which received one of the following topical applications: no treatment (control); water alone; isopropanol and water (1:1); Azone (2%) and water; or the standard vehicle of Azone (2%), isopropanol (49%), and water (49%). None of the topical preparations in this experiment contained [³H]dThd. Treatments were applied at t = 0, 12, and 24 h. At t = 48 h each mouse received 50 μCi of [³H]dThd by i.p. injection, was sacrificed 1 h later, and the dorsal skin biopsied.

RESULTS

In Vitro Studies The in vitro penetration of [³H]dThd was determined in 4 different vehicles (Fig 1). The vehicle chosen on the basis of maximum cumulative penetration over 48 h, consisted of 2% Azone, 49% isopropanol, and 49% water. At the end of the 48-h experiment 2.8% of the applied dose had accumulated in the aqueous reservoir beneath the skin and an additional 2.2% of the applied dose remained in the dermis. Thus an estimated 5% of the applied [³H]dThd dose actually traversed the basal layer of the epidermis. The cumulative 48-h penetration of [³H]dThd in Vehicle N, N-methylpyrrolidone, and water, was 1.5%, 0.2%, and 0.08%, respectively. The degree to which Azone enhanced in vitro [³H]dThd penetration was assessed by varying the Azone concentration in isopropanol and water (1:1). The maximum cumulative penetration of [³H]dThd was achieved with 2% Azone. Vehicles containing 0%, 0.25%, 0.5%, 1%, and 4% Azone achieved 38%, 59%, 54%, 65%, and 93%, respectively, of the [³H]dThd penetration that the 2% Azone vehicle achieved.

In Vivo Studies A time-dependent linearly increased labeling of the dorsal skin over 24 h is evident after a single topical application of [³H]dThd in the 2% Azone vehicle (Fig 2). The labeling is confined to the lower row of epidermal cells. The few labeled cells at sites distant from the site of [³H]dThd application was evidence for minimal systemic absorption (Fig 3). Doubling the SA of the topical [³H]dThd (SA = 25–47 Ci/mmole) significantly increased the autoradiographic labeling intensity of individual cells with topically applied [³H]dThd without changing the LIs. No epidermal labeling was evident with topical application of [³H]dThd in the isopropanol-water vehicle without Azone.

Comparing topical and systemic delivery using an intraperitoneal osmotic diffusion pump of [³H]dThd, the LIs both achieved a similar plateau of 85–95%, suggesting similar GFs (Fig 4). For any given LI, the intensity of AR labeling in terms of number of grains per cell was greater for systemic than for topical delivery of [³H]dThd. After 4 days of continuous systemic [³H]dThd labeling by intraperitoneal osmotic diffusion pump, an 85–95% dorsal skin LI was achieved. In contrast, after only 2 days of local [³H]dThd application, a LI of 95% was measured in topically treated dorsal skin. In these experiments, epidermal hyperplasia first became apparent 24–36 h after a single application of the Azone-containing vehicle. Vehicle-induced epidermal hyperplasia is presumably responsible for this accelerated labeling. In the topically treated mice by day 2, the abdominal skin has a LI of less than 20%. On the day 7, when dorsal and abdominal skin have similar LIs, the relative intensity of labeling, based on grain counts per cell, is approximately 5-fold greater in dorsal skin compared with abdominal skin (Fig 3). In contrast, the systemic delivery of [³H]dThd resulted in labeling the abdomen and back with equal intensity and percentage, and there was no evidence of hyperplasia in the dorsal epidermis (Fig 5).

Applying topical [³H]dThd once daily or every other day gives an equal degree of epidermal labeling. But daily application was associated with a slightly greater degree of vehicle-induced epidermal hyperproliferation compared with every-other-day treat-
ment. Of the 3 components comprising the vehicle only Azone was found to contribute to the induction of epidermal hyperpro-

DISCUSSION

Cell kinetic models provide a conceptual framework for both the study of proliferation control mechanisms and the rational design of therapy for diseases, such as cancer and psoriasis [5,6]. According to classic cell kinetic theory, those cells in the germinative basal layer not actively proliferating (G0) are either: (1) proliferating keratinocytes that are temporarily in a resting phase; (2) nonkeratinocytes (e.g., melanocytes); or (3) postmitotic differentiated keratinocytes still remaining in the basal layer. The term growth fraction represents the percentage of actively proliferating cells in the population actually being studied. In most human solid tumors that have been investigated, there are always cells that are never labeled by continuous [3H]dThd infusion, with the majority of tumors having a GF 30-70% [5,7]. In vivo data from long-term [3H]dThd infusion studies in mice and swine are consistent with an epidermal GF approximating 85-100% [2].

In the case of psoriasis there is general agreement that the GF in the proliferative compartment of lesional skin approximates 100% [2,8]. In contrast, the GF in normal or uninvolved skin has been estimated to range from 60-100% [2,9]. Estimating the numerical value of GF is a necessary prerequisite to calculating the cell cycle duration when the stage-duration method of analysis is used, i.e., Tc is calculated from the experimental values for length of S phase, LI, and GF [2]. The determination of GF in normal skin is critical to understanding the proliferative defect in psoriasis. If the true GF in normal skin is 100%, then psoriatic hyperproliferation is the result of a vastly accelerated rate of cell division. On the other hand, if the GF in normal skin is low, e.g., 20%, with a quiescent G0 fraction of 80%, then the Tc of

Figure 2. After a single topical application of [3H]dThd to the backs of

Figure 3. An autoradiograph at day 7 of a topically treated mouse dorsal

Figure 4. Determination of in vivo epidermal GF comparing topical and

systemic (continuous infusion of [3H]dThd by an intraperitoneal osmotic
diffusion pump) routes of [3H]dThd delivery.
normal cells might approximate the rapid rate seen in psoriatic cells. Hence, the transition from uninvolved to lesional skin in psoriasis would result in large part from the recruitment of the entire quiescent cell population in G1 into the proliferative GF. The current information indicates, however, that the GF of normal skin is at least 60% and thus the TC of each cell population must be substantially different [2, 9].

The present investigation is preliminary to exploring the use of topical [3H]dThd preparations for determining the GF of normal human skin in vivo. We have found that a combination of Azone, isopropanol, and water (2:49:49) provides effective [3H]dThd percutaneous penetration through excised human abdominal skin. Azone was included in these vehicle studies because of recent reports describing its penetration enhancing effects [10]. In the present study vehicles containing Azone produced cutaneous irritation and local augmentation of LI. This effect was minimized by lowering the Azone concentration and decreasing the frequency of topical application from daily to every other day. Previous studies have shown that Azone is extremely irritating to rodent skin, whereas in humans the irritancy is much less, comparable to mineral oil and even less irritating than Vaseline Intensive Care Lotion.† There are no published reports of Azone-induced hyperplasia in human skin.

The current use of intradermal [3H]dThd is generally 5 μCi/0.1 ml injections up to a maximum of 20 injections or a total of 100 μCi/subject. With the topical approach described here, 5% penetration rate of 20 μCi applied would give 1 μCi absorption after each topical application. The cumulative dose of a 10- to 20-day experiment would be well below a 100 μCi total intradermal dosage.

Our in vivo experiments revealed that within at least the first 24 h after a single application, there is a linearly increasing LI. In the mouse model it appears that there is a reservoir effect with [3H]dThd penetration over 24 h. To avoid vehicle-induced hyperplasia, the optimal approach in human skin may be to apply a [3H]dThd preparation at every-other-day or longer intervals. Augmenting the SA of [3H]dThd from 25 to 47 Ci/mmol increased the density of autoradiographic grains per cell, but did not significantly change the LI. Increasing the [3H]dThd SA should permit a decrease in the absolute number of microcuries per dose needed to achieve detectable labeling.

The present studies have demonstrated the effectiveness of topically applied [3H]dThd for continuous labeling of the epidermis. This may be a potentially useful approach to obtain the GF of human normal and uninvolved psoriatic skin in vivo. However, studies will have to be done to determine whether the Azone-vehicle-induced hyperplasia in mouse skin can be avoided or minimized in human skin.

Table 1. Hyperproliferative Effects of Vehicle Components in Hairless Mouse Skin

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>Labeling Index Mean (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment (control)</td>
<td>8.2</td>
</tr>
<tr>
<td>Water</td>
<td>5.2</td>
</tr>
<tr>
<td>Isopropanol:water (1:1)</td>
<td>5.6</td>
</tr>
<tr>
<td>Azone:water (2:98)</td>
<td>13.0</td>
</tr>
<tr>
<td>Azone:isopropanol:water (2:49:49)</td>
<td>15.5</td>
</tr>
</tbody>
</table>

* Treatments applied to dorsal mouse skin at t = 0, 12, 24 h; [3H]dThd injected i.p. at t = 48 h.

REFERENCES
