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SHORT COMMUNICATIONS

Thymidine phosphorylase in human epidermal keratinocytes

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The human epidermis is a self-renewing epithelial tissue which can salvage thymidine for DNA synthesis. Incorporation of radioactive thymidine into DNA is a widely used measure of keratinocyte proliferation [1, 2]. We have found that human keratinocytes catabolize thymidine and that this catabolism can severely limit incorporation of radioactivity into DNA [3].

The mammalian pyrimidine nucleoside phosphorylases, uridine phosphorylase and thymidine phosphorylase, can cleave the glycosidic bond of thymidine [4, 5]. These enzymes vary in their substrate specificities; thymidine phosphorylase cleaves only pyrimidine deoxyribonucleosides, whereas uridine phosphorylase cleaves both pyrimidine ribo- and deoxyribonucleosides. There are several inhibitors which specifically inhibit uridine phosphorylase, and these have been employed to differentiate between the two phosphorylases [4, 6, 7]. Using these inhibitors, we demonstrate here that the activity which catabolizes thymidine in human keratinocytes was a thymidine phosphorylase. Moreover, inhibition of keratinocyte thymidine phosphorylase by 6-aminothymine, an inhibitor of both pyrimidine nucleoside phosphorylases, increased the incorporation of radioactive thymidine into DNA of human keratinocytes by reducing the catabolism of thymidine.

Materials and methods

Human keratinocytes, isolated from neonatal foreskin, were grown in culture dishes prepared with a feeder layer of irradiated 3T3 cells as previously described [3]. Before the primary culture became confluent, cells were passaged onto irradiated 3T3 cells in 35-mm dishes; confluent secondary cultures of 5–7 cell layers were used for experiments. Microscopically, there was no evidence of fibroblasts. Representative keratinocyte cultures were tested for the presence of mycoplasma, and the results were negative. Cells were grown in Dulbecco's Modified Eagle Medium (Gibco Laboratories, Grand Island, NY) supplemented with 20% fetal bovine serum, hydrocortisone, epidermal growth factor, cholera toxin, penicillin and streptomycin. The concentration of thymidine in complete medium contributed by 20% fetal bovine serum was approximately 0.2 μM.

Soluble extracts of cultured cells and foreskin were prepared as previously described [3]. Briefly, cultured cells were washed free of medium, were scraped into phosphate-buffered saline containing 5 mM dithiothreitol, and were disrupted by sonication. Foreskins were dissected to remove as much subcutaneous material and dermis as possible. The minced tissue was homogenized in phosphate-buffered saline, 5 mM dithiothreitol with a Polytron homogenizer (Brinkmann Instrument Co., Westbury, NY). The crude extract from cells or tissue was centrifuged (20,000 *g* 4°, 30 min) to remove particulate material and stored at −70°. The Bradford assay was used to measure the protein concentration [8].

Thymidine catabolism was measured in reaction mixtures containing 0.5 to 10 μg of protein from crude extracts in phosphate-buffered saline (9.6 mM phosphate, pH 7.2) containing 5 mM dithiothreitol at 37°; the reaction was started by addition of [^{3}H]thymidine (20 Ci/mmol, New England Nuclear, Boston, MA) diluted with thymidine to give final concentrations ranging from 0.2 to 100 μM, 4 μCi/ml. If inhibitors were studied, they were present in the reaction mixture for at least 5 min before addition of radioactive thymidine. The final volume of the reaction mixture was 100 μl. The reaction was stopped by acidification of aliquots of the reaction mixture with 3% perchloric acid. Samples were chromatographed with carrier thymidine and thymine by ascending TLC on silica gel plates in a solvent system of chloroform, methanol and acetic acid, 18:5:1 [9]. Regions of the plate visualized by absorption at 254 nm that correspond to thymidine (R_f = 0.46) and thymine (R_f = 0.67) were cut from the plate, placed in vials, extracted with 1 N HCl in methanol, and counted with EcoScint in a scintillation counter. [^{3}H]-Uridine (17 Ci/mmol, New England Nuclear) was diluted with uridine to give a final concentration of 100 μM, 4 μCi/ml, to assay the catabolism of uridine in extracts. The TLC system described above separated uridine from uracil [9].

The incorporation of radioactive thymidine into DNA was measured in confluent cultures exposed to 1 ml of medium containing [^{3}H]thymidine (0.2 μM, 4 μCi/ml). At various times during the incubation at 37°, cultures were washed repeatedly with cold phosphate-buffered saline and, then, with cold 10% trichloroacetic acid. One milliliter of 3% perchloric acid was added to the dish, and the dish was heated to 90% for 60 min. The contents of the dish and two washings with 3% perchloric acid were transferred to a scintillation vial and counted with EcoScint.

5-Benzylacyclouridine was the gift of Dr. S. Cha at Brown University, Providence, RI, via Dr. R. E. Handschumacher at Yale University. 6-Benzylthiouracil was purchased from Sigma. 6-Aminothymine was the gift of Dr. D. Cooney at the National Cancer Institute and was synthesized by the method of Bergmann and Johnson [10].

Results and discussion

Human keratinocytes catabolize thymidine *in vivo* and *in vitro* [3]. To determine which pyrimidine nucleoside phosphorylase was responsible, we measured the catabolism of thymidine and uridine in extracts from cultured keratinocytes in the presence of pyrimidine nucleoside phosphorylase inhibitors (Table 1). The extracts catabolized both thymidine and uridine. Thymidine catabolism was more than five times greater than uridine catabolism. 5-Benzylacyclouridine, a specific inhibitor of uridine phosphorylase [11], inhibited uridine catabolism but did not affect thymidine catabolism at a concentration of 25 μM in extracts of cultured keratinocytes; 50 μM 5-benzylacyclouridine did not inhibit thymidine catabolism in extracts prepared from foreskin (data not shown). 6-Aminothymine, an inhibitor of both thymidine and uridine phosphorylases [12], inhibited thymidine catabolism in extracts of cultured keratinocytes and human foreskin (Table 1 and Fig. 1). There are no selective inhibitors of thymidine phosphorylase [13]; however, Niedzwicki *et al.* [13] reported that 6-benzylthiouracil is a potent inhibitor of thymidine phosphorylase from mouse liver. A concentration of 6-benzylthiouracil as high as 200 μM inhibited thymidine catabolism by an extract of human keratinocytes by only about 20%. It is unclear if this difference in sensitivity of 6-benzylthiouracil for thymidine phosphorylase from a crude extract from human keratinocytes and from

Table 1. Effects of inhibitors on the catabolism of thymidine and uridine in an extract of cultured human keratinocytes*

Nucleoside (100 μM)	Inhibitor† (concn)	Base formed in 15 min (nmol)	Percent of control
Thymidine		6.7 ± 0.4‡	100
Thymidine	BAU (25 μM)	7.0 ± 0.2	104
Thymidine	BTU (200 μM)	5.2 ± 0.2	78
Thymidine	6-AT (25 μM)	3.7 ± 0.1	55
Thymidine	6-AT (100 μM)	1.5 ± 0.1	22
Uridine		1.7 ± 0.4	100
Uridine	BAU (25 μM)	0.5 ± 0.0	29

* The extract of cultured keratinocytes in phosphate-buffered saline was incubated at 37° with or without the inhibitor for 5 min prior to addition of radioactive nucleoside (100 μM, 4 μCi/ml) in a final volume of 100 μl. The reaction was stopped after 15 min, and the products were analyzed by TLC.

† BAU, 5-benzylacyclouridine; BTU, 6-benzylthiouracil; and 6-AT, 6-aminothymine.

‡ Mean ± range of duplicates from one of two comparable experiments using extracts from different cultures.

a purified preparation of the enzyme from mouse liver reflects the state of purification of the enzyme or different enzymes from different tissues or species.

Thymidine phosphorylase is found in a number of human tissues including the liver [6, 14], spleen [6], B lymphocytes [15], platelets [12, 16], and, as we have found, skin. Several studies which differentiate between the two phosphorylases suggest that, like human skin, thymidine phosphorylase is more active than uridine phosphorylase in a number of human tissues [6, 14, 16]. In contrast, it appears that rodent tissues have considerably greater uridine phosphorylase activity than thymidine phosphorylase activity, especially in the liver [6, 14].

We have found that, unlike the human epidermis, the skin of mice, rabbits or guinea pigs has little or no capacity to catabolize thymidine [3]. Others have reported similar species differences; for example, thymidine phosphorylase is absent in rat platelets [16] but present in human platelets [12, 16]. Since some thymidine analogs, such as 5-bromodeoxyuridine and 5-iododeoxyuridine, are substrates for thymidine phosphorylase [17], animal models may not predict the activity of such agents for human cutaneous diseases.

The incorporation of 0.2 μM [^{3}H]thymidine into DNA of human keratinocytes *in vitro* reached a plateau by 2 hr (Fig. 2). We attribute this to the rapid catabolism of thymidine since radioactive thymidine incorporation became nearly linear for at least 12 hr when cultures were given 100 μM 6-aminothymine to inhibit thymidine catabolism. Thus, at concentrations of thymidine which are physiological in man, the availability of thymidine for salvage biosynthesis may be modulated by the activity of thymidine phosphorylase. Thymidine availability and thymidine phosphorylase activity may be determinants of cell proliferation in normal skin and in hyperproliferative epidermal diseases, such as psoriasis and basal cell carcinoma.

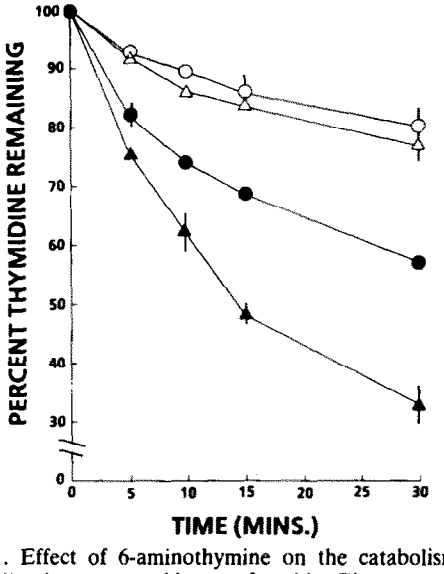

Fig. 1. Effect of 6-aminothymine on the catabolism of thymidine in extracts of human foreskin. The extract was incubated with phosphate-buffered saline in the absence (●, ▲) or presence (○, △) of 50 μM 6-aminothymine and [^{3}H]thymidine (4 μCi/ml) at a final concentration of 100 μM (●, ○) or 5 μM (▲, △). Samples of the reaction mixture were removed, and the percent of thymidine remaining was determined by TLC. Each symbol is associated with a bar that represents the range of duplicate samples from a representative experiment; repeated experiments with different extracts gave similar results.

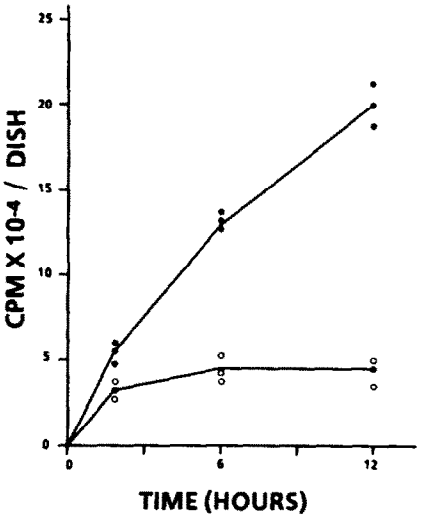

Fig. 2. Incorporation of [^{3}H]thymidine into DNA of human keratinocytes in confluent, stratified cultures in the presence (●) or absence (○) of 100 μM 6-aminothymine.

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Species differences in adrenal spironolactone metabolism: relationship to cytochrome P-450 destruction

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Spironolactone (SL) has been widely used as a potassium-sparing diuretic for approximately two decades [1, 2]. The mechanism of action of the drug involves competitive binding to renal mineralocorticoid receptors, resulting in sodium excretion and potassium retention. Among the side effects noted for SL is the inhibition of steroidogenesis in several tissues, including the adrenal cortex and testes [3–6]. The decline in steroidogenesis caused by SL is attributable, at least in part, to destruction of adrenal and testicular cytochromes P-450 [5–10], the terminal oxidases for several essential steroidogenic enzymes. The destruction of testicular cytochrome(s) P-450 by SL has been observed in all species studied, but effects on adrenal cytochrome(s) P-450 seem to be limited to those species that secrete cortisol as their major adrenal glucocorticoid, that is, species having an adrenal 17α-hydroxylase enzyme system [9, 10]. The mechanism(s) responsible for the species differences in the adrenal effects of SL has not yet been resolved.

The results of numerous studies indicate that most, if not all, of the actions of SL are mediated by metabolites of the drug [7, 8, 11–13]. Recent investigations suggest that 7α-thiomethyl-SL is the therapeutically active metabolite of SL [14, 15], but a different metabolite appears to be responsible for the destruction of adrenal and testicular cytochromes P-450 [16, 17]. It has been demonstrated that 7α-thio-SL is an obligatory intermediate in the actions of SL on cytochromes P-450 [17], but further metabolism of the intermediate within target tissues is also required. Because of the apparent importance of local metabolism in the actions of SL, studies were carried out to determine if species differences in the effects of SL on adrenal cytochromes P-450 [9, 10] are related to differences in adrenal metabolism of the drug. The results presented in this communication indicate that such a relationship does indeed exist.

Materials and methods

Adult male English Short Hair guinea pigs (800–1000 g), Sprague–Dawley rats (250–300 g), New Zealand White rabbits (1.5–2.5 kg), or mongrel dogs (17–22 kg) were used in all experiments. All animals were maintained under standardized conditions of light (6:00 a.m to 6:00 p.m) and temperature (22°), and received food and water *ad lib.* Animals were killed between 8:00 and 9:00 a.m.; adrenal glands were collected and microsomal fractions obtained by differential centrifugation as described previously [17].

For studies on the metabolism of SL or 7α-thio-SL, incubation medium contained 0.05 M Tris–HCl (pH 7.4), 5.0 mM $MgCl_2$, and adrenal microsomal protein (0.2 to 2.0 mg) in a total volume of 2.5 ml. Where indicated, an NADPH-generating system consisting of NADP (0.5 mM), sodium isocitrate (10 mM), and isocitrate dehydrogenase (0.15 units) was included in the incubation medium. Incubations were done in 25-ml Erlenmeyer flasks at 37° under air in a Dubnoff Metabolic Incubator. The reaction was initiated by the addition of SL (100 μM) or 7α-thio-SL (100 μM) in small volumes (5–10 μl) of ethanol. Incubation times varied from 10 to 60 min, depending upon the amount of microsomal enzyme activity in adrenals from the different species studied. Conditions which resulted in approximately 20–30% depletion of substrate (50–75 nmol metabolized) were employed in all experiments. The HPLC analyses used for measurement of SL and of 7α-thio-SL are sensitive to approximately 2 nmol of each compound. For all microsomal preparations, the incubation conditions employed ensured linearity of product formation or substrate (7α-thio-SL) metabolism with respect to incubation time and microsomal protein concentration. After incubation, 40 μg of progesterone was added to each flask to serve as an internal standard. The incubation media were extracted twice with 4 ml of ethyl acetate (HPLC grade) and the extracts were combined, filtered, and evaporated. Samples were then reconstituted in small volumes of HPLC grade acetonitrile for subsequent HPLC analyses.

Incubation conditions for the preincubation experiments were similar to those described above. However, after the preincubation period, the microsomal suspensions were recentrifuged at 105,000 *g* for 60 min, washed with KCl–Tris buffer, and centrifuged again. The final pellets were resuspended in buffer and used for cytochrome P-450 determinations. Cytochrome P-450 was measured as the dithionite-reduced CO complex as described by Omura and Sato