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Biochemical Evidence for Keratinization by Mouse Epidermal Cells in Culture

Abstract. *More than 70 percent of the urea-extractable proteins from mouse stratum corneum or from differentiated cells of mouse epidermis grown in culture are two proteins of molecular weight 68,000 (keratin 1) and 60,000 (keratin 2), which are present in equimolar amounts on polyacrylamide gels. These proteins are the subunits of the keratin filaments, because when isolated from stratum corneum or cells grown in culture they form native-type epidermal keratin filaments in vitro. These observations provide biochemical evidence that epidermal cells grown in culture synthesize the major differentiation products of the epidermis.*

Cultures of specialized cells have been extremely valuable for studies of cell biology, differentiation, and pharmacology and appear promising for investigations of chemical carcinogenesis. Primary epidermal cell cultures have been used for studies of differentiation and carcinogenesis (*1*). Recent reports have shown that human epidermal cell lines may also be valuable for such studies (*2*), which suggests that propagation of cells from diseased epidermis might shed light on the pathogenesis of the disease process. In previous reports of epidermal cell culture from any species, the determinants of differentiation have been the histochemical, morphological, or nonspecific chemical nature of the cells or cell products. This report presents specific biochemical evidence that the differentiation products of mouse epidermal cells in vitro are keratin proteins identical to those obtained from the fully differentiated stratum corneum of mouse skin.

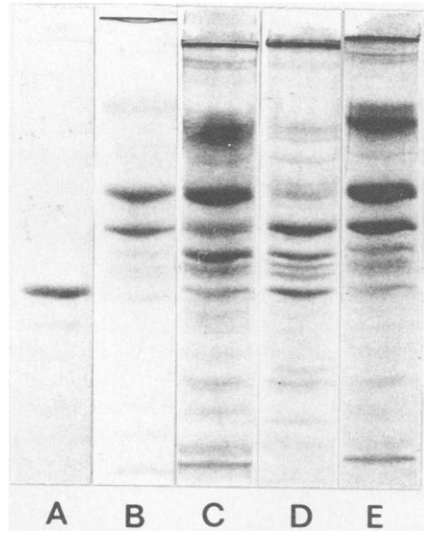

Fig. 1. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis of urea-soluble proteins. Mouse muscle actin, mouse stratum corneum, or cell pellets were extracted in 8*M* urea, 0.05*M* tris-HCl buffer (*p*H 9.0), and 0.1*M* 2-mercaptoethanol for 4 hours at 23°C and centrifuged to remove debris. The supernatant was equilibrated in cathode buffer containing 0.5 percent (weight/volume) SDS and 0.1*M* 2-mercaptoethanol and heated at 95°C for 2 minutes. A portion containing 50 to 100 μg of protein was electrophoresed for 4 hours at 2 mA per gel on 0.6 by 10 cm 9 percent T and 3 percent C gels (*3*). Gels were stained with fast green and scanned at 610 nm with an Isco model 659 gel scanner. (A) Mouse muscle actin, (B) mouse stratum corneum, (C) mouse epidermal cells before culture, (D) attached cells from mouse epidermal cultures 4 hours after plating, and (E) unattached cells from mouse epidermal cultures 4 hours after plating. The bands of high molecular weight in (B) to (E) are noncovalently bound aggregates of the two keratin proteins.

Epidermal cells from newborn BALB/c mice were isolated (*1*) and cultured in medium 199 (National Institutes of Health Media Unit) containing 11 percent fetal calf serum (Reheis Chemical Co., Kankakee, Illinois) and 1 percent antibiotic-antimycotic solution (Gibco, Grand Island, New York). Cells were plated at 1×10^5 per square centimeter into 60- or 100-mm plastic tissue culture dishes and maintained at 37°C in a humidified atmosphere of 5 percent CO_2 and 95 percent air. Four hours after plating, unattached cells were collected from each dish by centrifugation of the medium and a saline wash at 1400 rev/min for 5 minutes. At subsequent times unattached cells, sloughed into the medium from the monolayer, were collected as above, and attached cells were harvested by scraping and centrifugation. Cell pellets were extracted with 8*M* urea buffer (legend, Fig. 1) and electrophoresed on sodium dodecyl sulfate polyacrylamide gels (*3*). Authentic stratum corneum, which was removed from newborn mouse skin during the isolation of cells for culture, was extracted similarly. Gels were prepared and evaluated as described in the legend of Fig. 1. Proteins were isolated from preparative gels and assayed for their ability to form 80 Å filaments in vitro (*4*).

Figure 1B represents a gel containing bands for two major proteins extracted from stratum corneum migrating and having molecular weights of 68,000 [upper band, keratin 1 (K_1)] and 60,000 [lower band, keratin 2 (K_2)]. These two

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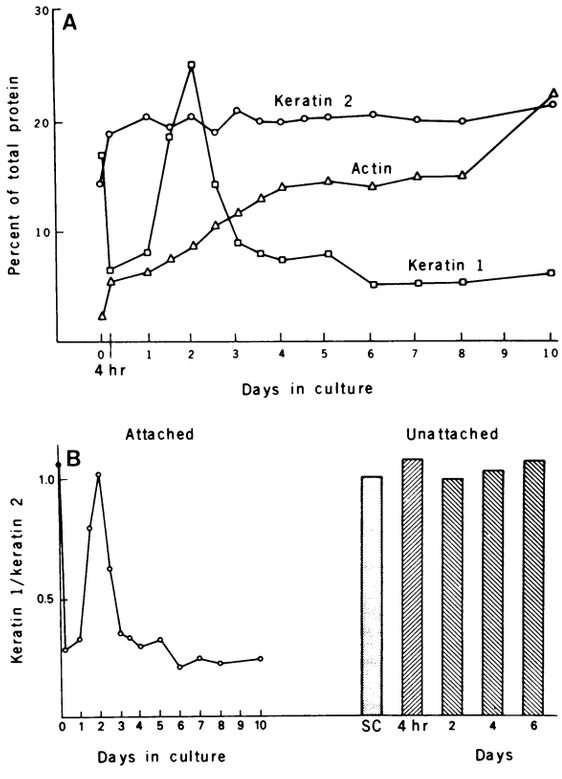

Fig. 2. (A) Gel scans of urea-extractable proteins from attached population of mouse epidermal cell cultures. Mouse epidermal cells were isolated and cultured as described in the text. At various times before (zero time) and after plating, attached cells were harvested by scraping and pelleted, and urea-extractable proteins were isolated, electrophoresed, and scanned as described in the legend to Fig. 1. (B) Molar ratio of K_1 to K_2 from gel scans of urea-extractable proteins from attached and unattached populations of mouse epidermal cell cultures and stratum corneum. The K_1 and K_2 were quantitated from gels prepared as described in the legend to Fig. 1. Attached cells (○) were harvested by scraping. Stratum corneum (*SC*) and 4-hour (*4 hr*) unattached cells were obtained as described in the text. Unattached material from days 2, 4, and 6 in culture represents extracted protein from cellular material sloughed in the medium during the previous 24 hours only.

bands constitute 75 percent of the urea-soluble protein or 50 percent of the dry weight mass of the stratum corneum and thus appear to be the subunits of the epidermal keratin filaments. The electrophoretic profile of preparations of epidermal cells extracted by the urea buffer before culture is shown in Fig. 1C. Although a number of additional bands are present, prominent K_1 and K_2 bands are evident which coelectrophorese with isolated and purified K_1 and K_2 from stratum corneum. Another protein with a molecular weight around 40,000 is present in the cell extracts and coelectrophoreses with authentic mouse muscle actin (Fig. 1A) isolated by standard methods (*5*). The isolation of actin from basal and cultured epidermal cells has been reported (*6*). When attached and unattached cells were studied 4 hours after plating (Fig. 1, D and E), striking differences were noted in these protein patterns. The attached population, largely composed of basal cells (*7*), had small amounts of K_1, but K_2 and actin were major components. The unattached population, composed largely of differentiated cells, showed little actin but yielded major K_1 and K_2 bands and in general was very similar to the pattern of stratum corneum.

Figure 2A presents gel scans from attached cells harvested during 10 days in vitro. Keratin 2 remains a major protein constituent of these cultures at all times whereas the K_1 content is initially very small, as shown in Fig. 1D. Keratin 1 production begins to increase approximately 24 hours after plating and rises very rapidly over the next 24 to 48 hours, falling off quickly after 72 hours and remaining low thereafter. This pattern is reproducible in different primary isolations but may vary in exact timing by as much as 12 hours. The increase in K_1 is presumably related to new synthesis, since it is an absolute increase in content and not related to a decrease in other protein species seen on the gels. The tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate appears to accelerate K_1 production, and retinyl acetate inhibits it (*7*). Also notable is the steady increase in actin content during the culture time (Fig. 2A). Previous data (*8*) have shown that the DNA content per dish (a measure of the number of nucleated cells) remains relatively constant while significant cell proliferation (*9*) occurs during the time when K_1 production is maximal (24 to 72 hours). This presumably reflects a balance of proliferation and differentiation and suggests that during this time there is a selective but temporary increase in a cell population that produces both K_1 and K_2. However, we cannot rule out the possibility that a transient change in gene regulation induces the synthesis of more K_1 in the entire population. At later times (after 4 days) the number of cells or the ability of each cell to produce K_1 reverts back to the level of the attached starting population (Fig. 2A) and remains constant thereafter in the attached population.

The data in Fig. 2B suggest that concomitant production of K_1 and K_2 leads to irreversible differentiation and sloughing of the cells in vitro, analogous to keratin production in vivo. Figure 2B shows the molar ratio of K_1 to K_2 at various times after plating in both the attached population and the cellular material sloughed into the culture medium. A K_1:K_2 molar ratio of ~1 is found in stratum corneum. This value is also found in the attached cells at the peak of K_1 production but is lower at all other times. However, at all times studied, the sloughed material, representing medium contents collected only over the previous 24 hours, has a K_1:K_2 ratio of 1, and these proteins are the major components (70 to 80 percent) of the urea-soluble protein of the sloughed material. These data suggest that stratum corneum–like material is continually sloughed into the medium even when

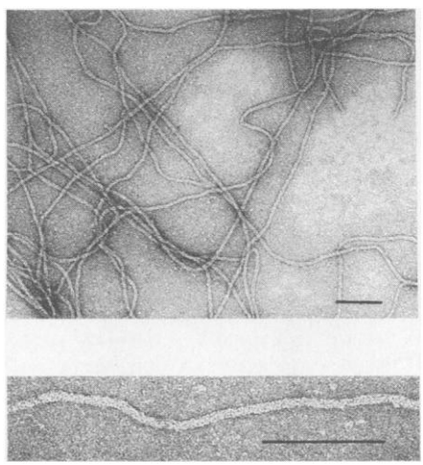

Fig. 3. Mouse epidermal keratin filaments polymerized in vitro. Urea-soluble proteins from 4-hour unattached cells (1 to 2 mg/ml in urea buffer) were dialyzed against 5 m*M* tris-HCl buffer (*p*H 7.6) for 15 hours at 23°C, during which time filaments formed in suspension (*4*). A portion was examined after negative staining with uranyl acetate (scale bars, 0.1 μm). Filaments of identical structure were formed from the soluble proteins of stratum corneum or from recombinations of K_1 and K_2 purified from either 4-hour unattached cells or 2-day attached cells. On examination by x-ray diffraction, all filaments gave a prominent meridional reflection at 5.18 Å and an equatorial reflection at 9.6 Å, indicative of the presence of regions of triple-chain coiled-coil α-helical conformation; this is the characteristic α-type x-ray diffraction pattern given by epidermal keratin (*11*).

most cells are not making K_1. It also seems likely that K_1-poor cells are not being sloughed into the medium although they are rich in K_2. Thus a minor subpopulation (representing approximately 20 percent of the K_2-producing cells) (Fig. 2B) must continue producing equivalent amounts of K_1 and K_2, which is followed by their death and detachment. Alternatively, there could be a delay in detachment of cells that had keratinized at an earlier time in vitro. This seems unlikely since medium from cultures as old as 10 days contained protein with the stratum corneum K_1:K_2 ratio. Neither K_1 nor K_2 proteins were found in attached cells or sloughed material from cultures of dermal fibroblasts prepared from the same donor mice by techniques previously described (*10*).

Critical for the specific identification of keratin proteins is their ability to produce filaments in vitro whose ultrastructural morphology is the same as that characteristic of filaments in situ (*4*). Figure 3 shows filaments produced when purified K_1 and K_2, isolated from epidermal cells in culture or from stratum corneum, are recombined under conditions known to generate keratin filaments but not other types of fibrous proteins (*4*). The keratin filaments formed in vitro are solid rods, 70 to 80 Å in diameter and up to 10 μm in length, have an α-type x-ray diffraction pattern (legend to Fig. 3), and contain K_1 and K_2 in the molar ratio of 1:2 or 2:1. These observations agree with the idea that a triple-chain segmented α-helical conformation constitutes the basic unit structure of keratin filaments (*4*).

The results reported here are direct proof of the production of normal keratin proteins by mouse epidermal cells in vitro. The two major protein bands obtained should therefore be useful markers in further studies of the control of differentiation in vitro. We hope that they will also be useful in studies of chemical carcinogenesis in mouse epidermal cells in vitro and in studies of the relationship between differentiation and carcinogenesis.

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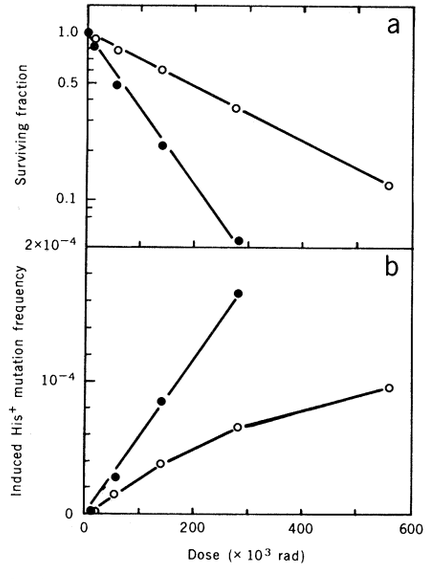
Thermorestoration of Mutagenic Radiation Damage in Bacterial Spores

Abstract. *The frequency of mutations in* Bacillus subtilis *spores irradiated anoxically with ionizing radiation decreased as a result of subsequent anoxic heating to the same extent as the increase in spore survival. This result indicates that DNA damage is the origin of the "thermorestoration" phenomenon.*

Bacterial spores exhibit a unique response to radiation. The viability of spores irradiated by ionizing radiation is reactivated by heat if anoxic conditions are used for both irradiation and heating. This phenomenon was first found in spores of *Bacillus megaterium* by Powers and his co-workers and was called "thermorestoration" (*1*). Later, Tanooka and Hutchinson (*2*) confirmed this effect in *B. subtilis* spores; however, they failed to show this effect with transforming DNA of spores possibly because of the extremely large radiation doses required to inactivate the DNA. The question of whether the thermorestoration of viability is a reflection of the reversal of DNA damage or has some other origin has long been unanswered. On the basis of the use of a newly developed mutation detection system with *B. subtilis* (*3*), I show in this report that DNA is a target molecule in the thermorestoration.

Spores of *B. subtilis* (strain HA101) carrying a suppressible mutation in the *hisB* gene were irradiated in N_2 gas with various doses of electrons and heated at 80°C for 20 minutes. Control spores were given only irradiation in N_2. Spores were then exposed to air and suspended in dis-



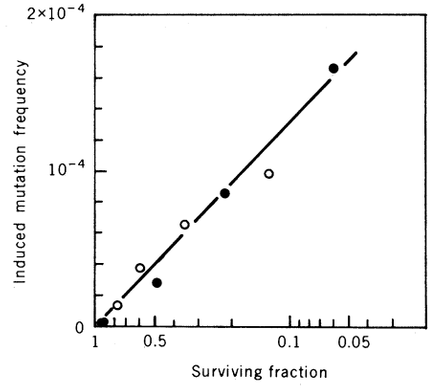


Fig. 1 (left). Effect of heat on the survival and the induced mutation frequency of irradiated spores. Dry spores of *B. subtilis* (strain HA101) (*his met leu*) were sealed in glass tubes that had been flushed with 99.999 percent N_2 gas and irradiated with 6-MeV electrons from a linear accelerator (Varian model V-7705). Spores were heated for 20 minutes at 80°C, then exposed to air, and suspended in sterile distilled water. Surviving spores (a) and His^+ mutants (b) were measured on the selective medium as described in (*3*): (○) heat, (●) no heat. Fig. 2 (right). A redrawing of Fig. 1 showing the induced His^+ mutation frequency as a function of the logarithm of the spore surviving fraction: (○) heat, (●) no heat.

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