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Detection of Ornithine Decarboxylase Gene Expression in 12-*O*-tetradecanoylphorbol-13-acetate-treated Mouse Skin using *In situ* Hybridization

TAKESHI KONO[1], MICHIO FUKUDA[1], MASAMITSU ISHII[1], NOBUYUKI MIZUNO[1], HIDEKI TAHARA[2], HIDEKI YOSHIDA[2], ISAO MATSUI-YUASA[2], SHUZO OTANI[2] and TOSHIO HAMADA[1]

Departments of [1]Dermatology and [2]Biochemistry, Osaka City University Medical School, Osaka, Japan

The localization of ornithine decarboxylase gene expression in mouse skin by tumour promoter, 12-*O*-tetradecanoylphorbol-13-acetate, was investigated using *in situ* hybridization. After 4 h of treatment with tumour promoter, the grains representing ornithine decarboxylase mRNA increased remarkably in number in the epidermis, especially in the follicular region. When the cells containing more than 5 grains were counted as positive, the number of positive cells increased by about 52-fold in the follicular epidermis, by about 19-fold in the interfollicular epidermis and by 4-fold in the dermis, as compared with controls. These results indicate that the epidermal cells are mainly responsible for the activation of ornithine decarboxylase by 12-*O*-tetradecanoylphorbol-13-acetate. *Key words: Tumour promotion; Skin cancer; Polyamine.*

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T. Kono, Department of Dermatology, Osaka City University Medical School, 1-5-7, Asahimachi, Abeno-ku, Osaka 545, Japan.

Ornithine decarboxylase (ODC), a rate-limiting enzyme of polyamine synthesis, is well known as an indicator of skin tumour promotion (1) and epidermal proliferation (2). The activity of ODC is remarkably enhanced by topical application of a tumour promoter, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (3). The significance and the mechanism of ODC induction have been studied intensively (1, 4–6).

Recently, several other authors (7–9) and we (10, 11) have demonstrated by Northern blot analysis of mRNAs extracted from the skin that an increase in ODC mRNA contributes to the acceleration of ODC activity. However, the detection of localization of the ODC mRNA has not yet been reported. In the present study, we have examined the ODC gene expression in TPA-treated mouse skin, using *in situ* hybridization with a mouse ODC-specific cDNA probe to detect the spatial localization of ODC mRNA.

MATERIALS AND METHODS

Animals

Female CD-1 mice aged 7 to 9 weeks were obtained from Charles River Breeding Laboratories (Wilmington, Mass, USA). The dorsal skin of the mice was shaved 3-4 days before experimentation, and only those mice not exhibiting hair regrowth over this period were used. TPA (17 nmol in 0.2 ml of acetone) or vehicle alone was applied topically to the shaved skin. The dorsal skin was excised from the mouse 4 h after application, when the ODC mRNA level was known to reach steady-state (8, 9).

Skin samples

All skin samples excised from the treated skin of the dorsa were processed for *in situ* hybridization using a modification of previous published techniques (12–14). The samples were immediately fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at 4°C for 2 h and immersed in 30% sucrose solution at 4°C for 4 h. The fixed tissue was embedded in OCT compound (Lab-Tek, Naperville, Ill., USA), snap-frozen in liquid nitrogen and then stored at −80°C until used.

Frozen skin samples were sectioned at 4 μm with a cryostat at −20°C, and placed on poly-*L*-lysine coated slides.

Preparation of cDNA probes

For detection of ODC mRNA, ECOR1-HindIII-Pst1-Sal1-Bam H1 fragments (290, 320, 400 and 420-bp) of total 1.8 Kb mouse ODC cDNA (kindly donated by Dr C. Kahana, Weizmann Institute of Science, Rehovot, Israel) (15) were used. The fragments were labelled to mean specific activity of approximately 3.5 dpm/g using [1',2',5-^{3}H]dCTP (50 Ci/mmol, Amersham, Bucks, England) by the multi-prime method (16).

In situ hybridization

The sections were immersed in 70% ethanol at 4°C for 10 min, and incubated for 25 min at 37°C with 1μg/ml of proteinase K (Sigma, St. Louis, Mo., USA) in PBS. The samples were washed with 2 × SSC (standard saline citrate, 0.3 M NaC1/0.03 M Na-citrate) at room temperature (RT)

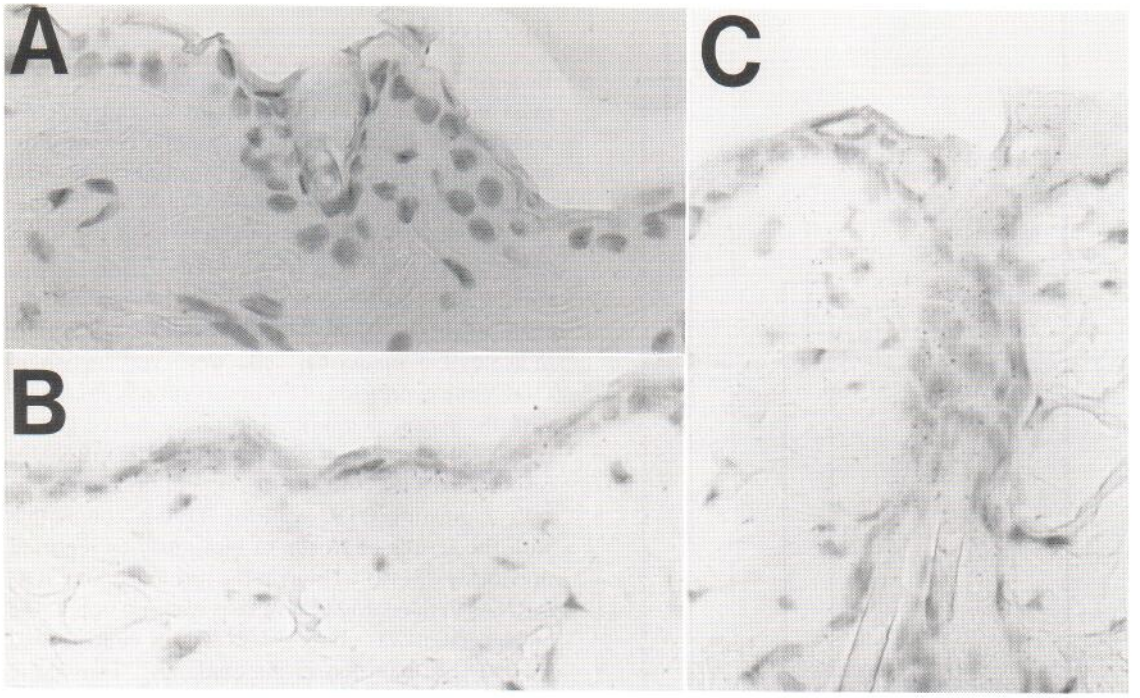

Fig. 1. Spatial distribution of the grains representing ODC mRNAs in the skin 4 h after treatment with acetone alone (A) or 17 nmol of TPA (B, interfollicular region; C, follicular region). Haematoxylin-eosin staining. ×150.

for 1 min, post-fixed in 4% PFA in PBS (5 min, RT), and rinsed with 2 × SSC (1 min, RT), followed by a 15-min acetylation in a freshly prepared solution containing 0.25% acetic anhydride and 0.1 M triethanolamine (pH 8.0). The specimens were washed with 2 × SSC (1 min, RT), immersed in 0.1 M Tris-HC1 (pH 7.0) containing 2 mg/ml of glycine (30 min, RT), rinsed twice with 2 × SSC (1 min, RT), dehydrated with successive incubation in 70% and 99% ethanol, and air dried.

Subsequently, the samples were pre-hybridized for 30 min at 42°C in a solution containing 200 μg/ml bovine serum albumin, 0.6 M NaCl, 50% deionized formamide, 20% dextran sulfate, 0.02% polyvinylpyrrolidone, 250 μg/ml yeast tRNA, 100 μg/ml sonicated salmon sperm DNA, 0.02% Ficoll, 1 mM EDTA, and 10 mM Tris-HC1 (pH 7.4). The hybridization mixture was an identical solution, into which the ^{3}H-labelled ODC cDNA probe was added (3.5 dpm/ml). Prior to hybridization, the mixture was heated at 90°C for 2 min, cooled on ice and applied to the slide. Hybridization was carried out at 42°C on a heat block for 16 h.

Following hybridization, the slides were rinsed thoroughly in 50% formamide/2 × SSC at 42°C for 2, 5 and 20 min followed by six washes in 2 × SSC at RT. The sections were dehydrated successively in 70% and 99% ethanol, and air dried.

Detection of bound probe and staining sections

Autoradiography was performed by dipping the slides into photographic emulsion (NR-M2, Konica, Tokyo, Japan), melted at 45°C diluted 2 : 1 in distilled water. After drying, the slides were stored and exposed in desiccant-containing boxes for about 20 days at 4°C. The samples were subsequently developed with a photographic developer (KONICADOL X, Konica) for 4 min, rinsed for 30 s with distilled water, and fixed for 5 min with a fixer (FUJIFIX, Fuji, Tokyo). Some sections were then stained with haematoxylin-eosin.

Those cells which expressed ODC mRNA were analysed in the interfollicular and follicular region of the epidermis and in the dermis separately.

Statistical analyses were performed using Student's *t*-test.

RESULTS

Fig. 1 shows spatial distribution of ODC mRNA in the untreated skin (A), interfollicular region (B) and follicular region (C) of TPA-treated skin as detected by *in situ* hybridization with a mouse ODC-specific cDNA. The majority of grains representing ^{3}H-labelled cDNA-mRNA hybrids coincide with the epidermal cells (B, C). Also some of the dermal fibroblasts contained ODC-specific mRNA transcripts. The number of signals was notably increased by applying TPA to the epidermis (B), especially in the follicular regions (C).

Fig. 2 illustrates a histogram of the proportion of grain-positive cells (cells containing over 5 grains in

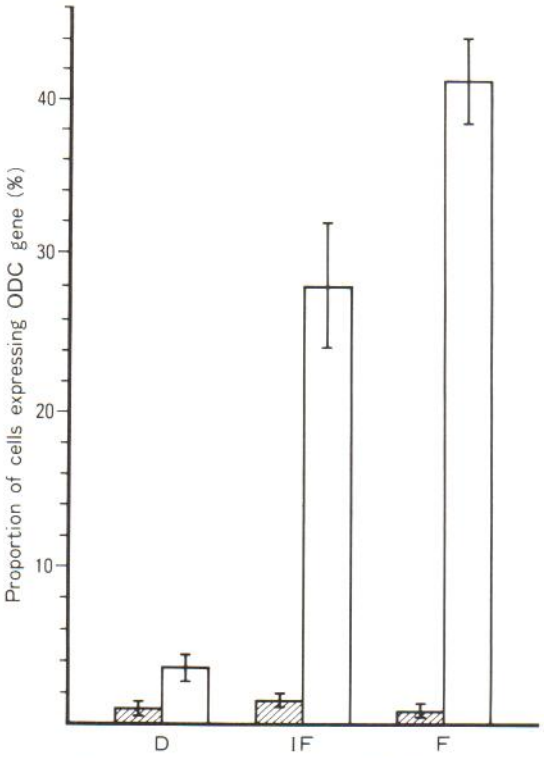

Fig. 2. Histogram of the proportion of ODC gene-expressing cells. Cells containing more than 5 grains in the cytoplasm were counted as positive. *D*, dermis; *IF*, interfollicular epidermis; *F*, follicular epidermis. □, Experimental group; ▨, Control group. Each value denotes the mean of 10 counts (100 cells each in 10 sections). Range bars indicate SE.

the cytoplasm were counted as positive). One thousand cells were counted (100 cells each in 10 sections). In the untreated skin, the proportion was highest in the interfollicular epidermis (1.5% ± 0.4%) followed in order by that in the dermis (0.9% ± 0.4%) and in the follicular epidermis (0.8% ± 0.4%).

By application of TPA to the skin, the proportion of positive cells increased remarkably in the epidermis. The increase was most prominent in the follicular epidermis (41.4% ± 2.8%, 51.8-fold the control). In the interfollicular region too, the proportion increased to 28.1% ± 3.8% (18.7-fold the control). The difference between the proportions of interfollicular and follicular regions was significant ($p<0.001$). By contrast, the increase in the rate in the dermis by TPA treatment (3.6% ± 0.8%) was significant ($p<0.001$), though smaller (4.0-fold the control) than that of the epidermis.

DISCUSSION

Because the epidermis and/or dermis is treated as a mass in Northern blot analysis of specific mRNA and because their complete separation is very difficult, no investigations into the localization of ODC mRNA have been reported previously. In this paper, we examined the local expression of the ODC gene at the mRNA level in *in situ* hybridization, using a mouse ODC-specific cDNA.

In the TPA-treated skin, the results of our study indicate that epidermal cells were very active in ODC gene expression. This observation is consistent with the recent demonstration that cultured keratinocytes are capable of synthesizing ODC mRNA in response to TPA (9). The increase in the number of cells expressing ODC gene by TPA is compatible with the results of Northern blot analyses reported previously (8–10). Though the presence of ODC mRNAs in the dermal fibroblasts was also noted, the enhancement was about 4-fold that of controls and was far less than that in the epidermis. Thus these results suggest that epidermal cells are predominantly responsible for the increase in ODC mRNA and enhanced ODC activity in TPA-treated skin.

In the case of TPA-treated skin, the highest number of density grains and the largest number of positive cells were associated with the follicular region of the epidermis. Probst & Krebs (17) have previously reported the association of ODC activity and DNA synthesis in hair follicles after hair plucking. These results appear to be consistent with previous reports which have shown strong hair-regrowth activity resulting from TPA treatment (18) or other activators of protein kinase C (19) and the importance of hair follicles in skin carcinogenesis (20).

In the present study, we were unable to more precisely detect localization of ODC mRNA in the epidermal layers and hair follicles due to the limited resolution caused by the diffusion of isotope. This problem warrants further investigation. A more sensitive and specific study of ODC mRNA in the skin by *in situ* hybridization using non-radioactive probes (12) has been under investigation in our laboratory.

In conclusion, we have demonstrated increased ODC gene expression resulting from TPA treatment of mouse epidermis, especially in the follicular region, using *in situ* hybridization.

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