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Expression of retinoic acid receptor genes in keratinizing front of skin

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We found, by an in situ hybridization method with riboprobes synthesized from human cDNA of the retinoic acid receptor (RAR), that the RAR genes (predominantly γ-subtype) are intensively expressed in the epidermis of normal and psoriasic human skins, and also in keratinizing fronts of 4-day-old mouse skins, nail matrices and hair follicles. Thus, target cells of retinoic acid in the skins are concluded to be keratinocytes, which is quite consistent with the fact that retinoic acid regulates keratinization of epidermis in vivo and also modulates expression of the keratin gene in vitro.

Retinoic acid receptor gene; Hybridization in situ; Epidermis; (Nail, Hair, Human, Mouse)

1. INTRODUCTION

Retinoic acid (RA) is known to play an essential role in cellular differentiation [1-3]. Recently, complementary DNAs of the RA receptor (RAR) genes were isolated and classified into three subtypes, RAR-α, -β and -γ [4-10]. Interestingly, the human and mouse RAR-γ genes were found, by a Northern blot analysis, to be expressed exclusively in skin [9,10]. In order to analyze the expression pattern of the RAR genes in skins, we conducted in situ hybridizations on sections of human and mouse skins with riboprobes synthesized from a human RAR-α cDNA. Since RA is known to be a very effective remedy for the common skin disorder of psoriasis, in which the rate of basal cell proliferation is greatly increased so that the epidermis thickens, we also observed the expression pattern of the RAR genes in psoriasic human skins. We demonstrated that the RAR genes are expressed in the epidermis of both normal psoriasic human skins and also in keratinizing fronts of the mouse epidermis, hair follicles and nail matrices, indicating that the main target cells for RA are the keratinocytes in the skin. Our findings are consistent with the fact that RA has been known to play important roles in the keratinization of the skin [2,3].

2. EXPERIMENTAL

2.1. *In situ hybridization*

Human and mouse (C3H) tissues were fixed for 3 h in 4% PBS-buffered (pH 7.4) paraformaldehyde at 4°C, and embedded in paraffin (Paraplast). Sections of 5 μm were collected on poly-L-lysine-coated slides. Section pretreatment and hybridization were performed according to Ingham et al. [11] with several modifications [12,13]. Sections were deparaffinized in xylene, rehydrated, acetylated, and prehybridized in 2 × SSC, 50% formamide, and 10 mM dithiothreitol (DTT) at 50°C. Twenty μl of hybridization mixture (50% deionized formamide, 4 mg/ml BSA, 4 mg/ml tRNA, 10% dextran sulfate, 2 × SSC) was applied to each slide. The slides were incubated for 15-17 h in a humid chamber at 50°C with a probe concentration of 1×10^5 dpm/μl for hybridization. The slides were washed in 50% formamide, 2 × SSC, 10 mM DTT at 50°C for 80 min, treated with 20 μg/ml RNase A for 30 min at 37°C in NTE buffer [11], washed further with 0.1 × SSC at 50°C for 1 h, dehydrated, and then air-dried. For autoradiography, the slides were immersed in Kodak NTB2 emulsion (diluted 1:1 with water), air-dried and exposed for 14 days at 4°C in a dry, light-tight box. The slides were developed at 20°C in Kodak D19 developer for 3 min and stained with hematoxylin and eosin for light microscopy.

2.2. *Probe preparation*

We used a human RAR-α cDNA (pTM61A, a gift from Dr M. Petkovich and Professor P. Chambon (CNRS, Strasbourg, France)) and its fragment containing the DNA binding (C-domain), ligand binding (E) or C-terminal (F) region, as a template to synthesize both anti-sense and sense RNA probes [13]. The riboprobes (spec. act. approximately 5×10^8 dpm/μg) were prepared from the both strands with [α-^{35}S]UTP (400 Ci/mmol, Amersham). After the removal of unincorporated labeled nucleotides, probes for the hybridization were subjected to limited alkaline hydrolysis [14] to reduce the transcripts to about 50-150 bp. We confirmed by Northern blot analysis that the riboprobe for the human RAR-α mRNA was cross-hybridized with mRNAs of the mouse RAR-α, -β and -γ. For control experiments, we used two types of anti-sense riboprobes synthesized from heterologous DNAs; a 341 bp fragment of a mouse nerve growth factor (NGF) cDNA (from Dr Noboru Tomioka of the Mitsui Toatsu Chemicals, Inc., Chiba, Japan) and a 500 bp fragment of an elastin cDNA (from Dr Shingo Tajima of Keio University, Tokyo, Japan).

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3. RESULTS

3.1. *Spatial expression pattern of the RAR genes in skin*

The most intensive accumulation of exposed silver grains was observed in the epidermis of the human skin. No significant difference was observed in the pattern of the grain accumulation between normal and psoriasic skin. Thus, we show a typical result for the psoriasic skin in fig.1. When we used the sense probe as a negative control to check the non-specific hybridization, the grain accumulation pattern observed was indistinguishable from that obtained with the anti-sense probe. We also observed a broad smeared band between 2 and 5 kb by Northern blot analysis of the total mouse skin mRNA with the sense probe (data not shown). The presence of some transcripts hybridized with the sense probe may be due to some regulatory transcription of the RAR genes, as pointed out previously [13].

Thus, for control experiments, we used two types of anti-sense riboprobes for NGF and elastin mRNAs. With these probes, we detected the NGF mRNA in an adult mouse submandibular gland and the elastin mRNA in an infant mouse aorta (data not shown). These control probes did not significantly hybridize with any of the regions where the hybridization signal was positive for the RAR mRNAs. Typical results with the riboprobe for the elastin mRNA are shown in figs 1c,d and 3b. These results indicated that the hybridization signals are specific for the RAR riboprobe. Judged from Northern blot analysis by Krust et al. [10], we concluded that at least the RAR-γ gene is expressed in the epidermis of the human skin.

3.2. *Expression pattern of the RAR genes in mouse skin*

In the mouse skin, intensive accumulation of grains was found in the hair follicles and nail matrices, in addition to the epidermis, as shown in fig.2. The location of the grains is restricted to the spinous and the granular layers of the epidermis of the skin, whereas no significant hybridization was observed in the basal and horny

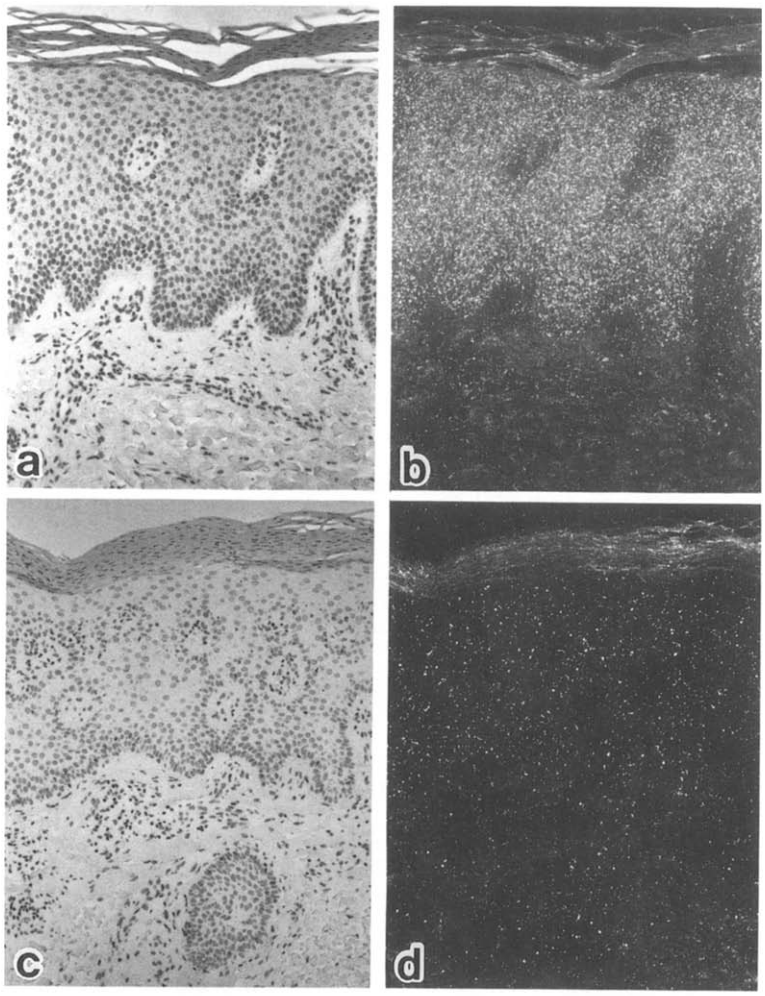

Fig.1. Expression of the RAR genes, as revealed by in situ hybridization with the anti-sense riboprobe from the RAR-α cDNA containing the C–F domains on the sections of a human psoriasic skin (a and b). Photographed under bright- (left) and dark-field (right) illumination. As a negative control, a result with the elastin probe was shown in c and d.

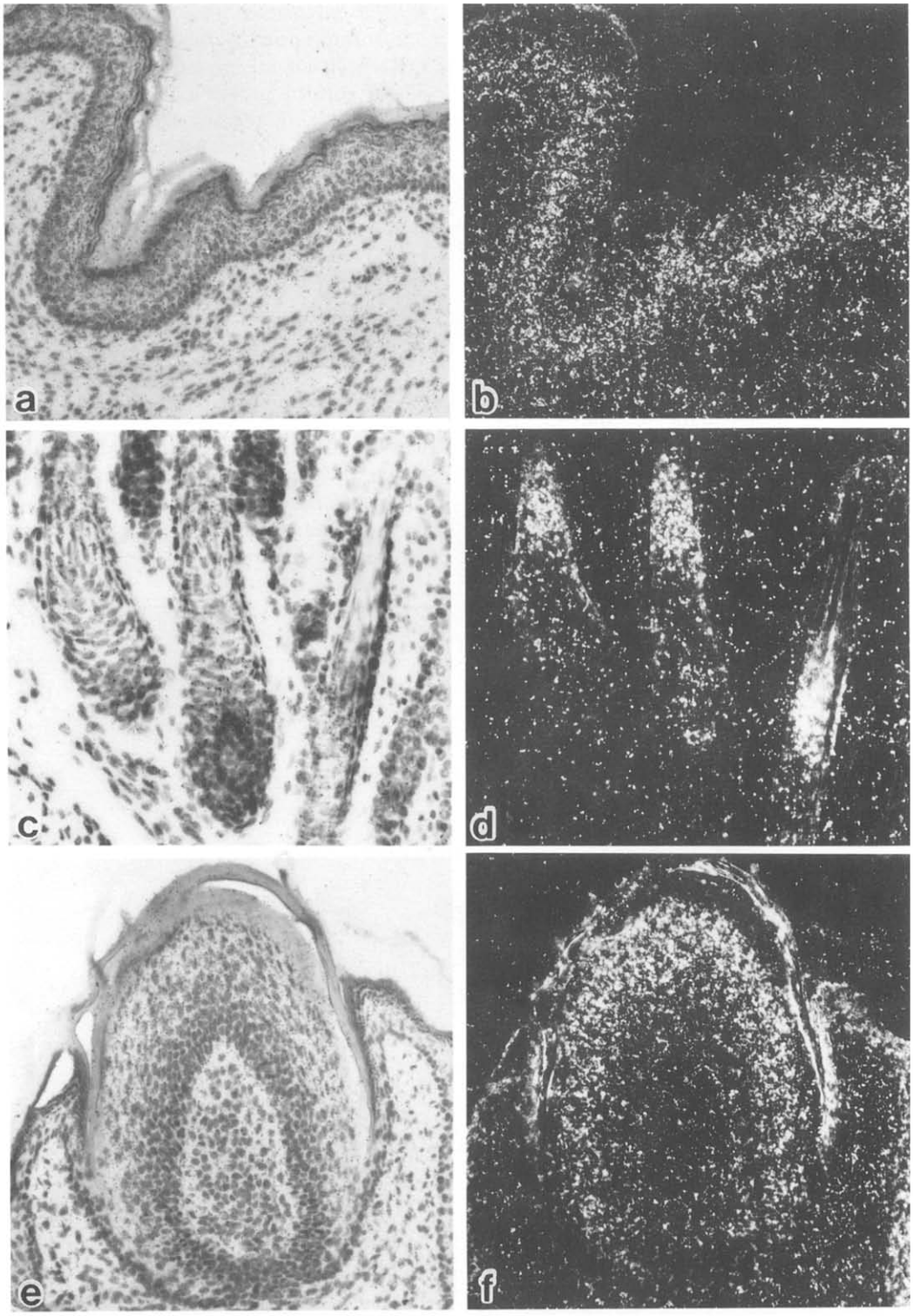

Fig.2. Expression of the RAR genes, as revealed by in situ hybridization with the anti-sense riboprobe from the RAR-α cDNA containing the C–F domains on sections of the 4-day-old mouse forelimb finger skin (a,b), hair follicle (c,d), and nail matrix (e,f). Photographed under bright-field (left) and dark-field (right).

layers. In the hair follicles, the silver grains accumulated specifically on the internal root sheath in the early stage of keratinization, as indicated by the presence of trichohyalin granules, whereas no accumulation was observed in the hair bulbs. In the nail-forming region of the forelimb, dense grains were observed in the keratogenous zone of the nail matrix, but were not observed in the deepest layer of the nail matrix or in the nail plates.

Although the same expression patterns were observed

throughout with both anti-sense and sense riboprobes synthesized from three different fragments of the RAR-α cDNA, i.e., C-, E-, and F-domain, the signal intensity depends on the probes used: with the probe synthesized from the E-domain, the signals were weak but significant in the epidermis (fig.3a). On the other hand, with the riboprobes synthesized from the C- and F-domains (containing the 100 bp 3′-noncoding region), the intensive accumulation was observed in the same regions (epidermis: fig.3c for the C-domain and 3d for the F-domain). The same was observed in adult human epidermis and various mouse tissues expressing the RAR genes, such as the brain (the hippocampus, Purkinje cell), testis and stomach of adult mice (data not shown). Since the nucleotide sequence homology of the C-domain among the RAR cDNAs is higher than that of the E-domain [9,10], the probes for the C- and E-domains should hybridize with mRNAs transcribed from the RAR gene family (so far α, β and γ) and predominantly with RAR-α mRNA, respectively. Thus, the difference in the signal intensity with the probe used implies multiple expression of the RAR gene family.

3.3. *Temporal expression pattern of the RAR genes during development of mouse skins*

To determine temporal change of the RAR gene expression, we performed in situ hybridization on sections of the mouse forelimbs at various stages (11-day embryo – a week old after birth). The RAR mRNAs were detected also in both epidermis and dermis rather uniformly in 15-day embryonic fingers. Then, the hybridization signals in the dermis became weak in 18-day embryos and disappeared at birth. The signals in the epidermis became most intensive in the 4-day-old limb skins and then weaker in the 7-day-old.

4. DISCUSSION

Our in situ hybridization experiments suggest that the controlled expression of the RAR genes is specifically related to keratinization in the epidermis, hair follicles and nail matrices. These results are quite consistent with the fact that RA and its derivatives are effective remedies for cutaneous diseases like psoriasis [2,3]. Furthermore, in vitro, RA is known to control expres-

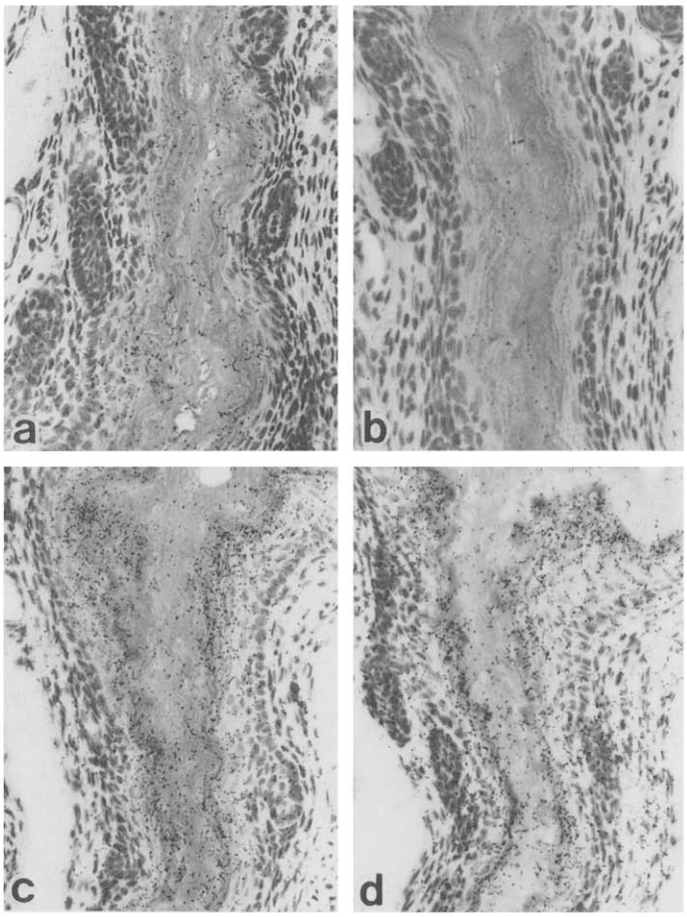

Fig.3. Localized expression of the RAR genes in cross section of the finger of 4-day-old mouse forelimb. The grains (black particles) were accumulated in a keratinizing front of the epidermis. (a) Probe for the E-domain, 14-day exposure; (b) for the elastin mRNA, 14-day exposure; (c) C-domain, 14-day exposure; (d) F-domain, 7-day exposure.

sion of keratin genes in the keratinocytes [2,3], depending upon the differentiation status. The epidermis is known to consist of at least 4 layers, each of which is composed largely of keratinocytes in different stages of differentiation. Only cells in the basal layer proliferate, but some of them cease to divide and start to undergo terminal differentiation, i.e., migrate toward the skin surface by synthesizing characteristic proteins essential for terminal differentiation. Essentially similar events should occur in hair- and nail-forming regions. It is interesting to note that the RAR genes are expressed predominantly in cells undergoing terminal differentiation, but only slightly in their stem cells. It is reasonable, therefore, to assume that RA plays a critical role in the control of terminal differentiation of the keratinocytes. Our reasoning is supported by the fact that treatment of skin with RA prevents keratinization [15]. The formation of a concentration gradient of RA in epidermis should be a intriguing working hypothesis to answer the question of how the differentiation of a keratinocyte is regulated after it has become committed to differentiate.

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