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Effects of Retinoic Acid Exposure *in utero* on Mouse Vibrissal Follicle Development

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With 2 tables

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Summary

It is known that topical all-*trans*-retinoic acid (RA) modulates growth and differentiation of skin and its cutaneous appendages. To examine whether a pre-natal exposure to a potentially non-teratogenic dosage of all-*trans*-RA had any effect on vibrissal follicle development, the histologic and immunohistochemical responses to RA during its morphogenesis in NMRI mouse were investigated. After a single oral dose of 30 mg/kg body weight of all-*trans*-RA on day 11.5 of gestation, no fetal malformations were detected and the histological features and the distribution of keratin (K) proteins in comparable stages of vibrissal development were similar for the untreated, vehicle-treated and RA-treated mice. The absence of teratogenic response and of adverse effects on the vibrissae under the experimental conditions indicates that this protocol may be useful for investigation of the effects of pre-natal exposure to RA on the post-natal development of experimental tumours in the mouse skin.

Introduction

Mouse skin is equipped with hair pelage follicles and, in the facial region, with large sensory hair follicles or vibrissae. Their potential quality is determined by the number, type and size of these hair follicles initiated during fetal life (Hardy and Vielkind, 1996). For the analysis of genetic and environmental effects on hair, the behaviour of the types of follicles in different species is of particular importance (Davidson and Hardy, 1952). Some useful tools to investigate morphogenesis and differentiation in skin are the keratin (K) proteins (Kopan and Fuchs, 1989), which are the major structural proteins of the vertebrate epidermis and its appendages, making up their cytoskeleton together with actin microfilaments and microtubules (Fuchs, 1995).

It has been established that compounds such as retinoids, including retinoic acid (RA), play a role on K expression in the keratinocytes (Stellmach et al., 1991), which express different types of RA receptors (Viallet and Dhouailly, 1994). Likewise some studies have shown that retinoids have profound influence on the morphogenesis, proliferation and differentiation of epithelial cells (Crave and Griffiths, 1996). Due to this role in epithelial cell differentiation (Lammer et al., 1985), RA is used for the therapy of dermatological (Sitzmann et al., 1995) and neoplastic diseases (Hill et al., 1995). Unfortunately, its clinical usefulness is limited by its teratogenic potential, which is well documented in laboratory animals (Kochhar et al., 1996) and humans (Rosa, 1983). The reports on the effects of retinoids on dermatology and oncology were carried out during the post-natal period after a topical application or an oral route of administration in animals (Verma, 1987; De Luca et al., 1993) and humans (Kligman et al., 1969; Peck et al., 1979), but no study on skin exposed *in utero* to RA has been found in the literature. Thus, the definition of an experimental design in which no congenital anomalies and no adverse effects on mouse embryonic skin were induced by pre-natal RA-exposure could be a first step towards the use of such protocol in research on the chemoprevention of adult mouse skin tumours using RA. Based on these facts, the aim of this study was to assess the *in vivo* effects of prenatal all-*trans*-RA exposure on its teratogenic activity as well as on embryonic vibrissal follicle development by analysing the morphological and K-distribution changes in NMRI mouse vibrissae.

Materials and Methods

Eight-week-old NMRI mice, weighing 30 to 35 g, were obtained from Antibioticos Laboratories S.A. (Leon, Spain). The animals were maintained in a cycle of 12 h light and 12 h dark, with a controlled temperature ($21 \pm 1^{\circ}C$) and $55 \pm 10\%$ relative humidity and were given free access to food and water. All experiments were performed following the guidelines of the European law on the Protection of Animals (Council Directive 86/609 EEC, 1986).

Mouse fetuses were obtained from pregnant mice which were treated orally with 30 mg/kg body weight of all-*trans*-RA (Sigma Chemical Company, St. Louis, MO, USA) in corn oil on day 11.5 of gestation (group RA-treated), treated orally with corn oil (group vehicle-treated) or untreated (group untreated). The day of the vaginal plug was designated as day 0.5 of pregnancy. All procedures involving manipulation of RA were carried out in the dark under dim yellow light to retard photo-degradation. Pregnant mice were killed by cervical dislocation from days 12.5 to 18.5 of pregnancy. Fetuses were removed from each uterus and examined under a dissection microscope.

The fetuses collected were fixed in Bouin's solution or in 70% ethanol, and five of each age of gestation and group were studied. Three-micrometre wax-embedded sections of each Bouin's fluid-fixed fetus were stained with haematoxylin and eosin (H-E) and periodic acid schiff (PAS). The vibrissal follicle morphogenesis has been described for the mouse by nine stages, previously defined by Davidson and Hardy (1952). For demonstration of K proteins, paraffin-wax sections were immunolabelled using the avidin biotin peroxidase complex (ABC) method (Peroxidase Standard, Vectastain, ABC kit; Vector Laboratories, Burlingame, CA, USA). Table 1 shows the work-

Table 1. Primary antibodies used for immunohistochemical labelling

Clone	Character/ species	Specificity	Dilution	Source
TROMA 1[a]	mo/R	CK 8	ud	R. Kemler[b]
AF 109	po/Rb	CK 1	1 in 2×10^5	BabCO, California, USA
AF 138	po/Rb	CK 5	1 in 10^5	BabCO, California, USA
MK6	po/Rb	CK 6	1 in 10^5	BabCO, California, USA
MK10	po/Rb	CK 10	1 in 4×10^4	BabCO, California, USA
AF 64	po/Rb	CK 14	1 in 10^5	BabCO, California, USA

[a] 70% ethanol-fixed tissues fetuses. [b] Dr. R. Kemler, Max Planck Institute, Freiburg, Germany. Unmasking of antigenic sites by heat pre-treatment. mo, monoclonal; po, polyclonal; CK, cytokeratin; M, mouse; R, rat; Rb, rabbit. ud, undiluted.

ing dilution and the characteristics of the primary antibodies used. The specificity of the immunoreactions were verified by staining negative and positive control tissue sections.

Results

After gross examination, no external malformations were found in the fetuses examined. The main morphological features of vibrissal development as well as the time-relations in histogenesis were similar in both control groups (vehicle-treated and untreated mice) and RA-treated mice. No qualitative and quantitative changes in the spatial and temporal expression patterns of Ks during the vibrissal follicle development were related to the treatment (Table 2).

At day 12.5 of gestation, the single-layered epidermis of the muzzle was covered by the periderm and some focal thickenings were developed, representing stage 1 of vibrissal follicle development (follicle plugs). One day later, mesenchymal cell condensation adjacent to follicle plugs, which grew deeper into the presumptive dermis, formed stage 2 (pre-papillae). In all the groups, the K expression of the vibrissae began on day 13.5 of gestation, showing a positive immunoreactivity for K14 (Table 2). At day 14.5 of gestation, the follicles elongated and surrounded the mesenchymal cell condensation (dermal papilla), forming stage 3 (papillae). In addition, a hollow cone, which later gives rise to the Inner Root Sheath (IRS), began to project upward through the tube of the external sheath and from the hair bulb (the deeper part of the follicle which appeared widened), making up stage 4 (hair cones). All vibrissae showed a positive reaction for K14 and K5 throughout the follicle epithelium except the hair bulb and the future cone-shaped IRS in stage 4 (Table 2). As this cone extended upwards, a group of epithelial cells appeared in the epidermis above the follicle forming the hair canal (stage 5 or hair canal). In this stage, the K expression in all groups examined was similar to that of stage 4, although K14-positive immunostaining was also observed in the hair canal (Table 2). In stage 6a (hair formation), a hair began to grow from the epithelial cells next to the dermal

Table 2. Stages of development of vibrissal hair follicles in NMRI control and RA-treated mice and their keratin expression

	Age from conception (days)						
Stages	12.5	13.5	14.5	15.5	16.5	17.5	18.5
Follicle plugs (1)	K−	K14+ K5−	K14+ K5+/−	K14+ K5+/−	K14+ K5+/−	K14+ K5+/−	K14+ K5+/−
Prepapillae (2)		K14+ K5−	K14+ K5+/−	K14+ K5+/−	K14+ K5+/−	K14+ K5+/−	K14+ K5+/−
Papillae (3)			K14+ K5+/−	K14+ K5+/−	K14+ K5+/−	K14+ K5+/−	K14+ K5+/−
Hair cones (4)			K14+[a] K5+/−[a]	K14+[a] K5+[a]	K14+[a] K5+[a]	K14+[a] K5+[a]	K14+[a] K5+[a]
Hair canals (5)			K14+[ab] K5+/−[a]	K14+[ab] K5+[a]	K14+[ab] K5+[a]	K14+[ab] K5+[a]	K14+[ab] K5+[a]
Hair formation (6a)			K14+[ab] K5+/−[a]	K14+[ab] K5+[a]	K14+[ab] K5+[a]	K14+[ab] K5+[a]	K14+[ab] K5+[a]
Opening of hair canals (6b)				K14+[ac] K5+[ac] K6+[c]	K14+[ac] K5+[ac] K6+[c]	K14+[ac] K5+[ac] K6+[c]	K14+[ac] K5+[ac] K6+[c]
Hairs in hair canals (7)				K14+[ac] K5+[ac] K6+[c]	K14+[ac] K5+[ac] K6+[c]	K14+[ac] K5+[ac] K6+[c]	K14+[ac] K5+[ac] K6+[c]
Hairs emerged (8)						K14+[ac] K5+[ac] K6+[c]	K14+[ac] K5+[ac] K6+[c]

+, positive; +/−, weakly positive; −, negative; no symbol: complete follicle primordium was positive for keratins (Ks) studied.
[a] outer root sheath (early/mature). [b] hair canal. [c] companion layer.

papilla, known as the hair matrix. In this stage, the K expression was identical to that described for stage 5 with negative reaction in IRS and hair (Table 2). From day 14.5 of gestation, blood cells were seen within the dermal sheath which surrounded the bottom-developing vibrissal follicle, representing the initial stage of blood sinus which appeared well differentiated 4 days later.

At day 15.5 of gestation, the hair canals began to open due to the keratinization of cells of hair canal (stage 6b or opening of hair canals) and they began to show a K6-positive companion layer (CL) made up by a single-cell layer of elongated cells which rested against the outermost face of the IRS. The CL was negative for K6 in the region of the hair bulb and the follicle above the sebaceous gland. The companion cells were also positive for K14 and K5 (Table 2). At stage 7 (hair in hair canals), the hair pierced the IRS and to reach the open hair canal. From day 15.5 of gestation onward, a few cells in the upper third of the vibrissal Outer Root Sheath (ORS) and epidermal basal cells close to these follicles showed positive reaction for K8, being identified as Merkel cells. At 16.5 day of gestation, sebaceous gland cells were seen for the first time, showing a positive immunoreactivity for K14. One day later, the hair emerged above the skin surface, being the last stage of vibrissal development (stage 8 or emerged hair). At stages 7 and 8, the K expression pattern was similar to that described for stage 6b (Table 2). All embryonic vibrissae showed negative immunolabelling for K1 and K10 in the three groups examined.

Discussion

Conflicting results on the effects of RA have been described in the literature because the extent of retinoid-induced damage depends on dosage, route of administration, the stage of development (Kalter and Warkany, 1961) and vehicle used (Lehman et al., 1988). The variety of results on RA-induced teratogenicity is even found when our data are compared with those obtained by other authors (Kochhar et al., 1996) who described several types of malformations in mice, using a similar protocol to that used in this study. Taking into account that the objective of this study was to obtain a potentially non-teratogenic dosage without adverse effects on skin and its appendages, our experimental design was established using a lower dosage than the 40 mg/kg dose employed by other studies which investigated the teratogenic activity in mice (Yasuda et al., 1987, 1989).

In contrast to *in vitro* studies which showed that RA led to the development of glomerular glands instead of hair vibrissal follicles (Dhouailly, 1993; Viallet and Dhouailly, 1994), the absence of RA-induced morphological changes using our protocol agrees with the results obtained in hair pelage follicles both in humans after topical application (Eichner et al., 1992) and in mouse teguments cultured *in vitro* with RA (Dhouailly, 1993; Viallet and Dhouailly, 1994). Particular attention was paid to the K expression during the development of mouse vibrissal follicle because, to the authors' knowledge, it has never been reported in the literature. As reported for the epidermis and hair pelage in previous researches (Banks-Schlegel, 1982; Fuchs, 1995), our data also indicate that during embryonic development the vibrissae undergo a programme of differentiation involving changes in the distribution of K proteins. Although in our case negative K1 and K10 expression was found in IRS, which differs from other results concerning hair pelage follicles of different species (Heid et al., 1988), in general the pattern of K expression during the vibrissal differentiation was similar to that described previously in hair follicles. The conservation of the pattern of K expression during the vibrissal differentiation in RA-treated embryos is in contrast to the changes observed in the epidermis both of *in vitro* RA-exposure mice (Lenoir-Viale et al., 1993) and of long-term topical RA treatment in humans (Eichner et al., 1992). As the RA-treated group did not show any changes in the patterns of expression of K5 and K14 proteins related to the proliferative activity of the epithelial cells (Byrne et al., 1994) when compared with the control group, our data may indicate that the protocol used in this study did not increase the hair growth, in contrast to the stimulating effect attributed to topical RA treatment in humans (Bazzano et al., 1986).

In conclusion, these preliminary results are encouraging concerning the possibility of using a non-teratogenic protocol of pre-natal RA-exposure, which induces no adverse effects on the morphogenesis of the skin and its hair follicles. Thus, it may represent a valuable protocol and a promising tool for use in studies on the protection afforded by pre-natal RA-exposure on the development of experimental skin tumours in the exposed offspring; a protective effect previously demonstrated after post-natal RA-administration in mice (Chen et al., 1994). The relevance for RA exposure *in utero* compared with other routes of administration used during the post-natal period is to improve the knowledge of the protective effect of this retinoid on skin carcinogenesis with respect to its local/systemic or temporal actions. Further studies into the effects of RA exposure *in utero* on mouse pelage hair follicles and the epidermis should be carried out to confirm these findings.

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