2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) Affects Keratin 1 and Keratin 17 Gene Expression and Differentially Induces Keratinization in Hairless Mouse Skin

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The environmental pollutant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) causes chloracne in humans by mechanisms that are as yet poorly understood. Because TCDD is known to affect keratinocyte differentiation *in vitro*, we have studied TCDD-dependent morphologic changes and the expression of murine keratin 1 (MK1; differentiation associated) and keratin 17 (MK17; presumably hyperproliferation associated) in HRS/J hr/hr hairless mouse skin. TCDD (0.2 μg in acetone) applied topically to the dorsal skin caused epidermal acanthosis and hyperkeratosis of the dermal cysts as well as an involution of the utricles and the sebaceous glands. By means of *in situ* hybridization with digoxigenin-labeled riboprobes of sections from untreated and vehicle (control)-treated skin, we localized MK1 mRNA to the epidermal spinous cell compartment. MK17 transcripts were detected only in the derivatives of the hair follicle-utricle epithelium and dermal cysts. No spatial overlap was observed between MK1 and MK17 expression. After TCDD application, MK17 was newly expressed in the upper spinous cell layers of the interfollicular epidermis, although it was suppressed in the involuting utricles. In contrast, MK1 expression in the interfollicular epidermis was not affected by TCDD. Furthermore, MK1 expression was induced in the epithelium of the utricle remnants and in some dermal cysts. These data suggest that increased keratinization of the part of the follicular epithelium corresponding to the dermal cyst epithelium of hairless mice most probably explains the pathogenesis of TCDD-induced chloracne. The results demonstrate, furthermore, that TCDD can differentially affect keratinocyte differentiation *in vivo* as well as *in vitro*. *Key words: chloracne/hair follicle/HRS/J mice. J Invest Dermatol 108:330–335, 1997*

The environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent member of a class of halogenated polycyclic compounds. In animals, TCDD produces pleiotropic responses such as thymus involution, immunosuppression, reproductive and endocrine effects, hepatotoxicity, developmental toxicity, and teratogenicity (Poland and Knutson, 1982; Neubert, 1992; Birnbaum, 1994). In addition, TCDD is a potent tumor promoter *in vitro* and *in vivo*. In addition to induction of hepatic adenomas, tumor formation may be promoted or inhibited in murine skin, depending on the experimental conditions (Poland *et al*, 1982; Poland and Knutson, 1982). In contrast to animal studies, only few effects have been demonstrated in humans (Mocarelli *et al*, 1992). The most consistent and common clinical sign of TCDD in humans is the occurrence of chloracne, which is characterized by keratinized cutaneous cysts and plugs (Schulz, 1957; Crow, 1970). The underlying molecular mechanisms and cellular events leading to these skin alterations have so far not been clarified.

The biologic and probably most of the toxic effects of TCDD are generally considered to be mediated by specific binding to the aryl hydrocarbon receptor (Poland and Knutson, 1982; Birnbaum, 1994), which is a ligand-activated transcription factor that belongs to the family of helix–loop–helix DNA-binding proteins (Whitlock, 1990; Dolwick *et al*, 1993). In transformed human keratinocyte cell lines and primary keratinocytes, TCDD has been shown to affect several genes encoding growth- and differentiation-regulating proteins (Sutter *et al*, 1991; Puga *et al*, 1992; Sutter and Greenlee, 1992; Okey *et al*, 1994), resulting in enhanced terminal differentiation (Osborne and Greenlee, 1985; Greenlee *et al*, 1987). Based on these and published histologic data (Puhvel *et al*, 1982; Moses and Prioleau, 1985), it seems likely that the *in vivo* effects of TCDD in human and animal skin are associated with altered patterns of keratinocyte differentiation.

The expression of specific keratins is one of the main characteristics of keratinocyte differentiation in mammalian skin (Moll *et al*, 1982a; Fuchs, 1993). The expression patterns for most of the keratins described so far are well defined for normal and pathologic proliferation and differentiation of keratinocytes (Moll *et al*, 1982a;

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Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; MK1, murine keratin 1, MK17, murine keratin 17.

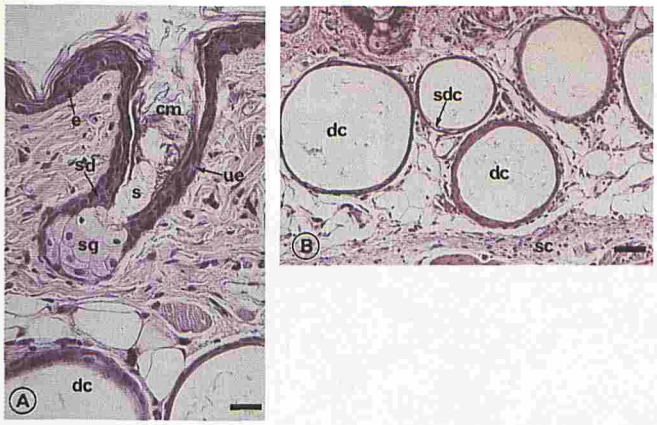

Figure 1. Histology of vehicle (control)-treated HRS/J mouse skin. HRS/J mice were treated with acetone as vehicle control. Paraffin-embedded sections were stained with hematoxylin and eosin. *(A)* Utricle with sebaceous gland. Note the accumulation of sebum and scales within the utricular lumen. *(B)* Dermal cysts with epithelium displaying various squamous and sebaceous differentiation patterns. e, Epidermis; cm, cornified material; dc, dermal cyst; s, sebum; sc, subcutis; sd, sebaceous gland duct; sdc, sebaceous cells in the epithelium of dermal cysts; sg, sebaceous gland; ue, utricular epithelium. *Scale bars: (A)* 23 μm; *(B)* 45 μm.

Fuchs, 1992). Keratin expression therefore seems to be a reliable marker with which to evaluate the effect of TCDD on keratinocyte physiology *in vivo*. To obtain further insight into TCDD-induced alterations on epidermal and hair follicle keratinocyte differentiation *in vivo*, we have studied the histology and the patterns of keratin 1 and keratin 17 gene expression in hairless HRS/J hr/hr mouse skin at different stages of TCDD-induced chloracne. Keratins 1 and 17 have been chosen for these studies, because keratin 1 has been reported to be associated with a differentiating phenotype of keratinocytes in human and murine epidermis (Fuchs and Green, 1980; Roop *et al*, 1987), whereas keratin 17 has so far not been detected in normal human or murine epidermis. Instead, it is associated with several physiologic and pathologic epidermal hyperproliferative processes and is normally expressed in human and murine hair follicles (Moll *et al*, 1982a; Weiss *et al*, 1984; Knapp *et al*, 1987; de Jong *et al*, 1991).

The hairless mice used in the current study carry a mutation in the hr locus and are known to respond to dioxin with skin lesions similar to those of human chloracne (Puhvel *et al*, 1982; Poland *et al*, 1984). Their skin contains specific structures (utricles and dermal cysts) that are homologs of different parts of the normal hair follicle (Montagna, 1952; Sundberg, 1994). These HRS/J hr/hr mice, therefore, appear to be a suitable model to study the effect of dioxin on the hair follicle, which is known to be the main skin structure involved in the development of chloracne (Crow, 1970).

METHODS AND MATERIALS

Animals and Their Maintenance HRS/J hr/hr hairless female mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Principles of laboratory animal care were followed as well as the current version of the German law on the protection of animals. Prior to the start of the experiment, the animals were adapted to the following maintenance conditions: constant 12/12-h day/night cycle at 21°C and relative humidity of about 50%, standard pellet feed (Altromin 1324; Altromin, Lage, Germany), and water *ad libitum*. The animals were free of any cutaneous lesions. At the beginning of the experiment (day 1), the animals were 14–18 wk of age.

Treatment of Mice and Collection of Samples 2,3,7,8-TCDD in toluene solution obtained from Ökumetric (Bayreuth, Germany) was dissolved in acetone (Merck, Darmstadt, Germany) 1:9 (vol/vol) to prepare a solution suitable for topical application (Connor *et al*, 1994). TCDD-treated and vehicle-treated animals were kept in separate cages under the same conditions. TCDD was applied topically to the back of the animals in 100 μl of the solution (0.2 μg of TCDD) every third day for 25 d (altogether eight applications). Untreated animals served as additional controls.

Dorsal skin was collected on day 1, 7, 13, 19, and 25 of the experiment. For every time point, three animals were sacrificed. Skin was fixed in 4% paraformaldehyde and embedded in paraffin (JUNG-Histowax; Reichert-Jung, Heidelberg, Germany) according to standard procedures. Five-micrometer sections were mounted on silane-coated glass slides (two slides were analyzed for each mouse) and used for *in situ* hybridization, as described previously or stained with hematoxylin and eosin (Mayer's hemalaun, Merck, Darmstadt, Germany) for routine histology.

cDNA Template Preparation A defined 255-bp fragment of the murine keratin 17 (MK17) cDNA clone (Knapp *et al*, 1987) was obtained as a gift from Dr. J. Schweizer, Deutsches Krebsforschungszentrum, Heidelberg, Germany. The fragment was ligated into a pSP72 vector (Promega, Madison, WI). Plasmids were prepared with the Quiagen plasmid midi kit (Quiagen, Studio City, CA).

For murine keratin 1 (MK1) cDNA preparation, total RNA was isolated from murine skin as described by Chomczynski and co-workers (Chomczynski and Sacchi , 1987) and transcribed into cDNA by using a reverse transcription–coupled polymerase chain reaction kit (Stratagene, Heidelberg, Germany). For the reverse transcription-coupled polymerase chain

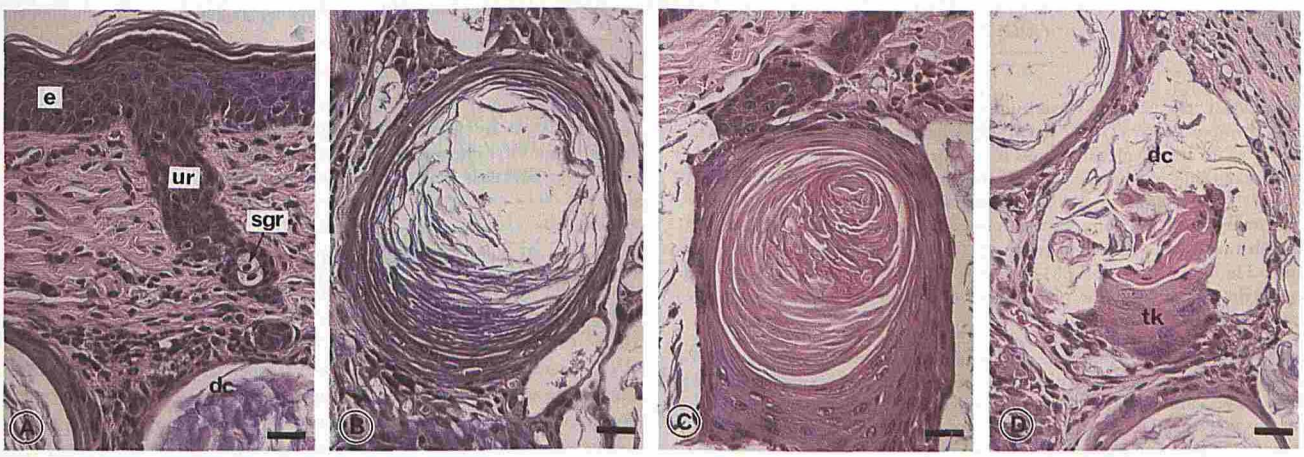

Figure 2. TCDD results in the involution of utricles and sebaceous glands. HRS/J mice were treated with TCDD every third day for 25 d. Paraffin-embedded sections were stained with hematoxylin and eosin. *(A)* Involuted utricle with a remnant of sebaceous gland during the early period of TCDD treatment (day 7). *(B)* Dermal cyst exhibiting keratinization of the "epidermal type" (day 13 of TCDD treatment). *(C)* Dermal cyst with keratinization of the "hair type" midway during TCDD treatment (day 13). *(D)* Zone of trichilemmal keratinization in the remnant of a dermal cyst of the "epidermal type" late during TCDD treatment (day 19). dc, Dermal cyst; e, epidermis; ur, utricular remnant; sgr, sebaceous gland remnant; tk, zone of trichilemmal keratinization. *Scale bars*, 23 μm.

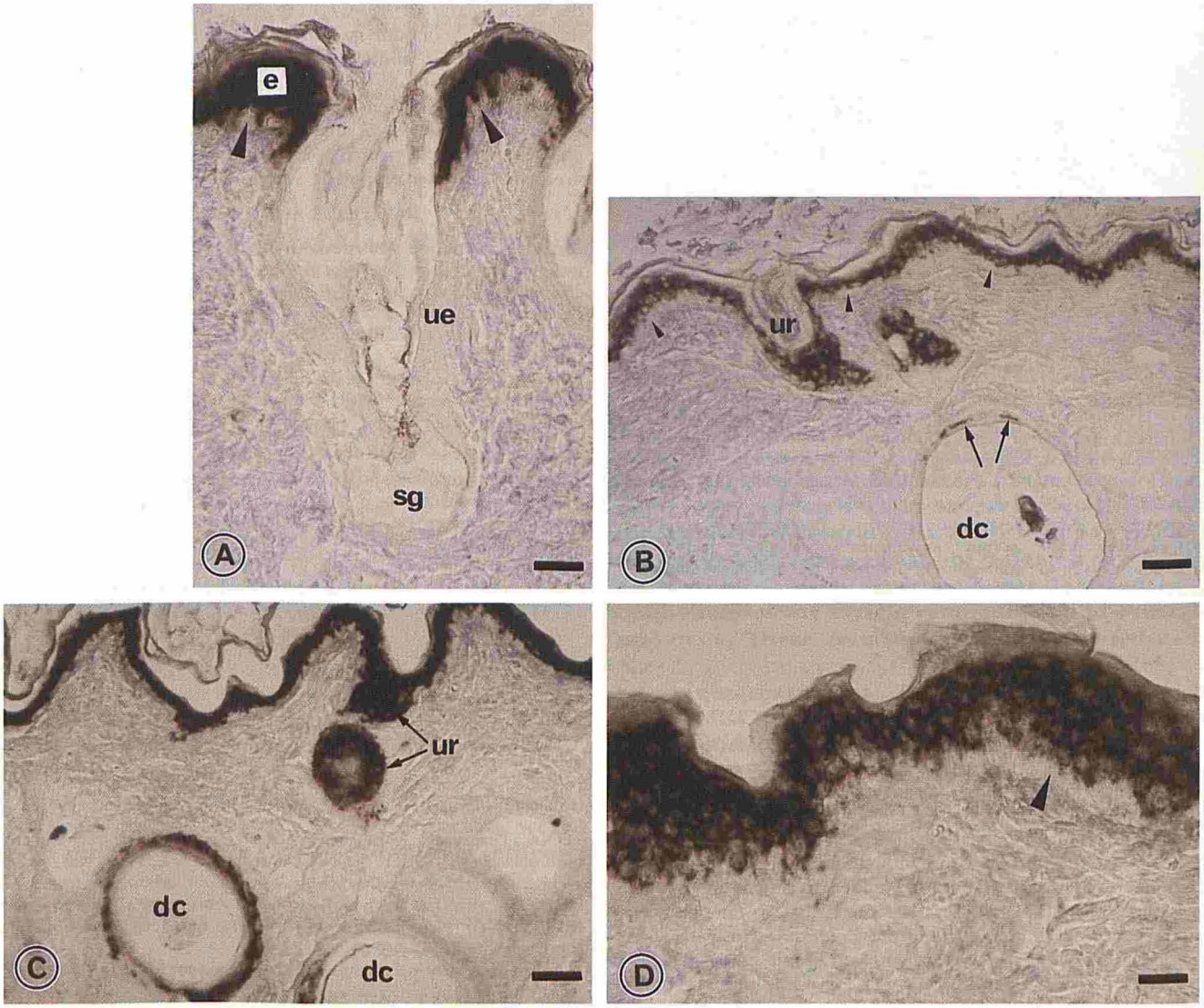

Figure 3. MK1 mRNA expression is increased by TCDD treatment. Vehicle (control)- and TCDD-treated skin were examined by *in situ* hybridization with a digoxigenin-labeled MK1 complementary RNA probe. *(A)* In acetone-treated HRS/J mouse skin, MK1 mRNA is expressed in spinous cells of the interfollicular epidermis. *(B)* In the early phase of TCDD treatment (day 7), MK1 mRNA is expressed in the epidermis and utricular remnants and sparsely in restricted portions of a dermal cysts (→). *(C)* The epithelium of dermal cysts expresses MK1 mRNA midway during TCDD treatment (day 13). *(D)* The entire epidermis is positive for MK1 mRNA at the end of TCDD treatment (day 25). For additional details, see **Fig 1** and **Fig 2**. ➤, Basal cell layer of the epidermis. *Scale bars: (A,D)* 23 μm; *(B,C)* 45 μm.

reaction of MK1, the primers were nucleotides 1664–1681 and 1881–1905 (accession number M10937) at 0.1 μM. Cycling was allowed to proceed for 2 min at 94°C for one cycle, followed for 1 min at 94°C and 1 min at 63°C for 35 cycles, ending with 7 min at 72°C. In a total volume of 50 μl, we used Ampli*Taq* DNA polymerase at 0.05 unit/μl (Applied Biosystems, Weiterstadt, Germany) and all four deoxynucleotide triphosphates (each at 200 μM) (Boehringer Mannheim, Germany). The 242-bp product was gel-purified and ligated into the pGEM-T vector (Promega, Madison, WI). The identity of the clone used for preparation of riboprobes was checked by restriction endonuclease analysis.

Preparation of Riboprobes Digoxigenin-labeled complementary RNAs were generated *in vitro* with 1 μg of the linearized plasmids by using the "DIG RNA labeling kit" (Boehringer Mannheim). T7 and Sp6 RNA polymerases were used to transcribe sense and antisense probes for MK1 and MK17, according to the manufacturer's protocol.

***In Situ* Hybridization** *In situ* hybridization was performed as described (Nürnberg *et al*, 1995), with 50 ng of freshly denatured complementary RNA probes per section at 50°C for 17 h. The MK1 and MK17 sense probes were used as negative control. For immunodetection of the *in situ* hybridization signal, the slides where incubated with sheep alkaline phosphatase-labeled anti-digoxigenin antibodies (DIG nucleic acid detection kit, Boehringer Mannheim) for 3 h at room temperature. The final staining was accomplished by incubation of the slides in nitroblue tetrazolium and β-chloroindolyl phosphate (Boehringer Mannheim) solution for 16–20 h in complete darkness at room temperature.

RESULTS

TCDD Results in the Involution of Utricles and Sebaceous Glands In the skin of untreated or vehicle (control)-treated mice, the sebaceous glands were connected to the hyperkeratinized utricles by short ducts (the proximal part of the utricular epithelium) consisting of polygonal keratinocytes **(Fig 1*A*)**. Dermal cysts were lined by thin atrophic epithelium that exhibited occasional sebaceous gland differentiation **(Fig 1*A,B*)**.

In the TCDD-treated skin, the utricles underwent gradual involution. A reduction in size of the sebaceous glands was clearly evident early on (day 7) **(Fig 2*A*)**, and involution was complete by the middle of treatment (day 13). Dermal cysts reacted to TCDD treatment (day 13) with the loss of sebaceous cells in their epithelium and markedly increased size associated with the accumulation of concentrically arranged layers of thin scales and an

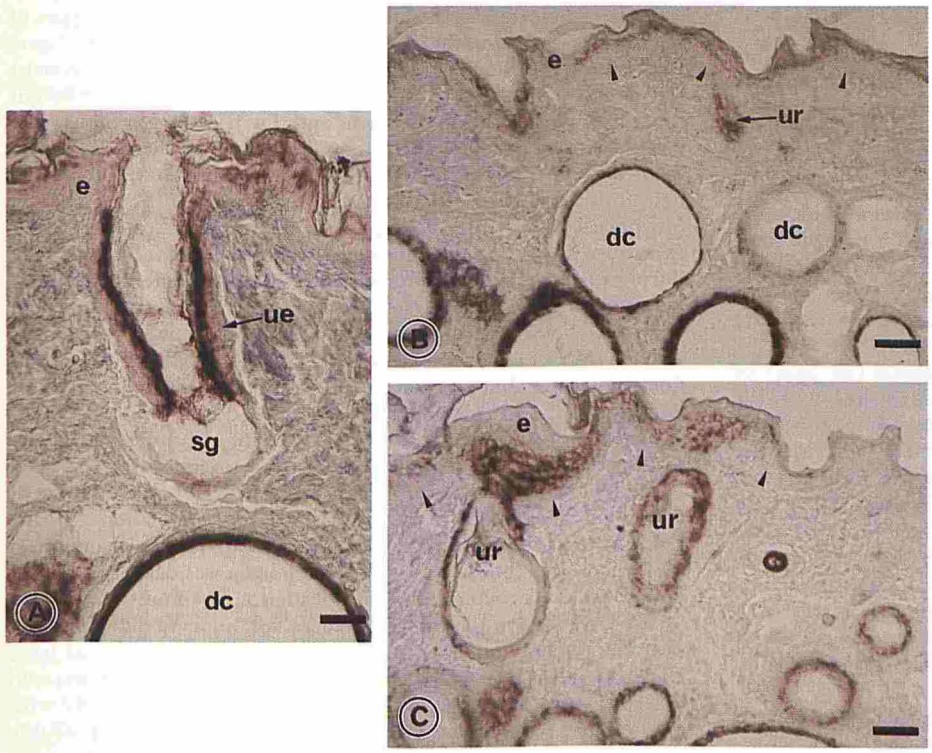

Figure 4. TCDD results in MK17 mRNA expression in the interfollicular epidermis. *In situ* hybridization with a digoxigenin-labeled MK17 complementary RNA probe was performed in vehicle control or TCDD-treated skin. *(A)* In acetone-treated HRS mouse skin, MK17 mRNA is present in the inner cell layers of the utricular epithelium and in dermal cyst epithelium but is absent in the interfollicular epidermis and the sebaceous glands. *(B)* Low to absent MK17 expression in utricles and in the interfollicular epidermis, with variable degrees of expression in dermal cyst epithelium early on during TCDD treatment (day 7). *(C)* MK17 mRNA in utricular epithelium and adjacent epidermis. *Scale bars: (A)* 23 μm; *(B,C)* 45 μm.

atrophic epithelium **(Fig 2*B*)** or with marked acanthosis of the cyst epithelium and the accumulation of eosinophilic keratinous material within the cyst cavity **(Fig 2*C*)**. In some cysts, the epithelium exhibited unusual patterns of differentiation such as keratohyalin-deficient keratinization, particularly during the late stages of TCDD treatment (days 13–25) **(Fig 2*D*)**.

MK1 mRNA Expression in Dermal Cyst Epithelium Is Increased by TCDD In untreated and vehicle (control)-treated mouse skin, MK1 transcripts were detected in all spinous cells of the interfollicular epidermis, reaching into the upper part of the utricular lining. Basal keratinocytes and the hair follicle derivatives (lower utricles, dermal cysts, sebaceous glands) displayed no signals **(Fig 3*A*)**.

By day 7 of TCDD treatment, MK1 transcripts were also detected in the lower parts of the utricular epithelium, although these structures underwent involution at this stage of the experiment. A weakly positive signal was noted in certain parts of the epithelium of several dermal cysts **(Fig 3*B*)**.

After day 13 of TCDD treatment, only a few remnants of utricular epithelium were detectable, although they still expressed MK1. In the epithelium of some dermal cysts, the level of MK1 expression reached that of the epidermis; in some cysts, it was completely absent. Staining was generally not evenly distributed over the entire dermal cyst epithelium, as shown in **Fig 3*C***.

At the end of TCDD treatment (day 25), MK1 transcription was detectable in only a few remaining dermal cysts. A high number of MK1 transcripts was noted in all cells of the acanthotic spinous layer of the epidermis **(Fig 3*D*)**.

TCDD Results in MK17 mRNA Expression in the Interfollicular Epidermis In the skin of untreated and vehicle (control)-treated mice, MK17 transcripts were absent in the interfollicular epidermis and the sebaceous glands, but they were clearly detectable in the utricles and the dermal cysts **(Fig 4*A*)**.

By day 7 of TCDD treatment, the level of MK17 expression was decreased in the involuting utricles. This held also for some dermal cysts, while others still actively expressed MK17 mRNA. Scattered zones of weak MK17 mRNA staining appeared in the upper spinous cell layers of interfollicular epidermis **(Fig 4*B*)**, sometimes in spatial association with utricular remnants **(Fig 4*C*)**.

By day 13 of treatment, only few remnants of utricular epithelium were present, and these lacked MK17 gene expression. The amount of MK17 transcripts in several portions of the epidermis was enhanced and located in the upper spinous cell layers, just beneath the stratum corneum.

At the end of treatment (day 25), MK17 transcripts were detectable in the spinous cell layer of most parts of the epidermis (data not shown).

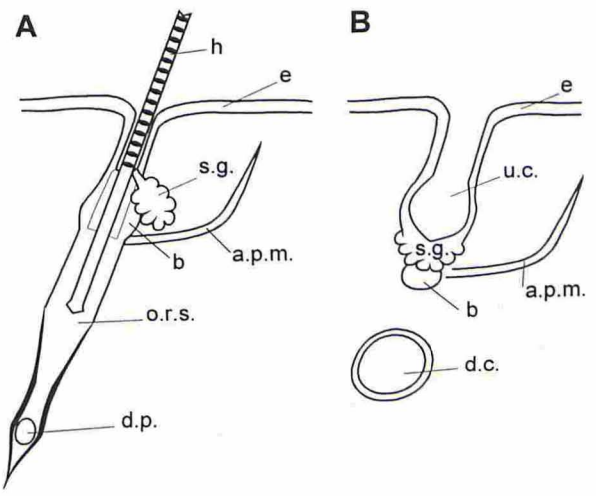

Figure 5. Scheme of a normal murine hair follicle and related structures in hairless HRS/J mice. *(A)* Normal hair follicle in catagen. *(B)* Hair follicle rudiments in hairless HRS/J mouse skin. a.p.m., Arrector pili muscle; b, bulge; d.c., dermal cyst; d.p., dermal papilla; e, epidermis; h, hair shaft; o.r.s., outer root sheath; s.g., sebaceous gland; u.c., utricular cavity.

DISCUSSION

HRS/J is an inbred mouse strain segregating for the hairless (hr) locus (Sundberg, 1994), which presumably determines, together with the Ah (dioxin)-receptor locus, the high susceptibility to dioxin-induced skin alterations of this mouse strain (Poland *et al*, 1984). The reason for the hair loss in hr/hr mice is not completely understood, even though the molecular basis of the underlying mutation has been defined (Stoye *et al*, 1988; Cachon-Gonzalez *et al*, 1994). It has been suggested that defects during the catagen stage of the first hair cycle after birth lead to the formation of two separate follicle derivatives: epidermis-associated utricles and disconnected dermal cysts (Montagna, 1952; Sundberg *et al*, 1991) **(Fig 5)**.

The localization of MK1 transcripts in suprabasal cell layers of the interfollicular murine epidermis **(Fig 3*A*)** is in line with the previously reported association of its protein expression with epidermal keratinocyte differentiation (Schweizer and Winter, 1983; Roop *et al*, 1988). In utricular epithelium, MK1 mRNA was found only in the portion most proximal to the epidermis **(Fig 3*B*)**, where an epidermal type of keratinization has been assumed (Kopan and Fuchs, 1989). Our finding of persisting MK1 expression in TCDD-treated epidermis as well **(Fig 3*A,D*)** suggests that TCDD-induced acanthosis in hairless mouse skin is not associated with increased recruitment of proliferating suprabasal keratinocytes, as seems to occur in hyperproliferative skin diseases such as psoriasis (Reichert, 1994).

MK17 expression is induced by TCDD in the interfollicular epidermis, primarily in cells of the upper spinous layer where keratinocytes have reached an advanced stage of differentiation **(Fig 4*B*)**. Cells in this layer expressed MK1 as well **(Fig 3*D*)** and thus are presumably not proliferating (Stoler *et al*, 1988). At early stages of TCDD treatment, MK17 expression was weakly detectable in the interfollicular epidermis, particularly in proximity to utricles **(Fig 4*C*)**. The outer root sheath of the hair follicle has been suggested to contain a specific keratinocyte subpopulation with high migratory ability that plays an essential role in epidermal wound healing (Lenoir *et al*, 1988). This cell population typically expresses MK17 and is localized to the suprainfundibular portion of the outer root sheath, a region that corresponds with the utricles in hairless mouse skin **(Fig 5)**.[1] Possibly, TCDD stimulates the MK17-transcribing keratinocytes to migrate from the utricular epithelium to the epidermal compartment, thus explaining the loss of MK17 expression in the utricles and the appearance of staining in the interfollicular epithelium close to the utricles **(Fig 4*C*)**.

TCDD appears to evoke two pathways in the utricular epithelium: (i) differentiation of the epidermal type and (ii) involution of the utricle. The involution of utricles seems to be the predominant effect, and neither the utricles nor the sebaceous glands apparently play a role in the formation of chloracne-like lesion in the skin of hairless mice. Our results suggest that instead, only the dermal cysts are the "key players" in the development of chloracne-like hyperkeratinized structures in the skin of these mice.

The dermal cyst epithelium in hairless mouse skin has been thought to originate from sebaceous glands (Montagna, 1952). Dermal cysts have therefore been suggested to respond to TCDD with squamous metaplasia (Poland *et al*, 1984; Puhvel *et al*, 1991). On the basis of our data, however, the cellular response of sebaceous glands and dermal cysts to TCDD is completely divergent. The epithelium of dermal cysts in u treated hairless mouse skin actively expresses MK17, as does the proximal outer root sheath,[1] whereas sebaceous glands fail to do so **(Fig 4*A*)**. These findings, together with previously published histologic data (Sundberg, 1994), lead us to the assumption that dermal cysts in hairless mouse skin originate from keratinocytes of the proximal cycling part of the hair follicle and not from sebaceous glands **(Fig 5)**. This is further supported by the fact that TCDD evokes various types of keratinization in dermal cysts **(Fig 2*B–D*)**, and the proximal part of the normal follicular outer root sheath is also known to consist of several keratinocyte subpopulations that exhibit different types of keratinization (Kopan and Fuchs, 1989). Thus, the epithelium of dermal cysts is represented by cells originating from different keratinocyte populations of the proximal part of hair follicles during the abnormal first catagen in the skin of newborn HRS/J mice. These cell groups seem to keep their specific differentiation potential after the hair follicles break during the first catagen after birth, resulting in different types of keratinization. Dermal cysts in untreated hairless mice undergo very slow keratinization, and completely keratinized cysts occur only in skin of aged mice (Sundberg, 1994). TCDD application does not alter but accelerates the keratinization program of dermal cysts, and this process is completed in 2–3 wk.

The dioxin-induced keratohyalin-deficient keratinization in dermal cysts **(Fig 2*D*)** is very similar (if not identical) to the so-called "trichilemmal keratinization" described for several cystic tumors of epidermal appendages and for certain cell populations in the proximal part of the hair follicle (Pinkus, 1969). The induction of this type of keratinization in restricted portions of dermal cysts also underlines the structural diversity of their epithelium.

We have shown that dioxin induces keratinocyte differentiation not only *in vitro* but also *in vivo*. All epithelial structures that have been studied in the skin of hairless mice (epidermis, utricles, and different types of dermal cyst epithelium) respond to TCDD with different patterns of differentiation and keratin expression, probably reflecting keratinocyte subpopulations within these structures with different intrinsic differentiation programs. Furthermore, our results show that the dioxin-induced development of specific chloracne-like lesions in the skin of hairless mice does not involve sebaceous glands, nor is it due to hyperkeratinization of the distal part of the hair follicle (utricles). TCDD-induced chloracne seems instead to be induced by an accelerated keratinocyte differentiation program in specific parts of the follicular epithelium, corresponding to dermal cysts in hairless mice.

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