Increased Frequency of Spontaneous Skin Tumors in Transgenic Mice Which Overexpress Ornithine Decarboxylase

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Abstract

Ornithine decarboxylase, a critical regulatory enzyme for polyamine biosynthesis, is highly inducible by growth-promoting stimuli in mouse epidermis but the enzyme level is only transiently elevated due to rapid turnover of the protein. Here we report that constitutive overexpression of the enzyme in the skin of transgenic mice causes several phenotypic abnormalities. Effects observed include development of dermal follicular cysts, excessive skin wrinkling, enhanced nail growth, alopecia, and spontaneous tumor development. These results indicate that up-regulation of polyamine biosynthesis can profoundly disturb skin homeostasis and alter susceptibility to neoplastic development.

Introduction

ODC^3 catalyzes the decarboxylation of L-ornithine to the diamine putrescine. Putrescine and the polyamines spermidine and spermine are cellular polycations essential for normal cell proliferation and differentiation. ODC expression in mammalian tissues is usually tightly regulated, although neoplastic cells often express high levels of the enzyme (1). Many factors other than the level of ODC activity, including other polyamine biosynthetic and degradative enzymes, the intracellular ornithine concentration, and polyamine excretion, exist to regulate intracellular polyamine concentrations. Nevertheless, one approach to study the possible deleterious effects of sustained high levels of polyamines on cell proliferation, differentiation, and neoplastic transformation is to up-regulate ODC expression constitutively. Several groups have used transfection and retroviral infection techniques to overexpress an exogenous ODC cDNA in various cell types (2-5). In addition, Halkemeyto et al. (6) have developed transgenic mice that overexpress the human ODC gene in certain tissues such as brain and testis. Only a modest increase in the basal level of ODC was reported for epidermis in these mice and no phenotypic effects in the skin of untreated mice were observed although the mice were more susceptible to a skin chemical carcinogenesis protocol (7). In the present study, we decided to target ODC overexpression to epidermis. This tissue was chosen because of its high ornithine content, which should facilitate polyamine accumulation if ODC is overexpressed (8), and its utility as a well-characterized model of multi-stage carcinogenesis (9-11). Moreover, we decided to target ODC expression to a specific subpopulation of epidermal keratinocytes via the use of a bovine keratin IV promoter (homologous to mouse and human keratin 6; hereafter referred to as K6) promoter-driven ODC transgene that should direct expression to cells of the outer root sheath of the hair follicle (12). By targeting this cell population within epidermis we hoped to determine the effect of increased polyamine levels on epithelial homeostasis and carcinogenic risk.

Materials and Methods

Production and Characterization of Transgenic Mice. Transgenic mice were generated by DNA microinjection of fertilized B6C3F2 oocytes by using standard techniques (13). The transgene was a 20-kb fragment derived from NotI digestion of a pBR322-based vector containing a K6 minimal promoter upstream of a murine ODC cDNA from -69 to +1682 bp of the ODC cDNA sequence (14), containing an introduced stop codon at position 427. Genomic DNA was isolated from the tails of potential founder mice and subjected to both Southern blot and PCR analyses to identify mice bearing the transgene. For Southern analyses, DNAs digested with EcoRI were electrophoresed on 1% agarose gels, blotted onto nitrocellulose filters, and sequentially probed by using a 1.75-kb ODC cDNA fragment and a DNA probe derived from the K6 promoter. Both probes were radiolabeled with 32P by random prime labeling and hybridized to the same 2.8-kb EcoRI fragment containing transgene sequence. For PCR analyses, the 5'-primer, P1, was a K6 promoter sequence (5'--GGCAAGGGAGCCATTACAC-3') and the 3'-primer, P2, was an oligonucleotide the sequence of which corresponded to an ODC coding sequence (5'--GGCGGATTTAGGAGAGGG-3'). The amplified 1.2-kb fragment, therefore, spanned the junction between the K6 promoter and the ODC sequence and was only detected in mice bearing the transgene. Control PCR reactions were performed by using two other primers designed to amplify exon 11 of the endogenous ODC gene (5'--GGGCTATTTAGGAGG-3' and 5'--CCACCAAGGCAAGCAATCA-3').

Histology and Immunocytochemistry. For histological analysis, tissues were fixed overnight in 10% neutral buffered formalin or Fekete's solution (60% EtOH, 3.2% formaldehyde, and 0.75 M acetic acid), embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin. For immunolocalization of ODC, skin sections were incubated with an anti-ODC antiserum and specific staining was detected with an ABC Vectastain kit (Vector Laboratories, Burlingame, CA) as described previously (15).

Biochemical Methods. To prepare tissue extracts for ODC activity and polyamine determinations, transgenic mice were killed, and the skin was excised and exposed to 55°C dH20 for 20 s. The epidermis was subsequently scraped off, and portions were placed either in buffer A [25 mM Tris-HCl (pH 7.5), 2.5 mM DTT, and 0.1 mM EDTA] or 0.2 N perchloric acid. The remaining dermis was minced thoroughly with scissors and portions were placed in buffer A or 0.2 N perchloric acid. Tissues were homogenized in a Polytron homogenizer and centrifuged at 20,000 x g for 20 min. The resulting supernatants were assayed for ODC activity (buffer A extract) or polyamine levels (0.2 N perchloric acid extract) as described (7). Normal littermates were handled similarly, except preshaved animals were depilated for 5 min immediately after killing. For ODC, results are expressed as units/mg protein, where 1 unit = 1 nmol C02 liberated/h. For polyamines, results are expressed as nmol polyamine/mg DNA.

Results and Discussion

A DNA fragment in which a mutant mouse ODC cDNA (containing a premature stop codon at position 427 resulting in a carboxyl-
Fig. 1. Production of transgenic mice which over-express ODC. A, a pBR322-based vector containing a keratin 6 minimal promoter upstream of a murine ODC cDNA (from -69 to +1682 bp of the ODC cDNA sequence (13), containing an introduced stop codon at position 427) was prepared. For DNA microinjection, the plasmid was digested with NotI, and the linearized 20-kb fragment was purified on a 1% agarose gel. B, genomic DNAs from four founder mice and three non-transgenic littermates were digested with EcoRI and subjected to Southern analysis. The probe used was a 1.75-kb ODC cDNA fragment. Lane 9 represents the plasmid DNA used for microinjection. A 2.8-kb band of varying intensity is present only in DNA derived from founder mice. C, the same blot shown in B was stripped and rehybridized with a probe derived from the K6 promoter. D, PCR analysis of tail DNA from founder mice and normal-appearing littermates. Primers derived from the K6 promoter (P1) and the ODC cDNA (P2) were used to amplify a 1.2-kb fragment only in founder mice (Lanes 1-6). Lanes 1-3, normal littermates, whereas Lanes 4-6 are reactions from founder animals. The same DNAs were used in a separate PCR reaction by using primers designed to amplify exon 11 of the endogenous ODC gene (Lanes 7-12). kb, kilobase pair.

Fig. 2. Skin of normal sibling (A) and transgenic mouse (B) showing the replacement of the s.c. fat tissue by the large follicular cysts that extend to the muscle layer. C, a higher power view of transgenic skin. Note that the cysts are variable in size and are lined by well-differentiated keratinocytes. D, immunohistochemistry (performed as described in Ref. 15 at an antibody dilution of 1:3000) shows expression (brown color) of ODC in the dermis confined to the cell layer lining the cysts (D), with no positive staining in the epidermis. A and B, × 125; C and D, × 250.
complete hair loss, including vibrissae, by 2 months of age. With
founders analyzed. Founder mice were characterized by smaller birth
weight, a normal first hair cycle, followed by progressive hair loss
beginning at 2-3 weeks of age, and excessive nail growth. Hair loss
was complete in six of eight founders, but the transgenic progeny of
the transgene were integrated into genomic DNA in each founder (Fig.
3). Southern analysis of tail DNA by using primers designed to amplify a
fragment spanning the K6 and ODC sequences of the transgene (Fig.
1A) was microinjected into the male pronucleus of fertilized B6C3F2
ooocytes to produce transgenic mice. The truncated ODC protein
produced would be expected to have a much longer in vivo half-life
than a full-length ODC (16). Eight founder mice were identified by
PCR analysis of tail DNA by using primers designed to amplify a
unit of ODC activity is equivalent to 1 nmol CO₂ liberated from L-ornithine/h at
37°C. The L-ornithine concentration used was 0.125 mm.

Table 1 Ornithine decarboxylase levels in transgenic mouse skin
A unit of ODC activity is equivalent to 1 nmol CO₂ liberated from L-ornithine/h at
37°C. The L-ornithine concentration used was 0.125 mm. The L-ornithine concentration used was 0.125 mm.

Table 2 Polyamine levels in normal versus transgenic skin
Polyamine levels were determined as described in Ref. 5.

Fig. 3. Western analysis of ODC expression in founder versus normal mice. Crude extracts of founder epidermis (Lane 6) or dermis (Lanes 2, 3, and 5) or dermis of littermate controls (Lanes 1 and 4) were run on a 10% SDS-PAGE gel, transferred to nitrocellulose, and incubated with an ODC-specific antibody at a 1:20,000 dilution. An extract of testosterone-induced mouse kidney was run as a positive control (Lane 7). kd, molecular weight in thousands.

Terminal truncation placed downstream of the K6 promoter (Fig. 1A)
was microinjected into the male pronucleus of fertilized B6C3F2
ooocytes to produce transgenic mice. The truncated ODC protein
produced would be expected to have a much longer in vivo half-life
than a full-length ODC (16). Eight founder mice were identified by
PCR analysis of tail DNA by using primers designed to amplify a
fragment spanning the K6 and ODC sequences of the transgene (Fig.
1D). Southern analysis of tail DNA revealed that multiple copies of
the transgene were integrated into genomic DNA in each founder (Fig.
1, B and C). Quantitative image analysis on a phosphorimager indi-
cated that transgene copy number varied from 4 to 24 for the various
founders analyzed. Founder mice were characterized by smaller birth
weight, a normal first hair cycle, followed by progressive hair loss
beginning at 2-3 weeks of age, and excessive nail growth. Hair loss
was complete in six of eight founders, but the transgenic progeny of
all founders, including those with only partial hair loss, exhibited
complete hair loss, including vibrissae, by 2 months of age. With
increasing age, the skin exhibited pronounced wrinkling and folding
and was excessively oily. Histological examination revealed that the
skin of transgenic mice contained large follicular cysts in the dermis
underlying an apparently normal epidermis save for the lack of hair
follicles (Fig. 2B). The sebaceous glands adjacent to the follicular
cysts were moderately hyperplastic. The cysts in older founders (>3
months) are very large and completely replace the s.c. fat layer. Most
founders (six of eight) were fertile and three males are being bred to
establish transgenic lines. Preliminary results from breeding exper-
iments indicate that the hair loss phenotype is dominant, and the
transgene is inherited in a classic Mendelian fashion (data not shown).

The ODC activity in the skin of transgenic mice was greatly
elevated over normal littermates (Table 1). When epidermis and
dermis from the same animals were analyzed separately, the majority
of increased ODC activity was present in the dermis. The specific
activity of ODC in epidermis ranged from 1.4 to 41.3 units/mg protein
in various founders, whereas the specific activity in the dermis con-
taining follicular cysts was both higher and less variable among
different mice (33-89.8 units/mg). The absolute values of ODC
activity in transgenic dermis were much higher than reported previ-
ously for induced levels of this enzyme in epidermis (17, 18). Immu-
nocytochemical analysis demonstrated high levels of ODC expression
in small, flattened keratinocytes lining the dermal follicular cysts.
There was no detectable overexpression of ODC in the epidermis by
using this method of analysis (Fig. 2D). To confirm that the elevated
ODC expression was actually due to transgene expression, Western
analysis was performed (Fig. 3). The only protein detected in extracts
of transgenic epidermis or dermis was approximately Mₐ, 49,000, the
predicted size of the transgene product based on the premature stop
codon engineered into the construct. No expression of the endogenous
gene product (Mₐ, ~54,000) was detected in either normal or trans-
genic epidermis or dermis. Polyamine levels, especially putrescine
and spermidine, were greatly elevated in transgenic mouse dermis,
and less so in epidermis relative to levels in control littermate tissues
(Table 2). For instance, in founder four, putrescine was elevated
37-fold over normal littermate values in dermis, but only a 4-fold
elevation was present in epidermis.

Transgenic mice developed spontaneous skin tumors at a high
frequency. For instance, three of eight founders developed at least one
grossly visible skin lesion. The F1 progeny of founders also developed
skin tumors at a similar frequency (i.e., about 40%), whereas we have
never observed a spontaneous skin tumor in normal littermates, even
in older animals. All of the skin lesions were squamous neoplasms
with varying degrees of dysplasia and aggressive character. The
histology of five of the spontaneous tumors is shown in Fig. 4.
Keratoacanthoma-like lesions were a common finding (Fig. 4, A-C),
whereas well-differentiated papillomatous lesions (Fig. 4, D and E)
were also observed. Tumors were frequently found on the tail, face,
and ears, as well as the dorsal and ventral skin. Squamous cell
carcinomas have not been observed.

Many of the tumors have been analyzed for ODC activity; the

Table 2 Polyamine levels in normal versus transgenic skin
Polyamine levels were determined as described in Ref. 5.

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ODC overexpression in mice and skin tumor frequency

Fig. 4. Histological sections of spontaneous tumors from transgenic mice. Many of the lesions are keratoacanthoma-like, characterized by dome-shaped endophytic lesions with moderately differentiated keratinocytes and a central area filled with keratin (k) from the mouth (A), the tail (B and F), and the ear (C). Bone tissue (b) and skeletal muscle are seen in panel B. D, detail of a squamous lesion from the dorsal skin with more aggressive cytological features. F, immunolocalization of ODC in the tumor shown in E. In contrast to Fig. 2D, the tumor was fixed overnight in Fekete’s solution and subsequently embedded in paraffin. Sections were cut and incubated with anti-ODC antibody at a dilution of 1:500.

results are summarized in Table 3. The absolute values of ODC-specific activity were extremely high in all tumors. It is worth noting that the mean value shown in Table 3 is 11-fold higher than the mean of a series of epidermal papillomas induced by a 7,12-dimethylbenz(a)anthracene/12-O-tetradecanoylphorbol-13-acetate-initiation promotion protocol recently analyzed by us (8). When the tumor illustrated in Fig. 4E was analyzed for ODC expression immunocytochemically, intense staining was observed throughout the tumor, particularly in the suprabasal cells. Western analysis indicated that ODC overexpression in most cases was due to elevated transgene expression, although significant coexpression of the endogenous ODC gene was detected in some tumors (data not shown). Most of the tumors arose in older mice and did not regress. Because of the relatively long interval between onset of transgene expression and tumor appearance, as well as the low multiplicity of tumors/mouse, it is likely that a second genetic or epigenetic event
has occurred that cooperates with ODC overexpression to facilitate tumor development. Consistent with this idea is the recent report (5) that constitutive overexpression of ODC is not sufficient to induce tumors in normal epithelial or fibroblast cells but is sufficient to enhance tumor development in initiated keratinocyte cell lines. There are several obvious candidates for a “second hit,” such as mutations in proto-oncogenes or loss of functional tumor suppressor genes. It will be of interest to determine the nature and diversity of genetic alterations that can cooperate with ODC overexpression in this model.

Our results indicate that ODC overexpression targeted to the outer root sheath cells of the hair follicle can profoundly alter skin structure and function. One of the earliest phenotypes observed is the disruption of the hair cycle. At present, we have little information regarding the time of onset of ODC overexpression, although small follicular cysts are present in transgenic skin as early as 2 weeks of age (data not shown). Because the first hair cycle is normal, it is likely that ODC transgene expression does not become significant until sometime after birth. Although there are numerous mutations in the mouse that cause hair loss (19), the functions of most of the genes involved are not known. The mice described here could be a useful model for characterizing genes regulated by increased polyamine levels, for studies on the properties of follicular stem cells (20), and for addressing the hypothesis that cells resident in hair follicles are the progenitors of carcinogen-induced epithelial tumors of the skin.

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Table 3  
ODC activity in spontaneous skin tumors arising in transgenic mice

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<th>Tumor</th>
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Mean ± SEM 83.6 ± 18.0

References
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