Severe Abnormalities in the Oral Mucosa Induced by Suprabasal Expression of Epidermal Keratin K10 in Transgenic Mice*

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Received for publication, May 24, 2002, and in revised form, July 8, 2002
Published, JBC Papers in Press, July 15, 2002, DOI 10.1074/jbc.M205143200

Previous studies have demonstrated that keratin K10 plays an important role in mediating cell signaling processes, since the ectopic expression of this keratin induces cell cycle arrest in proliferating cells in vitro and in vivo. However, apart from its well known function of providing epithelial cells with resilience to mechanical trauma, little is known about its possible roles in nondividing cells. To investigate what these might be, transgenic mice were generated in which the expression of K10 was driven by bovine K6β gene control elements (bK6β/hK10). The transgenic mice displayed severe abnormalities in the tongue and palate but not in other K6-expressing cells such as those of the esophagus, nails, and hair follicles. The lesions in the tongue and palate included the cytolysis of epithelial suprabasal cells associated with an acute inflammatory response and lymphocyte infiltration. The alterations in the oral mucosa caused the death of transgenic pups soon after birth, probably because sucking was impai red. These anomalies, together with others found in the teeth, are reminiscent of the lesions observed in some patients with pachyonychia congenita, an inherited epithelial fragility associated with mutations in keratins K6 and K16. Although no epithelial fragility was observed in the bK6β/hK10 oral epithelia of the experimental mice, necrotic processes were seen. Collectively, these data show that the carefully regulated tissue- and differentiation-specific patterns displayed by the keratin genes have dramatic consequences on the biological behavior of epithelial cells and that changes in the specific composition of the keratin intermediate filament cytoskeleton can affect their physiology, in particular those of the oral mucosa.

Keratin intermediate filaments (KIFs)1 are present in the cytoplasm of all epithelial cells as heteropolymers of type I and type II keratin polypeptides. Type I and type II keratin genes display highly regulated expression patterns in a pairwise and differentiation-specific fashion (1–3). The role of KIF in epithelial cells and tissues remained elusive until the discovery, through studies with transgenic mice and the finding of mutations affecting keratin proteins in dominantly inherited epithelial fragility syndromes (4–8), that keratins impart mechanical resilience to cells. This appears to be a function shared by the majority of the keratin family. Therefore, the changes in keratin expression observed during differentiation (or in certain situations such as in tumor growth or wound healing involving stratified epithelia) probably indicate subtle, cell type-specific differences in function among these polypeptides. Further keratin-specific functions should not be discarded.

Previous studies have demonstrated that K10 has specific functions. This keratin replaces K14 as skin keratinocytes enter the terminal differentiation program and become postmitotic (9). In addition, K10 expression is severely reduced under hyperproliferative situations, such as in wound healing and epidermal tumors. We have previously demonstrated that forced K10 expression in cultured cells induces cell cycle arrest in vivo

* This work was supported in part by Spanish MCYT Grant PB94-1230 and CAM Grant 08.1/0054/2001.1. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: KIF, keratin intermediate filament; hK, human keratin; mK, mouse keratin; bK, bovine keratin; AC, anterior column; BC, buttress column; PC, posterior column.

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transgenes to stratified epithelia in transgenic mice (15, 20, 21). We have previously reported two lines of transgenic mice expressing human keratin K10 (hK10) under the control of the bovine keratin K6β promoter (bK6β/hK10 mice) (25). Although they displayed no overt phenotype, a clear delay was found in tumor development when these mice were subjected to skin chemical carcinogenesis protocols (25). However, this is a relatively minor effect compared with that observed in bK5hK10 transgenic mice, which are almost completely resistant to tumor development (12). This difference is probably attributable to the expression of hK10 in different cell compartments. The three mK6 genes are normally absent from interfollicular epidermis, but they are rapidly induced upon hyperproliferative stimuli in suprabasal keratinocytes (13, 15, 16). Only one, namely mK6a, is expressed in the basal layer of the hyperproliferative epidermis (13, 16), where bK5 is expressed (26). In contrast, the bovine bK6β regulatory elements drive the expression of the transgene, similarly to the endogenous mK6b gene, in the suprabasal layers of the hyperproliferative epidermis (13, 15, 16). In this compartment, the keratinocytes display a very limited proliferative activity compared with the basal layer cells. In addition, differences in the level of K10 expression may also contribute toward explaining the observed differences in tumorigenic susceptibility between bK5hK10 and bK6β/hK10 transgenic mice. In support of this, heterozygous bK5hK10 mice do not display overt epidermal abnormalities, whereas hypoplastic and hyperkeratotic epidermises have been observed in homozygous bK5hK10 transgenic mice in parallel with increased expression of the transgene (12). This is also in agreement with our observations demonstrating that the effects of keratin K10 are clearly related to its expression level (10, 11).

In this work, we have tried to address the possible functions of K10 in nonproliferative cells by studying the consequences of hK10 expression in tissues normally expressing K6. As previously reported, bK6β/hK10 animals display no obvious phenotype even in homoygosis; we have thus generated new bK6β/hK10 transgenic mice lines bearing a higher copy number of the transgene in order to increase the expression of hK10 in those cells in which bK6β is active. In this context, it is important to point out that the bK6β promoter has an expression pattern very similar to the endogenous mouse keratin K6b (mK6b) (13–16).

All these high copy number transgenic mice display a clear phenotype that affects the oral mucosa and is characterized by the necrosis of the suprabasal cells of the tongue, palate, and gingival epithelium, in association with acute inflammation. This leads to severe shedding of the epithelium, causing periapical inflammation in animals with 15 or fewer copies of the bK6β/hK10 transgene (Fig. 1, A and B). Generation of Transgenic Mice Expressing bK6β/hK10—To monitor the proper expression of the bK6β/hK10 construct (Fig. 1A), transient transfection experiments were performed in MCA3D mouse keratinocytes, which express K6. Synthesis and incorporation of hK10 into the endogenous keratin cytoskeleton was observed in all transfection experiments, with no sign of cytoskeletal disruption (arrows in Fig. 1, B and B′). Similar results were obtained using bovine BMGE+H cells. No expression of the construct was detected when using cell lines that did not express K6 (VeroC, PtK2, and NIH3T3; not shown). Since the absence of keratin clumping and the proper incorporation of hK10 into the keratin cytoskeleton was observed in all experiments, these results indicate that K10 does not cause the collapse of the endogenous cytoskeleton, contrary to the reported effects of increased hK15 (30–32) and mutant mK6 (14) expression.

The linearized bK6β/hK10 construct was subsequently injected into fertilized oocytes. We have previously reported that in animals with 15 or fewer copies of the bK6β/hK10 transgene, no overt phenotype (besides a delay in tumor formation) is
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Overexpression of bK6βK10 Leads to Severe Necrosis and Acute Inflammation of the Oral Mucosa—Transgenic bK6βK10 pups appeared normal at birth, but within a few days they were smaller and weaker and had less milk in their stomachs (sometimes none) in comparison with their control littermates (Fig. 1C, arrows). These pups generally died between 3 and 5 days postpartum, weighing about half that of their littermate controls (1.4 and 2.9 g, respectively, on average). At death, the skin and nails of these mice appeared normal (Fig. 1C and data not shown) and showed no obvious developmental anomalies other than reduced size and frail appearance.

Since the bK6β transgene is constitutively expressed in several oral epithelia (15) and since one possible reason for the observed mortality of the bK6βK10 transgenics could be poor feeding, the anatomy of the oral cavity of these mice was examined. The dorsal surface of the tongue and, to a lesser extent, the ventral surface of the upper palate were covered with white plaques from the midregion to the pharynx (denoted by lp in Fig. 1D and data not shown). Nontransgenic littermates (Fig. 1D') showed no signs of these lesions. Similar features have been reported for mice lacking keratins mK6a and mK6b (13, 16) and are similar to the oral leukoplakia seen in some pachyonychia congenita patients (23, 34).

Histological studies of sections from the tongue of bK6βK10 mice demonstrated severe damage from the midregion to the posterior region of the dorsal epithelium. Extensive areas with consistent features of coagulative necrotic changes were seen, with loss of cell cytoplasm and degenerative nuclei. Some tissue observed (25). Given that the expression levels of transgenes under the control of the bK5 and bK6 promoter regions are frequently proportional to the copy number of the transgene integrated (12, 25, 33) (data not shown), attempts were made to generate transgenic mice with more than 25 copies of the transgene. Two founders were obtained bearing 50 and 75 copies, respectively (see Fig. 1D) and endogenous K6 (B') expression. Note that in all cases K10 is integrated into the endogenous keratin cytoskeleton (at least 200 cells were scored per experiment; experiments were performed in triplicate). C, appearance of transgenic (right) and wild type (left) 3-day-old littermate. Note the runt appearance of the transgenic animal. The arrows indicate the empty stomach in the transgenic compared with the milk-containing stomach in its wild type littermate. D, example of Southern blot showing the identification of the transgenic animals and the estimated copy number of the transgene after normalization and phosphorimaging scan. E, abnormalities in transgenic tongue; note the leukoplakia lesions observed from the midregion to the posterior region of the dorsal tongue in the transgenics (denoted by lp). E', normal tongue in wild type littermate.

FIG. 1. Structure of the transgene, expression in cultured cells, and gross abnormalities in bK6βK10 transgenic mice. A, structure of the bK6βK10 transgene used in these studies, including the bK6β gene regulatory region (shadowed) and the hK10 gene showing the exons and introns. B, expression of bK6βhK10 in MCA3D mouse keratinocyte cells 48 h after transfection. Transfected cells were analyzed by double immunofluorescence for hK10 (B) and endogenous K6 (B') expression. Note that in all cases K10 is integrated into the endogenous keratin cytoskeleton (at least 200 cells were scored per experiment; experiments were performed in triplicate). C, appearance of transgenic (right) and wild type (left) 3-day-old littermate. Note the runt appearance of the transgenic animal. The arrows indicate the empty stomach in the transgenic compared with the milk-containing stomach in its wild type littermate. D, example of Southern blot showing the identification of the transgenic animals and the estimated copy number of the transgene after normalization and phosphorimaging scan. E, abnormalities in transgenic tongue; note the leukoplakia lesions observed from the midregion to the posterior region of the dorsal tongue in the transgenics (denoted by lp). E', normal tongue in wild type littermate.

FIG. 2. Histological abnormalities in the epithelium of the tongue and palate of bK6βhK10 transgenic mice. Hematoxylin-eosin-stained sections of the tongue (A, A', and A') and palate (B and B') in wild type (A, A', and B) and transgenic mice (A' and B'). See the common filiform papillae arrangement of the dorsal epithelium in the tongue of the wild type (A) compartmentalized in the anterior column, posterior column, and buttress column (ac, pc, and bc, respectively in A'). The transgenic suprabasal layers of the epithelium in the tongue (A') and palate (B') appeared necrotic and infiltrated by neutrophil leukocytes, whereas the basal layers remained unaffected. A moderate increase in the number of cell layers in this region can also be observed in the transgenic samples. Dashed lines denote the epithelial boundaries in A' and B'. Bar, 100 μm.
The immunohistochemical analysis of the tongue of newborn bK6/H9252 transgenic mice. Peroxidase-hematoxylin-stained sections of the tongue (A–C) and palate (D–E) in wild type (A and D) and transgenic mice (B–D). The mK6 expression is restricted to the suprabasal epithelium of the palate (p in A) and the anterior and butts column of the tongue (t in A). K10 in the transgensics is strongly expressed in the same regions of the nonlesional regions of the tongue (B) as well as in the necrotic plaque of the tongue (C) and the palate (D). Dashed lines denote the epithelial boundaries. Bars, 100 μm.

The injured superficial areas expanded the tongue and palate epithelium with a mixture of neutrophils and cell debris disrupted from the several unaffected rows of basoloid cells normally attached to the basement membrane (Figs. 2A and 4B, semithin section). This shedding process of the necrotic suprabasal areas was probably induced by the intensive discharge of gelatinase and other proteases present in the granules of the abundant neutrophils. No major alterations were observed in the subjacent muscle of the tongue (Fig. 4B). Most of the pathological changes in the lingual, gingival, and palate epithelia are similar to those described for mice lacking mK6a or mK6b genes (13, 16). In these deficient mice, the lesions are associated with a decrease in (or even the absence of) KIF in the anterior compartment, which induces an important increase in the size of intercellular spaces (13, 16). Although a similar absence of KIF was not expected, it could not be ruled out a priori that other changes in the keratin cytoskeleton might occur in the bK6βK10 mice. To investigate such possible changes, ultrastructural analyses were performed.

Ultrastructural Pathology of the Tongue of bK6βK10 Transgenic Mice—The dorsal epithelium of the tongue in control and transgenic mice was studied by transmission electron microscopy. In wild-type animals, differentiating keratinocytes in the anterior column (AC) showed electron dense granules similar to those found in the granular layer of the epidermis (37). The butts column (BC) keratinocytes had a flattened shape and large bundles of densely packed KIF but no keratohyalin granules. Finally, the keratinocytes of the posterior column (PC) were rounded and had a well organized keratin cytoskeleton, although less bundled than in BC keratinocytes. An example of the different regions of a control filiform papilla, as observed with the electron microscope, is provided in Fig. 4A. Samples taken from the same region in bK6βH10 transgenic mice (semithin section in Fig. 4B) showed dramatic alterations in the AC and BC keratinocytes, readily visible through their cytolitic and distended appearance and unusually clear and swollen cytoplasm (Figs. 4D and 5A, asterisks). The AC cells appeared to have a normal complement of keratohyalin granules, but these were larger than those of wild type mice (Fig. 4C; wild type in Fig. 4A). Desmosomes and KIF were found in these cytolitic swollen cells (data not shown and Fig. 5A, arrows). Similar cytolytic events were observed in BC keratinocytes (Fig. 4C), which also displayed normally arranged KIF bundles and desmosomes (Fig. 5B). No major abnormalities were observed in the PC or basal keratinocytes (Figs. 4D and 5A), which do not express the transgene (Figs. 3, B and C). Neutrophil leukocytes were seen infiltrating the cytolytic areas of the AC and BC (arrows in Fig. 4D, pmn in Fig. 5A, and data not shown). Despite the severe cytolitic changes observed in these regions, the affected cells maintained intact desmosomes (white arrows in Fig. 5B), and the intercellular spaces were not enlarged (Fig. 4, C and D, and Fig. 5A). Since the hK10 expression is restricted to the AC and BC in agreement with the expression of endogenous mK6 (Fig. 3, compare B with A), these findings suggest that these cells are particu-
particularly susceptible to changes in the expression, either quantitative or qualitative, of keratin polypeptides. In this regard, the blisters occurring in mK6a/b-deficient mice have been attributed to the absence of KIF in these cells (13, 16), whereas KIF and desmosomes normally arranged were clearly observed even in cytolytic cells (Fig. 5, A and B). Data obtained with mK6a/b-deficient mice have been interpreted as an indication that the cells in the AC region of the filiform papillae are particularly sensitive to the mechanical stress of suckling (13, 16). However, some animals were maintained by parenteral feeding, and similar lesions still occurred (not shown), indicating that mechanical stress is not the only origin of the observed tongue and palate anomalies. Nonetheless, it is worth mentioning that some differences exist between the lesions observed in mK6a/b null mice and those of our transgenic animals. In particular, desmosomes and KIF are normally arranged, and intercellular edema is absent from the tongue lesions in bK6bK10 mice, whereas in K6a/b-deficient mice KIF is decreased or absent in the AC, and the intercellular spaces are enlarged (13, 16). This clearly points to a different etiology in these apparently similar lesions; in the deficient mice there is a clear epithelial fragility process, whereas in the present case the defects are more related to an acute inflammatory response.

**The Expression of Other Keratins Is Not Altered in the Tongue of bK6bK10 Transgenic Mice**—The above results do not rule out the possibility that the ectopic expression of hK10 can affect the composition of the keratin cytoskeleton of the tongue keratinocytes. To investigate this possibility, protein extracts prepared from whole tongues of low copy number (15 copies) nontransgenic and high copy number (75 copies) transgenic mice (Fig. 6) were studied by Western blotting. As a control, protein extracts from human skin were included. The anti-K10 antibody detects a single polypeptide in the extracts from human skin and transgenic tongues. In addition, the level of expression of hK10 was increased in those extracts from transgenic animals with high transgene copy number. The amounts of the characteristic keratin polypeptides of the suprabasal tongue keratinocytes, namely K13 and K6 (36), were not significantly different between the transgenic and control samples. Finally, compared with the controls, the protein levels of the basal keratins K5 and K14 were not altered in transgenic samples. Collectively, these results indicate that the expression of hK10 does not produce major alterations in the keratin expression profile. It is worth mentioning that the hK10 expression levels in high copy number transgenic mice are very similar to those of endogenous hK10 in human epidermis, indicating that the phenotype found in these mice cannot be attributed to an aberrant excessive overexpression of the transgenic protein.

**bK6bK10 Transgenic Mice Have No Hair or Nail Abnormalities**—Keratin K6 is constitutively expressed in the outer root sheath of the hair follicles and nail bed epithelium. Possible alterations in these cutaneous structures were therefore studied. No abnormalities were found between control and transgenic mice in the hair or interfollicular skin by day 4 after birth.
and from wild type (A) birth in K4-deficient mice (38), in clear contrast with the appearance of esophageal alterations only 2 months after the absence of alterations in the tongue of these null mice. However, it cannot be ruled out that the levels of hK10 transgene expression may vary among the different K6-expressing cells in the different tissues, thus promoting phenotypic changes (or not) or that these cells may display different intrinsic susceptibility to possible K10-induced effects.

Tooth Abnormalities in bK6bhK10 Transgenic Mice—Different tissues from bK6bhK10 mice were analyzed, and no ectopic expression of K10 besides that observed in K6-expressing cells was seen. The findings in oral mucosa closely resemble those observed in patients suffering from pachyonychia congenita. One of the variants of this disease, the Jackson-Lawler form or pachyonychia congenita type II, is characterized by the presence of abnormalities in the teeth of the transgenic mice (A) in newborn wild type (A and B) and transgenic mouse (A' and B'). Note the precocious eruption of the incisor in the transgenic (A') in comparison with the wild type (A). The gingival epithelium displays similar necrotic changes in suprabasal layers as described for the tongue and palate epithelium (arrows in A'). The inset in A' shows the degenerative region in the transgenic incisor. Higher magnification of these degenerative areas of the transgenic incisor (B'), compared with wild type littermate (B). Note the affected ameloblast (am) and odontoblast (od) layers as well as the absence of the dentine layer (denoted by de in B). p, dental pulp, od, odontoblast layer; de, dentine; en, enamel; am, ameloblast layer; ep, enamel pulp. Bars, 50 μm.

As with the hair follicles, no alterations were detected in the esophagus or forestomach (not shown). Again, the absence of abnormalities may be due to the early death of bK6bhK10 transgenic mice, which precludes the detection of lesions that might occur later. Supporting the early lethality hypothesis is the appearance of esophageal alterations only 2 months after birth in K4-deficient mice (38), in clear contrast with the absence of alterations in the tongue of these null mice. However, it cannot be ruled out that the levels of hK10 transgene expression may vary among the different K6-expressing cells in the different tissues, thus promoting phenotypic changes (or not) or that these cells may display different intrinsic susceptibility to possible K10-induced effects.

Tooth Abnormalities in bK6bhK10 Transgenic Mice—Different tissues from bK6bhK10 mice were analyzed, and no ectopic expression of K10 besides that observed in K6-expressing cells was seen. The findings in oral mucosa closely resemble those observed in patients suffering from pachyonychia congenita. One of the variants of this disease, the Jackson-Lawler form or pachyonychia congenita type II, is characterized by the presence of neonatal teeth (24, 39–41). We therefore investigated the presence of abnormalities in the teeth of the transgenic mice. These animals displayed severe teeth anomalies such as defects in position and precocious eruption of the incisors, in some cases associated with microdontia (Fig. 8A’ and data not shown; wild type in Fig. 8A). Hematoxylin-eosin sections revealed a clear decrease (even absence) of the thickness of the dentine layer (de in Fig. 8B, wild type; transgenic shown in Fig. 8B') associated with degenerative changes in the ameloblast and odontoblast layers (am and od in Fig. 8, B and B', wild type and transgenic, respectively). In addition, some degenerative changes were also observed (see inset in Fig. 8A’). Collectively, these abnormalities are clearly suggestive of specific roles of keratin-expressing cells in the process of tooth growth. In this regard, it has been demonstrated that keratins participate in the assembly of amelogenin during amelogenesis, supporting a possible involvement of these proteins in tooth development (42). This important and complex issue will be the subject of future studies.

Many of the phenotypic features observed in the bK6bhK10 mice resemble those characterized in pachyonychia congenita patients. This disease is an autosomal dominant ectodermal...
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