Collagenase Expression in Transgenic Mouse Skin Causes Hyperkeratosis and Acanthosis and Increases Susceptibility to Tumorigenesis

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In a series of transgenic mice, the human tissue collagenase gene was expressed in the suprabasal layer of the skin epidermis. Visually, the mice had dry and scaly skin which upon histological analysis revealed acanthosis, hyperkeratosis, and epidermal hyperplasia. At the ultrastructural level, intercellular granular materials were absent in the transgenic skin epidermis but contact was maintained through the intact desmosomes. Despite a diversity of underlying etiologies, similar morphological hyperproliferative changes in the epidermis are observed in the human skin diseases of lamellar ichthyosis, atopic dermatitis, and psoriasis. Subsequent experiments demonstrate that when the transgenic mouse skin was treated once with an initiator (7,12-dimethyl-benz[*a*]anthracene) and then twice weekly with a promoter (12-*O*-tetradecanoylphorbol-13-acetate), there was a marked increase in tumor incidence among transgenic mice compared with that among control littermates. These experiments demonstrate that by overexpressing the highly specific proteolytic enzyme collagenase, a cascade of events leading to profound morphological changes which augment the sensitivity of the skin towards carcinogenesis is initiated in the epidermis.

The skin, the largest organ of the body, is composed of two predominant layers. The outermost layer is a stratified, squamous, keratinizing epithelium called the epidermis and is separated by a basement membrane from the dermis, a layer rich in vasculature and nerves. The major cell type in the epidermis is the keratinocyte, which arises from a basal layer of proliferative cells attached at their bases to the basement membrane. As these epithelial cells progress in an ordered fashion towards the outer surface, they undergo and complete their differentiation into keratinocytes (35).

Tissue collagenase (EC 3.4.24.7) is a matrix metalloproteinase expressed in most tissues that undergo remodeling (22). Collagenase expression has also been observed in a number of disease states, including rheumatoid arthritis, periodontal lesions, and cancer invasion (6, 22). In the skin, dermal fibroblasts and epidermal keratinocytes synthesize the proteinase (21, 28). Under various conditions, the expression levels of collagenase in the epidermis are appreciably elevated above baseline (14, 27, 28). For example, keratinocytes at the leading edge of a wound secrete increased amounts of collagenase (9, 14) which allow the cells to migrate during the healing process (14). Similarly, cultured keratinocytes have been shown to enhance collagenase synthesis up to 34-fold after treatment with the tumor promoter TPA (12-*O*-tetradecanoylphorbol-13-acetate) (27, 28).

The role of increased collagenase synthesis in pathological conditions of the skin is difficult to discern, because it is often only one of many ill-defined effects produced by an initiating stimulus. In this study, a direct analysis of the effect of collagenase on the skin was made by bypassing the nonspecific

inflammatory cells which are the source of the proteolytic enzyme. These transgenic mice will provide a physiological model in which to study the sole effect of this enzyme in the normal skin.

MATERIALS AND METHODS

Generation of transgenic mice. The 10.35-kb haptoglobin-collagenase transgene was constructed, isolated, and purified as described previously (6). This construct has 1.05 kb of the haptoglobin promoter and 9.3 kb of the collagenase gene, including sequences that code for the signal peptide. CBA/J and C57BL/6J inbred mice were obtained from lines maintained by the Jackson Laboratory, Bar Harbor, Maine. Fertilized mouse eggs from F₁ (CBA/J × C57BL/6J) × F₁ crosses were isolated, and one pronucleus was injected with the isolated DNA fragment (8 ng/μl) in injection buffer (5 mM Tris-HCl [pH 7.4], 10 mM NaCl, 100 mM EDTA). Fourteen founder transgenic mice were identified by Southern blot analysis (6, 16).

Analysis of mRNA. Total RNA was prepared from mouse tissue by the guanidinium thiocyanate-cesium chloride method (5), and RNase protection analysis was performed as previously described (18, 23). Ten micrograms of each RNA sample was hybridized, and a protected fragment of 585 nucleotides was generated (6). A standard amount of RNA from transgenic mouse line 50 was hybridized as a positive control for all reactions.

Histological analysis. Skin was obtained from the back, abdomen, ear, and tail and fixed in neutral buffered formalin after the mice were sacrificed by cervical dislocation. Paraffin-embedded tissues were sectioned (4 μm) and stained with hematoxylin and eosin (Fischer Scientific) for light microscopy (10). Evaluation of the slides was performed by observers who did not know of the collagenase expression in the tissues.

In situ hybridization. The procedure used for in situ hybridization was essentially as described previously (10). A 1.0-kb collagenase cDNA was transcribed in vitro with T3 polymerase (Gibco BRL) and 250 μCi of ³⁵S-UTP (1,320 Ci/mM; New England Nuclear) to generate the radiolabeled single-stranded antisense mRNA probe. The probe was then hydrolyzed to an average size of 150 nucleotides by sodium bicarbonate treatment at 60°C. Tissue sections were deparaffinized and hydrated to 0.85% saline and subsequently treated with 20 μg of proteinase K (Boehringer Mannheim Biochemicals) per ml in 50 mM Tris-HCl and 5 mM EDTA (pH 7.5). After the slides were acylated for 10 min with 0.0025% acetic anhydride in 0.1 M triethanolamine (pH 8.0), the salts were removed and the sections were dehydrated to 100% ethanol. Prehybridization

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conditions were $4\times$ SET (0.6 M NaCl, 0.12 M Trizma base [pH 8.0], 8 mM EDTA), 0.02% Ficoll-400, 0.02% polyvinylpyrrolidone, 0.1% Pentex bovine serum albumin (BSA) (ICN, Costa Mesa, Calif.), 500 μ g of sheared and denatured salmon sperm DNA per ml, 600 μ g of yeast total RNA per ml, and 50% deionized formamide for 2 to 4 h at room temperature. A total of 35 ng of probe (5×10^6 cpm per slide) was added in 50% deionized formamide, $8\times$ SET, 0.04% Ficoll-400, 0.04% polyvinylpyrrolidone, 0.2% Pentex BSA, 200 μ g of sheared and denatured salmon sperm DNA per ml, 200 μ g of yeast total RNA per ml, 20% dextran sulfate, 0.1% sodium dodecyl sulfate, and 10 mM dithiothreitol and hybridized for 18 h at 50°C. Following hybridization, sections were treated with 5 U of RNase T1 per ml and 100 μ g of RNase A per ml in 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA, pH 8.0, at 37°C for 30 min. Slides were washed twice for 10 min in $3.5\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and for 2 h at 60°C in $0.1\times$ SSC and then were dipped in NTB-2 X-ray emulsion (Kodak, Rochester, N.Y.), exposed for 3 to 5 days, and developed. Under a red safe light (Kodak), the slides were developed in D-19 developer (Kodak) for 5 min, placed in water for 30 s to stop the reaction, and fixed for 10 min. All of these procedures were performed at 16°C. Slides were then kept under running water for 20 to 30 min, dehydrated to 100% ethanol, and counterstained with half-strength hematoxylin (Fisher Scientific) and eosin (Fisher Scientific). Coverslips were affixed to slides with Permount (Sigma).

Electron microscopy. Skin was removed from the bellies of 6-day-old transgenic and normal mice, cut into small sections, and fixed in 2.5% glutaraldehyde (Ted Pella) in 0.1 M cacodylate buffer at 4°C overnight. Samples were briefly rinsed with 0.1 M cacodylate buffer (Ted Pella) and fixed in 2% osmium tetroxide

FIG. 2. Expression patterns of the haptoglobin-collagenase transgene. RNase protection analysis was performed with an 842-nucleotide riboprobe, which results in a 585-nucleotide protected fragment (6). Only the animals which developed skin lesions expressed the transgene in the skin (Col 50 and Col 75). At least nine mice from each transgenic line were analyzed for RNA expression, and identical results were obtained.

(Ted Pella) in 0.1 M cacodylate buffer at 4°C for 2 h. Samples were dehydrated in gradient ethanols and embedded in Epon 812. The ultrathin sections were cut with an LKB ultramicrotome with a diamond knife. Sections were picked up onto a butvar-coated slot grid (1 by 2 mm) and stained with 2% aqueous uranyl acetate for 20 min and then with 0.2% lead citrate in 0.1 M NaOH for 5 min. The stained sections were examined with a Hitachi HU-500 transmission electron microscope at 75 kV.

Tumor studies on mouse skin. Mice between 8 and 12 weeks of age from line 75 were screened by Southern blot analysis (6, 16). Nineteen transgenics and 20 negative control littermates were identified, and their backs were shaved 48 h before tumor initiation. Animals were initiated on the back with a single topical application of 200 nmol of 7,12-dimethylbenz[*a*]anthracene (DMBA; Aldrich Chemical Co., Milwaukee, Wis.) dissolved in 200 μ l of acetone. One week later, animals were treated twice a week for the next 30 weeks with topical applications of 6 nmol (4 μ g) of TPA (L. C. Services Corp., Woburn, Mass.) dissolved in 200 μ l of acetone. Forty-eight hours after the last application, the mice were sacrificed and whole dorsal skin was excised. Skin tissue was stretched (dermal side down) and stapled to a plastic backing. The tissue was then fixed for 24 h in 10% neutral buffered formalin, and histological analysis of paraffin-embedded sections (6 μ m) was performed.

RESULTS

Seven transgenic mouse lines harboring a haptoglobin-collagenase transgene were established (6). Noticeably, heterozygous mice from lines 50, 52, and 75 were distinctly smaller in size and had wrinkled and scaly skin compared with their normal littermates (Fig. 1A). The skin lesions persisted throughout adulthood, with the most severe changes found in the tail (Fig. 1B).

Although the transgene was expressed in the lungs of mice from all seven lines (6), most importantly, expression was observed only in the skin of mice from the three lines which demonstrated skin abnormalities (Fig. 2 [data not shown for line 52]). Skin expression of collagenase is a function of the haptoglobin promoter, since the skin is an endogenous site of expression of the haptoglobin gene and was shown not to be related to transgene copy number (unpublished data). In situ hybridization localized collagenase expression to the suprabasal layer (stratum spinosum) of the epidermis in the transgenic mice from both line 50 and line 75 (Fig. 3). In addition, tail skin from transgenic mice of line 75 and wild-type litter-

FIG. 1. Transgenic mice from line 50 are smaller than normal mice and have wrinkled and scaly skin. (A) A 5-day-old transgenic mouse (top) compared with a normal littermate (bottom). (B) The skin from an 18-month-old transgenic mouse (top) has numerous lesions along the length of the tail. The tail skin of the normal littermate is without any abnormalities (bottom).

FIG. 3. Epidermal localization of the haptoglobin-collagenase transgene. Photomicrographs of adjacent, parasagittal sections through the skin of an 8-day-old transgenic mouse from line 50 hybridized to the transgene-specific probe are shown. Expression is observed in the epidermis (A). Bright-field microscopy localizes expression to the suprabasal (stratum spinosum) layer of the epidermis (B). Magnification, $\times 300$. The epidermis from littermates negative for the transgene did not demonstrate any hybridization (data not shown).

mates was incubated overnight in medium to assay for collagenase protein activity. Collagenase activity was detected in the medium obtained from cultured transgenic mouse skin but not in that obtained from the skin of normal littermates (data not shown).

All three skin-expressing transgenic lines were analyzed for gross phenotypic skin changes. Nine litters from line 75 were monitored from birth into adulthood. The earliest skin changes were seen postnatally at 5 to 6 days, with 20 of 20 transgenic mice eventually developing skin lesions. Visually, two subsets of transgenic animals within this line were identified. Thirty percent of the mice displayed extensive peeling skin lesions and died prior to 14 days of age. The remaining 70% of the transgenic mice survived and had a milder phenotype, with flaky skin on the limbs, belly, back, and tail and occasionally on the feet and eyelids. The majority of the surviving older animals examined from this line developed other gross skin abnormalities, including leathery skin within the chest and belly region, alopecia, and lightening of the agouti coat color to auburn shades. Line 50 exhibited a phenotypic pattern similar to that of line 75, with skin changes beginning as early as 4 days after birth. All transgenic animals from 10 litters of line 52 died prior to 10 days of age and displayed extensive peeling skin

lesions on the entire body surface that were identical to those on the most severe animals from line 75.

Histopathological examination of skin from all three lines revealed that the skin of the transgenic mice suffered from acanthosis, hyperkeratosis, and basal cell hyperproliferation without significant changes in the dermis (Fig. 4A to D). These changes were prominent in the skin of the tail, limb, abdomen, and back and less severe in the ear. The acanthosis involved a proportionate increase in all of the epidermal layers, and the skin phenotype became more severe with aging (Fig. 4E and F). To assess the differences in cellular proliferation, anti-bromodeoxyuridine antibody staining was performed on tissue sections (24) from line 50 and line 75, and the staining revealed a threefold increase in mitotically active cells in the transgenic epidermis compared with normal epidermis (Fig. 5). There were 21.8 ± 7.5 labeled nuclei per mm^2 in the epidermis from transgenic mice compared with 6.3 ± 1.3 labeled nuclei per mm^2 in the epidermis from control mice (Student's *t* test [$P < 0.01$]).

Further detailed morphological analysis was performed at the ultrastructural level on the skin of mice from line 50 and line 75. In comparison with those of normal skin, the basal cells of the transgenic mouse skin demonstrated undulations in the

FIG. 4. Histological analysis of mouse skin from age-matched littermates. (a and b) Skin from the bellies of a 6-day-old transgenic mouse and a normal littermate, respectively. Note the hyperkeratosis of the epidermis from the transgenic mouse (c) compared with that from the normal littermate (d). (e and f) Sections of skin from the bellies of a 14-month-old transgenic mouse and a normal mouse, respectively. The affected skin from the 14-month-old transgenic mouse revealed mitoses of the epidermal basal cells (inset in panel e), with some disorganization of the basal and spinous cells. Mitoses were rarely observed in normal skin sections, as has been previously described (20). E, epidermis; D, dermis. Hematoxylin and eosin stain was used. Magnification, $\times 117$.

basal lamina, with extension of the cell processes into the dermis. The number of tonofilaments within the epidermal cells of the transgenic mice appeared to be decreased, but the desmosomes remained intact (Fig. 6). Interestingly, the granular materials present in the intercellular spaces of the normal epidermis disappeared in the transgenic mice, yielding more loosely interconnected cells (Fig. 6).

Since hyperproliferation is one of the stages observed during the progression of neoplasia (7), the susceptibility of the collagenase-overexpressing mice to tumorigenesis was examined by subjecting 2-month-old animals in line 75 to a two-stage carcinogenesis protocol utilizing DMBA and TPA. Tumor incidence was accelerated in the transgenic mice, with tumors first developing after 6.5 weeks of tumor promotion. In contrast, in the negative littermate mice, tumors appeared nearly 4 weeks later. Sixty-eight percent of the collagenase-transgenic mice developed tumors after 12 weeks, whereas only 10% of the control mice had tumors (Fig. 7A). By 16 weeks, the percentage of mice bearing tumors reached a plateau among the transgenic group, with 89% of the animals developing tumors, compared with only 20% of the control group ($P < 0.01$). The

FIG. 5. Bromodeoxyuridine labeling of transgenic and control mouse skin. The transgenic mice and normal littermates were given bromodeoxyuridine intraperitoneally and sacrificed 30 min later. Skin was washed, fixed in 4% paraformaldehyde, sectioned, and stained with an anti-bromodeoxyuridine antibody conjugated with biotin (24). Representative skin sections are shown. Note the increased labeling in the skin from the transgenic mouse (B) compared with that in skin from the normal littermate (A). Magnification, $\times 111$. Arrows indicate several representative stained nuclei.

tumor response rate for these control animals is comparable to the rate cited in the literature for moderately responsive mouse strains (30).

The number of tumors per animal was also observed at various time points during treatments with TPA over the 30-week period (Fig. 7B). The mice with the collagenase transgene consistently revealed more tumors per animal, with the tumor multiplicity after 20 weeks averaging 8.11 tumors per mouse for the transgenics, a response which nearly quadrupled the control ratio of 2.15 tumors per mouse. Control experiments performed with DMBA alone and TPA alone revealed no tumor formation in both the transgenics and the controls (data not shown). Histopathologically, the tumors showed marked papillomatosis and hyperkeratosis without stromal invasion by the tumor cells and were diagnosed as papillomas (Fig. 8).

DISCUSSION

This study describes the phenotypic analysis of three transgenic mouse lines which overexpress collagenase in the skin. The mice developed wrinkled and scaly skin as early as 5 days postnatally. Histological analysis revealed a thickened epidermis with proliferation of the basal cells and hyperkeratosis. Furthermore, the epidermal hyperplasia caused by enhanced collagenase production increased the susceptibility of the skin to tumor formation in a two-stage carcinogenesis protocol.

Collagenase causes disruption of the epidermal architecture in the transgenic mice. However, there are no known fibrillar collagens within the epidermis, the usual substrate for tissue

FIG. 6. Electron microscopy of epidermis from a transgenic mouse and a normal mouse littermate. Two suprabasal keratinocytes from the bellies of 6-day-old mice are shown. Extracellular spaces (*) between the spinous cells in the control skin are occupied by fine granular material (B), which is lacking in those in the transgenic sample (A). Similar results were obtained for mice of various ages. Magnification, $\times 26,000$.

collagenase (19). Therefore, the mechanism leading to the observed phenotype mediated by collagenase could either be indirect by inducing expression of other degradative proteinases or be more likely by the direct action of collagenase on substrates in the epidermis. The specificity of collagenase may be wider than ascertained, and the enzyme may be degrading nonfibrillar collagens. One feasible substrate, type XIII collagen, has recently been identified in the epidermis (29); however, its structural role and susceptibility to collagenase degradation are as yet unknown. In addition, collagenase could degrade other noncollagenous proteins within the epidermis which are critical in preserving the cell-cell contact of this layer. In fact, collagenase has been reported to degrade such noncollagenous proteins as α -2 macroglobulin and tumor necrosis factor alpha (12, 31).

In a previous study, transgenic mice expressing collagenase in the lung developed emphysema, suggesting a role for the proteinase in this destructive disease process (6). In the present study, mice which expressed the transgene in the epidermis exhibited hyperproliferation. This demonstrates the different response that each tissue has to the same injury. The hyperplastic lesions in the skin probably occur as a result of the skin's ability to regenerate, whereas the more limited repair of the lung results in destructive emphysematous changes. Therefore, the development of pathological changes in a particular tissue is due not only to the initiating stimulus but also to the unique response of each tissue to the injury.

The present transgenic mouse model demonstrates that acanthosis, hyperkeratosis, and basal cell proliferation in the skin can be generated solely by the overexpression of a single proteinase. Interestingly, similar pathological changes are observed in a variety of human skin diseases, such as lamellar

FIG. 7. Enhanced susceptibility of the skin of collagenase transgenic mice to tumorigenesis compared with that of wild-type controls. Nineteen CBA/J $\times$ C57BL/J transgenic mice and 20 wild-type littermates (8 to 12 weeks old) were initiated with 200 nmol of DMBA. One week later, the mice were promoted with biweekly applications of TPA (6 nmol) for 30 weeks. (A) Percent mice with tumors. (B) Tumors per mouse.

FIG. 8. Development of papillomas and histological analysis of the tumors in the collagenase transgenic mice. (A) Papillomas on the back of a transgenic animal after initiation with DMBA and 30 weeks of promotion with TPA. (B) Histology of a typical papilloma from a transgenic animal after 30 weeks of promotion with TPA. Magnification, $\times 100$.

ichthyosis, atopic dermatitis, and psoriasis (20). Strikingly, the electron micrographic changes seen in the transgenic mice are similar to those seen in the epidermis of patients suffering from psoriasis, in which intercellular spaces are widely opened, with cell separation and adhesion limited to the desmosomes (20, 26). Inflammatory cells (macrophages and neutrophils) are commonly present in hyperplastic skin lesions (25, 32). These cells not only are a rich source of proteinases, including collagenase (4, 36), but they also release cytokines (32) which in-

duce collagenase production in keratinocytes (33). A multitude of changes occurs as a result of the inflammatory cell infiltrate (32). The transgenic mice described above do not exhibit a large infiltration of inflammatory cells in these lesions. Therefore, these mice bypass the nonspecific inflammatory response, suggesting that the single pathological alteration of hyperplasia could be caused by deregulated proteinase production. The mice should prove useful for our understanding of the specific hyperplastic change common to the diseases discussed above.

The phenotype of the collagenase-transgenic mice is observed in a number of experimental situations which predispose the skin to carcinogenesis (3, 7, 8, 27, 33, 34). Basal cell hyperproliferation as well as acanthosis and hyperkeratosis was seen in the skin of transgenic mice that express either the gene for transforming growth factor alpha (4, 8, 34) or oncogenes, including *BNLF-1*, *v-fos*, and *k-ras* (3, 13, 37). Similarly, when tumor promoters are applied to the skin, epidermal hyperplasia and hyperkeratosis are also observed (7). The electron micrographic features of the present transgenic mouse model are virtually identical to those seen in transgenic mice expressing transforming growth factor alpha in the skin, including such features as undulations in the basal lamina, with loosely interconnected cells (33). However, transgenic mice expressing transforming growth factor alpha developed spontaneous early papillomas (8, 33). These tumors occur in the areas of trauma and were subject to regression (8, 33). The *v-fos* and *k-ras* transgenic mice developed significant papillomas which were more dysplastic and did not regress (3, 13). In addition, it has recently been shown that in the *v-fos* transgenic mice, transcription of collagenase is increased, correlating with sites of *fos*-induced long-term cellular alterations (11). The growth factors (as well as oncogenes and tumor promoters) alter the expression of multiple genes and activate a number of different pathways that predispose the cells to carcinogenesis (2, 15). In contrast, the transgenic mice described in this paper cause hyperplasia by the simple alteration of just one enzyme, namely, collagenase. This suggests that the expression of proteinases could occur early in tumorigenesis, resulting in hyperplasia, but hyperplasia alone is not sufficient for complete transformation and perhaps requires the activation of other genes (17).

The increase in tumor susceptibility is intriguing and can occur during both stages of the carcinogenesis protocol. At the initiating stage with DMBA, the hyperproliferation in the transgenic animals could be associated with an increase in DMBA metabolic activation of a larger population of initiated cells. Tumor susceptibility in the transgenic mice could also be enhanced during tumor promotion. It has been shown that induction of epidermal hyperplasia correlates strongly with the ability of tumor promoters to induce neoplasia (7). In addition, differences in strain sensitivities to tumor formation in mice have been shown to be due to the tumor promoter stage, with sustained epidermal hyperplasia in particular strains increasing the susceptibility of mice to tumor promotion. Since the collagenase transgenic mice demonstrate chronic epidermal hyperplasia, these mice are therefore more likely to be sensitive to tumor promoters. Furthermore, the transgenic animals demonstrate, as previously hypothesized, that epidermal hyperplasia is important in the process of tumor development but clearly is not by itself sufficient for tumor formation.

The present study directly implicates collagenase in the development of hyperplastic skin lesions. A possible mechanism would be the disruption of intercellular contact by the degradation of collagenase-sensitive material between the suprabasal cells and basal cells. The resulting loss of contact inhibition (1) would lead to hyperproliferation of the basal cells and subsequently result in the observed alterations of the epidermis (26). Additional phenotypic features indicative of each factor or disease state would obviously arise from the activation of other molecular and cellular pathways. Previously, the number and diversity of these interactive pathways complicated the analysis of dermatological lesions at the molecular level. The strength of the present transgenic mouse model is that it isolates a single molecular component and the phenotypic ramifications of its overexpression in a specific organ. The

mice should be a valuable model, not only to study neoplasia and human skin diseases but also to provide insights into the role of collagenase in other epidermal disorders, such as wound healing and injury repair.

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