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Restrained Terminal Differentiation and Sustained Stemness in neonatal skin by Ha-Ras and Bcl-2

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Abstract

Nonmelanoma skin cancer (NMSC) is the most frequently diagnosed cancer in the United States. Deregulation of bcl-2 and ras family members is commonly observed in NMSC. It has been previously demonstrated that simultaneous bcl-2 and Ha-ras gene expression in keratinocytes results in resistance to cell death induced by ultraviolet (UV) radiation and enhanced multistep skin carcinogenesis. In the current study, we aimed to elucidate the central roles of Ha-Ras and Bcl-2 in maintaining epidermal homeostasis. To assess the effect of deregulated Ha-Ras and Bcl-2 on skin differentiation, we have generated skin-specific transgenic mouse model constitutively expressing both oncogenic Ha-Ras and Bcl-2. Ectopic expression of Ha-Ras and Bcl-2 in new born double transgenic epidermal keratinocytes induced abnormal epidermal differentiation accompanied by increased cell proliferation and suppressed apoptotic cell death, which resulted in thickened and wrinkled skin morphology in neonate skin. Expression of epidermal differentiation marker cytokeratin 1 was decreased. Expression of other differentiation markers loricrin and filaggrin was also decreased and delayed to be detected only in the upper stratum granulosum. Whereas, the proliferative markers cytokeratin 14 and cytokeratin 6, which are expressed in constitutively proliferative basal layer and stem cell niches such as hair follicle or neoplastic lesions, respectively, were highly expressed. The abnormal expression of epidermal cytokeratins suggests that Ha-Ras and Bcl-2 suppresses the terminal differentiation and sustains the stem cell-like features in epidermal keratinocytes.

Keywords

Ha-Ras; Bcl-2; keratinocyte; epidermal differentiation; stemness

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Introduction

The epidermis is a complex self-renewing tissue composed of functionally different stratified layers^{(1), (2)}. The epidermal homeostasis is maintained through the balanced proliferation, differentiation and cell death. In this context, proliferation is normally confined to basal keratinocytes, whereas suprabasal keratinocytes are quiescent and gradually acquire the features of terminal differentiation, which is characterized by expression of several epidermal differentiation markers, such as specific cytokeratins, cornified envelope precursor proteins, and transglutaminases. Terminally differentiated keratinocytes reach the most upper layer in the skin and form protective cornified envelopes containing condensed nonviable cells⁽³⁾.

Ha-Ras is a guanosine-5'-triphosphate (GTP) binding protein and a key signal transducer to regulate proliferation, differentiation and apoptosis in eukaryotic cells⁽⁴⁾. Previous studies have shown that oncogenic Ras is integral in initiating and maintaining the malignant phenotypes^(5, 6). Approximately 20% of all human cancers, including nonmelanoma skin cancer possess activating mutations involving Ha-Ras⁽⁷⁾. Bcl-2 is a cell death regulatory protein and is one of most widely studied oncoproteins⁽⁸⁾. The expression of Bcl-2 is tightly regulated during keratinocyte differentiation and is normally limited to the basal keratinocytes⁽⁹⁻¹¹⁾. It is known to be an important regulator of epidermal cell death susceptibility to the various cytotoxic environmental stresses including UV irradiation, the most important causative agent of skin carcinogenesis^(12, 13), ^(8, 10). Etiologically, the dysregulated expression of Bcl-2 is a putative mechanism of basal cell carcinomas (BCC), which constitute the majority of nonmelanoma skin cancer (NMSC)⁽¹¹⁾, ⁽¹⁴⁾, ⁽¹⁵⁾. However, the exact mechanism of Ras and Bcl-2 in epidermal homeostasis and skin carcinogenesis remains to be established.

Taken together, Ha-Ras and Bcl-2 are key proteins to regulate epidermal cell proliferation and differentiation, and the functional dysregulation results in skin carcinogenesis. In our previous study, we have demonstrated that constitutive expression of Ha-Ras and Bcl-2 in the epidermal keratinocyte resulted in increased skin tumor development, and co-expression of these oncoproteins exerted a synergistic increase in malignant transformation of skin papillomas⁽¹⁶⁾. In this study, we have demonstrated that dysregulated Ha-Ras and Bcl-2 suppress the epidermal differentiation and induce proliferative keratinocytes with sustained stemness, which predisposes to hyperplastic growth of the epidermis and to skin carcinogenesis.

Materials and Methods

Generation and genotyping of transgenic mice

HK1.Bcl-2 and HK1.Ha-Ras transgenic mice were generated as previously described⁽¹⁶⁾.

Immunohistochemical analysis

Epidermal samples from neonatal transgenic mouse skins were fixed in 10% buffered formalin for 10 hours. The fixed samples were dehydrated in an ascending series of alcohol and xylene and then embedded in paraffin. For immunostaining, 4µm-thick tissue sections

were deparaffinized in xylene and rehydrated in a descending alcohol series. Before staining with antibody, the tissue sections were treated with 3% H₂O₂ to block endogenous peroxidase activity and with a serum-free protein block (DAKO Corp., Carpinteria, CA) to prevent artificial color reaction. For the skin differentiation study, rabbit anti-mouse primary antibodies, including K1, K6, K14, involucrin, loricrin and filaggrin (Babco, Berkeley, CA) were used. For the proliferation and apoptosis studies in papillomas, mouse primary anti-human/mouse proliferating cell nuclear antigen (PCNA) (DAKO Corp.) and hamster primary anti-human Bcl-2 (Pharmingen, San Diego, CA) antibodies were used. The sections were incubated with primary antibodies for 1 hour, and then washed in PBS. The slides were next incubated with HRP-conjugated donkey anti-rabbit secondary antibody (DAKO Corp.) for 30 minutes. The markers were visualized using standard DAB techniques (DAKO Corp.) and counterstaining using Mayer's hematoxylin.

Western blot analysis

Epidermal proteins from neonatal transgenic mice were extracted as described (13). Briefly, the dorsal skins were immersed in boiling saline for 30 seconds, and the epidermal layers were removed from the dermal matrix. To solubilize the highly cross-linked cornified envelope proteins and cytokeratins, the epidermis was boiled and solubilized in extraction buffer (0.0625M Tris-base, 5% (w/v) SDS, 10% (v/v) glycerol, 20% (v/v) 2-beta-mercaptoethanol, 0.05% (w/v) bromophenol blue, pH 6.8) for 10 minutes. The protein extracts were electrophoresed in 10% SDS-polyacrylamide gels, and then transferred to nitrocellulose membranes (Micron Separations Inc., Westborough, MA). After blocking in 5% nonfat dried milk (Carnation), the blots were immunoblotted using the same primary and secondary antibodies used for immunohistochemistry. The immunoblots were then developed using the ECL system (Amersham life Science, Arlington Heights, IL).

Results

HK-1.Ha-ras neonate skin exhibited a distinctive thickened and wrinkled skin morphology characterized by huge epidermal hyperplasia and hyperkeratosis with desquamation of scaly skin (Fig. 1). However, the histological structure of Bcl-2 neonatal epidermis was very similar to control littermates, and it did not give any distinguishable change when it was co-expressed with Ha-Ras in double transgenic neonatal skin (Fig. 1).

Ha-Ras transgenic neonates have shown very distinctive change in the magnitude and distribution of epidermal cytokeratin expression (Fig.2A and B). We observed decreased expression of terminal differentiation marker cytokeratin 1 in Ha-Ras and Ha-Ras/Bcl-2 double transgenic neonatal skin (Fig. 2A). Further the suprabasal-specific expression pattern of cytokeratin 1 was also altered by dysregulated Ha-Ras expression. Comparing to the evenly distributed positivity in control, cytokeratin 1 expression in Ha-Ras and Ha-Ras/Bcl-2 double transgenic neonatal skin was slightly suppressed in the differentiation-initiating lower epidermal layer, and gradually increased in the suprabasal keratinocytes located at highly differentiated upper spinous layer (Fig. 2A). The total amount of cytokeratin 1 expression was also slightly decreased (Fig. 2B).

The expression of proliferative epithelial cell marker, cytokeratin 14 was also altered. Cytokeratin 14 was detected throughout the entire stratified layers of the Ha-Ras and Ha-Ras/Bcl-2 transgenic epidermis comparing to control neonate, which exhibited limited expression in the basal keratinocytes (Fig. 2A). The amount of cytokeratin 14 protein expression, however, was not significantly changed (Fig. 2B).

There was more dramatic change in the expression of cytokeratin 6, a proliferating basal-like stem cell marker. Cytokeratin 6 was essentially undetected in control neonate skin. However, its expression was increased in the entire epidermis of Bcl-2 and Ha-Ras neonate. Interestingly, we could observe an additive effect on cytokeratin 6 expression in the Ha-Ras/Bcl-2 double transgenic neonatal skin (Fig. 2A, 2B).

The terminal differentiation marker, involucrin was detected in the upper granular layer of the control and Bcl-2 neonatal epidermis. However, comparing to the condensed and upper-top layer-specific expression pattern in control and Bcl-2 neonate, the Ha-Ras and Ha-Ras/Bcl-2 neonatal skin showed decreased but evenly distributed involucrin positivity through the entire differentiating layers (Fig. 2A). However, the total amount of involucrin protein expression was shown to be increased in Ha-Ras and Ha-Ras/Bcl-2 neonatal skins (Fig. 2B).

Another terminal differentiation marker, loricrin was selectively detected in upper granular layer of control neonate skin. Ha-Ras and Ha-Ras/Bcl-2 neonatal skin has shown decreased loricrin positivity in Ha-Ras and Ha-Ras/Bcl-2 transgenic mice (Fig. 2A). The other terminal differentiation marker, filaggrin showed similar decreased expression in Ha-Ras and Ha-Ras/Bcl-2 neonatal skin. Especially strong positivity of filaggrin in the cornified layer of control neonate skin was barely observed in Ha-Ras and Ha-Ras/Bcl-2 neonate (Fig. 2A). Despite the significant change of immunological positivity in extent and distribution, the total amount of loricrin and filaggrin protein expression was not changed (Fig. 2B).

Discussion

To maintain epidermal homeostasis, the coordinated regulation of cell proliferation, cell death, and differentiation is necessary. As key regulators, we have previously demonstrated the integral functions of Ha-Ras and Bcl-2 in the maintenance of keratinocyte cell growth and differentiation using *in vitro* model(12). In the current study, we observed similar effects of these oncoproteins on murine neonatal skin differentiation. The ectopic expression of Ha-Ras in epidermal keratinocytes resulted in wrinkled and thickened epidermal structure associated with enormous hyperplasia and hyperkeratosis. However Bcl-2 overexpression did not cause any detectable morphological change except for common focal hyperplasia as shown in the previous study(13).

We discovered that the constitutive expression of Ha-Ras protein in the epidermis affects skin differentiation by altering the magnitude and distribution of cytokeratin expression. Decreased expression and delayed appearance of cytokeratin 1, a typical early stage marker in epidermal differentiation, both in Ha-Ras and Ha-Ras/Bcl-2 neonatal skins demonstrates that constitutive expression of Ha-Ras, but not Bcl-2 contributes to restrain the initiation of epidermal differentiation process. In contrast to cytokeratin 1, a basal layer marker

cytokeratin 14 is a main structural protein in proliferating keratinocytes, and is down-regulated in differentiating cells as shown in suprabasal keratinocytes in control neonatal skin (Fig. 2A). The Cytokeratin 14 was expressed through the entire epidermis including both basal and suprabasal layers in Ha-Ras and Ha-Ras/Bcl-2 neonatal skin. These findings are consistent with our previous study showing that BrdU-positive proliferating keratinocytes were detected not only in basal layers, but also in the significant portion of differentiating suprabasal layers in the Ha-Ras and Ha-Ras/bcl2 neonatal skin comparing to control skin which is restricted only in basal layers(16).

Loricrin and filaggrin are terminal epidermal differentiation markers, mainly expressed in uppermost and cornified envelop. The expression of these proteins was down-regulated in the oncogenic Ha-Ras expressing epidermis, and furthermore, they appeared only in terminally differentiated keratinocytes, but not detected in whole cornified envelop. The expression of another cornified envelope protein involucrin was also abnormally decreased in the Ha-Ras expressing epidermis comparing to control. The involucrin is a major component of the cornified envelope and involves extensive crosslink to various other structural envelope proteins to produce the flattened cornified envelope. Decreased expression of involucrin in Ha-Ras transgenic epidermis may cause insufficient crosslink of cornified envelop proteins and incomplete synthesis of stratum corneum and epidermal envelop, which might result in wrinkled skin with scaly desquamation found in Ha-Ras and Ha-Ras/Bcl-2 neonatal skin.

Taken together, these results demonstrate that dysregulated expression of Ha-Ras maintains the epidermal cells to be proliferative and suppresses the terminal differentiation, which results in the neoplastic epidermal growth. Furthermore, this abnormal expression and distribution of cytokeratins and epidermal differentiation-related proteins especially the epidermal envelop subunits including loricrin, filaggrin and involucrin can cause hyperkeratosis with desquamation of scaly skin.

In our previous study, we demonstrated that constitutively expressed oncogenic Ha-ras and Bcl-2 significantly affected neoplastic epidermal growth for papillomatogenesis, and the coexpression of these proteins with their distinct oncogenic mechanisms are functionally synergistic in the malignant transformation of chemically-induced skin carcinogenesis(16). Interestingly, we observed that both Ha-ras and Bcl-2 transgenic neonate skins exhibited an abnormal expression of cytokeratin 6, which is a hyper proliferative keratinocyte marker specifically expressed in the outer root sheath of hair follicles, or wound-healing tissues, which are putative niches for stem cells. Cytokeratin 6 is known to be one of stem cell markers, and expressed in many neoplastic cells including squamous cell carcinomas(17-20). The abnormal expression of cytokeratin 6 in both Bcl-2 and Ha-Ras transgenic neonates and additive expression in Ha-Ras/Bcl-2 double transgenic skin demonstrates that these oncoproteins play an integral role to maintain the stemness of epidermal keratinocytes during the epidermal differentiation. Further, dysregulation can cause increased susceptibility in skin carcinogenesis. A recent study has demonstrated that the activating N-Ras mutation which is prevalent in human myeloproliferative neoplasm and leukemia increases hematopoietic stem cells (HSCs) proliferation and self-renewal potential(21). In this study, the authors proposed the oncogenic Ras as a key mechanism for

HSC to maintain the Stemness and a precursor for pre-malignant clonal expansion. Similar study using chronic myeloid leukemia model demonstrated that depletion of Bcl-2 causes severe loss of therapeutic resistance, which is a hallmark of cancer stemness, and decrease of leukemia stem cell population (22). These findings would be very supportive evidence proving the function of Ha-Ras and Bcl-2 in the regulation of epidermal stem cell populations and neoplasia.

In summary, our data suggest that constitutively expressed oncogenic Ha-ras has profound effects on inducing cell proliferation and suppressing epidermal differentiation. Furthermore, Ha-Ras and Bcl-2 might also be involved in maintaining stemness of epidermal cells which can cause hyperplastic growth of the epidermis and skin cancers.

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Figure 1. Histological morphology of HK1.Ha-ras and HK1.Bcl-2 transgenic neonatal skin
Neonates (1day) expressing Ha-ras transgene exhibited a distinctive phenotype, which is thickened and wrinkled skin (3rd and 4th in left panel). Hematoxylin and eosin staining of neonatal skin sections (right panel, control, HK1.Bcl-2, HK1.Ha-ras, and HK1.Bcl-2/Ha-ras transgenic epidermis)

Figure 2. Epidermal differentiation in the neonatal epidermis

(A) Immunohistochemical analysis using antibodies against six epidermal differentiation marker proteins (cytokeratin 1, 14, 6, involucrin, filaggrin and, loricrin). The sections were harvest from neonate epidermis of control, HK1.bcl-2, HK1.v-Ha-ras, and HK1.bcl-2/HK1.v-Ha-ras transgenic mice. (B) Western blot analysis of epidermal protein extracted from neonatal skins.