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Activated Akt-1 in Specific Cell Populations During Multi-stage Skin Carcinogenesis

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Abstract. *The goal of the present study was to identify specific populations of cells that contain activated Akt-1, as determined by the presence of phosphorylated Akt at serine 473 (p Akt), during development of skin tumors using a murine multi-stage carcinogenesis model. Nucleated papillomas cells as well as both epidermal and follicular keratinocytes in hyperplastic skin contained increased pAkt compared to skin treated only with acetone or 7, 12 dimethylbenz[a]anthracene (DMBA). Although the numbers of both mast cells and neutrophils were significantly increased in the stroma of papillomas ($p<0.0005$; $p<0.0001$, respectively), only mast cells contained pAkt. The amount of total Akt protein was similar regardless of time or treatment group examined. The present results suggest that activation of Akt-1 may provide specific populations of epidermal keratinocytes that develop into skin tumors with the ability to resist terminal differentiation and have enhanced proliferation during multi-stage skin carcinogenesis. In addition, mast cells which contain activated Akt-1 may persist within the stroma of papillomas during skin tumor development and progression through this signaling pathway, thereby contributing to a pro-oxidant and pro-angiogenic microenvironment.*

Abbreviations: DAB: diaminobenzadine; DAPI, 4', 6-diamidino-2-phenylindole; DMBA: 7,12-dimethylbenz[a]anthracene; H_2O_2: hydrogen peroxide; IGF-1: insulin-like growth factor-1; IP3-K: inositidylphosphate-3-kinase; IOD: integrated optical density; MPO: myeloperoxidase; pAkt: Akt-1-phospho-Serine 473; SCC: squamous cell carcinoma; TPA: 12-0-tetradecanoylphorbol-13-acetate; UV: ultraviolet.

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Under normal conditions, keratinocytes, which are the specialized epithelial cells of the skin, undergo limited proliferation. As these cells migrate into the upper layers of the epidermis, they subsequently undergo terminal differentiation, which is a form of apoptosis. During multi-stage skin carcinogenesis, keratinocytes that contain mutations in the Ha-*ras* oncogene induced by exposure to 7, 12 dimethylbenz[a]anthracene (DMBA) undergo successive rounds of proliferation in response to a tumor promoter such as 12-0-tetradecanoylphorbol-13-acetate (TPA) (1,2). This leads to clonal expansion of these Ha-*ras* mutated cells, which also have significant alterations in their pattern of differentiation and a decreased level of apoptotic death (3,4). An initial study demonstrated that transgenic mice expressing insulin-like growth factor-1 (IGF-1) selectively targeted to the basal compartment of epidermis responded to exposure to ultraviolet (UV) light with a decreased epidermal apoptosis compared to the skin of non-transgenic mice, which was associated with significantly elevated Akt kinase activity (5). In addition, IGF-1 transgenic mice developed greater numbers of papillomas

when treated with a mouse skin carcinogenesis protocol. These results suggested that IGF-1 expression in the basal epidermis activated the inositidylphosphate-3-kinase IP3-K/Akt (protein kinase B) pathway, which was associated with both resistance of keratinocytes to apoptosis and with a tumorigenic phenotype (5). Another study demonstrated that transfection of PB keratinocytes, which usually have low rates of tumor formation when grown *in vivo*, with wild-type Akt resulted in rapidly growing, undifferentiated and highly invasive skin tumors (6). These observations were consistent with studies demonstrating that Akt acted as an oncogene when transfected into NIH 3T3 fibroblasts (7) and also confirmed a previous report that Akt null mice had significant defects in differentiation of the skin (8).

Although previous studies have demonstrated an association between increased Akt kinase activity and development of skin tumors (5,6,9), the specific cell populations that contain activated Akt-1 during multi-stage skin carcinogenesis are unknown. The present study used digital image analysis in conjunction with immunochemistry and immunofluorescence to identify cell populations within epidermis, dermis, papillomas and stromal tissue that contained activated Akt-1, as determined by detection of phosphorylation of Akt-1 at serine 473 (pAkt).

Materials and Methods

Treatment of SENCAR mice with DMBA and TPA. Female SENCAR mice (6-8 weeks old, 22-28 g; NCI, Bethesda, MD, USA) were housed in vivarium facilities at The Ohio State University that meet American Association for Accreditation of Laboratory Animal Care requirements. All procedures were approved by The Ohio State University Institutional Animal Care Utilization Committee prior to beginning the study. Animals were fed basal Teklad 22/5 rodent diet (Harland Industries, Indianapolis, IN, USA) *ad libitum* and kept in rooms maintained on a 12 h light/dark cycle. At 24 h prior to initial treatment with HPLC grade acetone, DMBA or TPA, the dorsal skin of mice was carefully shaved. Mice treated topically with 0.2 ml acetone served as the solvent control. DMBA (Sigma, St. Louis, MO, USA) was dissolved in acetone and applied at a final concentration of 25 nmol DMBA in 0.2 ml acetone to cover the entire back of mice. TPA (Alexis Biochemicals, San Diego, CA, USA), at a final concentration of 2 μg, was dissolved in acetone and applied in a volume of 0.2 ml to the dorsal skin of mice. SENCAR mice were treated with one of two protocols. For short-term experiments, the dorsal skin of SENCAR mice was treated with two topical applications of either 0.2 ml acetone or 25 nmol DMBA, with a 48-h time interval between treatments with acetone or DMBA, followed at 7 days by a single topical application of either 0.2 ml acetone or 2 μg TPA in 0.2 ml acetone. At 24 h after the final application of either acetone or TPA, mice were sacrificed by carbon dioxide euthanasia. Dorsal hair was removed using a depilatory agent, skin was isolated, and tissue samples were either fixed in 10% neutral buffered formalin and embedded in paraffin prior to sectioning for histological and immunohistochemical analysis, as described previously (10), or used to isolate protein for Western blotting analysis of total Akt protein, as described below.

For studies in which skin and papillomas were isolated at 15 weeks and 22 weeks of multi-stage carcinogenesis, SENCAR mice were either initiated with a single topical application of 25 nmol DMBA or treated with a single topical application of 0.2 ml acetone and, at 7 days later, mice were treated topically twice weekly with either 0.2 ml acetone or 2 μg of TPA in 0.2 ml acetone. Dorsal skin samples and papillomas were isolated at 15 weeks and 22 weeks. At 4 h following the final topical application of 2 μg TPA, mice were then sacrificed by carbon dioxide euthanasia. Skin and papillomas were isolated, and tissue samples were processed for immunohistochemical and Western blotting analysis.

Immunochemical staining methods. Following processing and paraffin embeddment, 4-μm serial sections of skin tissues were placed on histological glass slides. Immunohistochemical staining was performed using a DAKO Autostaining system, as previously described (11). Briefly, cross-sections of tissues were dewaxed, rehydrated and treated with preheated citrate buffer (pH 6.0) to recover antigenicity. Endogenous peroxidase activity was blocked with a 3% H_2O_2/methanol solution, followed by incubation in 10% goat serum/PBS blocking solution (Vector Laboratories, Burlingame, CA, USA). Skin sections were stained with rabbit anti-phospho-Serine-473-Akt-1 antibody (polyclonal anti-pAkt-1 [pAkt], 0.031 μg/mL, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The presence of the pAkt antigen was visualized using diaminobenzidine (DAB; 0.06% w/v, DAKO, Carpinteria, CA, USA) and tissues were counterstained with Methyl Green. The specificity of antibody staining was verified using pre-immune rabbit IgG (0.031 μg/ml, Santa Cruz Biotechnology). Using polyclonal anti-myeloperoxidase (MPO) antibody (0.4 μg/ml, Neomarkers, Fremont, CA, USA) and nuclear morphology, similar methods were used to identify and quantitate the numbers of activated neutrophils within the dermal compartment and within the stroma of papillomas. Mast cells were identified and quantitated based on staining with 0.2 % toluidine blue (in 0.7N HCl) (Sigma) using Vital New Red as a counterstain (Pfaltz & Bauer, Inc., Waterbury, CT, USA), as previously described (12,13).

Immunofluorescent staining methods. Sections of fixed, paraffin-embedded skin samples (4 μm) were mounted onto glass sides (SuperFrost/Plus, Fischer Scientific, Pittsburgh, PA, USA). Tissue sections were then deparaffinized using Histo-Clear (National Diagonistics, Atlanta, GA, USA) and rehydrated in a graded series of alcohols. Antigen was unmasked by heating samples in 10 mM citrate buffer (pH 6.0) in a microwave oven. To reduce autofluorescence, sections were treated in 3 changes of freshly prepared solutions of sodium borohydride ($NaBH_4$, 1 mg/ml in PBS, Sigma) for 10 min each. To block background staining, tissue sections were incubated for 30 min at room temperature with Image-iT™ FX Signal Enhancer (Molecular Probes, Eugene, OR, USA). Tissues sections were then incubated overnight at room temperature in a humidified chamber with rabbit polyclonal anti-phospho-Serine-473-Akt-1 (1:50 in PBS, Santa Cruz Biotechnology) and were then incubated at room temperature in the dark for 1 h with Alexa Fluor-633-conjugated goat anti-rabbit IgG (1:500 in PBS, Molecular Probes). After incubation with secondary antibody, tissues were washed in PBS, dehydrated and mounted using VectaShield Mounting Medium (Vector Laboratories) which contains the DNA dye 4', 6-diamidino-2-phenylindole (DAPI) that fluoresces in the 405 nm (blue) range and specifically stains

nucleated cells. Specificity of antibody binding was determined by incubation with only secondary antibodies followed by placement of coverslips using Vectashield with DAPI.

Digital photomicroscopy and image analysis. Fluorescent images were obtained using a laser scanning confocal microscope (Zeiss LSM 510 Meta), with excitation wavelengths of 633 nm for Alexa Fluor-633 (red) fluorescence and 405 nm for DAPI-associated (blue) fluorescence. Images were obtained using 40 x objective lens at identical settings of illumination. To quantitate pAkt immunohistochemical staining within specific cell populations, digital images of tissue sections were captured using a Diagnostic Instruments digital camera (Insight camera; 1600 x 1200 resolution) mounted on an Olympus research microscope. Images were then transferred to digital image analysis software (Image Pro Plus, Media Cybernetics, Silver Spring, MD, USA). Multiple images were captured from each tissue section using a 40 x objective in order to encompass the entire tissue sectional area. Images were captured under identical lighting and optical settings, and color segmentation was used to eliminate non-specific background staining and counterstaining. Epidermal and follicular compartments within the skin were delineated in each image, and pAkt immunostaining was quantitated within each compartment of the skin by calculation of integrated optical densities (IOD), which is a proportional measure of brown signal, based on DAB staining, as previously described (14-16). The capture and processing of digital images were performed by investigators who were unaware of the treatment group assignments. Intra-observer variability (coefficient of variation for 5 images made by one blinded observer) and inter-observer variability (three blinded observers, 5 images each) for these imaging procedures was less than 2% and 5%, respectively. In parallel experiments, serial sections of skin were also evaluated for the presence of neutrophils and mast cells using histochemical and immunochemical stains, as well as nuclear morphology, as described above. Multiple digital images of these leukocyte populations were captured and calibrated, as described above using an 80 x objective. Populations of neutrophils and mast cells were quantitated using a segmentation-based approach, as described above.

Protein extracts. Dorsal skin and papillomas were homogenized on ice in 2.5 ml homogenation buffer, pH 8.6 (Tris-HCl, 60 mM; EDTA, 5 mM; EGTA, 5 mM; sucrose, 300 mM; DTT, 5 mM; leupetin, 200 μg/ml; PMSF, 2 mM; aprotinin, 20 μg/ml; sodium molybdate, 10 mM), followed by sonication for 10 sec. Samples were clarified by centrifugation at maximum speed on a microcentrifuge at 4˚C for 15 min. Supernatants were collected and then centrifuged again for 5 min at maximum speed. Protein concentrations were determined using the BIO-RAD protein assay (BIO-RAD, Hercules, CA, USA) and bovine albumin serum (BSA) (Sigma) was used as a standard. Samples were stored at -80˚C until Western blot analysis was performed.

Western blot analysis. Equal aliquots of protein extracts (35 μg/sample) were analyzed by Western blot analysis using 10 % SDS-polyacrylamide gel and then blotted to Hybond-P polyvinylidene difluoride membrane (Amersham, Piscataway, NJ, USA) during an overnight transfer. The blots were blocked by incubation in 5% non-fat dried milk powder in phosphate-buffered saline containing 0.1% Tween-20 (PBS-T), and then washed with PBS-T. Blots were then incubated with rabbit polyclonal anti-Akt antibody (1:500 dilution) (Cell Signaling Technology, Beverly, MA, USA) in 1% BSA in PBS-T overnight at 4˚C with constant shaking. After extensive washing with PBS-T, blots were incubated for 1 h at room temperature with 1:2000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in PBS-T containing 5% non-fat dried milk powder. The enhanced chemiluminescence (ECL plus) Western Blotting Detection System (Amersham) was used to detect the signal, with exposure to Hyperfilm™ ECL (Amersham). To verify equal loading of protein samples, blots were re-probed with 1:5000 dilution of mouse monoclonal anti-β-actin (Sigma) for 1 h at room temperature, followed by extensive washing with PBS-T. Blots were subsequently incubated for 30 min at room temperature with 1:4000 dilution of peroxidase-linked sheep anti-mouse IgG (Amersham) and detection of the signal was processed as described above.

Statistical analysis. Significant differences in pAkt in specific regions of skin and in papillomas were determined using two-tailed Student's *t*-test (SigmaStat; Jandel Scientific, San Rafael, CA, USA) or one-way analyses of variance (ANOVA) on ranks for non-parametric comparisons (relative pAkt staining), with *post-hoc* Dunnet test to evaluate significant comparisons between treatment groups and acetone control skin. Comparisons of three or more groups were conducted using one-way ANOVA, with Student-Newman-Keuls *post-hoc* analysis. In all cases, results are expressed as mean±SEM, and significance was defined as $p<0.05$, unless otherwise described.

Results

Histology of mouse skin. Dorsal SENCAR mouse skin treated with acetone (Figure 1 A) or DMBA (Figure 1 B) had a uniform epidermal thickness of 1-2 epidermal cell layers, with few cells in the dermis. At 24 h after a single topical application of 2 μg TPA, the epidermal thickness had increased to 3-5 cell layers and there was also an increase in inflammatory cells within the dermis. Both changes were present regardless of previous topical treatment with either acetone or DMBA (Figure 1 C and D).

Skin treated for 15 weeks with either acetone alone (Figure 2 A) or with DMBA (Figure 2 B) followed by twice weekly applications of acetone was of uniform epidermal thickness, with 1-2 epidermal layers and low numbers of cells within the dermis. Skin isolated from mice treated once with DMBA followed by twice weekly applications of 2 μg TPA for 15 weeks (Figure 2 D) was hyperplastic, with a significantly increased epidermal thickness and visible increases in the numbers of cells within the dermis. Comparatively, at 15 weeks, the hyperplasia in skin treated with acetone followed by twice weekly application of 2 μg TPA (Figure 2 C) was not as great as that observed in skin treated once with DMBA followed by twice weekly treatments of TPA (Figure 2 D).

Quantitation of pAkt in compartments of skin at 24 h and 15 weeks. Sections of skin isolated from SENCAR mice at 24 h and 15 weeks were stained with anti-pAkt antibody and relative immunohistochemical staining intensities of pAkt

within the epidermal and follicular compartments were quantitated using digital image analysis (Figure 1 F, Figure 2 E). In general, regardless of the time at which skin samples were isolated, the epidermal compartment contained more cells than the follicular compartment of the skin, and more epidermal cells that contained pAkt (Figure 1 F and Figure 2 E). At the 24-h time point, there was a 3-fold increase in pAkt in the epidermis of skin treated with a single topical application of 2 μg TPA, regardless of pre-treatment of the dorsal skin with acetone or DMBA, ($p<0.01$) (Figures 1 C and D compared to Figures 1 A and B; Figure 1 F).

At 15 weeks, the highest pAkt levels in both epidermal and follicular compartments were in keratinocytes in skin treated with a single initiating dose of DMBA followed by twice weekly application of 2 μg TPA (Figures 2 D and E). At 15 weeks, the levels of pAkt in both compartments of the skin following treatment with DMBA/TPA were 2-fold higher than the levels of pAkt at the earlier time point of 24 h after treatment with DMBA/TPA (Figures 1 D and F compared to Figures 2 D and E).

Quantitation of pAkt in papillomas. The DNA dye DAPI in combination with immunofluorescence and confocal microscopy were used to identify the nucleated cells (blue fluorescence) that contained pAkt (red fluorescence) in papillomas isolated from SENCAR mice at 22 weeks of tumor promotion (Figure 3 A). Digital imaging analysis showed statistically significant increases ($p<0.0001$) in pAkt immunohistochemical staining in nucleated papilloma cells compared to pAkt in epidermal keratinocytes from the dorsal skin of mice treated topically with acetone (Figure 3 C). The level of pAkt in papillomas was similar to the level of pAkt staining detected in both the epidermal compartment of skin isolated at 15 weeks after treatment with DMBA/TPA (Figure 2 E and Figure 3 C) and in hyperplastic epidermis adjacent to papillomas isolated at 15 weeks and 22 weeks following DMBA/TPA treatment (data not shown).

Identification and quantitation of specific leukocytes in the dermal compartment of the skin and stroma. Dermal mast cells were identified and quantitated based on toluidine blue staining (Figure 4 A, arrows; inset, arrow). Regardless of the type of treatment of the dorsal skin, all dermal mast cells contained pAkt (Figure 4 B, arrows, inset, arrow). There were low numbers of dermal mast cells in skin isolated at 24 h regardless of the type of topical treatment. The numbers of mast cells were significantly increased in the dermis of skin isolated at 15 weeks from mice treated once with DMBA followed by twice weekly treatments with acetone ($p<0.05$), and in the dermis of skin isolated from mice treated once with acetone followed by twice weekly treatments with TPA ($p<0.01$). The stromal tissue of papillomas isolated at 22 weeks had the greatest numbers of mast cells ($p<0.0005$) (Table I).

Dermal neutrophils were identified and quantitated based on anti-MPO antibody staining (Figure 4 C, inset, arrow), which has previously been used as a marker of activated neutrophils (17). Although the numbers of dermal neutrophils were significantly increased in skin treated with either TPA or DMBA/TPA at the 24-h time point ($p<0.0001$) and in the stroma of papillomas ($p<0.0001$) (Table I), these cells did not contain pAkt (Figure 5 D, arrow; inset, arrow).

Western blot of total Akt-1 protein. The amount of total Akt protein levels in dorsal mouse skin and papillomas was constant, regardless of treatment of skin or time evaluated (Figure 5). To verify equal loading of protein, blots were reprobed with mouse anti-β-actin antibody.

Discussion

The present study identified specific cell populations in the epidermal, follicular and dermal compartments of the skin during multi-stage carcinogenesis which contained pAkt. The use of specific antibodies, immunochemistry, multi-color immunofluorescent probes and computer-assisted digital image analysis methods allowed quantitation and statistical analysis of pAkt in specific cell populations and provided a high degree of reproducibility (inter- and intra-observer variabilities were < 5 %).

Akt-1 (protein kinase B) is one signal transduction molecule that is activated during multi-stage skin carcinogenesis (5,6,9,18). Akt has been associated with enhanced keratinocytes survival, a proliferative phenotype, and with altered and/or diminished keratinocytes differentation (5,6). Previous studies reported that lapses in time of greater than a year between tumor initiation and tumor promotion do not significantly change the number of papillomas formed (19). These observations suggest that initiated keratinocytes have a survival advantage which allows them to persist in the skin for long periods of time despite the presence of apoptotic mechanisms, which, under normal conditions, would result in elimination of these mutated cells. The present study did not detect an increase in pAkt in skin treated only with DMBA which may be due to the very low number of initiated cells that contained pAkt that have not undergone clonal expansion. Akt-1 appears to become constitutively activated during tumor promotion, which was demonstrated in the present studies by the detection of pAkt in skin isolated at 15 weeks after a single exposure to DMBA and successive twice weekly treatment with TPA compared to the lack of significant elevation in pAkt in skin isolated at 15 weeks after treatment with TPA in the absence of tumor initiation with DMBA. Additional evidence supporting the constitutive activation of pAkt during tumor promotion comes from studies performed in our laboratory which found no significant difference in pAkt levels between skin treated

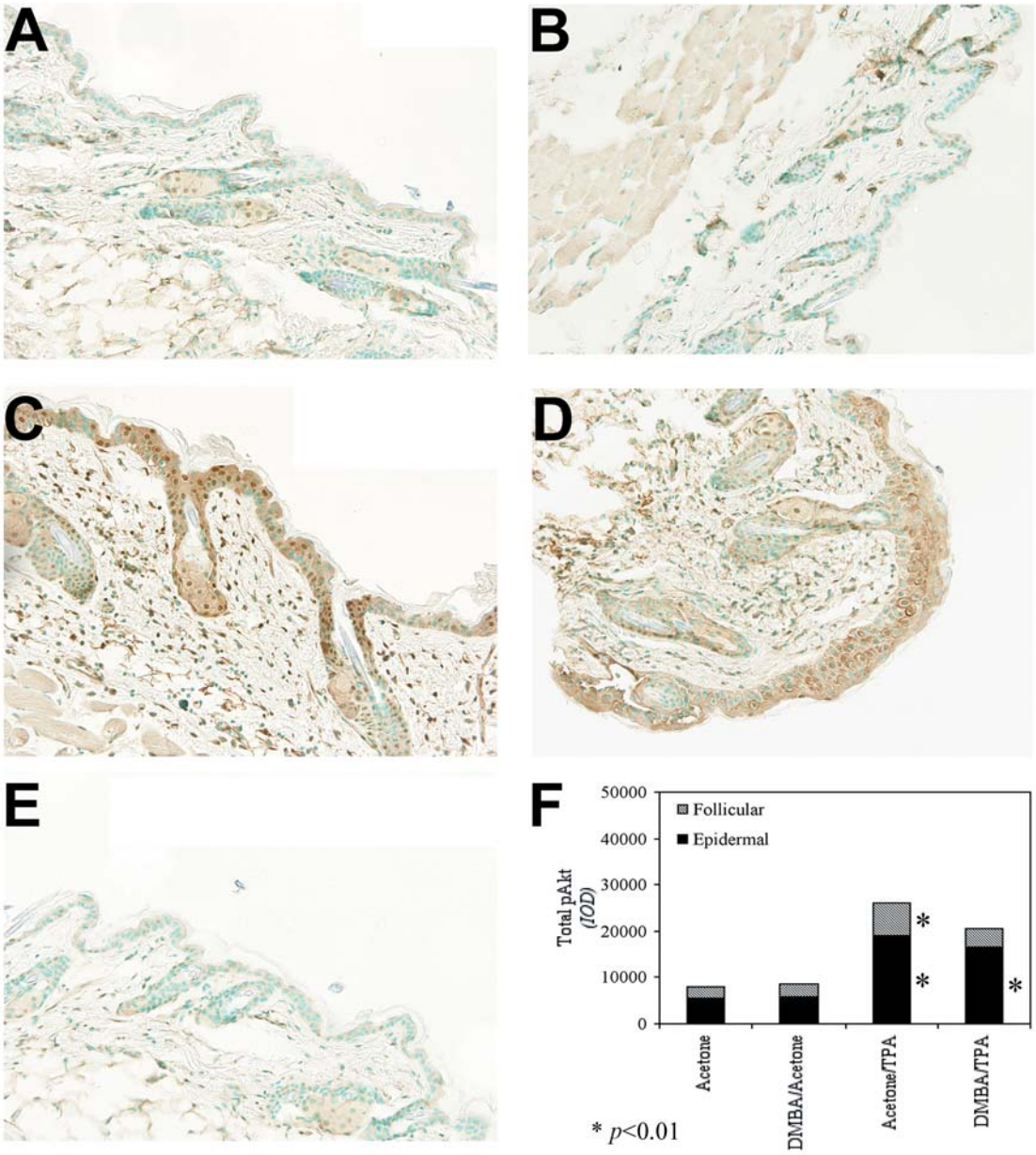

Figure 1. *Localization and quantitation of pAkt in skin at 24 h after topical treatment with acetone, DMBA, or TPA. There were few cells with pAkt in dorsal skin treated once topically with acetone (A), or DMBA (B), with significantly higher number of cells with pAkt in skin treated with acetone followed at 7 days later by a single topical application of 2 μg TPA (acetone/TPA) (C) and in skin treated once with 25 nmol DMBA followed at 7 days later by a single topical treatment with 2 μg TPA (DMBA/TPA) (D). Specificity of pAkt antibody binding was determined by evaluation of the lack of binding of non-immune IgG (E). (A-E, 400 x magnification). (F) Dorsal skin treated with either acetone/TPA or DMBA/TPA had significantly increased pAkt in the epidermal compartment (C, D and F). Only skin treated with acetone/TPA had significantly increased pAkt in cells in the follicular compartment (C and F). Data are expressed as mean±SEM; Quantitation of pAkt is expressed as integrated optical density (IOD). n=3-4 mice for each group; * statistically significant difference from acetone control group, p<0.01.*

topically with a single exposure or multiple treatments with DMBA (unpublished observations). Taken together, these results suggest that one of the critical factors in stimulating constitutive activation of Akt is the clonal expansion of Ha-*ras* mutated cells during tumor promotion.

Although the numbers of activated neutrophils were significantly increased at early times (24 h) after exposure to TPA and in the stroma of papillomas, this cell type did not contain pAkt. This observation is consistent with the known 3-7 day life-span of this cell type. Our earlier studies

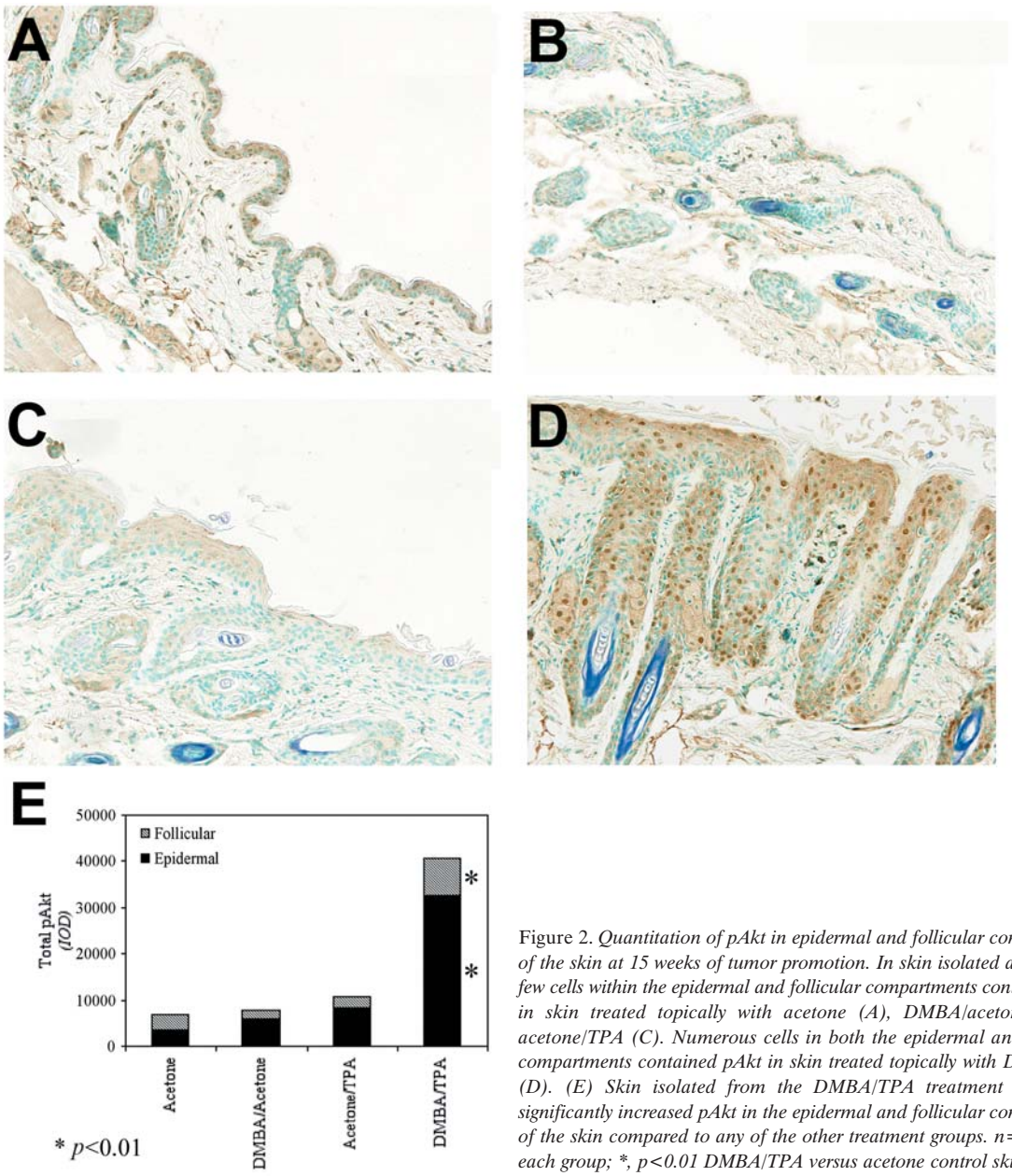

Figure 2. *Quantitation of pAkt in epidermal and follicular compartments of the skin at 15 weeks of tumor promotion. In skin isolated at 15 weeks, few cells within the epidermal and follicular compartments contained pAkt in skin treated topically with acetone (A), DMBA/acetone (B), or acetone/TPA (C). Numerous cells in both the epidermal and follicular compartments contained pAkt in skin treated topically with DMBA/TPA (D). (E) Skin isolated from the DMBA/TPA treatment group had significantly increased pAkt in the epidermal and follicular compartments of the skin compared to any of the other treatment groups. n=3 mice for each group; *, p<0.01 DMBA/TPA versus acetone control skin.*

demonstrated that the numbers of circulating peripheral blood neutrophils were elevated over the time of tumor promotion and that they produced elevated levels of reactive oxygen intermediates while in the circulation following topical application of dorsal epidermis with TPA (20,21). The amounts of reactive oxygen intermediates produced by neutrophils isolated from mice treated with either TPA or with DMBA followed by TPA was sufficient to induce DNA damage and mutation (20). Taken together with the present observations, it appears that, during skin carcinogenesis, bone marrow derived neutrophils are continually produced, are activated, and then migrate from the peripheral circulation into the dermal microenvironment of hyperplastic skin and papillomas, however, they are not a cell type that undergoes active proliferation nor do they have a prolonged life-span through Akt-1 activation during skin carcinogenesis.

In contrast, we found that mast cells were prevalent within the stroma of papillomas and did contain pAkt-1. These cells have been suggested to act as "co-conspirators" in skin carcinogenesis by their ability to release matrix metallo-

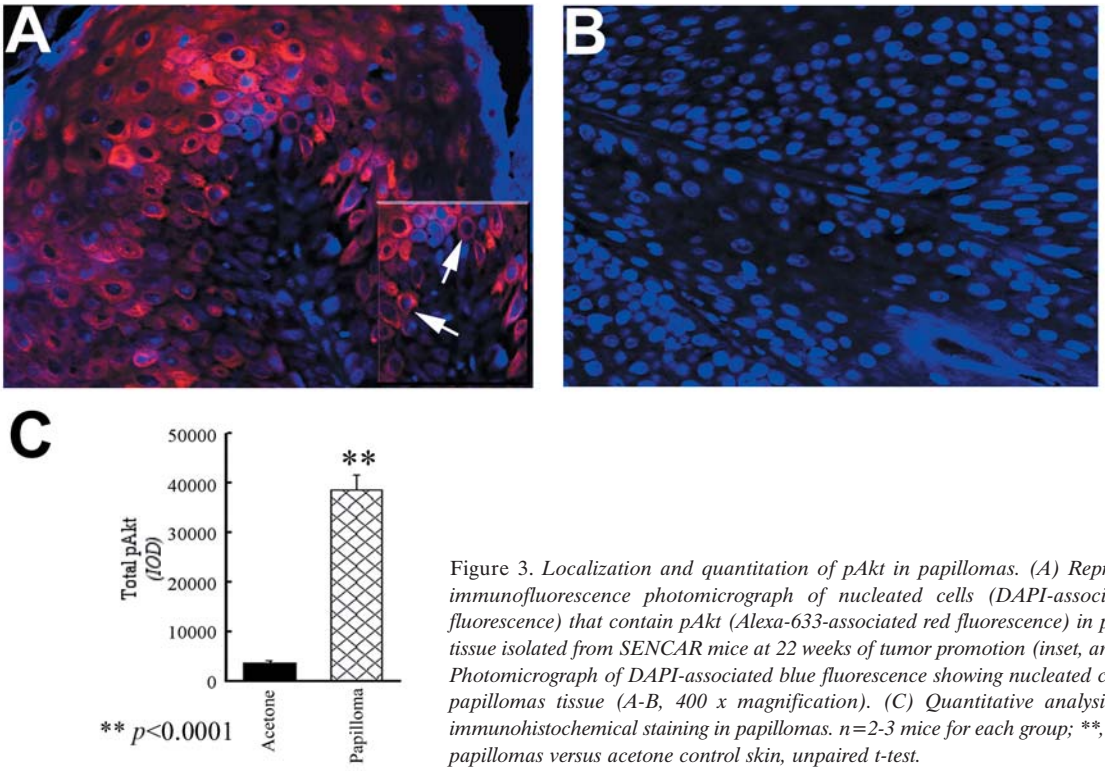

Figure 3. *Localization and quantitation of pAkt in papillomas. (A) Representative immunofluorescence photomicrograph of nucleated cells (DAPI-associated blue fluorescence) that contain pAkt (Alexa-633-associated red fluorescence) in papillomas tissue isolated from SENCAR mice at 22 weeks of tumor promotion (inset, arrows). (B) Photomicrograph of DAPI-associated blue fluorescence showing nucleated cells within papillomas tissue (A-B, 400 x magnification). (C) Quantitative analysis of pAkt immunohistochemical staining in papillomas. n=2-3 mice for each group; **, p<0.0001 papillomas versus acetone control skin, unpaired t-test.*

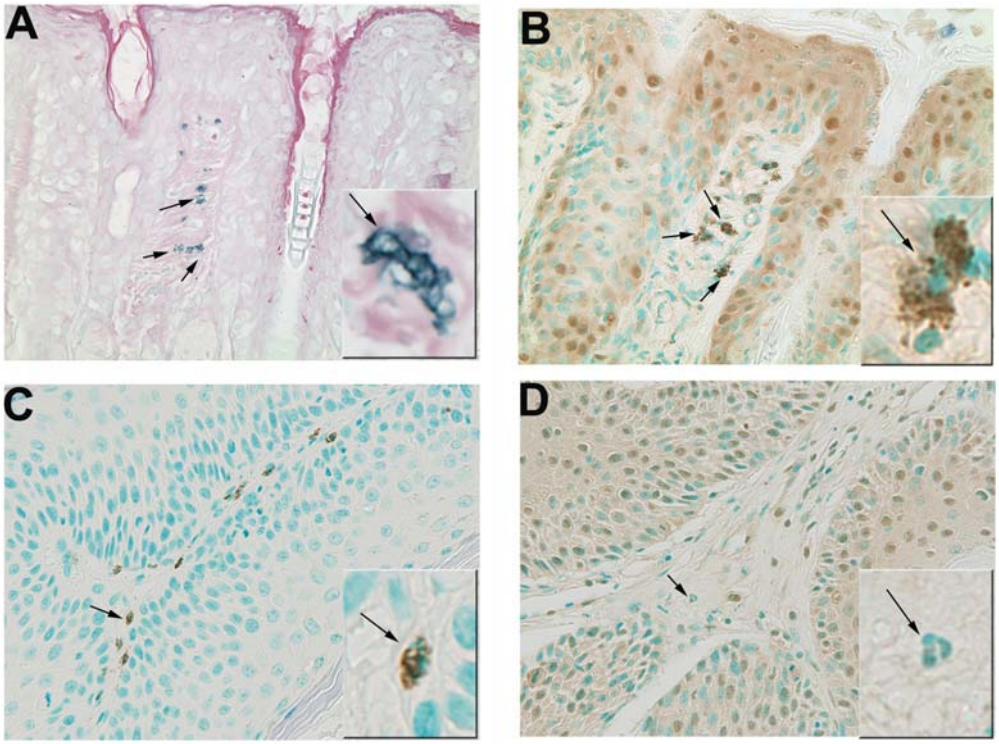

Figure 4. *Identification and localization of pAkt in dermal inflammatory leukocytes of dorsal SENCAR mouse skin. Mast cells in serial sections of skin treated with DMBA/TPA for 15 weeks were identified by toluidine blue staining (A, arrows, inset, arrow) and were found to contain pAkt (B, arrows, inset, arrow). Neutrophils were identified in serial sections of papillomas isolated from SENCAR mice at 22 weeks of tumor promotion using anti-myeloperoxidase (MPO) antibody staining (C, arrow, inset, arrow) and did not contain pAkt (D, arrow; inset, arrow). (A-D, 400 x magnification).*

Table I. *Quantitation of mast cells and neutrophils in SENCAR skin and papillomas.*

	Mast Cells/Treatment Group ± SEM		Neutrophils/Treatment Group ± SEM	
Treatment	24 hr	15 Weeks	24 hr	15 Weeks
Acetone/Acetone	5±1	6±1	4±1	3±1
DMBA/Acetone	5±0	9±1*	7±1*	4±1
Acetone/TPA	5±0	12±2**	189±21#	4±1
DMBA/TPA	5±0	8±1	78±6#	3±1
Papillomas	N/A	14±2***	N/A	21±3#

*$p<0.05$; **$p<0.01$; ***$p<0.0005$; #$p<0.0001$ *versus* Acetone control group.

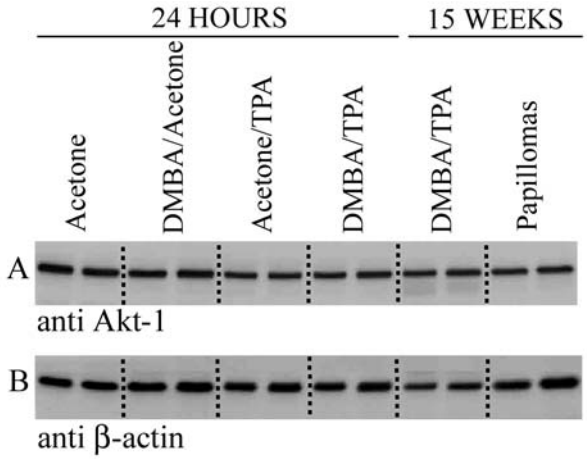

Figure 5. *Western blot analysis of total Akt-1 potein. (A) Analysis of total Akt-1 protein in extracts of dorsal SENCAR mouse skin at 24 h and in skin and papillomas isolated at 15 weeks demonstrated no change in total Akt-1 protein. Samples were run in duplicate. (B) To verify the equal loading of proteins, the blot was re-probed for β-actin.*

proteinases, which triggers the "angiogenic switch" leading to neovascularization (12,22). The present studies demonstrate that mast cells contain pAkt, which would both provide a survival advantage and would support their involvement in the pro-angiogenic microenvironment within hyperplastic skin and in papillomas over a prolonged period of time.

Infiltrating inflammatory cells have been suggested to have a role in inducing epigenetic alterations that occur during multi-stage carcinogenesis through their ability to produce a "pro-oxidant environment". Treatment of skin with either TPA or DMBA stimulates production of both oxygen and nitrogen reactive intermediates (10,21,23-26), resulting in formation of oxidative DNA adducts. Furthermore, we previously reported that both inflammatory leukocytes as well as epidermal keratinocytes release cytokines and other inflammatory mediators during tumor promotion, which in turn stimulates production of free radicals (10,27,28). Recent studies reported that reactive oxygen species, such as H_2O_2 (29-32), and reactive nitrogen species, such as peroxynitrite (33), can directly activate Akt-1. Taken together, it appears that there may be cross-talk between keratinocytes and inflammatory leukocytes through their production of reactive oxygen and nitrogen intermediates and production of cytokines, which may activate Akt leading to enhanced survival of these cell populations and stimulating sustained proliferation of specific epidermal and follicular keratinocytes during multi-stage skin carcinogenesis.

The present results suggest that specific populations of epidermal and follicular keratinocytes, which have been shown to ultimately form papillomas and carcinomas (34-37), contain constitutively activated Akt-1 during multi-stage skin carcinogenesis. In addition, mast cells within the dermis of hyperplastic skin and in the stroma of papillomas contain activated Akt-1, suggesting that this molecular pathway may provide these inflammatory cells and keratinocytes populations with the ability to persist in the tissue during skin carcinogenesis, which maintains a tumorigenic, pro-oxidant and pro-angiogenic microenvironment. Further studies are necessary to determine the effects of inhibiting Akt-1 activation in development of papillomas and their progression to malignant carcinomas.

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