Evaluation of Proliferating Cell Nuclear Antigen as a Surrogate End Point Biomarker in Actinic Keratosis and Adjacent, Normal-appearing, and Non-Sun-exposed Human Skin Samples

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Abstract
The incidence of nonmelanoma skin cancer, including both squamous cell carcinoma and basal cell carcinoma, is a significant health problem in the United States. Actinic keratosis (AK), the precursor of cutaneous squamous cell carcinoma, is a major risk factor for nonmelanoma skin cancer. In addition, AKs are tissue targets for the identification of biomarkers for use in chemopreventive studies. The biomarker addressed in this study is epidermal cell proliferation, as quantitated by proliferating cell nuclear antigen (PCNA). Shave biopsies were obtained from AKs, tissue immediately adjacent to AKs, normal-appearing, upper-medial arm skin, and non-sun-exposed skin from 19 subjects. When any degree of PCNA staining was considered positive (semiquantitative 1-4 scale), there was a significant difference and a progressively increasing mean PCNA labeling index (LI) in the total epidermis (basal and suprabasal layers), beginning with non-sun-exposed buttock skin, with the lowest LI (2.5 ± 1.6%), followed by upper-medial arm skin (12.3 ± 7.4%; P = 0.0015), skin adjacent to AKs (19.2 ± 12.2%; P = 0.0218), and finally, AKs with the highest LI (34.6 ± 20.1%; P = 0.0017). This same pattern was observed when the epidermis was separated into basal and suprabasal layers, with the exception of a nonsignificant result for upper-medial arm skin compared with adjacent skin in the basal layer (P = 0.3981). PCNA LIs were also analyzed separately by staining intensity (i.e., scores of 1-4). The PCNA LI in skin with varying degrees of sun damage and/or histological atypia is a candidate surrogate end point biomarker for skin cancer chemoprevention studies.

Introduction
Nonmelanoma skin cancer (NMSC), which includes both SCC and basal cell carcinoma, is the most frequently diagnosed cancer in the United States, with an estimated incidence of greater than 600,000 cases in 1994 (1, 2). SCC makes up approximately 20% of NMSC (3); although the metastatic potential and mortality rates associated with SCC are low, the incidence seems to be increasing and represents a major health issue (2). SCCs occur primarily on sun-exposed parts of the body and have been strongly associated with chronic sun exposure (1).

Solar keratoses or AKs are considered the premalignant precursors to SCCs and share many histological similarities with SCCs (1, 3). The presence of AKs is a major risk factor, as well as a marker of increased risk for NMSC (2, 4). The frequency of AKs increases with age and sun exposure.

Skin types that burn easily and tan poorly (types I and II) are at greatest risk of developing AKs (4). Individuals can have single or multiple AKs, with an estimated conversion rate to SCC of 1 per 1000 per year in individual lesions (4, 5). AKs also have the potential for spontaneous regression with reduction in UV exposure (4). Clinically, AKs appear as red, scaly, nonsubstantive papules on chronically sun-exposed areas (3). Sites adjacent to AKs have been shown to contain significant histological alterations, including nuclear heterogeneity, variation in cell size, and loss of polarity, suggesting extensive preneoplastic alterations of sun-damaged skin (6).

Enhanced cellular proliferation has been closely associated with the process of tumorigenesis in numerous tissues (7), including skin (8-17). A variety of techniques have been used to measure epidermal cell proliferation, including tritiated thymidine and bromodeoxyuridine uptake (11, 15, 16-29), flow cytometry (30, 31), PCNA (10, 12-14), and Ki67 (8, 9, 22). The measurement of both tritiated thymidine and BrdUrd LIs require nucleotide uptake into viable tissue, limiting the applicability of these techniques to clinical studies, whereas the use of monoclonal antibodies to endogenous cell cycle-specific proteins such as Ki67 and PCNA have become widely used. PCNA functions as an auxiliary protein to DNA polymerases and thus increases in G1, is maximally expressed in the S-phase, and decreases in G2-M (32, 33, 37-39).

Received 8/21/95; revised 12/22/95; accepted 12/22/95.

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1 This work was supported in part by NIH Grants CA-41108 and CA-27502.
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1 The abbreviations used are: NMSC, nonmelanoma skin cancer; SCC, squamous cell carcinoma; AK, actinic keratosis; PCNA, proliferating cell nuclear antigen; BrdUrd, bromodeoxyuridine; LI, labeling index; SEB, surrogate endpoint biomarker; bx, biopsy.
Human skin is a continually renewing organ with stem cells located in the basal layer of the epidermis. In normal skin, cell loss via the normal process of cell differentiation and sloughing at the surface equals cell production (40). Benign skin disorders (e.g., psoriasis and seborrheic keratosis), premalignant skin conditions, and skin cancers have increased epidermal cell proliferation rates (8–17, 22, 24, 27–31). Although most studies have reported the presence of few or rare proliferating cells in the basal and immediate suprabasal layers in normal skin, comparison of proliferation rates between studies is difficult due to the differences in the methods of expressing proliferative indices (8, 10–12, 14–21, 23, 25–29).

Preneoplastic lesions such as AKs are rational markers of risk for NMSC and can serve as targets for chemopreventive interventions (41). Additionally, development of SEBs based on biological properties and molecular alterations in AKs as well as in surrounding tissue may serve as markers of risk as well as targets for chemopreventive interventions. The relationship of SEBs in involved and uninvolved premalignant and malignant tissue is important to understand the biological changes that occur during the process of carcinogenesis and for their use as intermediate markers in chemoprevention studies.

In this study, we have attempted to validate the PCNA LI as a reliable biomarker for skin cancer chemopreventive agents by comparing PCNA LIs in 19 individuals with AKs in nonsun-exposed skin from the buttock, upper-medial arm skin, skin adjacent to an AK, and an AK lesion.

Materials and Methods

Patients and Tissue Preparation. Nineteen subjects with clinical AKs took part in this study. Informed written consent was obtained according to institutional and federal guidelines. Shave bxs of clinical AKs, adjacent skin within 4 cm of the AKs, upper-medial arm skin, and non-sun-exposed skin from the buttock area (13 of the 19 subjects) were obtained in this study. The area of skin to be biopsied was anesthetized with 1 % xylocaine with epinephrine (Ekins-Sinn Inc., Cherry Hill, NJ). Three- to 4-mm shave bxs were taken with a No. 15 scalpel, yielding primarily epidermis and a minimal amount of dermis. Immediately after removal, specimens were transported to the laboratory in MEM (Sigma Chemical Co., St. Louis, MO.), oriented epidermal side up onto premoistened Metricel membranes (Gelman, Ann Arbor, MI), and fixed in 70% ethanol for 24 h at 4°C.

Immunohistochemistry. All skin specimens were routinely processed at a temperature not exceeding 59°C and paraffin embedded, and 3-μm serial sections were cut. Just prior to staining, slides were deparaffinized through a series of graded alcohol. Immunostaining was performed using an avidin-biotin-based Vectastain avidin-biotin complex kit (Vector Laboratories, Burlingame, CA). All steps were carried out at room temperature. Nonspecific peroxidase activity was blocked with 3% H2O2 (Sigma) in methanol for 30 mm at room temperature, followed by two PBS rinses. The complex was visualized with H2O2, diaminobenzidine (DAB) (Sigma) at a 20 mg/mL concentration and was developed and used to manually grade the staining intensity of video prints. The numbers of cells graded 0–4 were counted for the basal and suprabasal layers separately. The basal layer was the single layer of cells adjacent to the basement membrane, and the suprabasal layer was all the cells above the basal layer. The two layers were combined for the total epidermis.

Statistical Analysis. PCNA LIs were calculated by dividing the number of labeled cells by the number of total cells (labeled and unlabeled) and multiplying by 100. Mean percentages of PCNA LIs were compared by Wilcoxon signed-rank tests, because not all LI distributions appeared normally distributed. Intraobserver reliabilities were computed by standard ANOVA methods.

Results

Demographic characteristics of the 19 study subjects appear in Table 1. The mean age of the subjects (7 females and 12 males) was 72.7 ± 6.3 (range, 60–84) years. Study subjects had between 2 and 200 AKs, which were located primarily on the dorsal surfaces of forearms and, in one case, on the hand. Eighteen of the subjects had skin type I (burns easily and never tans), 2 (burns easily and tans minimally), or 3 (burns moderately and tans gradually), whereas one subject had type 4 (burns minimally and always tans well).

Fig. 1 shows a PCNA-stained AK (Fig. 1A), tissue adjacent to the AK (Fig. 1B), upper-medial arm skin (Fig. 1C), and non-sun-exposed buttock skin (Fig. 1D). There is a progressive increase in PCNA LIs between non-sun-exposed skin, with the lowest LI, upper-medial arm skin, adjacent skin, and AK, with the highest LI.

All clinical AKs were histologically confirmed. Unexpectedly, the majority of the upper-medial arm samples had some evidence of sun damage (i.e., primarily cellular crowding). The adjacent samples, in addition to having significant evidence of sun damage, contained some of the features of AKs (i.e., primarily cellular crowding, budding, and slight cytological atypia). In contrast, non-sun-exposed buttock bxs were all judged normal.

Presented in Table 2 are the mean numbers of fields and...
cells quantitated for each tissue type. The greatest number of cells per field was found in non-sun-exposed buttock skin for the total as well as the basal and suprabasal layers. When comparing upper-medial arm, adjacent, and AK, there was a trend toward increasing numbers of cells per field, with the largest number of cells in AKs and the lowest number in upper-medial arm samples.

Results will be presented separately for four levels of PCNA staining: grades 1–4, 2–4, 3–4, and 4 (Tables 3–6). Table 3 shows the mean percentage of PCNA LIs (± SD) for the total epidermis (basal and suprabasal layers combined), basal and suprabasal layers in non-sun-exposed buttock skin, upper-medial arm skin, adjacent skin, and AK, when any degree of immunostaining was considered positive (i.e., grades 1–4). In the total epidermis, there was a stepwise and statistically significant increase in LIs, beginning with non-sun-exposed skin (2.5 ± 1.6%), followed by upper-medial arm skin (12.3 ± 7.4%; \( P = 0.0015 \)), adjacent skin (19.2 ± 12.2%; \( P = 0.0218 \)), and finally, AK, with the highest LI (34.6 ± 20.1%; \( P = 0.0017 \)). When the epidermis was separated into basal and
suprabasal layers, the same stepwise increase in mean percentage of PCNA LIs was seen, with the exception of a nonsignificant \((P = 0.3981)\) increase between upper-medial arm and adjacent skin in the basal layer.

Mean percentage of PCNA LIs for staining level 2–4 are shown in Table 4. A similar stepwise significant increase in mean PCNA LIs was seen when the faintly labeled grade 1 cells were excluded from the analysis. LIs were approximately one-half of those presented in Table 3. In the total epidermis, PCNA LIs were 1.6 ± 1.1\(\%\) for non-sun-exposed skin, 6.4 ± 3.8\% \((P = 0.0015)\), 10.0 ± 6.7% \((P = 0.0534)\), and 20.4 ± 15.5% \((P = 0.0017)\) in non-sun-exposed skin. In the upper-medial arm, PCNA LIs were 1.2 ± 1.0\% for non-sun-exposed skin, 8.6 ± 5.2% \((P = 0.0034)\), and 18.6 ± 11.6% \((P = 0.0017)\) in non-sun-exposed skin.

Table 3 summarizes data for PCNA LIs using a staining level of 2–4. Results for staining level 4 are summarized in Table 5. The stepwise increase in PCNA LIs was virtually lost in the total epidermis, basal, and suprabasal layers, with the exception of a significant difference between non-sun-exposed and upper-medial arm skin for both total \((P = 0.0303)\) and basal layers \((P = 0.0211)\) and between upper-medial arm skin and AK for the suprabasal layer \((P = 0.0283)\). In the total epidermis, PCNA LIs were 0.2 ± 0.3% for non-sun-exposed skin, 0.8 ± 0.8% for upper-medial arm skin, 0.8 ± 0.7% for adjacent skin, and 1.2 ± 1.0% for AK.

Intraobserver reliabilities were above 0.98 for all staining levels below grade 3, regardless of tissue type or layer (i.e., basal, suprabasal, or total) evaluated. Similarly, intraobserver reliabilities for grade 4 cells were 0.99, 0.79, and 0.91 for the basal layer, suprabasal layer, and total epidermis, respectively.

### Discussion

AKs are the putative premalignant lesions for cutaneous SCC and a risk factor for SCC development. In addition, they can serve as targets for chemoprevention trials, and they represent a resource for developing rational SEBs based on biological or molecular changes during the process of carcinogenesis. Unlike other organ sites, such as colon or breast, skin is easily accessible for repeated sequential bx's. In addition, tissue adjacent to AKs containing significant phenotypic alterations suggesting chronic sun exposure also can be sampled to determine the effect of a chemopreventive agent on an earlier step in the carcinogenesis pathway. Sampling of non-sun-exposed skin may also be useful in determining the extent of UV exposure and phenotypic, biological, and molecular alterations.

In continually renewing tissues such as skin, cell proliferation is a fundamental process, in balance with cell differentiation and cell loss. One of the hallmarks of neoplasia is a loss of normal growth control, making measurement of proliferative indices rational SEBs. Proliferation has been studied in skin using \(\text{[3H]}\)thymidine, BrdUrd, and Ki67 to assess proliferative patterns in normal skin, as well as in benign and malignant lesions (8–17, 22, 24, 27, 28–31). Differences in the methods...
of presenting proliferative indices make comparisons between studies difficult, but several studies reported LIs for normal skin to be between 1.4 and 5.8% (11, 16–19, 25–29) using [3H]thymidine or BrdUrd. Pearse and Marks (16) reported [3H]thymidine LIs in AK and adjacent and buttoc skin to be 17.4, 11.2, and 5.4%, respectively. Saida et al. (13) reported PCNA LIs for normal skin distant from neoplastic lesions to range from approximately 2 to 4% and LIs ranging from about 7 to 25% in AKs. Two studies gave more descriptive analyses of PCNA staining in AKs. Geary and Cooper (14) stated that the expression of PCNA in AK correlated with the distribution of atypical cells, whereas Pennys et al. (12) described increased labeling of nuclei in the basal layers of AKs.

In the current study, we determined cutaneous cellular proliferation rates as measured by PCNA LIs in 19 subjects with multiple AKs. The degree and difference in cell proliferation rates between AK lesions, tissue adjacent to AKs, upper-medial arm skin, and non-sun-exposed skin from the buttock area were studied in superficial bxs. Because of increasing degrees of direct UV exposure, there was a significant stepwise increase in mean percentages of PCNA labeling, progressing from non-sun-exposed skin, with the lowest mean LI, to upper-medial arm skin, to skin adjacent to AKs, and finally to AKs themselves, which had the highest mean PCNA LI.

The variable expression of PCNA throughout the cell cycle is reflected in varying staining intensities on immunohistochemically stained tissue sections. This variation in staining intensity complicates quantitation of PCNA LIs; decisions must be made on the level of staining to be considered positive. In colorectal bxs, intensely labeled cells are considered to reflect cells in the S-phase (43). An additional difficulty stems from the fact that PCNA has a long half-life (about 20 h) and may be detectable for 24–48 h after cells have stopped dividing (37, 38). In addition, PCNA has been shown to be involved in DNA repair, and this is likely to be a source of some PCNA staining, because skin is continually exposed to damaging UV radiation (34–36). To address this issue, we analyzed data separately by staining level to determine the impact of eliminating grade 1–3 cells on statistical significance and mean PCNA LIs in non-sun-exposed upper-medial arm skin, and adjacent skin and AKs. Our data show that including staining levels of grade 1 or 2 and above yielded similar differences with respect to statistical significance between tissues, whereas mean percentage of PCNA LIs were about 2-fold higher for grade 1 and greater. As would be expected, grade 3 and greater staining further reduced mean LIs and eliminated the significant difference between upper-medial arm and adjacent tissue skin for total, basal, and suprabasal layers. Grade 4 staining level LIs were greatly reduced, and the majority of the stepwise increase was lost between tissues, because there were relatively few grade 4 cells in any of the tissue sections.

As expected, assessment of buttock skin bxs revealed that these were histologically normal. Conversely, the majority of clinically normal-appearing upper-medial arm samples contained some signs of sun damage (primarily cellular crowding). Skin adjacent to AKs frequently demonstrated many features of AKs (primarily cellular crowding, budding, and slight cytological atypia), and although not meeting the diagnostic criteria of AK, they may represent very early AKs. Pearse and Marks (16) reported that skin adjacent to an AK demonstrated changes, such as epidermal thickening, and histological and cytological abnormalities, such as variation in cell size, budding, loss of polarity, and multinucleated cells. These authors suggested that AKs may develop in skin that has already undergone significant damage, supporting the concept that there may be a progression from sun-damaged skin through AK to SCC. In our study, there was a large amount of heterogeneity within each tissue in individual subjects with respect to both PCNA LIs and histological changes. It would be of considerable interest to evaluate this biological heterogeneity with respect to specific molecular alterations.

Cell proliferation is a fundamental biological process in tissues such as skin and has been shown to be disregulated in the process of tumorigenesis (44). Measures of proliferation will likely serve as useful SEBs in chemoprevention studies in skin and currently are being used extensively in prevention trials in tissues such as colon and esophagus (45).

References

Evaluation of PCNA as a Surrogate End Point Biomarker in AK

Evaluation of proliferating cell nuclear antigen as a surrogate end point biomarker in actinic keratosis and adjacent, normal-appearing, and non-sun-exposed human skin samples.

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