Expression of p53 Protein in Actinic Keratosis, Adjacent, Normal-appearing, and Non-Sun-exposed Human Skin

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Abstract

Nonmelanoma skin cancer, including both squamous cell carcinoma and basal cell carcinoma, is a significant and increasing health problem in the United States. The precursor lesion of cutaneous squamous cell carcinoma, actinic keratosis (AK), is a major risk factor for nonmelanoma skin cancer, and it is also a marker of long-term sun exposure. AKs themselves can serve as biomarkers in chemopreventive studies, but in addition, they may contain phenotypic and genetic alterations that are related to the process of UV-induced skin carcinogenesis. One of these alterations, the tumor suppressor gene p53, is altered early in UV-induced skin carcinogenesis in humans. p53 protein expression was measured by immunohistochemistry in biopsies from AKs, tissue immediately adjacent to AKs (AK-adjacent), normal-appearing upper medial arm skin, and non-sun-exposed skin from 19 subjects. There was a significant difference and a progressively increasing mean p53 labeling index in total epidermis (basal and suprabasal layers) between upper medial arm skin (0.9 ± 1.8%) and AK-adjacent (12.1 ± 14.4%; P = 0.0004) and between AK (27.7 ± 21.3%) and AK-adjacent skin (P = 0.04), whereas upper medial arm skin was marginally different from non-sun-exposed skin (0.1 ± 0.2; P = 0.05). This pattern of p53 expression was also seen when epidermis was separated into basal and suprabasal layers. We conclude that epidermal p53 protein expression is associated with histological evidence of chronic sun damage.

Introduction

NMSC, including both SCC and BCC, is the most frequently diagnosed cancer in the United States, with an estimated incidence of between 900,000 and 1.2 million cases in 1994 (1–3). Although the metastatic potential of SCC and mortality rates are low, SCC represents a significant health concern (2). The risk of SCC is strongly associated with chronic sun exposure, and SCCs appear primarily on sun-exposed parts of the body (1).

AKs are the premalignant precursors to SCCs (1) and share many histological similarities with SCCs (1, 4). The presence of AKs is a major risk factor for NMSC, although AKs can regress with a reduction in UV exposure (5). Clinically AKs appear primarily on chronically sun-exposed areas (5), and sites adjacent to AKs may contain significant histological alterations, suggesting extensive premalignant alterations in sun-damaged skin (6). Additionally, AKs may contain other phenotypic and genetic alterations that are related to the process of UV-induced skin carcinogenesis.

One of these alterations is the p53 tumor suppressor gene, located on the short arm of chromosome 17 (17p13.1). The 53-kDa nuclear phosphoprotein product of the p53 gene is essential in maintaining genomic stability by blocking DNA replication in response to DNA damage. Additionally, it recently has been proposed that p53 has a direct role in DNA repair (7, 8). WT p53 protein is normally present at low levels that are not readily detectable by immunohistochemical methods, and it binds DNA in a sequence-dependent manner (9). In response to DNA damage, such as UV irradiation (10–12), the half-life of WT p53 increases posttranslationally from minutes to hours (13). This up-regulation of WT p53 results in a transient G1 arrest, allowing cells to repair the DNA damage prior to entry into the S phase of the cell cycle (13). There also is a p53-dependent pathway whereby cells too damaged to repair themselves undergo apoptosis (14). Many p53 mutations result in increased protein levels through stabilization of the protein, allowing its detection by immunohistochemistry (13, 14), and the majority are missense mutations, producing a faulty protein that is defective in sequence-specific DNA binding and transcription (15, 16).

In humans, mutations in p53 have been reported to be between 0 and 60% in AKs (17, 20), 0 and 40% in Bowen’s (17, 21, 22), and 15 and 69% in SCCs (17, 18, 22–24). Additionally, mutations found in premalignant and malignant skin have been consistent with UV as the causative agent (i.e., CC-to-TT and C-to-T substitutions at dipyrimidine sites; Refs. 17–24). This high incidence of p53 mutations in NMSC and premalignant AK lesions strongly suggests that the p53 gene plays an important role in the etiology of NMSC. Additionally, the distribution of p53 mutations in skin cancer is different from those found in internal cancers (25). p53 mutations cluster between amino acids 241 and 280 for SCC and between 200 and 280 for AKs (14).

p53 protein expression, as measured by immunohistochemistry, has been demonstrated in AK, Bowen’s, and SCC (17, 18, 22, 26–36). The availability of antibodies to a number
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of epitopes, as well as the use of both frozen and fixed tissue, make these studies difficult to compare. p53 expression has been reported in 0–92% of SCCs (17, 18, 22, 26, 27, 29, 31, 32, 35, 36), 0–80% of Bowen’s (21, 25, 29, 33, 36), and 0–80% of AKs (18, 19, 26, 29, 31–33). Additionally, in apparently normal skin adjacent to AKs, SCCs, and BCCs, areas of increased p53 expression are evident (28, 30, 37–40). Recently, Jonason et al. (41) demonstrated the presence of clonal patches of p53-mutated cells within sun-exposed skin.

Here, we investigate the relationship between a quantitative measure of p53 protein expression and the level of UV-induced skin damage using biopsies taken from AKs, skin adjacent to an AK (AK-adjacent), upper medial arm skin, and non-sun-exposed buttock skin in 19 patients with multiple AKs.

Patients and Methods

Patients and Tissue Preparation. Nineteen subjects with clinical AK participated in this study. Informed written consent was obtained according to institutional and federal guidelines. Shave biopsies of clinical AKs, adjacent skin within 4 cm of the AK, upper medial arm skin, and non-sun-exposed buttock skin 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 biopsies 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 embedded in paraffin, and 3-μm serial sections were cut. Just prior to staining, slides were deparaffinized through a series of graded alcohol. Immunohistochemical staining was performed using a standardized streptavidin-biotin peroxidase system with a 3,3'-diaminobenzidine tetrahydrochloride chromagen and a hematoxylin counterstain (Ventana Medical Systems, Tucson, AZ) on an automated VMS 320 immunostainer (Ventana Medical Systems) as described previously (18). Anti-p53 PA1801 (Oncogene Science, Uniondale, NY) was used at a 1:200 dilution. Negative controls were run on each sample by substituting antibody diluent for PA1801. The T47D breast cell line with a p53 mutation was included as a positive control.

Histology. Pathology confirmation of clinical diagnosis was done for all biopsies. The histological criteria used to confirm the clinical diagnosis of AK on routine H&E sections were as follows: hyperkeratosis (orthokeratosis and/or parakeratosis), cellular crowding, bud-like proliferation of keratinocytes, and cellular atypia [pleomorphism (variation in size and shape of nuclei and nucleoli) and dyskeratosis (pyknosis of nuclei, early keratinization)]. Cases that showed cytological atypia involving the full thickness of the epidermis (SCC in situ) or evidence of invasion (SCC) were excluded (42).

Quantitation. Videoprints of longitudinally sectioned epidermis (Fig. 1) were generated using an Olympus microscope (X40 objective) equipped with a Sony CDD-iris camera (Sony Corp., San Jose, CA) and a color videoprinter (Sony). The videoprints were manually evaluated by one observer, and between 12 and 21 fields were counted for each tissue. Each field represented an approximate length of 0.15 mm. Any degree of positive (brown) staining was considered positive. The basal layer was the single layer of cells adjacent to the basement membrane, and the suprabasal layer was all of the cells above the basal layer. The two layers were combined for total epidermis.

Fig. 1. Examples of p53-immunostained AK (a), tissue adjacent to the AK (b), upper medial arm skin (c), and non-sun-exposed buttock skin (d). No p53 expression is seen in non-sun-exposed skin. A single positive cell (arrow) is seen in upper medial arm, whereas p53 expression (represented by dark gray to black nuclei) is increased in adjacent skin and in the AK (X40 objective).
Table 1  Mean percent p53 expression (± SD) in total epidermis and basal and suprabasal layers in non-sun-exposed skin (n = 12), upper medial arm skin (n = 19), skin adjacent to an AK (n = 19), and AK (n = 18)

<table>
<thead>
<tr>
<th>Layer</th>
<th>Mean % p53 (± SD)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total epidermis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonexposed</td>
<td>0.1 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Upper medial</td>
<td>0.9 ± 1.8</td>
<td>0.05</td>
</tr>
<tr>
<td>AK-adjacent</td>
<td>12.1 ± 14.4</td>
<td>0.0004</td>
</tr>
<tr>
<td>AK</td>
<td>27.7 ± 21.3</td>
<td>0.04</td>
</tr>
<tr>
<td>Basal layer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonexposed</td>
<td>0.2 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Upper medial</td>
<td>0.9 ± 1.9</td>
<td>0.9</td>
</tr>
<tr>
<td>AK-adjacent</td>
<td>7.1 ± 7.8</td>
<td>0.0005</td>
</tr>
<tr>
<td>AK</td>
<td>27.0 ± 23.7</td>
<td>0.01</td>
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<tr>
<td>Suprabasal layer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonexposed</td>
<td>0.1 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Upper medial</td>
<td>0.8 ± 1.8</td>
<td>0.6</td>
</tr>
<tr>
<td>Adjacent</td>
<td>15.3 ± 18.7</td>
<td>0.0004</td>
</tr>
<tr>
<td>AK</td>
<td>28.1 ± 20.5</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Statistical Analysis. p53 LIs were calculated by dividing the number of labeled cells by the number of total cells (labeled and unlabeled) and multiplying by 100. Mean % p53 LIs were compared by Wilcoxon signed-rank tests due to the fact that not all LI distributions appeared normally distributed.

Results

The study subjects consisted of 12 males and 7 females, with a mean age of 72.7 ± 6.3 years (range, 60–84 years). All subjects were Caucasian, with between 2 and 200 AKs on the dorsal surface of their forearms or hands. Four of the subjects had type I skin (burns easily, never tans), 8 had type II skin (burns easily, tans minimally), 6 had type III skin (burns moderately, tans gradually), and 1 subject had type IV skin (burns minimally, always tans; Ref. 43).

All clinical AKs were histologically confirmed using the criteria described in “Patients and Methods.” The majority of the upper medial arm samples had some evidence of sun damage (primarily cellular crowding). The AK-adjacent samples, in addition to having significant evidence of sun damage, contained some of the features of AKs (e.g., primarily cellular crowding, budding, and slight cytological atypia). In contrast, non-sun-exposed buttock biopsies were histologically normal.

The largest number of cells per 40× microscopic field was found in non-sun-exposed buttock skin for total epidermis (86 ± 43), as well as for the basal (25 ± 11) and suprabasal (61 ± 32) layers. There was a trend toward increasing numbers of cells per field between upper medial arm and AK-adjacent compared to AK for total epidermis (upper medial, AK-adjacent, and AK: 52 ± 21, 55 ± 25, and 75 ± 29, respectively) and the suprabasal layer (upper medial, AK-adjacent, and AK: 31 ± 11, 32 ± 16, and 50 ± 18, respectively), whereas the numbers of cells in basal layers were similar between the three tissue types (upper medial, AK-adjacent, and AK, 21 ± 10, 21 ± 9, and 19 ± 11, respectively).

Examples of a p53-immunostained AK (Fig. 1a), AK-adjacent (Fig. 1b), upper medial arm skin (Fig. 1c), and non-sun-exposed buttock skin (Fig. 1d) are shown in Fig. 1. In this example, no p53 expression is seen in non-sun-exposed skin, and a single positive cell (arrow) is seen in upper medial arm; in contrast, p53 expression is increased in AK-adjacent and AK.

Table 1 shows the mean percent p53 protein expression as a LI (mean ± SD) for total epidermis, as well as basal and suprabasal layers, in non-sun-exposed buttock skin, upper medial arm skin, AK-adjacent, and AK. In total epidermis, there was a significant and stepwise increase in mean percent p53 labeling between upper medial arm skin (0.9 ± 1.8%) and AK-adjacent (12.1 ± 14.4%; P = 0.0004) and between AK-adjacent and AK with the highest mean percent p53 LI (27.7 ± 21.3%; P = 0.04). Upper medial arm skin was marginally different from non-sun-exposed skin with the lowest mean LI (0.1% ± 0.2%; P = 0.05). When the epidermis was separated into basal and suprabasal layers, the same statistically significant increase in mean percent p53 LIs and mean difference was seen in the basal layer, whereas in the suprabasal layer, the significant difference between AK-adjacent and AK was lost.

There was a large amount of heterogeneity in p53 LIs between individual subjects for AK-adjacent and AK tissue samples, as demonstrated by the box plot graph of total epidermal p53% LIs in Fig. 2. The central box extends from the 25th to 75th percentiles, the horizontal line within the box represents the median LI, the vertical lines extending from the box represent the spread of the data, and the points outside are outliers. p53 LIs ranged from 0.5 to 69.0% for AK, from 0 to 54.1% for AK-adjacent, and from 0 to 7.1% for upper medial arm.

Discussion

Skin cancer has been proposed to progress in stages from sun-damaged epidermis to disordered keratinocytes, to AK, to cancer in situ, and, finally, to SCC and metastasis (20). This sequence of lesions provides an excellent opportunity to study the progression of molecular and phenotypic alterations that take place during UV-induced carcinogenesis. In addition, pre-malignant AKs can serve as targets for chemoprevention trials, and they represent a resource for developing rational surrogate end point biomarkers, such as p53 overexpression, based on the biological or molecular changes that occur during the process of carcinogenesis. Tissue adjacent to AKs often contains significant phenotypic alterations that are suggestive of chronic sun exposure, and it 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 also may be useful...
as a control for phenotypic and molecular alterations in relation to sun exposure.

Here, we determined epidermal levels of p53 protein expression, as measured by p53 immunohistochemistry, in 19 subjects with multiple AKs. The differences in expression of p53 protein in AK lesions, tissue adjacent to AKs, upper medial arm skin, and non-sun-exposed skin from the buttock area were studied in superficial shave biopsies. There was a stepwise increase in mean percent p53 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 percent p53 LI. Although p53 expression has been previously reported in AK and adjacent skin, our study is the only one where AK, skin adjacent to an AK, upper medial arm, and non-exposed buttock skin were sampled from the same individuals to quantitatively determine differences in p53 expression (37–40).

Although a progressive increase in p53 expression was seen, the significance of these results arecomplicated by the fact that the 1801 antibody recognizes both WT and mutant p53, and it cannot be assumed that overexpression of p53 is indicative of a mutation. WT p53 protein expression can be detected in normal skin by immunohistochemistry within 2 h of an erythemic dose of UV irradiation, peaking at 24 h and returning to undetectable levels by 3 days (44). The increased p53 expression seen in this study likely reflects a combination of mutant p53 protein and WT p53 protein due to DNA damage (11, 13).

Histological review of biopsies determined that non-sun-exposed buttock skin was normal, but the majority of clinically normal appearing upper medial arm samples contained signs of sun damage (primarily cellular crowding). Skin adjacent to AK frequently demonstrated many histological features of AKs (primarily cellular crowding, budding, and slight cytological atypia); although they do not meet the diagnostic criteria of AK, they may represent very early AKs. Taken together, the histology and p53 LI data suggest that, as histological alterations increase, so does the level of p53 protein expression.

There was also a large amount of heterogeneity in skin biopsies between individual subjects with respect to both p53 LIs and histological changes, but the sample size (n = 19) was not adequate to determine whether there was a relationship between histological change and p53 LI. There may be a number of potential explanations for the large variation in p53 expression, including severity of tissue damage, cumulative and acute sun exposure, or host/genetic factors like DNA repair capacity. A recent report by Jonason et al. (41) found that, in whole-mount preparations of skin, clonal patches of p53-mutated cells ranged from 60 to 3000 cells/cm². Patches were larger and more frequent in sun-exposed areas than in sun-shielded areas, whereas skin with intermittent exposure was intermediate. They also noted that samples taken in summer months contained a background level of p53-positive cells.

In several studies of skin cancer, the presence of p53 mutations has correlated poorly with measurement of p53 overexpression by immunohistochemistry (18, 19, 21). We reported previously that a higher percentage (80%) of AKs were immunopositive then were positive for p53 mutations (53%; Ref. 18). Two more recent studies microdissected skin samples on the basis of p53-positive immunostaining, an approach that may enhance the ability to detect mutations within a small subset of cells in a tissue sample (39, 40). Urano et al. (40) found no p53 mutations in normal epidermal samples from patients with BCC with “usual sun exposure” and positive p53 immunohistochemistry. In contrast, samples adjacent to BCCs had a p53 mutation. Similarly, Ren et al. (39) found that morphologically normal epidermis from patients with SCC having a “disperse pattern” (weaker staining pattern only affecting a proportion of nuclei) and not sharply demarcated by p53-immunopositive staining and no evidence of p53 mutations. Conversely, the majority of morphologically normal epidermis with a “compact pattern” (uniform nuclear staining in a sharply defined area) of immunopositive staining showed p53 mutations that were always different from the mutations found in the corresponding dysplasia, cancer in situ, or SCC. In our study, 14 of 19 AKs had a compact pattern, whereas only 4 of 19 adjacent samples had this pattern. The majority of upper medial arm samples had isolated single p53 positive cells with an occasional small focal area of scattered positive cells but no compact areas. Unfortunately, samples from the current study could not be analyzed for the presence of p53 mutations due to the small amount of tissue collected. Studies are ongoing to determine the frequency of p53 mutations and the p53 protein expression in AK, skin adjacent to an AK, and upper medial arm skin.

These kinds of studies will help to determine whether different levels or patterns of p53 immunostaining can be used as a surrogate for the presence of a p53 mutation or whether measures of p53 overexpression and mutational analysis will each provide some useful biological information. For use of p53 expression as a surrogate end point biomarker in chemoprevention studies, it will be essential to differentiate between normal and mutant proteins. The current study shows that there is differential expression of p53 in the progression of UV-induced skin carcinogenesis.

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