MITOCHONDRIAL DNA DELETIONS IN SKIN FROM MELANOMA PATIENTS

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INTRODUCTION

The incidence of melanoma, basal cell carcinoma, and squamous cell carcinoma has been increasing for many decades. Solar exposure is associated with the development of squamous cell carcinoma and seems to be associated with the development of melanoma, but the mechanism by which solar exposure causes cancer is not clear. The importance of understanding the way in which UVB affects the development of melanoma is underlined by the current promulgation of sunscreens to protect against UVB. If the way in which UVB affects the development of melanoma can be elucidated, then rational public health recommendations can be made. However, the effectiveness of protective topical applications is still controversial, as their use encourages individuals to prolong sun exposure or they do not affect the nonerythemic effects of UVB. Some sunscreens may reduce solar keratoses.

Compounding this issue is the increase in the rate of cancers and degenerative diseases with age. This increase may be due to a decrease in DNA repair with increasing age. The increase in mitochondrial damage may be a major factor in the development of age-related diseases and may contribute intrinsically to the aging process. Loss of mitochondrial function produces oxidative stress, which is one mechanism proposed to cause senescence. This loss in function has also been implicated in apoptosis and could affect natural cell turnover rates with increasing age.

Highly pigmented skin, especially with a higher proportion of eumelanin, has long been known to be more resistant to the carcinogenic effects of solar radiation than poorly pigmented skin or skin with a higher content of pheomelanin. The processes that mediate the protective properties associated with pigmentation are still unclear, but a substantial body of evidence suggests that the beneficial effects of pigmentation may be linked to either reduced DNA damage or enhanced DNA repair. From this standpoint we undertook a study of persistent DNA damage as reflected in mitochondrial deletions in skin derived from patients diagnosed with melanoma at the Memorial Hospital in New York City.

METHODS

Subjects

Sixty-seven study participants signed an informed consent form, and the study was approved by the institutional review board. The normal skin specimens donated for this study derived from patients diagnosed with melanoma who underwent wide skin excisions as part of their normal care at Memorial Hospital. Information on age, family history, and phenotypic characteristics (hair and eye color, tanning ability, freckling pattern, and propensity to sunburn) were extracted from self-administered questionnaires.

Phenotypic index (PI) was calculated by combining hair color, eye color, and tannability/propensity to burn characteristics as indicators of cutaneous phenotype as previously described (Table 1). During the analysis, the phenotypic indices were further grouped into three categories: low (PI 1–2), intermediate (PI 3), and high (PI 4–5).

Tissue Specimens

Skin tissues (100–325 mg) were obtained from wide skin excisions from melanoma patients undergoing standard care.
DNA Extraction
DNA was extracted from ≈25 mg of skin by using the QIAamp spin columns (Qiagen, Valencia, Calif) according to the manufacturer’s specifications and diluted to 100 ng/mL in 10 mM Tris, .1 mM EDTA (pH 7.5).

Real-Time PCR
Analysis of total mitochondrial DNA (mtDNA) copies and the common deletion was conducted on a Roche LightCycler instrument (Roche Applied Science, Indianapolis, Ind). Each capillary contained 1x LightCycler FastStart DNA Master HybProbe, 300 nM each forward and reverse primer, 200 nM probe and 0.1 unit heat-labile uracil-DNA glycosylase (Roche Applied Science) in a total volume of 10 mL. The common deletion of 4977 bp is located between nt 8470 and nt 13,447 of the so-called Cambridge sequence of Anderson et al. The primers and probes have been described by Pogozelski et al. The forward primer for the common deletion spans the mtDNA sequence from bp 8416 to bp 8437, and the reverse primer spans the sequence from bp 13,519 to bp 13,498. Nucleotides 13,461–13,480 of the Cambridge sequence within the resulting amplicon are complimentary to a probe tagged with FAM at its 5’ end and the quencher BHQ1 at its 3’ end. The total amplicon spans a region of the mtDNA that is rarely deleted. The specific primers used to detect the total mtDNA are located at nt 1307–1328 (forward) and nt 1433–1414 (reverse). The total mitochondrial probe binds to nt 1340–1359 of the Cambridge sequence. Each PCR included test samples and plasmid controls for both the common deletion and total mtDNA. The copy number per cell in the test samples was inferred by comparison of threshold cycles and the copy number of the control plasmids, assuming 16 pg of DNA per cell. Two concentrations of DNA (10 and 30 ng) were run for each sample. However some specimens required 60 ng in order to detect an amplicon. At the conclusion of each run, the reaction mixtures were collected by centrifugation and loaded onto 2% agarose gels for electrophoresis in 0.5x TPE buffer containing 5 mg/mL ethidium bromide. The resulting gels were photographed by transillumination with UVB light. A second, recently described mtDNA deletion, found to be induced by UVB irradiation of cultured human keratinocytes (Ahw), was quantified as described.

Statistical Analysis
Linear regression were calculated by least squares using the Excel spreadsheet (Microsoft Corp., Redmond, Wash) trendline utility. Correlation coefficients for the linear regressions and Student t tests were also calculated by using spreadsheet-embedded functions.

RESULTS
Patients and Skin Characteristics
A total of 67 specimens of skin tissue were collected. Questionnaire data were available in all 67 cases. The patients ranged in age from 16 to 85 years. The median age was 52.5 years, and the average was 55.1 years.

Distribution of mtDNA in the Patients
Figure 1 shows that the number of mtDNA copies per cell in the patients was extremely variable, ranging from <1 to >6500 copies per cell. The average and median cellular copy numbers were 2217.8 and 1409.0, respectively. The large variation in copy number may reflect the presence of distinct subpopulations within the overall sample. In Figure 2A, the results are stratified according to the number of copies per cell at age of diagnosis. There was no apparent age dependence for the six patients with fewer than three copies per cell. There was a slight increase of one copy per year for patients who had <1000 copies per cell. On the other hand, for patients with >1000 copies per cell, there was a definite decline in mtDNA copies per cell with age at diagnosis. The loss amounted to ≈63 copies per year. While this decline occurred in the studied subjects, the results suggest that the event may have occurred in all individuals and a class of individuals lost mtDNA copies in their skin as they aged. When cellular mtDNA content was examined according to diagnosis age, either above or below the median (54 years), the older patient group (age 56–85 years) had a significantly lower cellular mtDNA content (5% confidence level via t test) than the younger group (age 16–53 years), 2685 vs 1290 mitochondrial copies/cell respectively.
of 1.69 and a median of 0.13. Twelve of the patients exhibited additional bands on the gels in some of their samples, although all but three of them had amplicons in at least some of their replicates that were consistent with the common deletion. Most of the newly appearing PCR products were larger than the deletion, but one, found in four patients, was smaller than the common deletion. In a separate study of reactive oxygen species damage to mtDNA (unpublished results), we sequenced bands excised from gels that appeared in samples using the same primers used in the melanoma patient studied here. We found PCR products corresponding to deletions ranging in size from 4477 to 5021 bp that produced amplicons from 83 to 627 bp. The observed sizes of the novel PCR products found in the melanoma samples are consistent with these new deletions.

The common deletion has been reported to increase with age, and this is reflected in this study, as seen in Figure 2B in which as a percentage of total mtDNA genomes, the increase in deleted DNA copies in individual cells increases with age by ≈10-fold across the 72-year age span. When plotted as a percentage of total mitochondrial genomes, the regression showed a similar (11.7-fold) increase across the age range. The relationship between age and mtDNA appears not to be linear, as a more significant correlation coefficient for the data in Figure 2B was obtained with a polynomial regression model ($r^2 = .0011$).

**mtDNA Deletions and Phenotypic Index**

Mitochondrial DNA deletions were grouped according to patient phenotypic index to determine if we could observe any relationship between the number of deletions and degree of pigmentation. Figure 3 shows data on the relative levels of the common deletion and the $\Delta_{uv}$ deletion organized according to low, intermediate, and high phenotypic indices. Both types of mtDNA deletion were substantially more abundant in the intermediate PI group as compared with the low PI group, but mtDNA deletions in the intermediate PI group were also more abundant than in the high PI group. Although, statistical significance was not found, the pattern of differences as a function of phenotypic index for the two types of deletion was the same. We observed no difference between the low and high PI groups for either type of deletion.

**DISCUSSION**

We used mtDNA deletions as an indicator of persistent DNA damage in skin, and we found that mtDNA deletions tended to increase with age, as has been reported by others. While the phenotypic index is a qualitative measure, it reflects the individual's pigmentation characteristics, and individuals with lighter skin, red hair, and who are prone to burn after being exposed to sunlight are at higher risk for melanoma. Thus the phenotypic index gives an indication of risk from acute solar exposure during the course of an individual’s life history. Here, we quantified two different mtDNA deletions and found that, in both cases, deletions were substantially less prevalent in darker skinned (low-risk) patients than in those with lighter skin in the intermediate risk category. The finding that mtDNA deletions were less prevalent in the high-risk group than those in the intermediate-risk category was unexpected. In the high-risk group, damage to the mtDNA genome from solar radiation may have been more extensive, and the damaged mitochondrial genomes may have failed to
replicate. If so, damaged mtDNA would be more prevalent in the skin of high-risk individuals briefly after sun exposure but would not be seen after long periods of time. Since it takes years for melanomas to develop,\textsuperscript{17,18} this might explain the unexpectedly low incidence of mtDNA deletions in the high-risk patients. While the mtDNA copy number was also much higher in the high-risk patient group than in either the intermediate- or low-risk groups (915 copies/cell vs 376 and 388 copies/cell, respectively) there were fewer deleted genomes. Interestingly, al-

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\caption{Total and deleted mitochondrial copy number in skin specimens from melanoma patients as a function of age. The number of copies of mtDNA genomes were quantified by real-time PCR and plotted against donor age. A) Separate trend lines showing age trends are shown for mtDNA subpopulations >1000 copies per cell, <1000 but >3 copies per cell, or <3 copies per cell, as indicated. For the three groups analyzed, the trendline equations and corresponding correlation coefficients are: \(y=0.0181x+4.454\), \(r^2=0.0858\) (3 mitochondria/cell), \(y=-62.7x+7254.9\), \(r^2=0.3104\) (3 mitochondria/cell), \(y=1.025x+314.91\), \(r^2=0.0191\) (>1000 mitochondria/cell). B) Genomes containing the 4977-bp common deletion per cell were quantified and plotted against donor age. Values are the average of 3-4 replicates plus or minus standard error of the mean. A least squares trendline is shown (\(y=-0.0022x+1.9409\), \(r^2=4\times10^{-5}\)).}
\end{figure}
Figure 3. Relative levels of mitochondrial deletions in different risk groups. Copies of mitochondrial genomes containing either the common deletion (absolute copy number) or the Δmt deletion (relative copy number) were quantified by real-time PCR and normalized to total mitochondrial genomes. Average values plus or minus standard errors of the mean are shown for the common deletion samples where n =15, 10, and 11 for high-, intermediate-, and low-risk groups, respectively. Average values plus or minus standard error of the mean are also shown for the Δmt, where n=14, 8, and 9 for high-, intermediate-, and low-risk groups, respectively.

through the intermediate risk group exhibited many more mtDNA deletions, these patients were diagnosed with melanoma somewhat later in life (an average of 56.9 years vs 51.8 and 46.5 years for the high- and low-risk groups, respectively). This observation suggests that the acquisition of a certain level of mtDNA damage early in life may induce DNA repair mechanisms such that the higher levels of mtDNA deletions may be a marker of, or associated with, that novel protective response.

Implications for Improving Health Disparities

These findings outline a correlation between the accumulation of mtDNA deletions and cutaneous pigmentation that characterize persons of color. Inasmuch as pigmentation plays a role in moderating the development of skin cancer, quantitative analyses of mtDNA deletions will be useful as cancer risk biomarkers markers and may help elucidate mechanisms of skin carcinogenesis.

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REFERENCES

