Published in final edited form as:

Int J Radiat Biol. 2012 October ; 88(10): 682–687. doi:10.3109/09553002.2012.699697.

Chronic low dose UV exposure and p53 mutation: tilting the odds in early epidermal preneoplasia?

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Summary

Purpose—This review addresses how mutation of the *TP53* gene (*p53*) and ultraviolet light alter the behavior of normal progenitor cells in early epidermal preneoplasia.

Conclusions—Cancer is thought to evolve from single mutant cells, which expand into clones and ultimately into tumors. While the mutations in malignant lesions have been studied intensively, less is known about the earliest stages of preneoplasia, and how environmental factors may contribute to drive expansion of mutant cell clones. Here we review the evidence that ultraviolet radiation not only creates new mutations but drives the exponential growth of the numerous *p53* mutant clones found in chronically exposed epidermis. Published data is reconciled with a new paradigm of epidermal homeostasis which gives insights into the behavior of mutant cells. We also consider the reasons why so few mutant cells progress into tumors and discuss the implications of these findings for cancer prevention.

Keywords

Epidermis; Preneoplasia; Ultraviolet; p53 mutation; Stem cell

Introduction

Cancer is thought to develop from an initial oncogenic mutation in a single cell, which goes on to expand into a mutant clone. If such microscopic clones persist in the tissue, they may acquire further mutations that eventually result in the formation of visible preneoplastic lesions and tumors. These processes of clonal expansion and genetic heterogeneity have been hypothesized to recapitulate Darwinian evolution, a highly inefficient, random process in which only rare mutant clones succeed in developing into malignant lesions (Nowell 1976; Greaves & Maley 2012). Mutagenesis is a random process, generating many mutations which are “passengers” which do not alter cell behavior. Preferential clonal expansion depends on the founder cell developing a “driver mutation” that promotes its persistence and/or clonal expansion in the tissue. External selective pressures, in the form of environmental carcinogens, can also alter the fate of mutant clones (Cairns 1975; Brash & Cairns 2009). Evolutionary success in early preneoplasia results from cells receiving a driver mutation that increases their odds of survival and proliferation, giving them a selective

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Declaration of interests Statement

The authors report no conflicts of interest

advantage over their wild type neighbors in the carcinogen challenged tissue. These themes are well illustrated by p53 mutant clones in epidermis chronically exposed to ultraviolet radiation, which we consider here.

Ultraviolet radiation and p53 mutant clones (PMC)

Early studies identified p53 mutations in a majority of human non melanoma skin cancers (NMSC) and the commonest preneoplastic lesion, actinic keratosis, from sun exposed body sites (Ratushny et al. 2012). A high frequency of cytosine (C) to thymidine (T) base pair changes and the presence of some CC to TT double base pair changes, a signature of ultraviolet (UV) induced mutation, was observed, arguing that these mutations were caused by UV (Brash et al. 1991; Pierceall et al. 1991; Moles et al. 1993; Ziegler et al. 1993; Ziegler et al. 1994). These and subsequent studies have confirmed a characteristic distribution of mutations and frequently mutated codons in UV associated NMSC (Ziegler et al. 1993; Ziegler et al. 1994; Pfeifer & Besaratinia 2009). Several of the commonest mutations have been shown to result in proteins with a gain of function properties, able to cooperate with oncogenes to promote epidermal tumorigenesis, establish metastatic transformation and/or activate reporters repressed by wild type p53 (Caulin et al. 2007; Goh et al. 2011; Torchia et al. 2011). Taken together these observations argue that such mutations may give a selective advantage to mutant cells leading them to be found frequently in tumors in UV exposed epidermis.

A second key observation that has facilitated the study of p53 mutant cells in tissues is that some, though not all, p53 mutations result in protein stabilization in epidermal cells (Jonason et al. 1996; Ren et al. 1996; Ling et al. 2001; Rebel et al. 2005). Cells harboring such stabilizing mutations may stain with p53 antibodies while cells with wild type levels of p53 do not. p53 immunostaining, particularly in wholemount preparations of epidermis where the morphology and frequency of clones can be rapidly assessed, appeared to be a way to gain insight into early photocarcinogenesis in humans.

Analysis of immunopositive p53 mutant clones (PMC) in histologically normal human epidermis reveals several key characteristics. PMC are rare on sun protected skin but common (30/cm²) on chronically sun exposed sites, where PMC account for up to 4% of the epidermis (Jonason et al. 1996). The size of PMC varies widely, from 60 to 3000 cells, the clones in an individual varying 20 fold in size (Jonason et al. 1996). The cell clusters are cohesive but the shape of PMC is strikingly irregular, with some extending into hair follicles whilst others are confined to the interfollicular epidermis (IFE) (Jonason et al. 1996; Ren et al. 1997).

PMC can also be generated in murine epidermis following chronic exposure to low doses of UV, at or below the minimum level that causes skin reddening or erythema (Berg et al. 1996; Rebel et al. 2001; Zhang et al. 2001; Rebel et al. 2012). The clones generated in these experiments resemble those seen in human epidermis, and are confined to the interfollicular epidermis (Rebel et al. 2012). Larger interfollicular clones encircle but do not cross into hair follicle openings, consistent with the follicle and the IFE being maintained as separate cellular compartments (Rebel et al. 2001; Zhang et al. 2001; Ito et al. 2005; Levy et al. 2005; de Gruijl & Rebel 2008; Rebel et al. 2012). In two large studies of PMC in wholemount preparations of skin from animals irradiated for up to 11 weeks, the number and average size of PMC increased progressively, but the range of PMC sizes seen at each time point was very broad, extending from 3 to over 2000 cells at the 10 week time point (Zhang et al. 2001; Remenyik et al. 2003). Intriguingly, the number of PMC falls after cessation of treatment (Berg et al. 1996; Zhang et al. 2001; Remenyik et al. 2003).

These observations raise several important questions. Here we will first consider whether the data on PMC give insight into the behavior of p53 mutant cells in UV exposed epidermis, and then discuss the evidence regarding whether PMC are precursors of NMSC and the implications for cancer prevention strategies.

Models of normal and P53 mutant cell behavior

The majority of PMC arise within the IFE. The data on PMC in mouse and human epidermis was originally interpreted through the epidermal unit (EPU) model of IFE homeostasis. Drawing on the histological organization of murine epidermis, particularly in the ear, and cell kinetic studies with tritiated thymidine, the EPU model sought to explain the organization of the differentiated cell layers of the epidermis into regular columns (Figure 1 A, B) (Mackenzie 1970). It was hypothesized that if there was no lateral migration of cells, each column was supported by a single slow cycling stem cell, which divided asymmetrically to generate transit amplifying (TA) cells that underwent several rounds of division before exiting the cell cycle and leaving the basal layer by stratification (Allen & Potten 1974; Potten 1974; Allen & Potten 1976; Potten 1981). The EPU was thus a self maintaining clonal unit of about 12 basal cells and the overlying differentiated keratinocytes.

When interpreted within the EPU model, PMC data presents some challenges. Mutation of short-lived TA cells, which constitute most of the proliferative cells in the epidermis, would be predicted to generate small clones (up to 12 cells) which would be rapidly lost as they differentiate, unless p53 mutations block TA cell cycle exit. In this model, long lived PMC could only arise from mutated stem cells. These would expand to occupy a single EPU, but would have to “colonize” adjacent wild type EPU in order to expand: once a wild type stem cell had been displaced, the entire neighboring EPU would rapidly be occupied by mutant cells, so the clone would comprise multiple adjacent EPU (Figure 2A) (Ling et al. 2001; Zhang et al. 2001). However, PMC are actually highly irregular in shape with ragged edges that do not follow EPU boundaries and found in a continuum of sizes from 3 to over 2000 cells.

Whilst the EPU model was widely assumed to be correct, there is a significant body of evidence at odds with this hypothesis (Jones et al. 2007; Jones & Simons 2008; Doupe & Jones 2012). In brief, early studies of the epidermis of chimaeric mice revealed that the borders of mosaic patches were irregular and did not follow the boundaries of the supposed EPU, as should have been the case if the epidermis was organized into clonal units (Schmidt et al. 1987). Later, lineage tracing was used to follow the fate of proliferating cells, introducing a reporter gene encoded in a retrovirus which would be expressed in the proliferating cell and its progeny (Ghazizadeh & Taichman 2001). In such an experiment the EPU model predicts that only labeled stem cells and their progeny will remain in the tissue, the labeled cells being organized into EPU. However, when the epidermis was examined nine months after labeling, clones in the IFE were found to vary in size and did not fit within EPU (Ghazizadeh & Taichman 2001). Further small scale labeling studies also failed to confirm the existence of EPU as originally defined (Kameda et al. 2003; Ro & Rannala 2004, 2005).

Decisive evidence excluding the existence of EPU comes from large scale lineage tracing experiments using inducible genetic labeling in transgenic mouse epidermis (Clayton et al. 2007; Doupe et al. 2010). Such lineage tracing experiments reveal the behavior of cells proliferating directly in a way that surrogate assays, such as those used to argue for the existence of EPU, cannot. Inducible *cre* recombinase was used to express a fluorescent reporter gene in a representative sample of proliferating basal cells in adult mice. The animals were culled at time points out to 1 year post labeling and thousands of clones

imaged in three dimensions at single cell resolution. Average clone size increases linearly throughout the experiment, with a wide range of clone sizes despite loss in the number of clones progressively due to differentiation. Furthermore, the data exhibits the property of “scaling” with time. Scaling means that not only the average clone size, but the entire clone size distribution increases in direct proportion to time (e.g. if the proportion 4 cells at three weeks is 10%, the proportion of 8 cell clones at 6 weeks is also 10%). Such scaling is diagnostic of the clones arising from a population of functionally equivalent progenitor cells dividing at the same average rate, and excludes epidermal maintenance by stem and TA cells dividing at different rates (Clayton et al. 2007; Klein et al. 2007; Doupe et al. 2010; Klein & Simons 2011). At late time points, clones vary widely in size and are highly irregular in shape, resembling the appearance of PMC. Three dimensional reconstructions of 63 clones one year after labeling in ear epidermis revealed none that conform to the predicted shape of an EPU, but vary widely in size and are irregular in shape.

Further analysis reveals that the entire clone fate data set, in both tail and ear epidermis, gives an excellent fit to a remarkably simple “epidermal progenitor” model of cell behavior. The basal layer contains progenitor cells, which will go on to divide, and differentiated cells which have withdrawn from cycle and will soon stratify. Stratification of a differentiating cell is associated with division of a neighboring progenitor. The outcome of a progenitor cell division is random, generating either two progenitor cells, two differentiating cells or one cell of each type (Figure 1C). In normal epidermis, probabilities of producing two progenitor or two differentiating daughter cells are equal, so that on *average* an equal number of differentiating and progenitor cells is generated across the basal layer, ensuring homeostasis (Clayton et al. 2007; Doupe et al. 2010). In this paradigm, all proliferating cells have the same “life chances”. Following random labeling most proliferating cells will produce a short lived clone which undergoes terminal differentiation but a few “get lucky” producing proliferating daughters in successive divisions to generate a large clone that remains in the tissue long term. The irregular shape of clones reflects the random fate of progenitor cells (Klein et al. 2008; Doupe et al. 2010). The progenitor model does not account for the formation of regular columns of differentiated cells, but this may be a simple consequence of the packing properties of objects with the shape of the cornified keratinocytes, which spontaneously assemble themselves into columns (Menton 1976a, b; Honda et al. 1996).

It is important to stress that while lineage tracing studies have resolved the behavior of proliferating cells, they do not exclude the presence of a subpopulation of *quiescent* interfollicular stem cells, which as they might divide rarely, if at all, would make a negligible contribution to tissue maintenance. Indeed tritiated thymidine and bromodeoxyuridine label retaining assays, which detect the presence of slow cycling cells, indicate there is a subpopulation of quiescent keratinocytes in the epidermis that may represent interfollicular stem cells (Braun et al. 2003; Braun & Watt 2004). The self maintaining progenitor population would enable such cells to remain out of cycle in homeostasis, but they may be subject to p53 mutation and mobilized to proliferate by UV light.

Two studies have undertaken a quantitative analysis of data of PMC generated by chronic low dose UV radiation in mouse epidermis (Zhang et al. 2001; Remenyik et al. 2003). The first drew on the EPU model, arguing that if the epidermis is organized into clonal units supported by a single stem cell, expansion of a PMC would depend on the loss of a stem cell maintaining an EPU adjacent to the mutant clone, for example by UV induced apoptosis (Chao et al. 2008). This “frontier” model predicts that as PMC can only expand at their edges, their growth will be quadratic (Chao et al. 2008). However, the size distribution of PMC indicates that they in fact grow exponentially, except for the very largest clones whose growth rate is slower (Klein et al. 2010). Further analysis reveals that mutant cells have a

stochastic fate similar to that of the normal epidermal progenitor cells. However, instead of the rates of production of cycling and post mitotic cells being balanced as they are in homeostatic epidermis, UV exposure results in a small excess of proliferating cells over those lost through differentiation and apoptosis within PMC (Figure 3). Clone size distributions in sun-exposed human epidermis are also consistent with p53 mutation resulting in a net increase in the probability of mutant progenitors generating proliferating daughter cells (Jonason et al. 1996; Klein et al. 2010).

So how do these changes in mutant cell behavior result in PMC expansion? Chronic low dose UV irradiation below the minimal erythema dose induces a sustained increase in epidermal thickness (Remenyik et al. 2003). However, despite these changes, epidermal integrity is maintained, indicating the increase in cell production of proliferating wild type cells balances the increase in cell loss induced by UV (Remenyik et al. 2003). This suggests that compared to wild type cells, p53 mutant progenitors are relatively resistant to growth arrest, apoptosis and/or terminal differentiation that can be induced by UV treatment (Ziegler et al. 1994; Stout et al. 2005). If both wild type and p53 mutant progenitor cells respond equally to UV by generating more proliferating cells, the reduced loss of p53-mutated cells will result in exponential growth, as there is an effective imbalance between the rate of mutant cell loss and proliferation, giving the mutant cells a competitive advantage over wild-type cells (Zhang et al. 2005). The behavior of PMC remains stochastic, giving rise to irregular shaped clones that do not conform to the boundaries of supposed EPU (Figure 2B).

The competitive advantage of p53 mutant cells is only present during UV exposure. Following cessation of UV treatment, mutant cells revert to the balanced behavior of wild-type progenitors in homeostatic epidermis, the number of clones falling through differentiation, whilst the size of the remaining PMC increases through proliferation (Klein et al. 2010). The net effect is that the proportion of mutant cells in the epidermis remains the same, as the population of PMC mutant progenitors is self maintaining. This has important implications for prevention strategies, discussed below.

Inefficient progression of PMC to tumors

The relationship between PMC and NMSC has yet to be resolved. The spectrum of p53 mutations found in PMC in both UV exposed mice and in humans matches that observed in actinic keratosis and NMSC, consistent with these lesions evolving from PMC (Ziegler et al. 1994; Jonason et al. 1996; Zhang et al. 2001; Rebel et al. 2005). However, in adult human sun exposed skin, interfollicular PMC are very common. A recent deep sequencing study reveals 14% of human basal cells in sun exposed sites carry a p53 coding exon mutation, consistent with earlier immunostaining results (Jonason et al. 1996; Stahl et al. 2011). These observations argue that a very small proportion of cells in PMC evolve in to tumors (de Gruijl 2008; Stahl et al. 2011). A major factor contributing to scarcity of PMC progression is the low probability of mutant progenitors acquiring the additional mutations required for malignant transformation. Additional explanations for the scarcity of tumor development include the differentiation of all the proliferating cells in PMC which although less likely than for wild type cells will still occur frequently, resulting in the clone being eliminated before additional mutations are acquired (Klein et al. 2010). The nature of the p53 mutation harbored by the clone is also likely to be a factor. There is increasing evidence that while some p53 mutations are effective in driving squamous tumor formation in the epidermis, others, including the commonest germ line p53 mutations in Li Fraumeni syndrome, are not (Marin et al. 2000; Caulin et al. 2007; Petitjean et al. 2007; Wijnhoven et al. 2007; Gonzalez et al. 2009; Goh et al. 2011).

Implications for cancer prevention

NMSC is rarely fatal, but has reached epidemic levels in Caucasians and carries a substantial healthcare cost (Mosterd et al. 2008; Stang et al. 2008; Doherty et al. 2010). PMC are the commonest known oncogenic lesions present in human epidermis, and whilst the process of converting PMC to NMSC is very inefficient, mouse models indicate the size of the p53 mutant population in the epidermis is likely to predict future cancer risk (Jiang et al. 1999). It follows that reducing the number of mutant cells in the epidermis even if only a few of these will evolve into AK or tumors, is a plausible strategy for decreasing the incidence of NMSC.

The finding that PMC grow exponentially during UV exposure, but appear revert to homeostatic behavior following cessation of UV highlights the risks of long term sub minimal erythema dose exposure to UV. A given dose of UV administered over a long period will potentially have a greater impact than acute exposure, as chronic exposure drives the growth of existing clones (Zhang et al. 2001; Klein et al. 2010). Once mutant cells are acquired, the resulting clones will persist through winter periods, but expand in summer when UV exposure increases. Given the exponential nature of the increase in mutant cell numbers, older people will gain the most benefit from avoiding UV exposure.

Whilst epidermal PMC were first described almost 30 years ago, many questions about their significance remain to be addressed, in particular how frequent p53 mutation is common in human skin and yet very few of the mutant cells progress to NMSC. Further research in this area is likely to give insights into more effective means of preventing the commonest cancer in Western populations.

Acknowledgments

We thank Ben Simons, Allon Klein, David Doupe and Doug Brash for illuminating discussions. PHJ is supported by a Medical Research Council grant-in-aid and AR by a Cambridge Cancer Centre Research Fellowship.

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Figure 1. Models of normal epidermal homeostasis

A, B: The epidermal proliferative unit (EPU) model. A: It was hypothesized that the epidermis was maintained by slow cycling epidermal stem cells which divide asymmetrically to generate a stem cell and a transit amplifying (TA) cell. TA cells undergo 3-4 rounds of cell division after which they exit the cell cycle and differentiate. B: Stem and TA cells were thought to be arranged in EPU, a separate clonal unit lying under each stack hexagonal column of suprabasal cells. Stem cells occupy the central position, while transit amplifying cells are pushed peripherally before differentiating and moving to a suprabasal position.

C: The epidermal progenitor model. Interfollicular stem cells are quiescent in normal tissue making no detectable contribution to maintaining the epidermis. The tissue is supported by a single population of functionally equivalent progenitor cells. Stratification of a differentiated basal cell (marked 1) is associated with division of a nearby progenitor cell (2). There are three possible outcomes of progenitor division (3), the generation of two progenitor cells, two differentiating cells or one cell of each type. The outcome of a given progenitor division is random (depicted by the dice), but the probabilities of producing two progenitor and two differentiated daughters are equal, so that an equal number of progenitor and differentiated cells is produced across the progenitor population, ensuring homeostasis.

Figure 2. Models of PMC expansion

A: EPU model prediction. In this paradigm only mutation of the stem cell lying at the centre of the EPU will generate a long lived PMC, resulting in the entire EPU being populated by mutant cells (shaded fill). For the clone to expand it must colonize an adjacent EPU, perhaps following the death of an adjacent stem cell. The clone would be expected to consist of multiples of EPU.

B: In the epidermal progenitor model, basal cell behavior is independent of the boundaries of supposed EPU. The stochastic nature of progenitor cell behavior is predicted to result in ragged, irregular clones. This prediction fits with the observed shape of PMC in both mouse and human interfollicular epidermis.

Figure 3. Effect of UV exposure on PMC fate

A: p53 mutant progenitors (indicated by X) in UV exposed epidermis behave in a similar manner to wild type progenitor cells, but the combined effects of UV exposure and mutation are to produce a small net imbalance (ca. 10%, Δ) in the outcome of mutant progenitor division in favor of proliferation. B: When UV exposure ceases, the mutant cells revert to homeostatic behavior, becoming a self maintaining preneoplastic population.