CUTANEOUS MALIGNANCY

Milestones in Skin Carcinogenesis: The Biology of Multistage Carcinogenesis

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Centuries ago, an obscure report on an unusual skin cancer launched the modern era of cancer research. Ever since, skin research has made key contributions to our understanding of the biology and biochemistry of cancer pathogenesis. In 1775, Percival Pott published his essay entitled *Chirurgical observations Relative to the Cataract, the Polypus of the Nose, the Cancer of the Scrotum, [etc.]* describing the origin of the ''soot-wart'', a cancer of the inferior surface of the scrotum occurring with high frequency in English chimney sweeps (Brown and Thornton 1957). Pott attributed the cause of this tumor to ''the lodgement of soot in the rugae of the scrotum'', thus providing in one description the first recognition of environmental and occupational cancer, the first identification of a human carcinogen, the first opportunity for cancer prevention, and a protocol for surgical treatment. Included in that 797 word essay was a remarkably detailed description of the course of the disease from a local confined lesion to invasive and then disseminated lethal cancer. These insights were little noticed for almost 150 years until (Yamagiwa and Ichikawa, 1918) reported that the chronic topical application of coal tar on rabbit ears produced cutaneous squamous cell carcinomas, the first chemical carcinogenesis experiment. By describing the evolution Figure 1 of individual lesions, Yamagiwa and Ichikawa concluded that cancer developed through multiple phenotypic stages and progressed even after the carcinogen was removed. Over the ensuing 20 years, many biologists and chemists sought to identify the active carcinogen in coal tar, culminating in 1932 with the report by a group led by Ernest Kennaway that 3,4 benzpyrene was the potent polycyclic aromatic hydrocarbon in coal tar that produced cancers when topically applied to mouse skin. Incorporated into this project was the isolation or synthesis of multiple carcinogenic hydrocarbons, all recognized by their action as skin carcinogens (summarized in Kennaway, 1955).

At the same time, the availability of synthetic carcinogens and a reliable squamous cancer model emboldened a group of pathologists and biologists to methodically explore the pathogenesis of the neoplastic skin lesions. For the next two decades, these investigators discovered concepts regarding skin cancer development that dominate our current understanding of cancer development in almost all epithelial tissues. In particular, the work of Cecil Mottram, Friedewald and Rous (Rous later won the Nobel Prize for his work on the viral etiology of skin cancer), and Berenblum and Shubik led to the following conclusions on the biology of cancer: cancer occurs through multiple stages with distinct operational mechanisms and required sequence; cancer is more frequent when proliferating tissues are exposed to carcinogens; the extent of exposure or potency of the carcinogen determines the latent period for tumor development; tumor development does not require continuous exposure to carcinogenic agents and the effect of a subtumorigenic carcinogen exposure is permanent; tumors will develop after a subtumorigenic carcinogen exposure if the target tissue is subjected to regenerative hyperplasia as caused by repeated wounding or application of an irritant (Berenblum and Shubik, 1947, 1949; Mottram, 1944a, b; Berenblum and Haran, 1955; Berenblum, 1957; Friedewald and Rous, 1944). It was Friedewald and Rous who coined the terms ''initiation'' and ''promotion'' to distinguish the irreversible action of a carcinogen and the non-carcinogenic reversible activity of an irritant, still a major part of the cancer lexicon today. One of the important additions to the skin cancer protocols provided by Berenblum was the use of topical croton oil, a non-carcinogenic skin irritant that induced the outgrowth of tumors from skin that had been treated with a subtumorigenic dose of carcinogen (initiated).

Croton oil (Crotonis Oleum) is prepared from the seeds of *Croton tiglium*, a tree belonging to the natural order Euphorbiales and family Euphorbiaceae. Croton oil had been used for centuries as folk medicine and was known to induce inflammation and peeling of skin when applied topically and diarrhea when taken internally. Its potency to promote tumors on skin catalyzed interest among cancer biologists to identify the active compounds contained within the complex mixture.

In a series of brilliant chemical purifications and analyses, Van Duuren and Hecker almost simultaneously identified phorbol myristate acetate (12-O-tetradecanoyl-phorbol-13-acetate), commonly

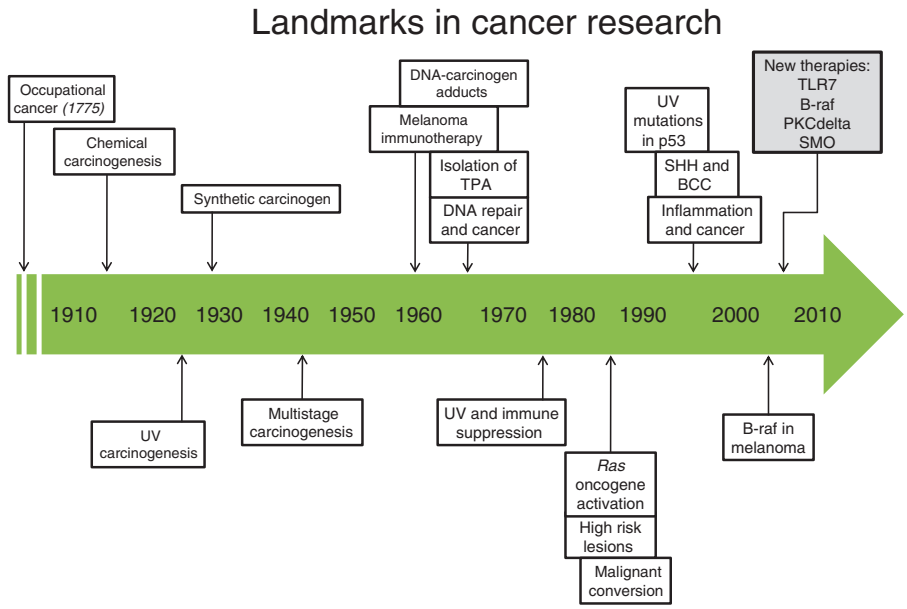

Figure 1. Milestones in cancer research. Shown is a timeline of important landmarks in cancer research first defined by studies of skin cancer pathogenesis or experimental skin carcinogenesis. As shown in the more recent era, such discoveries continue to be made and now provide therapeutic breakthroughs as well as insights into causation.

known as PMA or TPA, as the most potent skin tumor promoter in the oil (Van Duuren and Orris, 1965; Hecker, 1968).Using the now pure tumor promoter, a large cadre of scientists embarked on studies to understand its mechanism of action. To their surprise, PMA/TPA had a myriad of pleiotropic effects on cells and tissues, including growth stimulation or apoptosis, stimulation or inhibition of differentiation, stimulated secretion and migration, and many other cellular changes depending on the cell type and context under study. In skin and keratinocytes, PMA/TPA had a biphasic influence, stimulating immature basal cells to proliferate while accelerating differentiation in committed cells. Remarkably, skin tumor cells were much less responsive to TPA-induced terminal differentiation than normal keratinocytes (Yuspa *et al.*, 1982, 1986). The mystery surrounding these pleiotropic responses to PMA/TPA became transparent when Blumberg and colleagues discovered specific cellular receptors for the tumor promoting phorbol esters in brain and skin (Delclos *et al.*, 1980; Dunphy *et al.*, 1980). Shortly thereafter, the group of Nishizuka identified PMA/TPA as a potent ligand for binding and activation of protein kinase C (Castagna *et al.*, 1982). The discovery of PKC as the PMA/TPA receptor launched a multitude of studies in the ensuing 30 years, defining PKC as a multi-isoform family of protein kinases. Made possible by initial observations on skin cancer, these subsequent studies revealed intimate details of the PKC signal transduction pathway and its essentiality for many critical biological functions in cells and tissues (Griner and Kazanietz, 2007). By its dual function on skin keratinocytes to induce differentiation or proliferation in subpopulations of normal keratinocytes and its additional proinflammatory action, PMA/TPA provided the necessary irritation and regenerative hyperplasia to promote the outgrowth of differentiation-resistant initiated cells, as suggested 20 years earlier by Berenblum, Mottram, and Rous.

The isolation and synthesis of 3,4 benzpyrene by Kennaway encouraged multiple groups to investigate the carcinogenicity of other polycyclic hydrocarbons on mouse skin. Iball (1939) compiled a list of comparative potency of cyclic hydrocarbon molecules for inducing skin tumors (Iball Index). It made obvious that carcinogens were highly specific, with stereoisomers often varying greatly in potency. How they worked and their target remained an open question. In 1964, Brooks and Lawley published a landmark report addressing this question (Brookes and Lawley, 1964). Mouse skin painting of radiolabeled agents selected from the Iball Index resulted in covalent binding to DNA, demonstrating a strong correlation of carcinogenic potency with binding to DNA but not RNA or protein. Furthermore, kinetic studies showed that carcinogen–DNA binding persisted for extended periods, and peak binding was delayed after application, suggesting that metabolic activation was required. These findings bolstered the mutation theory of cancer pathogenesis, and shortly after, Bates and Yuspa showed that 7,12 dimethylbenza(a) anthracene, the most potent carcinogen on the Iball Index, could bind to the parental strand of replicating epidermal DNA and serve as a template for daughter strands (Bates *et al.*, 1970), a requirement for fixing a mutational event. In the decade of the 1970s, much effort was directed toward understanding the metabolic activation of carcinogens and how to intervene in the process as a mechanism of cancer prevention. With a

focus on 3,4 benzpyrene, the Sims laboratory discovered that the DNA-binding product from topically treated mouse skin was consistent with the 7,8-dihydro-7,8-dihydroxybenzo(a) pyrene 9,10 oxide as the ultimate carcinogenic species (Grover *et al.*, 1976). This and similar studies led to the elaboration of the complete metabolic activation pathway for benzpyrene and subsequently other polycyclic aromatic hydrocarbon carcinogens (Levin *et al.*, 1982). The remarkable specificity of the metabolic process was demonstrated by the marked differences in carcinogenic potency by optical enantiomers of the 7,8-diol-9,10-epoxides of 3,4 benzpyrene painted on mouse skin (Slaga *et al.*, 1979).

In 1981, the first indication of the phenotype of initiated cells came with the discovery that a small subpopulation of keratinocytes isolated from initiated mouse skin was resistant to differentiation in culture induced by elevating extracellular calcium, a potent differentiation signal (Yuspa and Morgan, 1981). The same phenotype was later determined to characterize keratinocytes treated with carcinogens *in vitro* or isolated from papillomas (Kilkenny *et al.*, 1985; Yuspa *et al.*, 1986). Nevertheless, the molecular change that determined this phenotypic divergence remained obscure. With the identification of DNA as the ultimate target for carcinogenesis and the recognition of the remarkable specificity of ultimate carcinogenic species, attention turned to identifying the genetic targets that converted normal keratinocytes into initiated cells. This was a particularly daunting task in the late 1970s, as at that time there were no databases that cataloged the number and sequence of genes in the genome, let alone how many were potential targets for tumor initiation. The number and locations of initiated cells in the skin was also completely obscure, making this particular problem like finding a single needle in a whole field of haystacks. As is often the case in science, parallel experiments in several laboratories working neither on skin nor on chemical carcinogenesis provided the solution: it was possible to identify the needle, in the form of DNA, by transferring it from tumor cells into (almost) normal cells, and selecting for those that became transformed. These experiments, carried out in the late 70s and early 80s, had an electrifying effect on the whole community of cancer researchers. The groups of Weinberg, Wigler, and others demonstrated in a technical tour de force that it was possible to transfer naked DNA from human tumor cells into mouse (NIH/3T3) fibroblasts, endowing them with transformed properties (Shih *et al.*, 1979; Shilo and Weinberg, 1981; Perucho *et al.*, 1981). While the existence of cellular ''proto-oncogenes'', akin to those found in transforming retroviruses, had been shown to exist in the human and mouse genomes in the Nobel Prize-winning work of Varmus and Bishop, this work showed for the first time that DNA, and DNA alone, was capable of causing cancerous changes in the absence of exogenous carcinogens or other agents. These seminal experiments also demonstrated for the first time that there was a qualitative change in DNA from tumors, as the same experiment transferring the same amounts of DNA from normal tissues did not cause transformation.

The culprits ultimately identified in these experiments turned out to be individual members of the *Ras* gene family (*HRAS, KRAS,* and *NRAS*), which had been previously known to be hijacked by a series of transforming retroviruses (Parada *et al.*, 1982; Santos *et al.*, 1982; Taparowsky *et al.*, 1982). A crucial difference between the transforming versions of these human genes and their normal cellular counterparts was the acquisition of specific point mutations leading to constitutive activation and independence from normal growth control signals. These provocative observations raised the possibility that chemical carcinogens, known to cause single point mutations in DNA, could in fact cause the first initiating step in carcinogenesis by mutating and consequently activating one of these *Ras* genes. The mouse skin model of two-stage carcinogenesis provided an ideal vehicle to address these questions. In a series of transfection experiments using DNA isolated from mouse skin tumors and corresponding cell lines (Balmain and Pragnell, 1983), a single member of the *Ras* gene family, *Hras*, was identified as the transforming agent. This constant activation of only one family member was in contrast to the emerging data on human transforming genes at the time, which suggested that all *RAS* gene family members could be activated in different tumor types, with *KRAS* being the most commonly mutated target gene. Much later, it transpired that the mouse data did in fact presage what would be found in human cancers, where it has been shown that although *HRAS* is less frequently activated generally in human cancers than *KRAS*, it appears to have a specific role in transformation of human squamous epithelial cells of the skin, lung, head and neck, and bladder (Agrawal *et al.*, 2011; Wang *et al.*, 2011; The Cancer Genome Atlas Research Network, 2012).

It seemed then that the elusive initiating event for mouse skin carcinogenesis had been identified, but several additional studies were required to fulfill Koch's postulates and demonstrate causality. First, if this was the initial event in skin cancer development, it should be detectable in the earliest forms of skin cancer induced in the mouse model – benign papillomas. This turned out in fact to be the case (Balmain *et al.*, 1984), and furthermore it was demonstrated that *Ras* mutations have additional roles beyond initiation, as gene copy number increases are seen during progression to malignancy (Quintanilla *et al.*, 1986). Second, if the initiating carcinogen causes the *Hras* mutation, by extension of the arguments presented above regarding the specific nature of the interaction between carcinogens and DNA, it would be postulated that any mutations detected would be specific for the particular carcinogen used. The possibility that this correlation applies to *Ras* gene mutations was first suggested from a study of carcinogen-induced rat mammary tumors (Sukumar *et al.*, 1983). Mammary carcinomas induced by treatment with the carcinogen MNU (*N*-Nitroso *N*-Methyl Urea) were

shown to contain activating G–A transition mutations at codon 12 of *Hras*, but similar tumors induced by treatment with 7,12-dimethylbenz[a]anthracene (DMBA) did not harbor this mutation, suggesting that the mechanisms by which the mutations arose were carcinogen specific. Studies of skin carcinogenesis identified the nature of mutations induced by DMBA (Bizub *et al.*, 1986; Quintanilla *et al.*, 1986) and demonstrated that within the same skin model system, the mutations found in *Hras* were indeed specific for the initiating carcinogen (Brown *et al.*, 1990). Tumors initiated by exposure to methylating agents MNU and MNNG harbored codon 12G–A transitions, whereas those initiated by DMBA contained almost exclusively A–T transversions at *Hras* codon 61. The latter observation was compatible with studies showing that DMBA, in contrast to alternative polycyclic hydrocarbons such as Benzo(a) pyrene, was capable of extensive adduct formation with adenosine residues in DNA (Dipple, *et al.*, 1983). These data, together with evidence showing that isolated DNA clones encoding normal HRAS acquired transforming properties after incubation with a reactive ''ultimate carcinogen'' derived from Benzo(a) pyrene (Marshall *et al.*, 1984), helped to establish the chain of events linking the chemical nature of the initiating carcinogen to the mechanisms by which normal RAS proto-oncogenes become activated to transforming alleles.

There remained, however, the issue of biological causality. While *Hras* mutations could be detected in early skin lesions, and the mutations were compatible with their being induced specifically at the time of initiation, were these mutant genes in fact capable of initiating tumorigenesis in keratinocytes as opposed to fibroblasts cultures, and *in vivo* rather than in cell culture systems? These questions were comprehensively addressed by demonstrating that (a) keratinocytes infected *in vitro* with viruses encoding transforming *Hras* genes could generate benign papillomas on grafting on to nude mouse skin (Roop *et al.*, 1986) and (b) direct infection of mouse skin keratinocytes *in vivo* with a retrovirus capable of expressing mutant Ras replicated all of the features of chemical carcinogenesis, including permanence of the initiated state, dependence on treatment with tumor promoters, and progression of benign tumors to malignant carcinomas (Brown *et al.*, 1986).

These data established the molecular nature of the initiating event in skin carcinogenesis, but several major questions remained to be resolved: what and where is the cell of origin of skin tumors, and do all initiated cells, or all papillomas, have the same capacity for malignant progression? Hennings and Yuspa found evidence for heterogeneity in the propensity for papillomas to progress to malignancy. Papillomas that developed soon after beginning promotion treatment appeared to progress to carcinomas at a high frequency (Hennings *et al.*, 1985). However, it was not clear whether these ''high-risk'' papillomas had arisen from a specific subpopulation of initiated (stem?) cells, or because of stochastic acquisition of a secondary genetic change after initiation. Evidence in favor of the former scenario came from experiments involving physical removal of cells in the interfollicular epidermis by abrasion after DMBA initiation (Morris *et al.*, 2000). This procedure led to a 50% decrease in the number of benign papillomas, but the total number of malignant carcinomas remained the same, suggesting that cells capable of conversion to malignancy may reside preferentially in the hair follicle region that was spared by the abrasion process. The advent of transgenic mouse technology, with the capacity to direct expression of mutant *Ras* and other genes to specific target cells within the skin, allowed further refinement of the concepts of initiation and progression in the skin model. When promoters of genes expressed in interfollicular suprabasal differentiating cells (Keratin 10 and Keratin 1) were used to express mutant *Hras in vivo*, this led to the development of benign papillomas that were extremely sensitive to promotion by wounding or TPA treatment, but failed to progress to malignancy (Bailleul *et al.*, 1990; Greenhalgh *et al.*, 1993). However, the use of a modified Keratin 5 promoter to target the same mutant *Hras* to basal cells within the hair follicle, including the bulge region containing the putative stem cells (Cotsarelis *et al.*, 1990), led to the development of promoter-independent lesions that progressed to carcinomas (Brown *et al.*, 1998). While a range of stem and progenitor cells may be capable of generating papillomas, only a select subset of target cells, possibly within the hair follicle stem cell compartment, may be capable of generating high-risk papillomas that undergo malignant conversion at high frequency.

Further evidence for heterogeneity in the papilloma population came from genetic mapping studies using crosses between skin tumor susceptible and resistant mice. This analysis identified some loci harboring polymorphisms that influenced susceptibility to development of carcinomas, but the same loci did not obviously affect papilloma numbers (Nagase *et al.*, 1995; Wakabayashi *et al.*, 2007), suggesting that early and late stages are under separate genetic control. All of these experiments, taken together, indicate that while malignant tumors obviously develop from premalignant lesions, this is not simply a stochastic process of progression, and subsets of papillomas have a particularly high propensity to become carcinomas. Loss of the p53 tumor suppressor gene does not appear to increase the number of initiated cells or benign papillomas, but specifically has an important role in promoting malignant progression (Kemp *et al.*, 1993). Clarification of the genetic and biological basis for these observations may have profound implications for our understanding of premalignancy, and consequently for the development of strategies for recognition of high-risk lesions in human populations. Of course the story does not stop here. Studying cancer pathogenesis in skin continues to yield important insights into the pathogenesis of cancer in other lining epithelia of internal organs both at the molecular level and regarding the required interactions among tissue compartments, signaling pathways, and organismal responses. These are

the major lethal cancers of humans to which skin research has made enormous contributions.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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