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Multiple Stages in Radiation Carcinogenesis of Rat Skin

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Epithelial cell cancers are induced in rat skin by ionizing radiation in a manner that is consistent with the dual action (i.e., two alterations) hypothesis of radiation effects on DNA. This hypothesis states simply that two initial alterations, presumably in the DNA, are necessary to start a normal cell on the pathway to cancer. The initial radiation-induced alteration in the DNA is repairable as indicated by the reduction in tumor incidence with increasing time between dose fractions; the repair halftime is estimated to be 3.0 ± 1.0 hr. Theoretical predictions of a specific dependence of tumor incidence on linear energy transfer (LET) have been verified experimentally for two specific LET values. However, the theoretical formulation provides no guidance regarding the observed reduction in the carcinogenic action of radiation with age at the time of exposure. Analysis of the tumor DNA for oncogene activation indicated k-*ras* and c-*myc* oncogenes were activated in highly anaplastic rat skin cancers, whereas only one of these oncogenes, usually c-*myc*, was activated in comparatively benign basal cell carcinomas and in squamous cell carcinomas.

Introduction

Rat skin exposed to ionizing radiation exhibits a variety of tumor types, including squamous carcinomas, basal cell carcinomas, sarcomas, and sebaceous cell tumors. These cancers presumably arise from cells of the appropriate type within the various tissue structures occurring in skin. Rat skin has proved to be a sensitive and reproducible system for studying the dose-response and time-response characteristics of radiation-induced epithelial cancer and for investigating how the early biological events associated with the absorption of the radiation might cause a cell to embark on the pathway to cancer (*1*).

Basal cell carcinomas are by far the most common cancer seen in human skin. These lesions occur predominantly in white-skinned individuals who lack sufficient pigmentation for filtering out the ultraviolet component of sunlight. These cancers rarely (< 5%) metastasize beyond their local site of origin. Ultraviolet light is thought to be the causative agent of most human basal cell carcinomas. Other skin cancers occurring in human skin include squamous carcinomas and melanomas, but the causative agents associated with these cancers are generally unknown. In early radiation workers whose skin was heavily exposed to X-rays, squamous carcinomas were a common occurrence. An extensive comparison of cancer incidence in animals relative to human epidemiological data is available for skin (*2*,*3*).

The multistage theory of carcinogenesis was originally invoked to explain the temporal steepness of cancer incidence data in human populations (*4*). Modified versions of this theory have been postulated to explain certain aspects of experimental carcinogenesis in animals, e.g., initiation and promotion in mouse skin, and more recently the occurrence of multiple oncogene activations in experimental tumors. Simple elements of this theory are illustrated in Figure 1. Each transition, of which six are shown, is considered to be genetic alteration that is transmitted to daughter cells and represents another step in the progress of the cell to the final neoplastic state. As illustrated, two of the transitions are alterations caused by

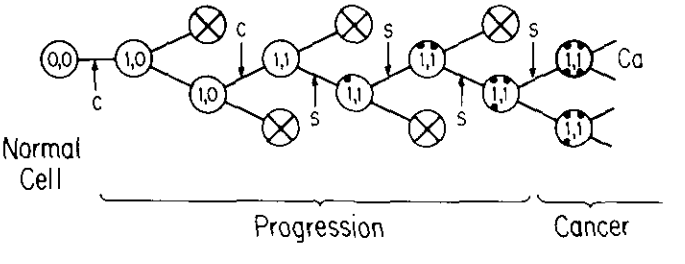

FIGURE 1. Diagram of the stages in carcinogenesis. Genetic alterations labeled C or S are envisioned to occur in association with cell division. The C alterations are produced by action of a carcinogen; the S alterations are spontaneous.

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action of the carcinogen. The remainder are spontaneous in the sense that they do not require direct carcinogen action. There is a potential for any of the cells at intermediate stages to proliferate into clones, thereby amplifying enormously the accumulated alterations and cells at risk for the next transition. Whether such amplification actually occurs is controversial and has only been shown conclusively for mouse skin papillomas.

Time-Response Function

A single dose of ionizing radiation applied to rat skin produces epithelial tumors that begin to appear about 10 weeks after irradiation and continue to appear at an accelerating rate essentially until the end of the normal lifespan. The consistency of this time pattern is remarkable and is the basis for constructing dose-response relationships. A time-independent dose-response relationship is possible if the overall tumor yield function can be expressed as a product of a function of time with a function of dose (*5*). This important idea can be expressed analytically as follows:

$$Y(D,t) = f(D)\, g(t) \qquad [1]$$

where $Y(D, t)$ is the overall cancer yield in tumors per animal as a function of dose D and time t. The functions $f(D)$ and $g(t)$ depend only on dose and time, respectively. For compatibility with the multistage theory of carcinogenesis, we have chosen the following form for $g(t)$ (*4,5*):

$$g(t) = c(t-w)^n \qquad [2]$$

where t is time and c, w, and n are constants. This form, sometimes referred to as a Weibull function, has frequently been fitted to temporal cancer incidence data, especially epidemiological studies of cigarette smokers. While noninteger values of n are conceivable, strict consistency with the multistage model requires the use of integer values. Based on experimental data in rat skin, we believe n is about 2 for single doses of ionizing radiation. The data in Figure 2 indicate that Equation 2 is applicable for a substantial portion of the lifespan.

A carcinogenically relevant alteration in the target cell DNA presumably occurs at the time of or within a few hours after irradiation. If cancer cells existed at that time, the time of tumor occurrence would be distributed around the median induction time with the earlier tumors being the more rapidly growing ones, and the later tumors being the more slowly growing ones. However, tumor growth rates as a function of the time of detection (generally when the tumors are about 1 mm in diameter) indicated an equivalent distribution of growth rates among the late and early tumors (*6,7*). Moreover, the distribution of histological types of tumors was approximately the same in early- and late-occurring tumors.

These results are consistent with the idea that the initial carcinogenic alteration caused by action of the radiation establishes a potential cancer cell that does not become an actual cancer cell until many cell divisions later. When radiation doses were given weekly for duration of life, the increased exponent for the multiple doses was much greater than would be expected if the effects of each individual dose were simply additive in each time increment. Simple additivity gives an expected exponent of about 3, i.e., 1 more than about 2, whereas the actual exponent was greater than 6 for 1.5 Gy/week (note: 1 Gy = 100 rads). For 0.75 Gy per week, the exponent was only 2.9, but these data are based on a relatively small number of tumors. In the multistage theory the increased exponent is interpretable as increased clonal growth of one or more of the intermediate stages.

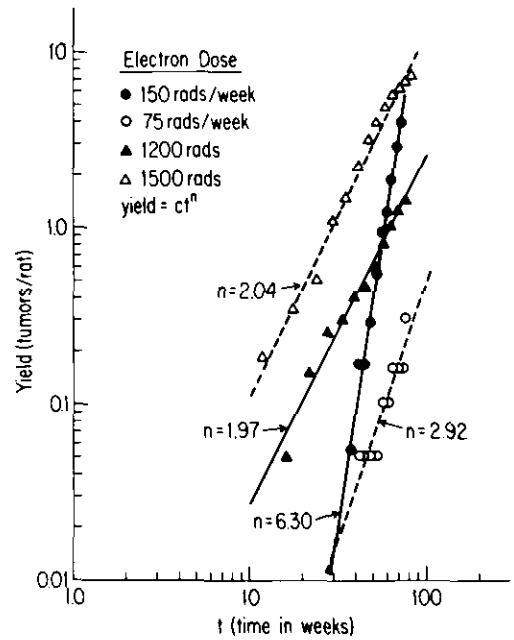

FIGURE 2. Examples of temporal tumor onset data. Rat skin was irradiated with electron radiation that penetrated about 1.0 mm. The onset time of each tumor was taken to be the earliest time of visual detection. Generally each tumor was examined and confirmed histologically. Equation 2 was fitted to the data with n = 1.97 or 2.04 for single doses and n = 6.3 for multiple doses with w = 0.

The data in Figure 2 indicate that split dose repair continued to be operative for at least 52 exposures. In spite of the exponent on the time function being close to 6 for weekly 1.5 Gy doses, the tumor yield was still less than 1.0 tumors/rat at an accumulated dose of 78.0 Gy. A single exposure of 16.0 Gy would produce the same yield (Fig. 2), which means that about 80% of the carcinogenic effectiveness of the radiation was lost because of repair in the multiple exposures.

Dose-Response Function

The most widely used analytic form of the dose-response function, $f(D)$, to describe the effect of radiation on cell lethality and chromosomal aberrations is the linear quadratic function derived from the dual action theory (*8,9*). The hypothesis underlying the dual action theory states that the yield of any biological end point

requiring two radiation-induced events is proportional to the square of the radiation dose in a microscopic region of space that essentially defines the target region within the cell (*9*). The form of the expected function is:

$$f(D) = AD + BD^2 \quad [3]$$

Equation 3 is derived from biophysical considerations of the way radiation dose is distributed statistically in small regions of space or from various biological hit theories that are based on breaks in the DNA being the primary event in the overall mechanism (*9*). Radiation, of course, kills many of the cells in the irradiated tissue, and yet there is no evidence that the cancer yield is reduced by this lethality except at extremely high doses where compensating repopulation is incomplete. If, as hypothesized, the alterations relevant to carcinogenesis are transmitted to daughter cells during repopulative cell division, then cell lethality may not reduce the number of cells at risk, because the carcinogenically altered cells may participate in the repopulative regeneration to the same extent as normal cells.

Proportionality between the coefficient A and the linear energy transfer (LET) is one of the more important implications of the linear quadratic theory. We examined this question by exposing rat skin to an argon ion beam at the Lawrence Radiation Laboratory Bevalac Accelerator (*10*). The LET of this beam is so high (125 kev/μm) that only a few tracks per nucleus are sufficient to produce several hundred rads of dose. The results were a striking confirmation of the hypothesis and are also shown in Figure 3. The dose-response relationship for argon ions is very nearly linear in the region below about 9 Gy. The data from Figure 3 can be used to provide estimates of the relative biological effectiveness (RBE) for tumor induction as a function of radiation dose. The resulting RBE is about 2.5 at the peak yield dose and increases to about 5 at the lowest dose where data is available (about 1.0 Gy).

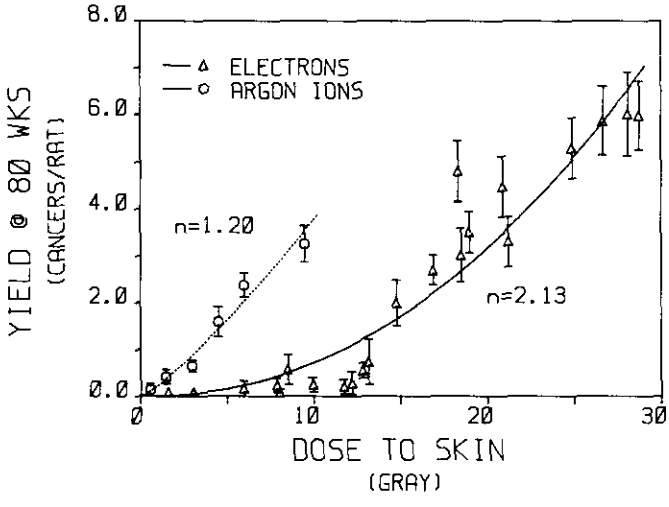

FIGURE 3. Tumor yield as a function of surface dose at 100 weeks after irradiation for rat skin irradiated at 28 days of age with monoenergetic electrons or argon ions (*10–13*). The n values refer to the best-fitting power function based on a least squares fit. This is perhaps the most complete set of data presently available on the dose-response function of radiation carcinogenesis.

Recovery and Repair in Radiation Carcinogenesis of Skin

Generally, mammalian cells are capable of repairing part of the damage caused by the low LET ionizing radiation, and results in rat skin indicate that carcinogenic alterations are subject to similar repair processes (*11–14*). If multiple events are involved in carcinogenesis, and if one or more events are repaired before subsequent ones occur, the risk of cancer induction may be substantially reduced.

Experiments were conducted to determine how two or more individual radiation doses add together to produce their overall carcinogenic effects. One possible expectation was that multiple doses produce time functions of tumor yield that are additive in all time increments. That such a possibility was not the case was found on the basis of results where two doses of radiation were applied to the rat skin (Fig. 4). The results indicated that the skin cells were capable of repairing a substantial part of the radiation damage leading to cancer induction and the repair halftime was found to be about 3 hr (*15,16*).

There are numerous candidates for the initial lesion in DNA that sets a cell on the path to cancer. Some of the most frequently cited are DNA strand breaks, base damage in the form of adducts, base deletion, and DNA-DNA cross-links. Breaks in the deoxyribophosphate strand structure are one important way that ionizing radiation damages DNA, and we attempted to determine whether their induction and repair kinetics were correlated with our knowledge about carcinogenesis in the rat skin (*17*). If such breaks occur in only one strand (single-strand breaks), they are readily repairable, presumably correctly because of the availability of an unbroken homologous template. However, if breaks occur in both strands (double-strand breaks), the consequence may be a break in the chromosome, which may not be repairable or which may repair in a way that causes chromosomal rearrangements (*18*).

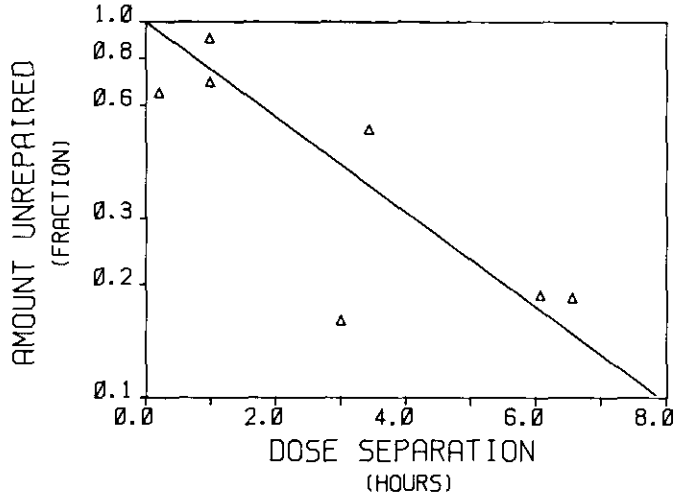

FIGURE 4. A plot of the percentage unrepaired (P) versus time between fractions.

Since two single-strand breaks on opposite strands could produce a double-strand break, it was important to determine whether the kinetics of single-strand break repair correlated with repair of carcinogenically relevant damage. It was known that mammalian fibroblasts in tissue culture generally are able to repair radiation-induced single-strand breaks with a halftime of about 20 min, which is much less than the halftime for the repair of the carcinogenic effect (180 min). Since the rate of single-strand break repair could differ *in vivo*, we applied *in vitro* techniques to measure the rate of repair of DNA single-strand breaks in the rat epidermis (*19*). Using alkaline unwinding, we obtained data indicating that single-strand breaks were produced in proportion to dose and that the repair halftime was about 21 min, not very different from values found for a variety of cell lines *in vitro*. The discrepant repair halftimes indicate that single-strand breaks are not consistent with the requirements of an initial lesion in carcinogenesis.

Age and Radiation Carcinogenesis

In the multistage theory of carcinogenesis, the events necessary to produce a tumor cell might occur spontaneously, which means that they might accumulate with age. If spontaneously occurring events relevant to carcinogenesis accumulate with age, one would expect a given dose of radiation to produce tumors more readily in older animals than in younger ones. To test this expectation, we exposed rats to single doses of radiation at various ages. The yield of tumors was determined at identical times after irradiation (*20*). The results contradicted the expectation that spontaneous events accumulate with age. In fact, fewer, not more, tumors formed as the rats were irradiated at progressively older ages. These results are shown in Figure 5.

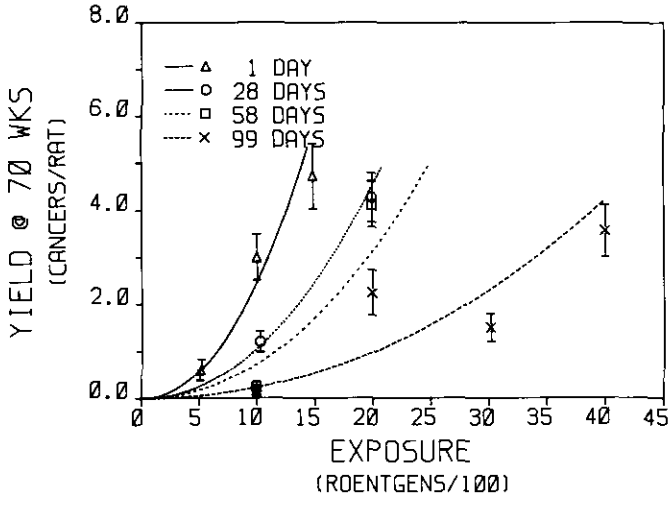

FIGURE 5. Cancer yield in rat skin as a function of X-ray exposure at different ages. Yields were determined uniformly at 70 weeks after irradiation. Error bars are SDs estimated from the total number of tumors.

Model of Radiation Carcinogenesis

One of the major theories concerning the molecular mechanism of radiation action on living cells is the dual action hypothesis. In this hypothesis, events or hits resulting in molecular damage are postulated as the starting point for several measurable end points of biological damage. We have been attempting to explain radiation carcinogenesis data in terms of a two event or dual action postulate (Fig. 6). In this formalism an interaction is assumed to occur between two primary events somehow forming an aberrant cell that progresses in a stepwise manner to acquire malignant properties (*21,22*). The interaction is envisioned to proceed quickly when the events are in close geometrical and temporal proximity. Furthermore, the events are assumed to be repairable so that an interaction may be averted if one event is repaired before the second one occurs. Unfortunately, the identity of the primary event is unknown, although it is presumably a molecular alteration in a DNA molecule, since the neoplastic properties must be propagated to daughter cells. As mentioned earlier, one plausible candidate is a break in the deoxyribophosphate strand structure, which could be the initial event in a cascade that leads to additional mutational and karyotypic changes.

Certain conclusions about the nature of the initial events can be derived from information now available. We must assume that the hypothetical events are a direct or indirect result of the molecular absorption events (ionizations) produced by the radiation. This assumption means that the geometrical distribution of the hypothetical lesions in the cells must be directly related to the distribution of the primary ionizations.

Consequently, the distribution of carcinogenic events (hypothetical) is determined by the physical location of the ionizations, and the latter can be markedly altered by varying the linear energy transfer (LET) of the radiation.

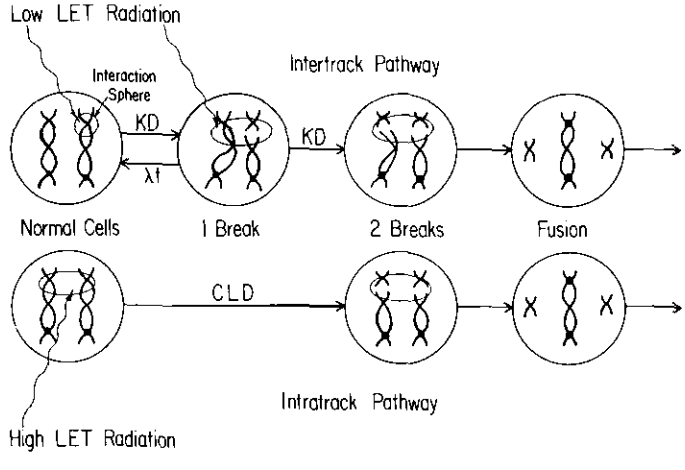

FIGURE 6. A model showing how low and high LET radiation might lead to the same chromosomal lesion by intertrack and intratrack pathways, respectively. Low LET proceeds in two steps with repair; high LET proceeds in a single step without repair. The interaction sphere is the region of the DNA molecule within which radiation energy must be absorbed to produce a lesion.

As an ionizing particle, e.g., electron, passes through a cell, it leaves a track of ionizations that are spaced in a manner that depends on the velocity, mass, and charge of the particle. The LET is proportional to the number of ionizations per unit length of tract. At extremely low LET values, where many individual tracks are necessary to produce a given dose, most ionizations are associated with different particle tracks. For example, high energy electrons produce only about 3 ions in traversing an epidermal nucleus, and as many as 3000 tracks are required to produce a dose of a few hundred rads. As the LET increases, the number of tracks necessary to produce a given dose declines proportionally until at very high LET values, e.g., 100 kev/μm or higher, hundreds of rads can be delivered by only one or 2 tracks per nucleus. In the latter circumstance the primary ionizations and any events derived from them follow a geometrical alignment along particle tracks. Consequently, at high LET the chance that members of an interacting pair of events are contained within the same track is quite high. As the LET increases, the chance of events being within an interaction distance of one another increases proportionally, and intratrack interactions are proportional to LET as well as dose. Moreover, since events in a given track are produced essentially simultaneously, intratrack interactions proceed quickly without the possibility of significant repair.

At low LET values, many individual tracks are necessary to produce a given dose, and the two members of any interacting pair of events were likely to be produced by events in different tracks. Since events in different tracks are independent, the probability of two occurring within an interaction distance is the product of the individual occurrence probabilities. Primary events are assumed to be proportional to ionizations (either single ionizations or clusters) and to dose. Hence, the yield of interactions between events in different tracks would be proportional to dose squared. Once two events interact, it is assumed that an irreparable lesion is formed. Without specifying the nature of the primary events, the above considerations lead to a dose-response function that is the same as the one derived from Dual Action Theory (*9*).

It is recognized that the approach outlined here is overly simplified in that it neglects a number of potentially important factors, such as the cytotoxic effect of the radiation and the likelihood that a variety of biological or hormonal factors may modify the expression of neoplastic and potentially neoplastic cells. Certainly, cytotoxicity cannot be ignored at doses above the peak yield where further dose increases lead to unregenerated tissue destruction and fewer tumors. Accordingly, the model outlined can only be fitted to data below the peak.

As an approach to elucidating the molecular basis of carcinogenic alterations, a group of 12 large radiation-induced rat skin tumors were analyzed for activation of oncogenes from the *ras* and *myc* complementation groups (*23*). These tumors were of the following histologic types: four squamous cell carcinomas, three poorly differentiated carcinomas (clear cell), one each of basal cell carcinoma, sebaceous carcinoma, sarcoma, mixed (mostly squamous) carcinoma, and fibroma (benign connective tissue tumor). DNA was extracted from the tumors and transfected onto NIH 3T3 cells. Positive transfections were found for the three clear cell carcinomas, the sebaceous carcinoma, the sarcoma, and the squamous carcinoma. Southern blot restriction analysis revealed rat derived restriction fragments homologous to the k-*ras* oncogene.

Southern hybridization of the original tumor DNA to the third exon human c-*myc* probe indicated gene amplification and polymorphism in 10 of the 12 tumors for the restriction enzymes, bam HI, eco RI, and hind III. Neither enhanced band intensity nor restriction fragment polymorphism was seen when the DNA was probed with the first exon c-*myc* probe. These results are consistent with an internal rearrangement of the c-*myc* gene, but whether the breakage leading to the rearrangement was caused by direct action of the radiation has not been established.

Double oncogene activation (k-*ras* and c-*myc*) was found in 4 of the 12 tumors examined. These were particularly large tumors showing significant amounts of local invasiveness and included 3 clear cell tumors and one sebaceous carcinoma. Alteration of the c-*myc* gene was found in 4 of 5 squamous carcinomas with the fifth showing k-*ras* activation. The sarcoma showed activation of k-*ras* but not activation of c-*myc*.

It is presumed that activation of the k-*ras* oncogene requires a point mutation at a specific base pair location in codons 12, 61, or a nearby codon. It is doubtful that radiation doses as employed in these experiments (12 Gy) could have produced an alteration in such a small target (one base pair) with the frequency that altered tumor DNA is actually observed. Further studies are required to link the genetic alterations found in the tumors with the original DNA alterations produced by the ionizing radiation.

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