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4.0 Scientific Background

Summary of Progress

An extensive experiment involving approximately 400 rats exposed to the neon ion beam at the Bevalac in Berkeley, CA and to electrons is nearing completion. The carcinogenicity of energetic electrons (2.0 Mev) was determined for comparison with the neon ion results. Based on tumor yields in skin irradiated with argon ions (LET=125 kev/μ) and electrons (LET=0.34 kev/μ), we are able to evaluate the parameters C and B in the equation:

$$Y(D) = CLD + BD^2; \quad (1)$$

where Y(D) is cancer yield (cancers/rat), L is linear energy transfer (LET) and D is dose. The question being asked was whether the tumor yield for some other value of LET could be predicted from equation 1. Neon ions (LET=30 kev/μ) were chosen for this critical test of equation 1, because of the possibility that both linear and dose squared responses might be observed simultaneously. Preliminary results indicate that the neon ion prediction was incorrect and that the values of C and B estimated for argon ions need to be adjusted. The reasons for this adjustment are: 1) the argon ion data used to estimate C and B was not sufficiently precise; and 2) electrons are inherently less effective than either argon or neon ions for inducing tumors by the two track mechanism. The latter observation is the first instance, to our knowledge, indicating an LET dependence of the dose-squared coefficient, B. For double skin thickness irradiations with electrons, there was an unusually large excess of connective tissue tumors, fibromas and sarcomas. Presumably the latter tumors are occurring, because more connective tissue is exposed by deeply penetrating, i.e., energetic, beams. However, no such excess of connective tissue tumors was found for neon ions even though the neon penetration was equivalent to that of electrons.

Our experiments have established that DNA strand breaks per unit dose in the rat epidermis are reduced by about 60% if the radiation penetrates to about 0.2 mm in comparison to a penetration of 1.0 mm. These results imply that about 60% of the DNA strand breaks in the epidermis are produced by indirect radiation action. The penetration effect was found to occur in explanted skin which means it is not dependent on systemic factors. In the explanted epidermis there was a reduction in the incidence of DNA strand breaks by about 50% but the penetration effect remained. An experiment was performed to determine if the DNA in the epidermis could be broken by irradiation of underlying tissue only. The exposure was accomplished by allowing electron radiation to enter a double thickness skin fold from one direction in such a manner that the exit dose was zero. Thus the underlying tissue could be irradiated to any desired dose while the epidermis received no radiation dose. Measurement of the DNA single strand breaks in the epidermis was accomplished by previously described techniques. The unirradiated epidermis exhibited DNA strand breaks with an incidence directly related to the dose to the underlying tissue.

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The activation of oncogenes in the radiation-induced rat skin cancers followed a pattern of greater malignancy with more oncogene activation. Four highly malignant cancers exhibited activation of K-ras and c-myc oncogenes, while the remaining 8 cancers exhibited only one or the other of these two oncogenes. Of 5 squamous carcinomas, 4 showed K-ras activation and 1 showed c-myc activation. Several cancers were biopsied, a few-several times, at various stages of development. These studies showed that c-myc amplification was a relatively late event in the progression of radiation-induced squamous and basal cell carcinomas. Highly invasive clear cell cancers (4/4) exhibited activation of both K-ras and c-myc oncogenes.

A high percentage of radiation-induced rat skin cancers including squamous and basal carcinomas exhibit amplification of the c-myc oncogene. The significance of this amplification and how it relates to the radiation exposure is not clear. Large and small skin cancers were probed by in situ hybridization for amplification of c-myc oncogene. In one instance up to 5 biopsies of the same cancer were probed at different stages of development. Comparisons were made with the results of Southern blots performed on DNA from the same tumours. In situ hybridization was performed with biotinylated c-myc probes, visualized with an ABC-AP system (Vectern Inc.). The results indicated that c-myc amplification in situ was correlated with the southern blot results, but that only about 30% of the cancer cells were amplified. The c-myc positive cells were distributed randomly within regions of the tumor and exhibited a more solid nuclear structure in comparison to the more vacuolated c-myc negative cells. No c-myc signal was detected on normal skins or on normal cells near the tumors. C-myc amplification appears to be cell or cell cycle specific within the cancer.

Detailed Description of Progress

As in past reports we will describe progress in three areas corresponding to the specific aims of the proposal: 1) carcinogenesis and DNA strand breaks in rat skin following exposure by the neon ions or electrons; 2) DNA strand breaks in the epidermis as a function of radiation penetration; 3) oncogene activation in radiation-induced rat skin cancers.

1.0 Skin Carcinogenesis with the Neon Ion Beam

Rats were transported to the Bevalac at Berkeley, CA and exposed to neon ions. By pinching the skin into a fold, as many as 20 rats were irradiated simultaneously. Control rats were exposed to a high energy electron beam for comparison. Charles River Breeding Farms, CD-1 rats were exposed as follows:

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Group	Electrons Dose(Gy)	# of rats	Neon Ions Dose(Gy)	# of rats
1	0	26	0	10
2	5.0	91	2.0	20
3	7.0	43	2.8	20
4	9.8	18	4.0	20
5	13.7	15	5.6	20
6	19.2	14	8.0	20
7	26.9	14	11.3	20
8			16.0	20
		221		150

Preliminary results for onset of epithelial cancers in skin, indicate a pattern that is consistent with the predictions of the linear quadratic equation based on data from an earlier experiment with argon ions. Generally epithelial cancer yield as a function of time after exposure was fitted with a power function of the form

$$Y(t) = G\ t^n$$

where t is elapsed time and n and G are constants. The argon ion data have been fitted with n=2.2. Comparable data for neon ion exposure have been fitted with the same power function (n=2.2) even though the data show a tending to plateau at longer times ($\geq$ 80 weeks). These are preliminary results and have not been confirmed histologically, although we expect the final results to vary little from the data shown.

Cancer yield per unit dose at 52 weeks for argon ions, electrons and neon ions is now available. Present data for electron radiation supplemented with earlier data were expressed as yield per unit dose in order to estimate CL as the y-intercept and B as the slope in the equation:

$$Y(D)/D = CL + BD \qquad (1a)$$

A best fitting line was derived from a least square fitting procedure to the argon ion data only. The curve for neon ions was derived solely from the argon ion result by assuming B and C remain the same and only L changes from 125 kev/μ to 30 kev/μ. These changes reduced the estimate of the y-intercept from 0.055 tumors/rat/Gy for argon to 0.013 tumors/rat/Gy for neon. The actual neon ion data are positioned around this predicted line as strong confirmation that Equation 1a correctly accounts for the effect of LET on cancer induction in the rat skin system. The value of the slope, B, for argon and neon is 0.0060 tumors/rat/Gy^2.

Equation 1a, however, fails to predict correctly the response to electron radiation. The predicted line for electrons based on equation 1a (LET = 0.34 kev/μ) is shown just below the line for neon. The actual data for electrons (open squares) is much lower and to the right of the predicted line. The electron data are best fitted with a slope of B 0.0027 tumors/rat/Gy^2. The ratio of expected and observed slopes is 2.2

which implies the neon (and argon) are, 1.49 ($1.49 = \sqrt{2.2}$) fold more effective than electrons in producing 2 track alterations relevant to carcinogenesis.

Another way to analyse these results, especially relevant to low dose extrapolation, is to consider the dose, D_e, where the linear and dose squared terms make equal contributions to the cancer yield. Based on the formula $D_e = (C/B)\ L$, these results are summarized in Table 1. The data in Table 1 indicate that the neon ion dose-response ought to be predominately linear below 2 Gy and the argon ion data ought to be predominantly dose squared above 8.3 Gy.

Table 1. Values for D_e in Gray.

				argon ions	neon ions	elections
	C	B	C/B	De	De	De
original estimate	.0077	.0036	.194	24.3	5.8	.066
new data high LET	.0004	.0060	.067	8.3	2.0	-
new data low LET	-	.0027	.148*	-	-	.050

* assumes C low = C high

The induction and repair of DNA single strand breaks in rat epidermis was measured for electrons and neon ions with somewhat contrasting results. A series of alkaline elution profiles for different doses of electron radiation was performed at time 0 and after 30 minutes of repair. Essentially complete repair is seen at all doses except 16 Gy. Plotting elution slopes (measure of DNA damage) as a function of electron dose gives a typical dose response and repair. By 2 hrs all DNA breaks were repaired at all doses. Rather than a monotonic increase in slope with dose, there is actually a decrease and response at 8 Gy. The effect at 4 Gy of neon ions was somewhat larger than seen for 4 Gy of electrons. At 2 hr there was little, if any, repair, but nearly complete repair occurred by 4 hrs (not shown). These results indicate quite an unusual dose response for single strand break induction and repair in rat epidermis after exposure to neon ions.

2.0 Radiation Penetration and DNA Strand Breaks

For a fixed dose to the epidermis, the incidence of cancers in rat skin declines with the penetration of the radiation. The mechanism of this effect is being explored in a series of experiments involving the measurement of DNA strand breaks or the suppression of DNA synthesis in the rat epidermis. The penetration of the electron beam into the skin is controlled by varying the energy of the beam and interposing appropriate absorbers between the beam and the skin. DNA strand breaks are measured by alkaline elution. Briefly the epidermal cells are labeled prior to irradiation by 3 daily intraperitoneal injections of 1.0 μCi ^{3}HTdR. The cells are removed from the skin in single cell suspension by overnight incubation in 0.5% trypsin at 4°C and are then layered on polycarbonate filters, lysed, and eluted at pH=12.1 for about 18 hrs. Fractions are collected each hour and are counted in a scintillation counter coupled to a laboratory computer. The data from the scintillation counter are stored and analyzed by the computer which plots the elution curves. The suppression of DNA synthesis was determined by counting the number of 3H-labeled cells on autoradiographs. The rats were injected intraperitoneally with 1.0 μCi/g ^{3}HTdR immediately after irradiation with various doses of radiation.

The summary of results is shown in Table 2 which lists the breaks/unit DNA in the epidermis of young mice and rats (28 days of age), old mice (270 days of age) and a mouse epidermal cell line derived from newborn skin (PAM cells) for deep and shallow radiation. The PAM cells were growing as a monolayer on the surface of a culture dish so that radiation penetrating beyond the cell layer entered only the plastic dish material.

Table 2. Comparative DNA Strand Breaks at 12 Gy.

Cell	Breaks/Unit Shallow	DNA Deep	Ratio Shallow/Deep*
Epidermis (Young Mouse)	0.65	2.31	0.28
Epidermis (Old Mouse)	0.91	2.10	0.43
PAM Cells (Newborn Mouse)	3.24	3.46	0.94
Epidermis (Young Rat)	0.80	1.42	0.56

* deep = 1.0 mm, shallow = 0.2 mm.

The data in Table 2 show a difference in the number of breaks in the epidermis depending on the penetration for both rats or mice. The ratio of DNA breaks (shallow/deep) was 0.28 in young mice and 0.56 in young rats. It is not known if these differences are meaningful in terms of the biological effectiveness of the radiation in the epidermis of the two species. Generally for a given radiation dose (all doses in Table 1 were 12 Gy) the mouse epidermis exhibited more breaks per unit DNA than the rat epidermis. The number of breaks in the PAM cells was even greater than in the mouse epidermis, and there was no evidence of a different number of breaks for deep and shallow penetrations. These data indicate that the DNA strand break dependence on penetration does not occur for PAM cells growing on plastic which proves that plastic is not capable of producing the effect.

Several experiments were performed in an attempt to establish how the penetration effect depends on the immediate local environment of the irradiated cells. A mouse epidermal cell line (PAM cells) derived from the skin of newborn mice was obtained from the National Cancer Institute and tested under various conditions. With these cells growing directly on plastic dishes a penetration protocol was performed analogously to the irradiations _in vivo_. The PAM cells were irradiated as if they were a monolayer of cells growing on the surface of an animal. The medium was poured off and the irradiations were performed identically as for the skin irradiation.

There are two important points to note: 1) the elution slope of the control DNA _in vitro_ is a little steeper than the comparable slopes _in vivo_ and 2) the elution slopes for the deep and shallow penetration are relatively the same but steeper than for _in vivo_ exposure. Apparently the PAM cell DNA is somewhat more easily broken than rat or mouse epidermal DNA in the sense that a given radiation dose produces more breaks per unit DNA in PAM cells than in the epidermal DNA _in vivo_. These results serve to validate the dosimetry in that no penetration effect can possibly occur if the residual range of the ionizing particle is in the plastic of the dish.

Direct measurement of strand break repair in young rats and mice and in old rats and mice indicate essentially no difference between the rates of repair in the two species. Although repair rates were generally faster in younger animals, no substantial difference in repair between deep and shallow penetrations was seen. These data indicate that DNA repair differences are not likely to be the explanation of the penetration effect on DNA break incidences. The repair halftime for old rats and mice is approximately 50 minutes. The halftime for repair in the younger rats and mice ranged from 25 to 35 min.

At the shallow penetration even 20 Gy was not enough to suppress DNA synthesis more than a few per cent, while at the deep penetration there was measureable supression at 10 Gy and a 50% reduction at 20 Gy. Cells scored as having lost the ability to synthesize DNA were unable to incorporate sufficient quantities of ^{3}HTdR to produce 5 or more grains in a 1 week autoradiographic exposure. These data indicate that strand breaks and suppression of DNA synthesis in the epidermal basal cells do

not depend solely on the direct radiation dose. Some other factor related to irradiation of the underlying tissue is involved. The nature of this factor is unknown but could be either a protective compound released from unirradiated tissue in the shallow exposure or a damaging compound released from irradiated tissue in the deep exposure.

To determine whether the penetration effect on DNA strand breaks is mediated by systemic factors, measurements were performed on explanted skin. The numbers of DNA strand breaks per unit dose was less in the explants than in intact skin but the penetration effect was still apparent. Whatever factor mediates the penetration effect, it seems likely to be local and not dependent on circulation of blood or lymph.

3.0 Multiple Oncogene Activation in a Radiation Carcinogenesis Model

Ionizing radiation is a thoroughly studied environmental agent and cancer induction is a significant late effect of radiation. Ionizing radiation creates a number of lesions in DNA including, base modification, single and double strand breaks, and ribose ring damage. A significant proportion of the radiation-induced mutations in somatic cells are associated with chromosomal deletions and gene rearrangements. These alterations are the result of breakage of the chromosomes followed by loss of genetic material (deletions) or rejoining of broken chromosomes (rearrangements) (17). Radiation has also been found to be a point mutagen in certain model assay systems. The relationship of these genotoxic effects of radiation to molecular mechanisms of radiation carcinogenesis is not yet understood. Several types of tumors develop in rat skin following localized exposure to single or fractionated doses of ionizing radiation (9), including squamous cell carcinomas, basal cell carcinomas, sarcomas, clear cell carcinomas, and sebaceous cell tumors. The histologic type of the tumor presumably reflects the cell type of origin.

Carcinogenesis is a progressive process involving multiple, independent steps (56). At the cellular level, establishment of the transformed phenotype may be a multi-step process and activation of several, independent genes may be required. Land et al. (28) have used primary rat embryo fibroblasts to show that two activated cellular oncogenes, c-ras and c-myc, collaborate to produce morphologically transformed cells with tumorigenic potential. There is evidence from animal systems to suggest that certain oncogenes may be activated by the direct action of the initiating carcinogen. Consistent activation by a point mutation of a single member of the ras oncogene family in different tumors produced by a single agent has been demonstrated in several experimental models (32,57,26,27). c-myc and other oncogenes have been shown to be activated by a process involving chromosomal translocations, enhanced expression, and/or gene amplification (58).

We initially examined 12 advanced rat skin tumors for activation of oncogenes from the ras and myc complementation groups (29). These tumors were of the following histologic types: five squamous cell carcinomas, three poorly differentiated carcinomas (clear cell), one each of basal cell carcinoma, sebaceous carcinoma, sarcoma, and fibroma.

Tumor DNA was purified by phenol extraction and transfected into NIH3T3 cells by the calcium phosphate coprecipitate method (59). The transfection efficiency of the 6 positive radiation tumor DNAs ranged from 0.020 to 0.110 foci/µg DNA. The positive tumor DNAs were from three poorly differentiated clear cell carcinomas, a sebaceous carcinoma, a squamous cell carcinoma, and a sarcoma. DNA from one of the primary transfectants was positive in a second round of transfection. The transformed phenotype of the transfectants was confirmed by anchorage independent growth and tumorigenicity in nude mice. Southern blot analysis of DNA from primary and secondary transfectants, as well as, from nude mouse tumors arising after injection of transfectant cells revealed the presence of rat derived restriction fragments homologous to the K-ras oncogene against the mouse background. Similar experiments using N- and H-ras probes, revealed only the endogenous mouse fragments in transfectant DNA.

Southern hybridization of the original radiation induced tumor DNAs to a third exon human c-myc probe revealed gene amplification and extensive restriction fragment polymorphisms in 10 of the 12 tumors after digestion with Bam HI, Eco RI, or Hind III. The c-myc gene was amplified 5- to 20-fold by densitometric analysis. When the tumor DNAs were hybridized to the first exon of c-myc, there were no differences in either band intensity or restriction fragment pattern between tumor and normal rat DNA. This result implies that an internal rearrangement of the c-myc gene had occurred in these tumors as has been seen in other tumor systems, such as, Burkitts lymphoma. Hybridization of the tumor DNAs to v-abl, c-myb, K-ras, or the first and second exons of N-myc showed no differences in band intensity or number.

In an attempt to determine whether the extensive pattern of restriction polymorphisms seen with c-myc was due to activation of another related gene, a series of hybridizations were carried out at various stringencies. The results from these experiments indicate that all five polymorphic bands are closely homologous to the c-myc 3rd exon, and that the higher molecular weight bands may be more altered forms of the normal myc gene than the smaller size bands.

Northern and dot blot hybridization of poly A^+ RNA isolated from normal rat epidermis and 6 rat skin tumors revealed evidence for enhanced c-myc expression in amplification positive tumors. The levels of c-myc gene expression in the tumors were 2- to 6-fold higher than control rat epidermal levels. No correlation was seen between any parameters of oncogene activation and age of animals, tumor growth rate, or dose or dose rate of radiation.

Based on a summary of myc and ras oncogene activation in radiation-induced rat skin tumors, there appears to be a tissue-specific component in oncogene activation in this system. Concurrent activation of K-ras and c-myc oncogenes was found in 4 of the 12 tumors examined. Each of the poorly differentiated clear cell carcinomas and a sebaceous carcinoma exhibited activation of both ras and myc oncogenes, whereas myc alterations alone were observed in 4 out of 5 differentiated squamous cell carcinomas. A sarcoma was found to have an activated K-

ras, but not an activated c-myc gene, whereas a benign fibroma contained only an activated c-myc oncogene. As mentioned above, there was a positive correlation between myc oncogene amplification and myc gene expression. The finding of concurrent multiple oncogene activation in a panel of primary tumors induced by a single etiological agent provides evidence in favor of the hypothesis that activation of oncogenes from two or more complementation groups may contribute to carcinogenesis in animals and man.

Preliminary data suggests that myc amplification is a late stage event. Several oncogenes were studied. These include c-abl, H-ras, K-ras, c-myc, c-fos and N-myc. In an original panel of 12 large, late stage tumors, it was shown that myc amplification occurred in 10. The histology included 1 fibroma, 1 sarcoma and 10 squamous cell carcinomas. The two tumors not showing amplification included the sarcoma and one squamous cell carcinoma. Smaller tumors were later probed for amplification. These showed a rare tendency for amplification, 4/22 for tumors under 1 cm and 6/17 for tumors greater than 1 cm contained an amplified c-myc gene. Southern blot analysis was performed on a panel of tumors produced by high LET radiation in the form of accelerated neon ions. This panel of 21 tumors contained mostly sarcomas (9) with 2 lipomas, 5 fibromas, 2 squamous cell carcinomas, and 1 clear cell, undifferentiated and mixed cell carcinoma. All of the tumors studied have very rapid growth rates, some as high as 1 mm per day, c-myc was found amplified in both squamous cell carcinomas and in one fibroma and one sarcoma.

Research on this project during the past year has clarified a number of questions resulting from previously reported data. This has been possible due to the availability of new molecular probes and refinements in methodological approach. Rat specific probes for the c-myc oncogene were obtained from Drs. Tsichlis and Steffen. Use of these probes in Southern blots of DNA from 12 radiation induced rat skin tumors confirmed the observation of c-myc gene amplification found using the human 3rd exon probe previously reported.

An unexplained finding using the human probe was the extensive pattern of restriction length polymorphisms seen in several of the tumor DNA samples. Most surprising was the fact that in 3 or 4 of these samples, the same bands were seen. After extensive experiments using various probes and hybridization conditions, we were able to rule out such obvious artifactual explanations as plasmid or bacterial contamination, restriction enzyme digestion artifacts, or errors in technique. One possible explanation was that these fragments were indicative of a complex yet non-random series of gene rearrangments at the c-myc locus in radiation induced tumors. Such a complex pattern of gene rearrangements has not been previously reported. We have now been able to eliminate this possibility, since hybridization of the same tumor DNA samples (as well as re-hybridization of some of the original filters) with the cloned rat c-myc gene probes does not show this pattern of extensive non-random RFLPs. Instead, we observe gene amplification along with one or two polymorphic bands in several cases. In particular, the DNA from tumor #7, from which a genomic library has been constructed, was extensively analyzed using the rat probes. This tumor DNA exhibits a clear

restriction fragment length polymorphism using several enzymes, as well as an approximate 10-fold amplification. The extensive banding pattern of the tumor DNAs reported earlier therefore appears to be as artifact due to cross hybridization of the human specific probe with a myc related gene, and is not the result of complex non-random rearrangements.

A major current effort of this project is to determine the significance of genetic alterations at the c-myc locus with respect to the effects of the radiation exposure. We have examined 39 samples of rat skin tumors induced by 12 Gy of electron of radiation; the same radiation used to generate the original panel of 12 tumors. This group of tumors was on average 10 fold smaller than the original panel of tumors. Southern analysis of these tumor DNAs revealed amplification of c-myc is strongly correlated with tumor size. When all the data were combined, a statistically significant correlation was found between tumor size and C-myc amplification. Other oncogenes, such as, K-ras, H-ras, abl, fos and m-myc were not so correlated.

These results suggest that activation of the myc oncogene in radiation-induced skin tumors is a function of tumor progression and is not an early event directly linked to the effects of carcinogen exposure. In order to further examine this hypothesis we have performed a series of biopsy experiments. Eight different tumors have been biopsied by removing one third of the tumor tissue for DNA analysis, one third for histologic examination and allowing 1/3 to remain on the animal. In all but one case the remaining tumor continued to grow. Three tumors have been biopsied 3 times so far, at 6 week intervals and indicate a consistent pattern of c-myc amplification occurring about ten weeks after the initial appearance of the tumor on average.

In situ hybridization has provided an efficient tool to localize a specific nucleic acid sequence in tissue sections. The technique is based on the formation of a highly specific hybrid between an appropriately labelled probe of nucleic acid and its complementary sequence in the specimen. Using this technique can yield both molecular and morphological information about individual tumor cell. More recently the application of c-myc probe labelled with a biotin and an avidin-biotinylated alkaline phosphatase detection system (Vectastain ABC-AP) has brought more advantages which included rapid detection, improved microscopic resoltion, and avoidance of a radiation hazard. Results are presented by using this method to detect the oncogene amplification and distribution in rat skin tumors induced by ionizing radiation, to characterize the tumor cell types which contain high oncogene amplification, and to explore oncogene activation during tumor development.

A variety of rat skin tumor sections were prepared on the polylysine-treated slides and baked prior to in situ hybridization. After in situ hybridization and staining the tumor slides were observed under the microscope. Photomicrographs of in situ hybridization sections from representative tumor types show non-random distribution of c-myc amplification. Compared to control the red grains are found in several tumor sections in which biotin labelled c-myc oncogene probe was

employed. The grains are considered to be a hybrid of the amplified oncogene in tumor tissue and a labelled oncogene probe. The background in normal tissue section is a very low level. The density of grains in tumor tissue was dependent on both the type of tumor examined and the stage of tumor progression in five biopsy samples. The results showed that certain types of tumors and certain stages in tumor progression have a high level of grains. The high level of grains on tumour slides implied high oncogene amplification. All the grains observed in section are in the tumour cell, not in normal cells. The grains observed were usually distributed in the nucleus of tumor cells. No grains were found in cytoplasm or extracellular area. This pattern of distribution again confirmed that the grains were the result of binding of oncogene probe to the cellular oncogene. The use of RNase before hybridization did not change the distribution of grains in tumor sections; however the use of DNAase before hybridization eliminated all the grains in the sections.

It is shown in the Table 3 that the results of in situ hybridization to seven different radiation induced tumor tissues excised from rat skin. The grains were found in tumour sections and the density of grains varied as the tumour type, which correlated to the DNA amplification as measured by Southern blotting. More grains were observed in tumors with high c-myc amplification revealed by southern blotting experiments, such as squamous cell carcinoma (RAD7) in which DNA amplification was 20x exhibited the most grains. But the counting of grains is not always quantitatively related to the result of Southern blotting. More grains were found in the clear cell carcinoma (RAD8) than in poorly differentiated clear cell carcinmoma (RAD5); the former tumour has a low DNA amplification fold 9x and the later one had a high DNA amplification fold 15x. The statistical analysis were based on the counting of grains in tumor section using minitab program (version 7).

It is shown in the Table 4 that the results of in situ hybridization to five biopsies of squamous cell carcinoma (RAD 106) excised from rat skin at the different interval after exposure to radiation. The tumor developed sequentially from T1(1) to T1(5), which roughly reflects the tumor progression process, but the tumour promotion and induction process may still be too limited to base major conclusions on these samples. Different biotin labelled oncogene probes was used in the five biopsies. Using c-myc oncogene as a probe only T1(2), and T1(3) samples showed a positive result; the grains in T1(3) were more dense than that in T1(2). But no grains were observed in all tumour sections without denaturing the DNA in tumour tissue, which indicated that the hybridization between the amplified oncogene and the labelled probe occurred in double stranded instead of single stranded DNA. Using v-H-ras oncogene as a probe, grains were found in T1(2), T1(3), and T1(4) tumour sections. The pattern of v-H-ras amplification in tumor development is apparently different from that of c-myc. Using v-K-ras oncogene as a probe no any grains were found in any tumor sections.

Characterization of the cell types of tumour revealed that only specific cell type in tumour sections showed the grains. Under microscopic examination it was found that these cells were relatively small and not vacuolated in compared with the surrounding tumor cells. These

cells are probably involved in the tumor progression.

The results here confirmed that the application of in situ hybridization using biotinylated oncogene probes in tumor sections was a reliable method to investigate the oncogene activities in carcinogenesis and made possible the study of what role oncogene activation would play in tumor development. But initially the loss of tumor tissue sections from slides was a difficult problem due to the high temperature treatment and tiny contact area of tissue to the slide. Both adhesion of tumour tissue section to the slide and a smooth coverslip are strongly required for a successful experiment using in situ hybridization. Polylysine coated slide and siliconized coverslip using Sigmacote have proved to be an efficient way to keep the tumour tissue on the slide.

The use of tritium labelled oncogene probes in in situ hybridization did not find any significant oncogene amplification. The change to using the biotin labelled probe has greatly increased the detection of oncogene amplification. This also avoids an isotope disposal problem and is relatively rapid in contrast to the long exposure time of the autoradiographic method.

Table 3. C-*myc* Amplification in Rat Skin Tumor Induced by Radiation Detected by *in situ* Hydridization Using Biotin Labeled Probe

No.	Tumor type	DNA amplification (Southern blotting)	Grain count* (%)	Mean**	Adjusted mean***
			(In situ hybridization)		
RAD1	Poorly differentiated clear cell carcinoma	>5(+)	59%	1.34	2.90
RAD4	Sebaceous carcinoma	15(+)	53%	2.07	3.70
RAD5	Poorly differentiated clear cell carcinoma	15(+)	44%	1.57	4.70
RAD7	Squamous cell carcinoma	20(+)	62%	1.60	2.90
RAD8	Clear cell carcinoma	9(+)	28%	0.70	3.70
RAD9	Sarcoma	1(-)	3%	0.03	0.03
Control			5%	0.05	0.05

* The number of cell in which at least one grain occurred over the number of total cell counted.
** The unit of grain mean is grains/cell.
*** The mean adjusted for the existence of two cell populations.

Table 4. Oncogene Amplification Detected by the *in situ* Hybridization in Five Biopsy of Rat Skin Tumor (Squamous Cell Carcinoma) Induced by Radiation

Biopsy #	Time from Irradiation (week)	Tumor Size (cm^3)	Growth Rate	Oncogene Amplification c-*myc*	H-*ras*	K-*ras*
1	13	0.2	0.1	-	+	-
2	27	2.2	3.6	++	+++	-
3	33	15.0	3.2	+++	++	-
4	39	2.7	-2.2	-	-	-
5	42	1.6	-2.5	-	-	-

The grain was observed under a microscope:
(+): low grain.
(++):intermedium grain.
(+++):high grain.
(-): no grain.

During the past year we have begun to analyze the molecular mechanism responsible for activation of the K-*ras* gene in these tumors.

Oligonucleotide hybridization analysis has localized the mutation to the 12th codon, and specific probes are now being used to identify the activating mutation.

Earlier Work as Background of the Research

This report includes descriptions of results from previous studies. These studies are based on the approach that a thorough understanding of radiation carcinogenesis in a single organ system is the most productive way to approach a general understanding of radiation carcinogenesis. We have found that a useful model to analyze radiation carcinogenesis in rat skin is the modified dual action hypothesis (1,2). In this hypothesis two initial events resulting in heritable molecular changes are postulated as the starting point for several measurable endpoints of biological damage including cancer induction (3).

We have been attempting to explain radiation carcinogenesis data in terms of the two-event or dual action postulate (1,4,5). In its application to carcinogenesis an interaction is assumed to occur between two primary events, forming an aberrant cell, which progresses in a presumably stepwise manner to acquire malignant properties probably in association with cell division. The interaction between initial events is envisioned to proceed quickly when they are in close geometrical and temporal proximity. Furthermore, the events are assumed to be reparable 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. It is presumably a molecular alteration in a DNA molecule, because the neoplastic properties must be propagated to daughter cells. One plausible candidate is a break in the deoxyribophosphate strand structure, i.e., a double strand break. Such breaks could be the initial event in a cascade that leads to additional mutations and karyotypic changes.

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 implies that the geometrical distribution of the hypothetical lesions in the cells must be directly related to the distribution of the primary ionizations (6,7,8). It follows that the distribution of carcinogenic events is determined by the physical location of the ionizations, which in turn can be markedly altered by varying the LET of the radiation. As an ionizing particle (e.g., electron) passes through a cell, it leaves a track of ionizations, which 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 track. At extremely low LET values, where many individual tracks are necessary to produce a given dose, most ionizations in a given nucleus are produced by different particle tracks.

For example, high-energy electrons produce only about 3 ions in DNA while traversing an epidermal nucleus, and as many as 3000 tracks are required to produce a dose of a few Gray (LET is near the theoretical minimum of 0.3 kev/micron). At higher LET, the number of tracks

necessary to produce a given dose is less and at very high LET values (e.g., 100 kev/micron or higher) several Gray can be delivered by only one or two tracks per nucleus. In the latter circumstance, the primary ionizations and probably any events derived from them follow a geometrical alignment along particle tracks. Because as the LET increases, the chance of events being within an interaction distance of one another increases proportionately, intratrack interactions are proportional to LET as well as dose. Moreover, because events in a given track are produced almost simultaneously, intratrack interactions proceed quickly, without the possibility of significant repair.

At low LET values, the two members of any interacting pair of initial events are likely to have been produced independently in different tracks. The probability of two events occurring within a specifi interaction distance is proportional to the product of the individual occurrence probabilities. Since events are assumed to be proportional to primary ionizations (either single ionizations or clusters) and to dose, the yield of interactions between events in different tracks is proportional to dose squared.

Without specifying the nature of the primary events, the considerations just mentioned lead to the following dose-response function when the time of exposure is so short that repair during the exposure can be neglected

$$Y(D) = CLD + BD^2 \qquad (1)$$

$$\text{or} \quad Y(D)/D = CL + BD \qquad (1a)$$

and to the following function when the dose rate is much less than the repair rate (i.e., r/D is much less than K) and the repair constant in the equation $L=L0\exp(-Kt)$

$$Y(D) = CLD + (BDr)/K \qquad (2)$$

The three measurable parameters are as follows: B in dose response studies with low-LET radiation, C in dose response studies with high-LET radiation and K in fractionation studies with low-LET radiation. Relevant experiments were carried out in rat skin and provided the following initial results: $C=2.5\times10^{-3}$, tumor micron/Gy/kev/rat; $B=1.3\times1^{-2}$, tumors/rat/Gy^2; and $K=0.24\ hr^{-1}$. For the dose, De, where linear and dose squared terms make equal contributions to overall yield, the linear quadratic equation gives:

$$De = (C/B)\ L \qquad (3)$$

where L is the LET of the radiation in kev/micron. Skin contains a variety of cell types, and the tumors induced by ionizing radiation exhibit a distribution of histologic types that reflect the major types of cells found in the skin (9). For example, the various types of tumors occur with the following overall relative frequencies: squamous carcinomas, 30%; basal cell carcinomas, 20%; keratosebaceous tumors, 35%; sebaceous tumors, 10%; and sarcomas, 5%. The distribution of tumor types is relatively invariant and does not depend on the type of

radiation, the geometrical distribution of the radiation, or the temporal pattern of dose application. A slight excess of squamous cell carcinomas is seen at doses above the peak yield dose.

If multiple events are involved in carcinogenesis, one might expect carcinogenic alterations to be subject to comparable repair mechanisms (10,11,12,13,14,15,16). A series of experiments was designed to establish in rat skin whether or not splitting a given dose into two fractions separated in time affects the carcinogenicity of a given dose (11,12,13). The results showed that the cancer yield was reduced with increasing time between doses, indicating that the tissue was capable of repairing radiation damage relevant to carcinogenesis (11). The repair half-time of the repairing event relevant to carcinogenesis was estimated to be about 3 hr (12).

Having observed repair for two doses, we asked whether or not a similar degree of repair occurs irrespective of how many individual doses are given (2). Daily fractions of electrons were given to the same region of skin. A given dose D was split into n equal fractions of magnitude D/n. Because the time between fractions is long in comparison with the repair half-time, the expected yield, Y from n fractions equals n times the yield from one fraction, Y(D/n), i.e., Yn=nY(D/n). Yn is measurable, and dividing it by the fraction number and plotting against the dose per fraction (D/n) gives Y(D/n), i.e., the single dose yield for a dose of D/n. The tumor yield per fraction plotted against the dose of electrons per fraction gives a curve with a dose exponent of 2.4.

Oncogenes in Radiation Carcinogenesis

Ionizing radiation is one of the most throughly studied environmental carcinogens in humans and animals. Rat skin carcinogenesis by ionizing radiation has proved to be a useful model for elucidation of several biological and theoretical parameters of tumorigenesis by radiation (17,18). So far no genotoxic or biochemical effects of radiation have been directly related to initiation or progression of neoplasia in the target tissue.

Radiation has been shown to cause DNA single and double strand breaks, chromosomal aberrations, DNA base damage, formation of oxygenated adducts, and other manifestations of direct and indirect damage to the genome (19,20). Radiation is cytotoxic and causes cellular damage in membranes and other organelles. The correlation of molecular lesions to biological effect has been difficult, and has been historically based on comparative dose response relationships. Using such model, a viable hypothesis was formulated that gene rearrangements resulting ultimately from double strand DNA breaks may be a critical event in radiation induced cancer (2,17). Because of recent advances in molecular biology of cancer, it has become possible to directly address this hypothesis and examine the structure and activity of specific genes most likely to be activated by this type of radiation induced lesion. Our preliminary results on activation of the myc oncogene are consistent with this hypothesis.

Activation of cellular oncogenes may be a critical step in tumorigenesis by various agents. Evidence in support of this view has been building from early experiments demonstrating the presence of dominant transforming retroviral related genes in some human tumors (21,22), to more recent work with oncogene activation in experimental animal tumor models (23-29). The finding that multiple active oncogenes are required to transform primary cells (28) suggests that activation of certain oncogenes may be relevant to particular stages of tumor progression and/or that the activation of no single gene is sufficient for cell transformation. Our findings of concurrent action of K-ras and myc genes in the same tumor (29) support the concept that multiple oncogene activation may be required for tumorigenesis in some instances.

Despite the promise of oncogene activation as a central mechanistic pathway to explain carcinogenesis by chemicals, radiation, viruses, and "spontaneous" mutations, recent research in the field has raised sufficient questions to prevent the formulation of a single hypothesis to account for all the data. Objections have in fact been raised to the idea that cellular oncogenes have any role in human carcinogenesis (30). It is possible that no particular single gene (such as, ras) is necessary or sufficient for tumorigenesis, but instead that a number of alternative combinations of abnormally activated oncogenes can lead to transformation of cells of a given species and tissue.

This hypothesis is consistent with previously published data, and provides a theoretical framework for examination of oncogene activation in a large panel of tumors. By comparing the frequency of activation of specific oncogenes, such as, K-ras and myc in radiation induced tumors of different histologic origin, we were able to conclude that there are at least 2 major pathways to oncogene activation.

There is evidence from animal systems to suggest that certain oncogenes may be activated by the direct action of the initiating carcinogens. Several laboratories have reported consistent activation of a single member of the ras oncogene family in different tumors produced by a single agent. Carcinogen specificity has been reported in mouse thymomas (25,26) where radiation induced tumors exhibited an activated K-ras gene while the same tumor type induced by NMU contained active N-ras. Barbacid and his colleagues have postulated that the single G→A transition mutation in the 12th codon of the activated H-ras oncogene in rat mammary tumors induced by NMU is a direct result of the type of carcinogen-DNA adduct (guanine O^6) produced by the carcinogen (3). This conclusion is supported by a recent report that rat mammary tumors induced by DMBA, an agent which does not form O^6 guanine adducts, do not contain 12th codon H-ras mutations (32). It should be noted that although a smaller than normal percentage of DMBA tumors were positive in the NIH/3T3 transfection assay, the oncogene found in the transfectants was also H-ras, activated by mutation at the 61st codon.

In view of this evidence, the issue of the role of carcinogens in oncogene activation is not entirely clear. Considering the general consensus that carcinogenesis is a progressive multistage process, activation of ras genes by carcinogen induced point mutations seems unlikely

to be the only mechanism for tumorigenesis in any system. Furthermore, the argument that the molecular lesions known or expected to result from carcinogen action on DNA should be observed in activated oncogenes found in the end-stage tumors does not always hold true. The type of single base pair mutation found in the active K-ras oncogene of radiation induced mouse thymomas is not likely to have been caused directly by exposure to ionizing radiation which does not produce point mutations with sufficient frequency to account for the effect (33).

On the other hand our preliminary data suggest the possibility that a known effect of radiation on target DNA - double strand breaks leading to chromosomal rearrangements and gene amplification - may be directly linked to myc gene activation found in these tumors. Activation of the myc oncogene has been shown to occur via gene amplification, rearrangement and enhance transcriptional activity in other systems (34-37). We propose to extend our preliminary findings to elucidate the precise molecular lesion(s) associated with myc activation in radiation induced skin tumors.

The myc oncogene is especially interesting because it has been studied extensively in human tumors. The myc oncogene in Burkitt's lymphoma is consistently involved in a specific translocation of the second and third exons from chromosome 8 to chromosome 14 just downstream from an active immunoglobulin promoter region (38). Similar translocations leading to aberrant expression of myc have been seen in mouse plasmacytoma (37,39). The myc gene has been mapped to chromosome 7 in rat (40).

The temporal aspect of oncogene activation during carcinogenesis has been addressed in a chemically induced murine skin tumor model (23). Balmain and co-workers found that H-ras was activated in preneoplastic papillomas, as well as in later stage carcinomas of mouse skin treated with the 2-stage initiation-promotion protocol. However, other reports indicate that ras gene activation may be a late stage event in some tumors, such as, human melanomas (41). The few reports of oncogene activation as a function of tumor progression deal either with only ras genes by transfection or with expression of several oncogenes having unknown relevance to the final tumor phenotype. The advantage of the rat skin radiation tumor system is the potential to study the time course of activation of oncogenes known to be active in the final malignant stage.

One potential difficulty in the use of irradiated skin to look for activation of oncogenes in pre-malignant tissue, is that negative results may be due to the fact that only a small proportion of cells are destined to progress to malignancy. However the in vitro work of Kennedy and Little (42) suggest that the primary carcinogenic effect of radiation may affect a large proportion of cells and that rare events are selected in a small number of cells that actually become malignant.

Despite rapid progress in the molecular biology of oncogenes and the mechanisms by which such genes may contribute to transformation, the connection between the wealth of biological and biochemical information regarding environmentally induced cancer and the molecular mechanisms

leading to carcinogenesis is poorly understood. Research using the new tools of molecular biology applied to well established models of experimental carcinogenesis can provide the data needed to make this critical connection. The elucidation of the causal relationship between carcinogen effects and the mechanism of oncogene activation is of major importance in the theoretical understanding of carcinogenesis, as well as identification of putative carcinogens, determination of a mechanistic foundation for risk assessment analysis, and possible approaches to cancer prevention, early diagnosis, and intervention.

By mapping the activated myc oncogene in radiation induced tumors, it will be possible to analyze for consistent alterations (such as location of break points, occurrence of deletion, control elements, exonic rearrangements, etc.) in the activated gene. This detailed understanding of the molecular architecture of an activated myc gene in radiation induced tumors should provide critical information regarding the putative causal connection between the effects of radiation exposure and oncogene activation.

Recovery and Repair in Radiation Carcinogenesis of Rat Skin

Generally mammalian cells are capable of repairing at least part of the damage caused by low LET ionizing radiation, and we have studied this effect extensively with respect to its importance in carcinogenesis (43,44,45). Certainly if multiple events are involved sequentially in carcinogenesis and one or more events are repaired before a subsequent a one occurs, the risk of carcinogenesis would be reduced.

Another important question in radiation carcinogenesis is whether the carcinogenically relevant alterations are repairable as radiobiological lesions before being repaired or eliminated during aging. In an extensive series of studies, we have shown that such repair occurs and is quite effective if the LET of the radiation is low but does not occur for heavy ion irradiation where the LET exceeds 100 kev/micron. For multiple exposures of low LET radiation, the time between exposures is important determinant of the carcinogenic effect of the radiation. We estimated on the basis of two doses that the reparable alteration was being removed with a halftime of about 3 hrs (2,12).

In other experiments we showed that the effectiveness of this repair remained undiminished for as many as 52 exposures (2). In an initial series of experiments a relatively small number of daily fractions (maximum) were given to the same region of dorsal skin of rats. Considering each individual fraction as a separate dose we were able to extend the dose response curve from 4.0 Gy to about 0.75 Gy. There was no discontinuity in the data between these multiple doses and the larger single doses. The best fitting power function to the overall data exhibited an exponent of 2.4 in the range of 0.75 Gy to about 12 Gy. These dose response data for induction of tumors are the most extensive available in any mammalian system at the present time.

In another series of experiments a more fundamental question was addressed relating to whether something fundamentally new happens when

the radiation exposure is extended long enough that some of the early tumor cells are exposed. Radiation doses were given weekly for a year and tumors were plotted. There were two important findings of these studies; (1) repair continued to be effective for at least about 40 exposures (over 90 Gy was necessary to produce the same tumor yield that 12 Gy would produce in a single exposure) and (2) the exponent of the power function fitted to the temporal onset data jumped from about 2 for single exposures to more than 6. The increase in the time exponent tended to reverse the effects of fractionation repair, and may indicated that irradiation of early tumor cells accelerates progression to cancer.

The Geometry of Dose Distribution

Most models of carcinogenesis based on genetic alteration as an undelying mechanism assume that the yield of cancer is proportional to the number of cells exposed, but the data in rat skin contradicts this plausible assumption. There are 2 general explanations for departures from proportionality with number of cells exposed: (1) the close proximity of unirradiated cells prevents carcinogenic alterations from being expressed or (2) cancer in not monoclonal and instead arises from clusters of cells. Since there is no evidence that cancer is anything but monoclonal, the first explanation seems more probable than the second.

Hence a series of experiments was designed to establish experimentally whether proximity of radiation dose affects carcinogenicity (46,47,48,49,50). Important practical considerations for risk assessment are involved, for most risk calculations assume a priori that risk of cancer is proportional to the number of cells exposed. However our work suggests this may not be true when the size of the exposed tissue is less than a few hundred microns. Proximity of unexposed tissue seems to reduce the yield of tumors in the exposed tissue. Mechanistically this is an interesting observation and implies an interaction between cells that is capable of affected the carcinogenic outcome in irradiated tissue. At the present time there is little understanding of the basis for this effect.

In our own studies we have approached the question of geometrical distribution to 2 ways; (1) by localizing the dose in the x-y sense, i.e. laterally on the skin surface and (2) by limiting the penetration of the radiation. For lateral localization the tumors displayed a remarkably delayed onset, in comparison to generalized exposure. Moreover if the shielded tissue were irradiated within 1 hour the effect was abolished, although even here there was a temporary delay in the onset of tumors. We interpretedthese findings to mean that unirradiated cells immediately adjacent to the irradiated cells were able either to lower the effective dose by providing a reservoir of radioprotective substances or were able to retard the progression of potential cancer cells. From geometrical considerations alone, the protection extended for a distance of 200 microns.

Even more impressive was the protective effect of shallow penetration. In fact if the radiation penetrated less than about 180 microns no tumors at all were produced when the same dose to a penetration of

about 1000 microns would produce tumors in 100% of the animals. The inhibition was applicable to all tumor types including squamous carcinomas which presumably arise in the epidermis, adnexal tumors and sarcomas. Overall the yield of tumors was more close related to the dose at a depth of about 300 microns than to any other depth. This was intriguing and suggested that the hair follicle stem cells might be the target for radiation in producing its carcinogenic effect on skin because they are normally found at a depth of about 300 microns. However selective irradiation of these cells with an the Bragg peak of an alpha particle beam failed to produce tumors (57). There is no obvious explanation for these puzzling findings except to suggest that the epithelium may be responding as an organ with the least exposed portion limiting the overall response.

Cell Proliferation and Carcinogenesis

Cell proliferation has long been suspected of playing a role in carcinogenesis, but its specific role remains to be established. Apart from the obvious requirement that a cancer mcell must retain a capacity for cell division, little direct evidence exists that cell division plays a role in carcinoganesis. Indirect evidence and logic suggest that cell division could play a role in carcinogenesis by converting initial alterations into a form that is genetically transmissible to daughter cells (51,52).

To test whether cell proliferation affects tumor yield, rat skin was exposed inthe growing phase of the hair cycle when the epithelial cell population in the hair folliclesand to some extent inthe epidermis are in a rapid state of proliferation. The yield of tumors was not substantially different than for skin irradiated in the resting phase of the hair cycle (see previous reports, 1975). We further examined this question by artificially stimulating cell proliferation in the hair follicles and surface epidermis, respectively by hair plucking and cellophane tape stripping. The stimulation of proliferation was continued for 6 months without any affect on tumor yield. Perhaps the cells that were stimulated into proliferation were not the ones at risk for carcinogenesis. More study is required to resolve this issue.

We were able to show that the location of the target cells for carcinogenesis was not substantially different for skin in the growing stage in comparison to skin in the resting stage. In spite of great differences between the depth of anagen (1.0 mm) and telogen (0.3 mm) follicles, thelocation of the sensitive cells remained unaffected. This result can be explained by hypothesizing that the cells in the anagen hair follicle below about 0.3-0.4 mm are essentially not at risk for carcinogenesis because these cells are resorbed as part of the normal hair growth cycle.

Age and Radiation Carcinogenesis

In multistage theory carcinogenic events occur spontaneously and if not repaired or removed should accumulate with age. Accordingly one would expect older animals to be more sensitive to the effects of a

carcinogen than younger animals. An experiment was conducted where rats of different ages (0, 28, 100, and 200 days) were exposed to single doses of x-rays and then followed for equal amounts of time after irradiation. The results showed clearly that the carcinogenic effectiveness of the radiation declined with age, i.e. progressively more dose was necessary to produce the same tumor yield as the animals became older. The results contradicted the the expectation that spontaneous events accumulate with age.

The rate of single strand break repair was found to decline with increasing age somewhat in parallel to the trend for reduced carcinogenicity. The former is an intriguing observation but could be coincidental. At the present time we have no evidence of a link between reduced carcinogenicity and reduced DNA strand break repair. This question deserves further investigation, but it isnot difficult to imagine a repair process that might insert carcinogenically relevant alterations in the DNA with low frequency to explain such a result.

Ultraviolet in Combination With Ionizing Radiation

Our own followup studies of people who were treated as children for ringworm of the scalp (tinea capitis) have shown a large excess of basal cell carcinomas most especially in regions of skin near the hair line, eg. ears, forehead, nose, etc., where substantial exposure to ultraviolet might be expected (54,55). These results suggested that the combined effect of x-ray followed by ultraviolet radiation might be far more carcinogenic than the effects of either agent individually.

We examined this effect in rats by exposing the skin to electron radiation followed by multiple weekly doses of germicdal (254 nm) or solar spectrum (>290 nm) ultraviolet light (55). The incidence of pyrimidine dimers was measured directly in the epidermis and was directly proportional to the number of keratoachanthomas formed. The combined effect of UV and ionizing radiation seemed to depend on dose. At low doses of ionizing radiation and UV the effects were mildly synergistic, while at higher doses there was either no effect or an inhibitory effect of the UV. Whenever the UV was stopped the inhibitory effect was removed suggesting the occurrence of some sort of sterilizing effect of the UV on the developing ionizing radiation induced tumors.

It was interesting that the sterilizing effect of the UV extended to all tumor types including those that presumably arise in the hair follicles. Since not more than 20% of the UV penetrates through the epidermis, and virtually none penetrates deeply enough to reach the hair follicle stem cells at 300 microns. For the sterilizing effect to be a direct one the affected cells must be in or near the epidermis and yet other evidence suggests the sensitive cells are much deeper. Perhaps the damaging effect of the UV extends beyond the immediate tissue actually absorbing the dose. Further study will be necessary to elucidate the correct explanation for these findings.

5.0 Description of Proposed Experiments

5.1 The Dose Response and Repair for Intermediate LET Neon Ions

Experiments currently in progress to test the carcinogenic predictions of the linear quadratic equation at intermediate LET will be continued. Results from these experiments indicate that the constant, B, in the quadratic equation, $Y(D)=CLD+BD^2$ has different values for heavy charged particles, such as, neon and argon ions than for electrons. This new finding needs to be verified in a second tissue, namely, the dermis. In addition we urgently need to establish whether split dose repair is applicable in the dose squared (2 track) portion of the neon ion dose response relationship. We already know that repair does not occur in the linear region of the high LET dose response relationship as evidenced by the cancer incidence for split doses of argon ions. At the doses utilized in the argon ion experiment, the dose response relationship for cancer induction was predominantly linear. The D_{eq}, dose where linear and dose-squared contribute equally to the overall response, for argon ions was estimated to be 8.3 Gy and split dose protocols were carried out at 3 and 6 Gy. Based on new data, the D_{eq} of neon ions is estimated to be 2 Gy which means that the entire observable response of the neon beam is in the predominantly dose squared region. The protocol and preliminary results of the existing dose response experiment is shown below:

Table 5.1

Protocol and Results of an Existing

Neon Ion Versus Electron Dose Response Comparison

Neons Ions			Electrons		
Dose (Gray)	Yield/Dose Expected	Yield/Dose Observed	Dose (Gray)	Yield/Dose Expected	Yield/Dose Observed
2.0	.024	.040	5	.014	.006
2.8	.029	.035	7	.019	.006
4.0	.036	.018	9.5	.026	.015
5.6	.046	.039	13.6	.038	.035
8.0	.060	.063	18.2	.050	.055
11.3	.080	.077	26.8	.073	.075
16.0	.108	.126			

Yield/Dose Units=cancers/rat/Gy @ 52 wks

A new fractionation experiment is proposed to determine if the dose squared term is subject to repair for neon ion exposure. The fractionation experiment is important partly because the effect of fractionation is to make the dose squared term relatively smaller in comparison to the linear term in the equation $Y(D)=CLD+BD^2$. The following protocol is designed to establish whether split dose repair occurs in the dose

squared response region for neon ions and whether the linear term exists with the expected magnitude and is not subject to repair as expected. For n fractions the expected yield of cancers per unit dose is governed by the following equation if repair is complete between fractions:

$$Y(D)/D = CL + (1/n)BD$$

i.e. fractionation is expected to reduce the effective coefficient of the dose squared term by the number of fractions. Even 2 fractions should be enough to demonstrate a different slope in fractionated versus single dose exposures. We propose 4 fractions but the actual number will be somewhat dependent on the availability of the neon ion beam.

Table 5.2

Neon Ion Fractionation Protocol and Expected Results

Dose	Dose/Fraction	Yield/Dose Expected (tumors/rat/Gy)	No. of Rats
8.0 Gy	2.0 Gy	.026	50
16.0 Gy	4.0 Gy	.031	50
		Total	100

The protocol in Table 5.2 will extend the dose response information for cancer induction by neon ions down to well below 2.0 Gy. The neon ion experiment will be performed at the Lawrence Radiation Laboratory, Berkeley, CA.

5.2 DNA Damage and Radiation Penetration

A puzzling finding in the rat skin studies is that penetrations just barely reaching beyond the epidermis are far less carcinogenic than deeper penetrations. The same phenomenon has been observed in mouse skin. The effect applies in rat skin to all of the tumors induced, including the squamous carcinomas that presumably arise in the epidermis. Because of the practical importance of these observations in the nuclear industry, the National Commission on Radiation Protection (NCRP) has appointed a committee (# 80) to consider how applicable these animal results might be for human skin.

In one approach to this problem we postulated that the target cell for carcinogenesis may be hair follicle stem cells and not the epidermal stem cell. A test of this idea proved negative when selective irradiation of the hair follicle stem cells (the hair germ) with an alpha particle bragg peak did not produce tumors (57). We concluded that induction of skin tumors required irradiation of both of the major epithelial elements, namely the epidermis and the hair follicles. Nevertheless when both the epidermis and hair follicles were irradiated, the dose in the vicinity of the hair follicle stem cells seemed more critical to induction of tumors than the dose to any other region. One possible way to explain these results is to suggest that stem cell totipotency and regenerative hyperplasia may be playing a major role in radiation

carcinogenesis of skin. Recent results have made this explanation unlikely. We are currently exploring a diffusible molecule explanation.

An experiment is proposed to study whether this penetration effect is based on regeneration in the tissue. Rat skin will be irradiated with electrons of 2 different energies, one that penetrates only about 200 microns and another that penetrates over 1000 microns. Following irradiation the following 3 endpoints of radiation damage will be measured: (1) cell lethality and regeneration in the epidermis and hair follicles, (2) DNA single strand breaks, and (3) repair of DNA single strand breaks. Cell lethality will be measured by the technique of labeled doublets on whole mounts. Regeneration will be measured cytofluorometrically by counting total cells per unit area and determining the rate of accumulation of cells during blockade with the stathmokinetic compound, vinblastine. DNA breaks and the repair of such breaks will be measured by means of an alkaline elution procedure found to be useful for measuring such breaks in a variety of mammalian organs (45).

These experiments will be extended with a new reverse penetration protocol. A skin fold will be irradiated with electrons that just fail to exit the second thickness, leaving the epideris on the exit side unirradiated. DNA strand breaks and carcinogenicity will be measured.

The following experiment is designed to determine whether the penetration effect for DNA strand breaks is mediated by movement of a protective compound or a damaging compound. Rat skin DNA is labeled by i.p. injection of ^{3}HTdR on 2 consecutive days. The labeled rats are exposed to electron radiation of 2 different energies that provide skin penetrations of either 1000 μ or 200 μ. The skin will be frozen and then the epidermis will be removed mechanically. Freezing is intended to prevent the movement of what is hypothesized to be a diffusible compound. This hypothetical compound could be either a damaging compound that is generated in the deeper tissue and migrates to the surface and damages the DNA of the surface cells when the radiation penetration is high, or it is a protective compound that migrates from the deeper tissue and protects the surface cells when the penetration is low. Exposure time and preparation time will be minimized to prevent the migration of any relevant compounds prior to freezing the tissue. The frozen epidermis will be removed mechanically from the dermis by scraping with a scalpel after warming the surface layer briefly to just above freezing. This is essentially a mechanical removal with minimum opportunity for the movement of chemical substances from dermis to epidermis.

There are 3 possible results of this experiment that can be interpreted as possible underlying mechanisms: 1. the DNA strand break pattern remains unchanged, 2. the quantity of DNA breaks for high penetration radiation approaches the level seen for low penetration, 3. the quantity of DNA breaks for low radiation penetration approaches the number of breaks in the high penetration group.

The implication of these several possible results is as follows: Result #1 implies that the effect is not dependent on migration of compounds or the migration is extremely rapid and occurs within the time

required for exposure and tissue preparation. Result #2 implies that a DNA damaging agent is migrating from deeper regions of the skin and causing damage to the surface cells. Result #3 implies that a radioprotective compound is migrating from deeper in the skin and protecting the surface cells from damage at low radiation penetration.

The results of the above experiment should provide definitive proof of the type of underlying mechanism of the penetration effect of radiation carcinogenesis in rat skin. It is important to understand this mechanism in order to determine the likelihood of a similar phenomenon occurring in human skin.

5.3 Oncogene Activation in Radiation-Induced Skin Cancers

Our preliminary studies have indicated that all of radiation induced epithelial tumors in rat skin exhibit amplification of the myc oncogene while the most malignant tumor has amplification of myc and also activation of k-ras, presumably by mutation. It is especially interesting that the amplified myc oncogenes so far tested exhibit a considerable amount of restriction enzyme polymorphism. This means than the gene, in addition to being amplified, has been internally rearranged in some way. The latter effect is one that might be expected to occur as a direct result of radiation induced double strand breaks. Whether or not these rearrangements are related to the amplification process or occur independently of it is not known.

The activation of oncogenes represents an exciting avenue of research into the molecular biology of carcinogenesis. Radiation carcinogenesis in rat skin has been studied here at N.Y.U. intensively and the system represents a useful, reproducible model in which to study oncogene activation. The question to be addressed is whether activation of dominant transforming oncogenes is a common characteristic of radiation-induced rat skin tumors at all stages of their development.

Specific approaches will be as follows:

1) To extract DNA from a panel of rat skin tumors selected from different stages of development and to test the DNA for the presence of an activated or rearranged myc gene and for the presence of other activated oncogenes by either transfection of NIH/3T3 cells or by probing with oncogene and oligonucleotide probes. These experiments are completed;

2) To extract DNA from regenerating rat epidermis for evidence of oncogene activation prior to the occurrence of a palpable tumors. The latter study will be done at a time after irradiation when regenerative clones are likely to be present in the epidermis; such clones should be present about 3 or 4 weeks after moderately high doses of electron radiation. These experiments are partially completed;

3) To extract and analyze DNA from neon ion induced cancers for activation of myc and ras oncogenes. These experiments are proposed for the current project year;

4) To determine how the presence of c-myc or v-K-ras oncogenes under the influence of specific keratin promoters (K14 and K10) affect the yield of skin cancers induced by ionizing radiation. We already know that c-myc is amplified and transcribed in all radiation-induced epithelial skin cancers of the rat so far tested and that v-K-ras is present with amplified c-myc in particularly malignant clear cell carcinomas. By inserting these oncogenes transgenically under the control of keratin promoters, we will be able to test the hypothesis that mutation of K-ras is a likely initiation event and amplification of c-myc is a likely promotion event in the development of basal cell and squamous carcinomas and clear cell carcinomas in rat skin exposed to environmental carcinogens. These experiments are proposed for the current project year; and

5) In situ hybridization will be utilized to determine which cells in radiation-induced cancers exhibit amplification of c-myc, K-ras and H-ras oncogenes. Preliminary evidence indicates that only about 30% of the cells in c-myc positive cancer exhibit amplification. We will determine how c-myc amplification depends on the stage of tumor development and whether such amplification is associated with wound healing.

The DNA will be prepared from individual tumors and normal epidermis by phenol extraction and ethanol precipitation. Purified DNA will be used for transfection of NIH/3T3 cells by the calcium phosphate co-precipitate method. Transformed foci will be counted 2-3 weeks after transfection and transformation. Restriction endonuclease mapping will be used to compare transforming genes as a function of radiation dose and type of tumor.

Methods

Preparation of DNA from Tumors

Frozen tumors will be ground to a fine powder in a stainless steel mortar and pestle in liquid nitrogen. After suspension of the powdered tissue in TRIS-EDTA buffer, cells will be disrupted by addition of SDS to 0.2% final concentration and Streptomyces griseus protease (20 mg/ml final concentration) for 2 hours at 37°C. The suspension will then be extracted with buffer saturated phenol and chloroform:isoamyl alcohol 24:1. The DNA will be precipitated with ethanol and redissolved in TRIS-EDTA buffer. Concentration and purity of DNA will be estimated by spectrophotometric analysis at 260 nm and 280 nm. Molecular weight and restriction endonuclease digestibility will be determined by horizontal slab gel electrophoresis in 0.3% agarose gels.

DNA Mediated Gene Transfer

The calcium phosphate-DNA co-precipitate method of Wigler et al. (46) works reliable in our hands and will be followed exactly. Thirty six hours after treatment each flask will be trypsinized and subcultured into three 25 cm^2 flasks. Medium containing 0.05 mg/ml gentimycin will be changed twice a week for three weeks, at which time foci will be picked and/or flasks will be fixed with methanol for one minute and stained with 2% Giemsa for 30 minutes. Scoring will be done by microscopic evaluation of foci to distinguish spontaneous overgrowths and type I foci from transformed type III foci (47). Foci to be picked for subculture will be chosen on the basis of their morphology under phase contrast microscopy. In a blind experiment, all putative foci will be picked, and confirmation of their transformed phenotype will depend on soft agar and secondary transfection experiments. To recover single foci for subculture, tops of culture flasks will be cut away with a hot scalpel, and a sterile glass cloning cylinder, coated with sterile silicone grease will be placed over the foci. Cells within the cylinder will be removed with a few drops of trypsin and replated. All transfections will include at least two controls, one of which receives no DNA and one of which is treated with normal rat liver or mouse DNA. T24 bladder carcinoma DNA will be used as a positive control. All experiments will be scored blind with prior randomization of flasks at the time of subculture. Experiments will be done with 3-5 flasks/treatment group, including controls.

Southern Blot Hybridization

The DNA will be digested to completion with Eco RI or one of several other restriction endonucleases. After resuspension in TRIS-borate buffer, pH 8.0, DNA will be applied (10-30 µg) to a 0.8% agarose horizontal slab gel. Gel electrophoresis will be performed according to McDonnell et al. (48). After electrophoresis, the DNA fragments will be transferred to a nitrocellulose filter according to the method of Southern (49), and Maniatis et al. (50). Gels will be denatured in 0.5 M NaOH, 1.5 M NaCl, rinsed in 1 M TRIS, 3 M NaCl and placed on a paper wick dipped in 20x standard saline citrate (SSC). A nitrocellulose filter will be placed directly over the gel, and several layers of paper placed over the filter. After 16-24 hours, the filter will be rinsed and baked at 80°C under vacuum for 2 hours and stored under vacuum until use. Probe DNA molecules will be radiolabeled to a specific activity of $3\text{-}10 \times 10^7$ Cerenkov cpm/ug DNA by nick-translation in the presence of 32P-dCTP (51). Filters will be hybridized with radiolabeled probes in the presence of dextran sulfate using a modification of the procedure of Wahl et al. (52), washed in 0.5 xSSC - 0.1% SDS at 65°C to remove non-specifically bound probe molecules, dried, and subjected to autoradiography at -70°C using XRA-5 film (Kodak) and Cronex Lightning Plus intensifying screens (Dupont). Under these conditions, DNA fragments corresponding to single-copy genes can be detected in 10 ug samples of total rat or mouse genomic DNA. Comparative densitometry will be used to determine the extent of gene amplification.

Hybridization with Oligonucleotide Probes

After DNA sequence data has been obtained for the protooncogene, oligonucleotides will be prepared in order to screen tumor DNAs for mutations of rearrangements at specific sites. The probes will be from 17 to 21 nucleotides with the central base corresponding to the mutation site. Probes will be synthesized using an Applied Biosystems 380A DNA Synthesizer, and hybridization will be performed as described by Wallace and his colleagues (53,54). Briefly, tumor DNA will be digested with an appropriate restriction enzyme, run on an agarose gel and blotted to nitrocellulose filters as described above. Oligonucleotide probes will be labeled by T4 polynucleotide kinase and 32P-ATP. Hybridization will be done in 0.9 M NaCl, 6mM EDTA, 90 mM TRIS pH 7.5, 0.1% DSDS, at $55^{o}C$ for 2 hours, followed by washing at $55^{o}C$ in 6x SSC, 0.5% SDS at $4^{o}C$ for 15 minutes. The exact hybridization and wash conditions will be modified to provide optimum resolution in the results between the normal and mutated genes.

In situ Hybridization

The slides weie dipped for two 10 min in xylene and another 10 min in absolute ethanol, then for sequential 5 min in an ethanol-double distilled water (DDW) mixture containing 95%, 80%, 75%, 60%, 30% respectively. Washed in DDW for 5 min and fixed in Carnoy's solution about 5 min. Dipped in 0.1% Triton X-100 in phosphate-buffered saline solution (PBS) pH 7.2 for 2 min. Washed in PBS and placed in 0.2 N HCI for 20 min. Washed in DDW and placed in 2 XSSC at 70oC. Washed in DDW for 5 min and treated with proteinase K solution on the surface of tissue section slide, then incubated in humid chamber at 37oC. for 15 min. Washed in PBS containing 0.2% glycin and dipped in PBS for 1 min. Dipped in PBS containing 4% paraformaldehyde at room temperature for 20 min. Washed in PBS for 3 successive 5 min. Dipped in 95% deionized formamide in 0.1XSSC at 65oC. water bath for 15 min. Dipped for 2 min in mixture of ice and 0.2XSSC. Dipped for every 5 min through a graded ethanol-DDW series containing 50%, 70%, 80%, 90% and 100% ethanol and air dry. Treat 20 ul prehybridization mixture (200 ug/ml sperm DNA, 100 ug/ml Polyadenylate, 50% (v/v) Deionized formamide, 10% (w/v) Dextran sulfate, 3XSSC, 0.02% BSA, 0.02% Ficoll, 0.02% PVP) on the section slides, covered it with pretreated coverslip and incubated at 37oC. for 1 hr. Removed coverslip and washed in 2XSSC. Treated 10 ul hybridization mixture (20 ug/ml biotinylated c-myc oncogene probe plus prehybridization) on tissue section, covered the pretreated coverslip and incubated at 85oC. for 10 min in a humid chamber. Kept hybridization reaction for 30 min at room temperature after removing from the water bath. Washed in 2XSSc for 5 min and removed the coverslip. Dipped for 10 min in 50% deionized formamide in 0.1 XPBS at room temperature. Dipped for 5 min in 0.05 % Triton X-100 in PBS at room temperature. Dipped for 20 min in 1XTTBS (0.1 M Tris HCl pH 7.5, 0.1 M NaCl, 2 mM MgCl2, 0.05% Triton X-100, 3% crystalline grade BSA). Treated 100 ul of Vectastain ABC-AP reagent (Vector Laboratories Inc. Burlingame, CA) on section slides and incubated for 30 min.at room temperature. Dipped in 1XTTBS for 15 min and change the buffer twice. Treated 100 ul of substrate solution freshly prepared and incubated for 1 hr in the dark at room temperature. Washed in TTBS briefly. Dipped in

the hematoxylin for 15 sec and washed in DDW. Dipped in a graded ethanol-DDW series and xylene. Mounted for microscopic examination.

Preparation of Oncogenes Under Keratin Regulation

The plasmid K-6-ras-2 bearing a full length cDNA of the activated human c-Ki-ras-2 under the control of a 370 b.p. segment of the human K-6 keratin regulatory region was constructed. The ki-ras sequence was excised from clone pSW11-1 (ATCC) with Pst1 and BamH1 sites present in the pUC 13 polylinker. The K-6 regulatory region was excised from plasmid K-6 CAT (gift of M. Blumenberg) with Pst1 and HindIII. Vector pUC19 (BRL) was digested with Pst1 and BamH1. Ligation of the construct was accomplished by first incubating the K-6 and pUC19 fragments for 1 hour with T4 ligase before addition of the ras fragment. After addition of ras the reaction continued for another 3 hours. The DNA from the ligation was isolated, blunt ended with mung bean nuclease, extracted, and the blunt ended product ligated for 24 hours.

The reaction mixture was then used to transform DH5α^{TM} competent cells (BRL). Cells were plated on LB-Amp plates containing X-gal (20 mg/ml) and IPTG (200 mg/ml). After 16 hours of incubation transformed (surviving) colonies bearing recombinant plasmids were selected by lack of α-complementation and consequent formation of white colonies on the chromogenic x-gal substrate.

Measurement of DNA Strand Breaks in Epidermis

Male albino CD rats are obtained at 21 days of age from the Charles River Breeding Farm (Brookline, MA). The hair is clipped at 23 days of age and intraperitoneal injections of 3H-thymidine (New England Nuclear, Boston, MA) at a specific activity of 20 Ci/mmol(2.0 uCi/g body weight) are administered every 12 hours for 4 days prior to irradiation. After irradiation the animals are killed by cervicle dislocation. The dorsal skin is mechanically depilated, excised from the animal, cut into 2 cm by 2 cm pieces, and floated epidermis-side-up in a 0.4% trypsin solution for 14 hours at 4°C. A single cell suspension is made from the epidermis of each rat by mincing, gently, stirring, and filtering. Neutral filter elutions are performed according to published methods. Approximately 400,000 cells are layered in PBS onto a 2.0 µm pore size polycrbonate membrane filter (Nucleopore, Pleasanton, CA), lysed with a solution containing 2% SDS and 0.5 mg/ml proteinase K, and eluted at pH 9.6. Two ml fractions are collected every 64 minutes using an 8 channel peristaltic pump and counted in 15 ml of Aquasol containing 0.88% (v/v) glacial acetic acid. The DNA on the filters was denatured by heating in 1 N HCl at 60°C for 1 hour. The apurinic sites are converted to strand breaks with 0.4 N NaOH before counting in Aquasol. Two ml aliquots of the PBS and lysing solutions are also counted in Aquasol.

The following formula has been adapted from the formalism developed by Rydberg(72) for the alkaline unwinding technique:

$$\ln F = -(K/M)t^{b}$$

where F = fraction of DNA retained on the filter
M = the average molecular weight of the DNA

b, K = constants

The time exponent b can be less than 1 experimentally which has the effect of introducing curvature into the elution curve. Generally b has arbitrarily been set equal to 1.0 with the understanding that the resulting linear curves are an approximation to the data. Taking the ratio of slopes, -K/M, the K's cancel and we are left with the ratio of molecular weights. Thus the following equation permits the calculation of the number of strand breaks per unit DNA, wHere the unit of DNA is the mean molecular weight of the DNA in control cells. The formula is:

$$(\text{dsb's/unit length DNA}) = \text{slope}_{\text{irrad.}}/\text{slope}_{\text{control}}$$

Irradiation procedures

At 28 days of age, while the hair follicles are in telogen, each rat is anesthetized with 35 mg/kg Nembutol and placed in a shielded box exposing the dorsal skin through a 2 cm by 5 cm window. The dorsal skin is irradiated generally with a 0.8 MeV electron beam produced by a 25 MeV Van de Graaff accelerator here at NYU, or are transported for irradiation at distant sites. Radiation dose is measured by thin window ionization chambers with a collecting gap of 1.0 mm. The chambers are constructed of tissue equivalent plastic and a Victoreen vibrating reed electrometer is used to measure the current output of the chamber on both + and - polarity. To estimate the penetration of the beam into the skin, various thickness tissue equivalent absorbers are placed over the chamber and appropriate depth dose curves are determined.

List of Documents Submitted Separately

DOE Designation	Type	Title
1. DOE/ER/60539-3	Progress Report	Progress 05/01/90-04/30/91

Graduate students on project:

	Name	Status	Topic
1.	Steve Hosselet	Ph.D. candidate	Transgenic rats
2.	Jin Yi	M.S. candidate	In situ hybridization
3.	Mary Felber	Ph.D. candidate	High LET oncogene expression

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