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PROGRESS REPORT

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Project Title: The Oncogenic Action of Ionizing Radiation on Rat Skin

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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:

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

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 connec-

tive 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.

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:

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^{o}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 DNA Shallow	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 carci-

noma, 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 radia-

tion 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.

Excised rat skins were fixed in 10% buffered formalin and then embedded in paraffin and cut in 5 u section and placed on the polylysine-coated slides, then bake the slides for 6 hrs at 60°C. The cover glasses were immersed in Sigmacote (Sigma Chemical Co. St.Louis, MO) brifely and dried overnight at room temperature. The slides were 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 prehybridi-

zation 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 85^{o}C. 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 1X TTBS (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.

A variety of rat skin tumor sections were prepared on the polylysine-treated slides and baked prior to <u>in situ</u> hybridization After <u>in situ</u> hybridization and staining the tumor slides were observed under the microscope. Photomicrographs of <u>in situ</u> hybridization sections from representative tumor types show non-random distribution of c-<u>myc</u> amplification. Compared to control the r d 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 <u>in situ</u> 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 differen-

tiated 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* (%) (In situ hybridization)	Mean**	Adjusted mean***
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 mechan-

ism 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.

The following document was submitted separately along with the renewal proposal.

DOE designation	Type	Title
1. DOE/ER/60539-3	Progress Report	Progress 5/1/90-4/30/91

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

REFERENCES

1. Kellerer, A. and Rossi, H. A generalized formulation of dual radiation action. Radiat. Res., 75: 471-488, 1978.

2. Burns, F.J. and Albert, R.E. Radiation carcinogenesis in rat skin. In: Radiation Carcinogenesis (Eds, A.C. Upton, R.E. Albert, F.J. Burns and R.E. Shore) Elsevier, New York, 1986, pp. 199-214.

3. Chadwick, K.H., and Leenhouts, H.P. The rejoining of DNA double strand breaks and a model for the formation of chromosomal rearrangements. Int. J. Radiat. Biol., 33: 517-529, 1978.

4. Goodhead, D.T. Deductions from cellular studies of inactivation, mutagenesis, and transformation. In: Radiation Carcinogenesis: Epidemiology and Bio. (Eds., J.D. Boice, Jr., F. Fraumeni, Jr.) Raven Press, New York, pp. 369-385, 1984.

5. Goodhead, D.T., Munson, R.J., Thacker, J., Cox., R. Mutation and inactivation of cultured mammalian cells exposed to beams of accelerated heavy ions. IV. Biophysical interpretation. Int. J. Radiat. Biol., 37: 135-167, 1980.

6. Barendsen, G.W. Comparison of the effectiveness of different radiations for the induction of reproductive death, chromosome aberrations, morphological transformation and specific mutations in cultured mammalian cells. In: Proc. 7th Int. Cong. Radiation Research (Eds., J.J. Broerse, G.W. Barendsen, H.B. Kal, and A.J. Van der Kogel) Section C: Somatic and Genetic Effects, C5-02 Martinus Nijhoff Publishers, The Hague.

7. Little, J.B. Radiation carcinogenesis in vitro: implications for mechanisms. In: Origins of Human Cancer (Eds., H.H. Hiatt, J.D. Watson, J.A. Winston) Vol. IV, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 923-939, 1977.

8. Vanderlaan, M., Burns, F.J., Albert, R.E. A model describing the effects of dose and dose rate on tumor induction by radiation in rat skin. In: Biological and environmental effect of low-level radiation, Vol. I, Vienna: International Atomic Energy Agency, pp. 253-263, 1976.

9. Albert, R.E., Phillips, P., Bennett, P., Burns, F., Heimbach, R. The morphology and growth characteristics of radiation-induced epithelial skin tumors in the rat. Cancer Res., 29: 658-668, 1969.

10. National Academy of Sciences. The effects on populations of exposure to low levels of ionizing radiation. Advisory committee on the biological effects of ionizing radiation (BEIR), National Research Council, National Academy of Sciences, Washington, DC., 1980.

11. Burns, F.J., Albert, R.E., Sinclair, I.P., Vanderlaan, M. The effect of a 24-hour fractionation interval on the induction of rat skin tumors by electron radiation. Radiat. Res., 62: 478-487, 1975.

12. Burns, F.J. and Vanderlaan, M. Split dose recovery for radiation induced tumors in rat skin. Intl. J. Radiat. Biol. 32: 135-144, 1977.

13. Burns, F.J., Albert, R.E., Vanderlaan, M., and Strickland, P. The dose-response curve for tumor induction with single and split doses of 10 MeV protons. Radiat. Res., 62: 598, 1975.

14. Wallace. S.S. Detection and repair of DNA Base Damages produced by ionizing radiation. Environ. Mut., 57 769-788, 1983.

15. Elkind, M.M. Repair processes in radiation biology. Radiat. Res., 100: 425-449, 1984.

16. Little, J.B. and Williams, J.R. Effects of ionizing radiation on mammalian cells. In: Handbook of Physiology, Section 9 (Eds., D.K. Lee, H.L. Falk, S.D. Murphy, S.R. Geiger) American Physiological Society, 1977, pp. 127-156.

17. Leenhouts, H.P. and Chadwick, K.H. Radiation induced DNA double strand breaks and chromosome aberration. Theor. Appl. Genet., 44: 167-172, 1974.

18. Upton, A.C. Role of DNA damage in radiation and chemical carcinogenesis. In: Environmental Mutagens and Carcinogens, Proc. Third International Conference on Environmental Mutagens (Eds., T. Sugimura, S. Kondo and H. Takebe) U. Tokyo Press, Tokyo/Alan R. Liss, Inc., New York, 1982, pp. 71-80.

19. Chang, E.H., Furth, M.E., Scolnick, E.M. and Lowy, D.R. Tumorigenic transformation of mammalian cells induced by a normal human gene homologous to the oncogene of Harvey murine sarcoma virus. Nature, 297: 479-483, 1982.

20. Goldfarb, M., Shimizu, K., Perucho, M., and Wigler, M. Isolation and preliminary characterization of a human transforming gene from T24 bladder carcinoma cells. Nature, 296: 404-409, 1982.

21. Parada, L.F., Tabin, C.J., Shih, C. and Weinberg, R.A. Human EJ bladder carcinoma oncogene is homologue of Harvey sarcoma virus ras gene. Nature, 297: 474-478, 1982.

22. Pulciani, S., Santos, E., Lauver, A.V., Long, L.K., Aaronson, S.A. and Barbacid, M. Oncogenes in solid human tumors. Nature, 300:

539-542, 1982.

23. Balmain, A., Ramsden, M., Bowden, G.T., and Smith, S. Activation of the mouse cellular Harvey-ras gene in chemically induced benign skin papillomas. Nature 307: 658-660, 1984.

24. Balmain, A. and Pragnell, I.B. Mouse skin carcinomas induced in vivo by chemical carcinogens have a transforming Harvey-ras oncogene. Nature, 303: 72-74, 1983.

25. Guerrero, I., Calzava, P., Mayer, A., and Pellicer, A. A molecular approach to leukemogenesis; mouse lymphomas contain an activated c-ras oncogene. Proc. Natl. Acad. Sci., 81: 202-205, 1984.

26. Guerrero, I., Villasante, A., Corces, V., and Pellicer, A. Activation of a c-K-ras oncogene by somatic mutation in mouse lymphomas induced by gamma radiation. Science, 225: 1159-1162, 1984.

27. Garte, S.J., Hood, A.T., Hochwalt, A.E., D'Eustachio, P., Snyder, C.A., Segal, A., and Albert, R.E. Carcinogen specificity in activation of transforming genes by direct-acting alkylating agents. Carcinogenesis, in press.

28. Land, H., Parada, L.F., and Weinberg, R.A. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature, 304: 596-602, 1983.

29. Sawey, M.J., Hood, A.T., Burns, F.J., and Garte, S.J. Activation of K-ras and c-myc oncogenes in radiation induced rat skin tumors. Molec. Cell Biol., 7: 932-935, 1987.

30. Duesberg, P.H. Activated proto-oncogenes: sufficient or necessary for cancer. Science, 228: 668-677, 1985.

31. Sukumar, S., Notario, V., Martin-Zanca, D., and Barbacid, M.R. Induction of mammary carcinoma in rats involves malignant activation of H-ras-1 locus by single point mutation. Nature, 306: 658-661, 1983.

32. Zarbl, H., Sukumar, S., Arthur, A.V., Martin-Zanca, D., and Barbacid, M. Direct mutagenesis of Ha-ras-1 oncogenes by N-nitroso-N-methylurea during initiation of mammary carcinogenesis in rats. Nature, 315: 382-385, 1985.

33. Goodhead, D.T., Munson, R.J., Thacker, J., and Cox, R. Mutation and inactivation of cultured mammalian cells exposed to beams of accelerated heavy ions. IV. Biophysical interpretation. Int. J. Rad. Biol., 37: 135-167, 1980.

34. Hayward, W.S., Neel, B.G., and Astrin, S.M. Activation of a cellular oncogene by promoter insertion in ALV-induced lymphoid leukosis. Nature, 290: 475-480, 1981.

35. Yander, G., Halsey, H., Kenna, M., and Augenlicht, L.H. Amplification and elevated expression of c-myc in a chemically induced mouse colon tumor. Cancer Res., 45: 4433-4438, 1985.

36. Dalla Favera, R., Wong-Staal, F., and Gallo, R.C. Oncogene amplification in promyelocytic leukaemia cell line HL-60 and primary leukaemic cells of the same patient. Nature, 299: 61-63, 1982.

37. Stanton, L.W., Watt, R, and Marcu, K.B. Translocation, breakage and truncated transcripts of c-myc oncogene in murine plasmacytomas. Nature, 303: 401-406, 1983.

38. Dalla-Favera, R., Bregni, M., Erikson, J., Patterson, D., Gallo, R.C., and Croce, C.M. Human c-myc oncogene is located on the region of chromosome 8 that is translocated in Burkitts lymphoma cells. PNAS, 79: 7824-7827, 1982.

39. Harris, L.J., D'Eustachio, P., Ruddle, F.H., and Marcu, K.B. DNA sequence associated with chromosome translocations in mouse plasmacytomas. Proc. Natl. Acad. Sci., 79: 6622-6626, 1982.

40. Sumegi, C., Spira, J., Bazin, H., Szpirer, P., Levan, G., and Klein, G. Rat c-myc oncogene is located on chromosome 7 and rearranges in immunocytomas with t (6:7) chromosomal translocation. Nature, 306: 497-498, 1983.

41. Albino, A.P., leStrange, R., Oliff, A.I., Furth, M.E., and Old, L.J. Transforming ras genes from human melanoma: a manifestation of tumor heterogeneity. Nature, 308: 69-72, 1984.

42. Kennedy, A.R., Murphy, G., and Little, J.B. Effect of time and duration of exposure to 12-O-tetradecanoyl-phorbol-13-acetate on x-ray transformation of C3H10T 1/2 cells. Cancer Res., 40: 1915-1920, 1980.

43. Burns, F.J., Albert, R.E., Sinclair, I.P., and Bennett, P. The effect of fractionation on tumor induction and hair follicle damage in rat skin. Radiat. Res., 53: 235-240, 1973.

44. Burns, F.J., Albert, R.E., and Sinclair, I.P. The effect of a 24-hour fractionation interval on the induction of rat skin tumors by electron radiation. Radiat. Res., 62: 478-487, 1975.

45. Albert, R.E., Burns, F.J., and Heimbach, R.D. The association between chronic radiation damage of the hair follicles and tumor formation in the rat. Radiat. Res., 30: 590-599, 1967.

46. Burns, F.J., Albert, R.E., Bennett, P., and Sinclair, I.P. Tumor incidence in rat skin following proton irradiation in a sieve pattern. Radiat. Res., 50: 181-190, 1972.

47. Albert, R.E., and Burns, F.J. Tumor and injury responses of rat skin after sieve pattern X-irradiation. Radiat. Res,. 67: 142-148, 1976.

48. Albert, R.E., Burns, F.J., and Heimbach, R.D. The effect of penetration depth of electron radiation on skin tumor formation in the rat. Radiat. Res., 30: 515-524, 1967.

49. Albert, R.E., Burns, F.J., and Heimbach, R.D. Skin damage and tumor formation from grid and sieve patterns of electron and beta radiation in the rat. Radiat. Res., 30: 525-540, 1967.

50. Albert, R.E., Newman, W., and Altshuler, B. The dose response relationships of beta-ray induced skin tumors in the rat. Radiat. Res., 15: 410, 1961.

51. Reuber, M.D. Development of preneoplastic and neoplastic lesions of the lever in male rats given 0.025 percent N-2-

fluorenyldiacetamide. J. Natl. Cancer Inst., 34: 697-723, 1965.

52. Burns, F.J., Vanderlaan, M., and Albert, R.E. Proliferation of early tumor foci in irradiated rat skin. Radiat. Res., 59: 18, 1974.

53. Ormerod, M.G. Radiation-induced strand breaks in the DNA of mammalian cells. In: Biology of Radiation Carcinogenesis (Eds., J.M. Yuhas, R.W. Tennant, J.D. Regan) Raven Press, New York, pp. 67-92, 1976.

54. Burns, F.J. and Sargent, E.V. The induction and repair of DNA breaks in rat epidermis irradiated with electrons. Radiat. Res., 87: 137-144, 1981.

55. Strickland, P., Burns, F.J., and Albert, R.E. Induction of skin tumors in the rat by single exposure to ultraviolet radiation. Photochem. Photobiol. 30: 683-688, 1979.

56. Boutwell, R.K. The function and mechanism of promoters of carcinogenesis. Crit. Rev. Toxicol., 2: 419-443, 1974.

57. Quintanilla, M., Brown, K., Ramsden, M., and Balmain, A. Carcinogen-specific mutation and amplification of Ha-ras during mouse skin carcinogenesis. Nature (London), 322: 78-80, 1986.

58. Dalla-Favera, R., Martinotti, S., Gallo, R.C., Erikson, J., and Croce, C.M. Translocation and rearrangements of the c-myc oncogene locus in human undifferentiated B-cell lymphomas. Science 219: 963-967, 1983.

59. Wigler, M., Pellicer, A., Silverstein, S., Axel, R., Urlaub, G. and Chasin, L. DNA-mediated transfer of the adenine phosphoribosyltransferase focus into mammalian cells. Proc. Natl. Acad. Sci., 76: 1373-1376, 1979.

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