Final Project Report

Project Title: Cellular and Molecular Mechanisms in Radiation Carcinogenesis

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The study was designed to compare cellular and molecular events in the carcinogenic process after exposure to neutrons with those observed after gamma radiation. To accomplish this goal, mouse mammary epithelial cells which were transformed by either neutrons or gamma rays and isolated using methods developed in this laboratory were studied early after transformation (early preneoplastic) or during progression toward the neoplastic phenotype.

Specifically, the aims of this research were:

- 1) Determine whether the loss of specific tumor suppressor gene activity is associated with specific altered growth phenotypes.
- 2) Characterize the genetic changes involved in the alteration of suppressor gene activity following neutron irradiation and compare these with those which occur water gamma ray irradiation.
- 3) Determine whether the altered expression of specific oncogenes are associated with specific alterations in growth phenotypes. In addition, compare the time at which changes occur and the spectrum of these changes to those observed in radiation altered cells obtained at similar times after gamma irradiation.
- 4) Determine changes in specific cellular growth regulatory mechanisms associated with altered growth phenotypes, and compare these to those altered growth variants obtained at similar times following gamma irradiation. Initial studies will focus on mechanisms involved in the response of cells to TGF-B and in the acquisition of angiogenic activity.

Initial studies examining a series of oncogenes and tumor suppressor genes including Rb, c-myc, neu, and cyclin D1 indicated that alterations in these genes were associated with late preneoplastic or full blown neoplastic cells. No differences in the spectrum or time at which these alterations were observed when cells transformed by gamma rays or neutrons were compared. The exception to the late appearance of genetic alterations was alterations in expression of p53. In the majority of transformed cells accumulation of p53 was observed with immunostaining relatively early after transformation. This led our laboratory to focus on p53.

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Results of studies of p53 alterations indicated that the accumulation of p53 was a result of point mutations in the p53 gene which resulted in a more stable protein configuration. Interestingly, all the mutations observed were point mutations. A total of 11 cell lines derived from subculturable EF have been analyzed for p53 status as a function of time in culture and neoplastic potential. Studies designed to investigate the preneoplastic to neoplastic transition in relation to mutations in p53 initially focused on EF42. This is because the *in vitro* and *in vivo* growth characteristics of this altered growth variant were particularly well defined. The results of our analysis of EF42 has been published and are briefly described below.

The growth variant, EF42, was derived from the mammary tissue of a 16 week old virgin female BALB/c mouse 4 weeks after irradiation with 1 Gy of gamma rays. This variant was initially identified as a single epithelial focus consisting of 50-60 cells, arising from a total cell sample of 200 clonogenic cells plated on 10 dishes. Because each 60 mm dish contained only 20 clonogens, it is reasonable to assume that this focal growth was derived from a single irradiated cell (A similar argument can be made for all of the altered growth variants identified as EF that we have obtained). EF42 was subsequently isolated as a morphologically homogeneous colony containing 2×10^3 near diploid cuboidal epithelial cells. At passages 3-15 injection of EF42 into gland-free fat pads resulted in the development of normal ductal outgrowths. Ductal dysplasias and eventually mammary adenocarcinomas developed in these apparently normal outgrowths 8-12 months after injection. Injection of cells into fat pads at *in vitro* passage 25 and higher resulted in tumors in 6-8 weeks. These data confirm both the preneoplastic nature of EF42 at low passages and its ability to progress to the neoplastic phenotype with time *in vivo* or *in vitro*.

Figure 1. Summary of Genetic Alterations in EF42 during Neoplastic Progression

clearly evident. Upon further analysis, numerical heterogeneity was evident within the population as early as we were able to examine the cell population (Data not shown). Immunohistochemical analysis at passage 11 revealed an accumulation of p53 protein in approximately 10% of the population. At passage 27, when the cells were tumorigenic, the percentage of p53-positive cells approached 100%.

To characterize the p53 mutations in EF42 we first identified mutations in clonal isolates of EF42 in which virtually all cells appeared to contain mutant protein (based on immunohistochemistry). A more detailed discussion of the derivation of these clonal isolates is contained in the next section of the progress report. Three mutations were identified using direct sequencing of PCR products: a transversion at codon 173 from TGC to TGG leading to a substitution of tryptophan for cysteine (C173W); a transition at codon 278 from GAC to GGC, resulting in a substitution of glycine for aspartic acid (D278G); and a transversion from GTT to GAT at codon 269 resulting in a substitution

of aspartic acid for valine (V269D). Since each of these mutations created new restriction sites, we next analyzed EF42 using PCR-RFLP analysis. Results are summarized in figure 1. At passage 11, both the C173W and D78G were present at a frequency of 8%. V269D was only detected in the clonal isolates derived from EF42. The growth advantage conferred to cells with the D278G mutation was striking. At passage 15, D278G was present in 35% of the population and by passage 27 more than 90% of the population was homozygous for this mutation. In addition to the loss of heterozygosity, amplification of c-myc and cyclin D1(not shown) was observed in these tumorigenic cells as well as in tumors derived following their injection into fat pads. Clonal isolates of EF42 carrying these individual mutations were used to assess the biological significance of each of these mutations (see the next section).

Although it was clear from the data that p53 mutations represent critical early events in the neoplastic progression of EF42, our data argued that these mutations were not directly induced by radiation but arose in the progeny of irradiated cells several generations later. Because of the clonal origin of EF42 and the clear growth advantage conferred by p53 mutations in these cells (particularly D278G), p53 mutants should have comprised virtually 100% of the cells at the earliest passages examined if it had been directly induced. Rather, p53 mutants comprised only 8% of the cell population at passage 11 and were undetectable at passage 6 using the PCR-RFLP or the more sensitive probe shift assay. To our knowledge these data represent the first evidence for delayed mutations in a gene directly involved in the carcinogenic process.

Analysis of additional subculturable EF from both gamma ray and neutron irradiated mice support this conclusion. For these other subculturable EF we have used SSCP analysis of PCR products to detect the presence of p53 mutations followed by sequencing. All of these mutations were found to be point mutations rather than large scale alterations normally associated with mutations directly induced by radiation. In our hands this approach is capable of detecting most p53 mutations when present in greater than 15-20% of the cell population. We have analyzed 6 gamma ray induced subculturable EF at various *in vitro* passages and degrees of neoplastic potential, and have found evidence for point mutations in p53 in 4 of these 6 and in 3 of 5 neutron induced subculturable EF. Importantly in all cases, these mutations were not detectable at low passages but only became detectable with time in culture and increasing neoplastic potential. In no case could we detect mutations before passage 10 and most became detectable at passages 17-25.

BIOLOGICAL SIGNIFICANCE OF P53 MUTATIONS

To examine the biological significance of these mutations we first concentrated on a series of clonal derivatives of EF42. A summary of genotypic alterations and the tumorigenic potential of these derivative lines is shown in figure 2. Interestingly, these various derivatives each with distinctive phenotypic and genotypic characteristics evolved from a single clonal isolate developed originally from EF42 at passage 11 in the following manner. At passage 11, preneoplastic EF42 cells were plated at low density to allow selection of distinct individual colonies. A single colony containing approximately 100 cells was isolated based on its cuboidal epithelial morphology and cloned using

limiting dilution in 96 well plates. This resulted in the clonal line 11A. Like EF42, 11A rapidly became pleiomorphic and within 5 passages two sublines could be identified. The subline 11A-1 consisted of elongated cells all of which stained positive for accumulation of p53. The subline 11A-2 contained two morphologically distinct cell types, small strongly staining cells and less intense staining larger cells. These two morphologically distinct cell types were subcloned by limiting dilution and designated Cl-1 and Cl-2. Further analysis indicated that 11A-1 was non-tumorigenic upon injection into the fat pads of BALB/c mice and was pseudotetraploid. The near diploid 11A-2, Cl-1 and Cl-2 were tumorigenic under these conditions although Cl-2 was only weakly tumorigenic. SSCP analysis of PCR products and subsequent direct sequencing indicated that 11A-1 contained the p53 mutation C173W which had also been observed in parental EF 42. 11A-2 contained two mutations. The first mutation was the D278G mutation found in EF42. A second new p53 mutation at codon 269 (V269D) was also found. This mutation was a transversion from GTT to GAT resulting in the substitution of aspartic acid for valine. As discussed above these mutations created new restriction sites which facilitated a more quantitative analysis of these mutations. Because 11A-1 and 11A-2 were not individually derived by limiting dilution we screened 11A-1 for the presence of D278G using PCR-RFLP analysis as well as the more sensitive probe shift assay-none was found. PCR-RFLP analysis also indicated loss of heterozygosity at codon 173. A similar analysis of 11A-2 failed to detect C173W. These data suggest 11A-1 and 11A-2 diverged after independent assortment of these mutant alleles. To analyze whether D278G and V269D were associated with two distinct populations within 11A-2, we performed PCR-RFLP analysis of the lines Cl-1 and Cl-2. Cl-1 cells contained D278G, whereas Cl-2 showed no alterations at this codon. The mutation V269D was heterozygous in Cl-1 but appeared homozygous in Cl-2. Further analysis indicated these two mutations were located on different alleles. The absence of V269D in 11A-2 tumors is the result of preferential loss of cells homozygous for V269D *in vivo*, as well as the loss of the allele bearing this mutation.

These results clearly demonstrate a critical role for p53 mutations, particularly D278G in neoplastic progression. The acquisition of p53 mutations is followed by clonal expansion *in vitro* and clonal selection and progression *in vivo*. The genetic instability of the p53 mutants is demonstrated by the loss of heterozygosity and amplification of c-myc and cyclin D1 in these cells. Studies using chromosome specific painting probes for chromosomes 2, 3, and 11 have demonstrated ongoing cytogenetic instability in these cells.

Based on these results we have proposed a model which identifies genomic instability as the earliest cellular event in the multi-step process of radiation induced cancer. In this

model, mutations in p53 are a secondary consequence of this radiation-induced instability. It is predicted that this initial instability would put virtually all genes at risk but its major impact for carcinogenesis occurs when mutations in certain critical genes, such as p53 occur. This model is linked to the growing body of studies describing the delayed appearance of mutations and chromosomal abnormalities in the progeny of irradiated cells. In these studies the latent expression of chromosomal aberrations and mutations in these progeny has been interpreted to be a manifestation of genomic instability induced by radiation. Most evidence for this radiation-induced instability has come from studies demonstrating delayed chromosomal instability. In fact, such chromosomal instability might be considered a hallmark of this effect. Evidence for an increased mutation rate in the progeny of irradiated cells has also been seen but the data are less extensive. The progeny of irradiated human lymphoblastoid cells have displayed unusually high rates of coincident mutations at several unselected loci. Other investigators have also reported delayed mutations in the hprt locus in CHO cells following irradiation. Interestingly, the spectrum of structural changes observed in directly induced and delayed mutations were found to differ significantly. Directly induced mutations were mainly, as expected, large deletions while the majority of delayed mutations appeared to be point mutations. In papers describing their studies of radiation-induced instability, several of these investigators have proposed that radiation-induced genomic instability may have an important role in the process of tumor development. However, such a view has largely been speculative and the specific role that radiation-induced instability might play in the complex process of carcinogenesis was not discussed. To our knowledge, our data represent the first evidence for delayed mutations in a gene directly involved in the cancer process. More importantly, the data suggest a specific, and perhaps unique, role for radiation-induced instability as a critical early event associated with initiation of the carcinogenic process.

Results of these studies have led to a new direction in this laboratory and in the field of radiation carcinogenesis. Our laboratory is now examining in more detail radiation-induced instability and its relation to radiation-induced mammary cancer. These more recent studies not funded by the DOE have revealed that instability can be induced by both neutrons and gamma rays. Interestingly, however, neutrons appear to be more efficient and the kinetics of development differ for neutrons and gamma rays. Following gamma irradiation the instability is not immediately manifested but takes 20-25 population doublings for the instability to develop in the progeny of irradiated cells. Following neutron irradiation, the instability is seen virtually immediately.

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