INTRODUCTION

Over the last several years research in this laboratory has focused principally on mammary cancer development following radiation exposure. These studies have used the female Balb/cAnNbd mouse because of its relatively low natural incidence (8.0%) of mammary adenocarcinomas and because this mouse strain is sensitive to induction of mammary tumors following radiation exposure. From these studies dose response relationships have been obtained for tumor development following exposure to Cs-137 gamma rays and fission spectrum neutrons. In addition, data on time-dose relationships following fractionated and protracted neutron and gamma ray exposures have also been published (1, 2).

To understand the mechanisms of radiation induced mammary carcinogenesis and the basis for the observed time-dose relationships, this laboratory has shifted emphasis to studies aimed at delineating cellular changes which precede tumor development. For these studies an in vivo/in vitro model of mammary carcinogenesis is being used. By using in vitro cell culture methods, this model permits the detection and isolation of cells from mammary tissue following irradiation in vivo which have altered growth phenotypes. This latter approach follows closely methods originally described by Terzaghi and Nettesheim for analysis of cellular events in tumor development with rat tracheal epithelial cells (3).

DETECTION OF PHENOTYPICALLY ALTERED CELLS IN IRRADIATED MAMMARY TISSUE

In female Balb/c mice mammary tumors generally occur late in life with few tumors appearing before the mice reach 15 months of age. Irradiation with either gamma rays or neutron does not appear to change this time of appearance but rather increases incidence
of mammary tumors. Prior to the development of mammary tumors, hyperplastic lesions characterized as ductal dysplasia can be detected in both non-irradiated and irradiated mice. Such dysplasias begin to appear about 12–14 months after irradiation. Studies by other investigators have demonstrated that these dysplasias are preneoplastic lesions (4). No morphological changes in mammary tissue can be detected prior to the development of these dysplasias. Recent studies in this laboratory have concentrated on characterizing cellular and molecular changes which precede the development of these preneoplastic lesions in situ. Initial results of these studies have been published (5) and are summarized in Table I. Following in vivo irradiation, mammary epithelial cells were isolated using enzymatic dissociation methods at intervals of 1, 4, 16, and 52 weeks after irradiation. These cells were then plated under conditions in which control mammary cells had limited proliferative ability. Under these same conditions irradiated cells continued to proliferate and formed discrete proliferating epithelial foci (EF). These EF were further characterized based on their ability to be subcultured (EFs). The growth potential of these EFs was characterized further by injecting cells at the fourth in vitro passage into gland-free fat pads and classifying the outgrowths obtained as normal ductal, dysplastic or tumorigenic. Injection of these subculturable EF derived from mammary tissue irradiated 1–16 weeks prior to cell dissociation gave rise to normal ductal outgrowths in all instances when mammary outgrowths were obtained. With continued in vitro passage, they became tumorigenic. The range of passage levels at which these subculturable EF became tumorigenic varied among the individual EFs, ranging from passage 20 to passage 40. Injection of EFs derived from mammary tissue irradiated 52 weeks prior to cell dissociation resulted in dysplastic outgrowths (3 of 4) or tumor formation (1 of 4). These dysplastic outgrowths were similar to those eventually arising in situ and, as with many of the in situ dysplasias, leukocytic infiltration and angiogenesis was often associated with these lesions (4, 5). These EFs became tumorigenic with in vitro passage more rapidly (passages 4–12) than did those EFs derived at earlier times after irradiation.

These results demonstrated that very early after irradiation cell populations are present within the irradiated mammary tissue at a high frequency with altered in vitro proliferative potential. Further these studies have shown that cells continue to acquire new phenotypes with time both in vivo and in vitro. With the acquisition of of these additional phenotypic

<table>
<thead>
<tr>
<th>Time in situ (weeks)</th>
<th>1 Week</th>
<th>4 Weeks</th>
<th>16 Weeks</th>
<th>52 Weeks</th>
</tr>
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<tbody>
<tr>
<td>% EF</td>
<td>2.5 (2/79)</td>
<td>57.1 (44/72)</td>
<td>13.3 (20/150)</td>
<td>15.6 (5/32)</td>
</tr>
<tr>
<td>% EFs</td>
<td>0/2</td>
<td>20.5 (9/44)</td>
<td>45.0 (9/20)</td>
<td>80.0 (4/5)</td>
</tr>
</tbody>
</table>

* Number of dishes with EF 12 weeks after plating/number of dishes plated at 5x10^4/60 mm dish.
* Number of subculturable EF/Total # of EF examined.
changes cell populations emerge with characteristics more closely associated with the neoplastic phenotype.

This experimental approach has recently been extended by comparing the effects of gamma rays and neutrons at high and low dose rates on the induction of EF and EFs. The data from these recent experiments suggest that the induction of EFs correlates at least qualitatively with the tumorigenic effects of the radiation. Fewer EFs were obtained from mammary tissue of animals irradiated with low dose rate gamma rays compared with high dose rate groups while low dose rate neutron exposures resulted in an increased frequency of subculturability (Figure 1).

**CHARACTERIZATION OF SPECIFIC CELLULAR ALTERATIONS**

The ability to subculture radiation altered cell populations has allowed the development of a number of cell lines useful for delineating important molecular genetic and cellular alterations involved in mammary cancer development. These cell lines are being used to approach the problem from two directions. First, changes occurring as cells progress in vitro from non-tumorigenic to tumorigenic are being examined. Second, early passages of cell lines isolated at different time after irradiation are being compared as a means of examining progression in vivo. Studies thus far have concentrated principally on two cell lines. The cell line EF42 was derived from mammary tissue from a mouse irradiated with 100 cGy of gamma rays 4 weeks prior to cell dissociation. The cell line EF137 was derived from mammary tissue from a mouse irradiated 16 weeks prior to dissociation. Both cell lines
produce normal ductal outgrowths following injection into gland-free fat pads over passages from 3 to 18. Injection of passages 20 or higher results in tumor development. The growth characteristics of EF42 are summarized in Table 2. These cell lines were cloned and most studies have been performed on clones isolated from these EF. For clones from both cell lines in vitro progression leading to the emergence of cells capable of forming tumors in vivo was associated with the emergence of cells with specific, unique chromosomal alterations, generally in the form of metacentric indicator chromosomes (MIC). The presence of these MICs in the majority of cells in tumors derived from these EF as well as in the cells grown in vitro suggests a role for these alterations in tumorigenesis. Analysis of protooncogenes also indicated amplification and overexpression of c-myc in tumorigenic cells and their derived tumors. No evidence has been found for alterations in other protooncogenes or alterations in their expression in these cell lines at passages when they were not tumorigenic.

Because of the possible role of tumor suppressor genes in tumor development (6, 7), alterations in the retinoblastoma (Rb) and p53 tumor suppressor genes have also been conducted with these cell lines. Alterations in the Rb gene have been detected in both cell lines prior to their development of tumorigenicity. These include reduced or absent expression of the Rb protein, a reduced level of Rb mRNA and an under represented banding pattern. All of these data are consistent with a loss of heterozygosity. In clones derived from EF42 such changes have been observed in intermediate passage (11–16), non-tumorigenic cells and in high passage (>20), tumorigenic cells and in tumor cells. No changes have been detected in low passage (<10) cells. Interestingly, in clones from EF137 the changes in Rb expression are observed even at the lowest passages tested (passage 6). Since EF137 was isolated later after irradiation than was EF42, it is tempting to suggest that the loss of Rb expression in EF137 had already occurred in vivo prior to its isolation. In EF42 this loss of expression apparently occurred during in vitro passage prior to the development of the neoplastic phenotype. The timing of this loss of expression suggests that alterations in the Rb gene may be involved in radiation-induced mammary tumor development in intermediate stages of progression. Southern and Northern analysis of the p53 gene provided no evidence for gross alterations regardless of the tumorigenic potential of the cells. Subsequently, the expression of p53 was examined by immunohistochemistry and immunoprecipitation with monoclonal antibodies capable of detecting wild type and mutant forms. At high passages,

Table 2. Growth Parameters of EF #42

<table>
<thead>
<tr>
<th>In vitro passage number</th>
<th>11</th>
<th>25</th>
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<tbody>
<tr>
<td>Morphology</td>
<td>large, cuboidal</td>
<td>small, elongated</td>
</tr>
<tr>
<td>Growth pattern</td>
<td>monolayer, contact inhibition</td>
<td>piled up, loss of contact inhibition</td>
</tr>
<tr>
<td>DNA content</td>
<td>2.1C</td>
<td>2.1C; 3.6C</td>
</tr>
<tr>
<td>Population doubling time (hr)</td>
<td>25.5</td>
<td>15.5</td>
</tr>
<tr>
<td>Saturation density (cells/60 mm)</td>
<td>$5.4 \times 10^6$</td>
<td>$1.10 \times 10^7$</td>
</tr>
<tr>
<td>Outgrowth in vivo</td>
<td>normal ductal</td>
<td>adenocarcinoma</td>
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the mutant form was detected by immunoprecipitation in clones of both EF42 and EF137. Further, strong nuclear staining with antibody specific for the mutant form was detected using immunohistochemical techniques in greater than 95% of these cells. Interestingly at low non-tumorigenic passages (6–10) a small number of cells (1–5%) also stained positive. These presence of these positively stained cells at low passages suggest that alterations of p53 may be an early event in mammary cancer development and provides a selective advantage. The nature of the alterations are being examined.

In addition to the molecular studies, we are also investigating alterations in growth factor responses and/or production. Because of its potential importance, one of the growth factors under study is the angiogenesis factor. To determine angiogenic activity in the various cell lines available, an in vitro system originally developed in Folkman’s laboratory is being used (8). For this assay bovine capillary endothelial cells are trypsinized from gelatinized dishes and resuspended in a collagen solution of Vitrogen and media (1:1). Aliquots of this suspension are plated in 24 well plates along with boluses of test cells. The cells mixtures are then incubated at 37 C in 10% carbon dioxide. Each well is examined for endothelial cell reorientation, migration, growth, and differentiation. Results of these studies suggest that for gamma irradiated cells the acquisition of angiogenic activity is a relatively late event which occurs in vitro with the acquisition of tumorigenic potential. At low passages only those cells isolated 52 weeks after irradiation show angiogenic activity.

CONCLUSIONS

Data presented demonstrate that multiple cellular events are responsible for radiation induced mammary cancer development. Evidence thus far suggest that important early events in this process may involve alterations in tumor suppressor genes such as RB and p53. From the frequencies of EF and EFs obtained in these studies, it is apparent that the frequency of radiation altered cells is much higher than would be expected based upon tumor incidence data. This suggests an important role for cell-cell interactions and other host factors in regulating expression and perhaps progression of these phenotypically altered cells.

REFERENCES


