**RADIOGENIC NEOPLASIA IN THYROID
AND MAMMARY CLONOGENS**

**Progress Report
January 1, 1993 - December 31, 1993**

Kelly H. Clifton

**Department of Human Oncology
University of Wisconsin Comprehensive Cancer Center
600 Highland Avenue
Madison, WI 53792**

July 30, 1993

**Prepared for
THE U.S. DEPARTMENT OF ENERGY
Agreement No. DE-FG02-87ER60507**

NOTICE

This report was prepared as an account of work sponsored by the United States Government. Neither the United States nor the Department of Energy, nor any of their employees, nor any of their contractors, subcontractors, or their employees, makes any warranty, express or implied, or assumes any legal liability of responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product or process disclosed or represents that its use would not infringe privately owned rights.

Progress Report

1 January 1993 - 31 December 1993 (Prepared 30 July 1993)

Abstract

The induction of cancer by ionizing radiation is a matter of great practical importance to the nuclear industry, to national defense, to radiological medicine and to the general public. It is increasingly apparent that carcinogenesis is one of the leading dose-limiting effects of radiation exposure (Co90). Quantitative information at the cellular level is essential to an understanding of the mechanisms of radiogenic neoplastic initiation and the stages of promotion and progression to overt neoplasia. We have developed two experimental models, the rat thyroid and rat mammary clonogen transplant systems, for the quantitative study of radiation carcinogenesis at the cellular level *in vivo* (C185). Past experiments with these systems have shown a) that both rat mammary and thyroid glands contain subpopulations of putative clonogenic epithelial cells which when transplanted are self-perpetuating and can give rise to functional multicellular glandular structures, b) that these clonogens are the progenitor cells of radiogenic thyroid and mammary cancer, c) that such cells are capable of a high degree of intracellular repair after irradiation, d) that radiogenic initiation, the first step in the carcinogenic process, is a common event at the target clonogen level, and e) that the promotion-progression of initiated clonogens may be inhibited by normal epithelial cells (C190).

The most important steps taken or completed during the current grant year include: a) demonstration of the high age-dependent radiosensitivity of prepubertal rat mammary clonogens to radiogenic damage which may influence their susceptibility to neoplastic initiation, and b) demonstration of the feasibility of using a molecular test for clonogenicity in which Simple Sequence Repeats in the DNA serve as identifying signals of the genotypic origin of the cells. We have also c) set up a large carcinogenesis experiment to test the effect of close intercellular contact in thyroid glands *in situ* on promotion-progression of radiogenically initiated clonogens, d) achieved considerable further concentration of thyroid clonogens, and e) begun to explore whether thyroid cells can be induced to give rise to three dimensional multicellular structures in culture in reconstituted basement membrane. These are discussed below.

Narrative

Introduction: Our aims during this renewed grant period are to perform long-term carcinogenesis studies and short term cell studies to address the following questions:

- a. Does intimate cell-cell contact within the intact thyroid gland suppress progression to cancer in radiogenically initiated thyroid clonogens *in situ* in part via local mechanisms which do not involve classical hormonal feedback?

- b. Does age at the time of exposure influence the frequency of radiogenic mammary cancer? If so, is this influence correlated with age-related differences in the concentrations or the total numbers of clonogens per mammary gland, or on the rates of proliferation at the times of exposure? Are the neoplastic initiation-inducing effects of low LET x-rays and of high LET neutrons affected by these factors in the same manner? Investigation of these questions will be in part supported by another project grant.
- c. Can thyroid clonogens be further concentrated by a combination of physiological and physical procedures including fluorescence activated cell sorting for more complete characterization?
- d. Can thyroid clonogens be induced to form three dimensional glandular FU (follicular units) in culture? If so, can the procedures for inducing FU formation in culture be sufficiently standardized to be used as an assay endpoint for estimation of clonogen concentrations?
- e. Can the recently demonstrated inbred rat strain-specific differences in DNA Simple Sequence Repeats (SSRs) (We90) be used to rigorously and directly test the hypothesis that the FU formed in graft sites from inocula of monodispersed thyroid cells, and the carcinomas that arise within them, are clonal in origin, as is most consistent with the mass of our previous data (Cl90)?

Progress in each of these areas is summarized below.

Cell-cell interactions during promotion-progression of initiated cells : This aspect of our program was initiated in response to the observations that a) the number of carcinomas per graft site of irradiated thyroid cells did not increase in proportion to the increase in the numbers of irradiated cells grafted (Mu84), and b) that the addition of unirradiated thyroid cells to graft inocula of irradiated cells further decreased the carcinoma incidence (Wa88). These data suggested a cell number-dependent suppression of neoplastic promotion-progression of radiation initiated thyroid clonogens. An experiment was designed to investigate whether this suppressive effect was mediated by a cell number-dependent re-establishment of the thyroid-pituitary hormonal feedback system, by locally acting cell-cell interactions, or both. A grafted cell number-related decrease in thyrotropin levels was found to be correlated with a lower carcinoma frequency in an experiment in which localized cell-cell interactions were controlled (Do93). However, the data do not rule out such interactions. Furthermore, in another project, grafted cell number-dependent suppression of promotion-progression has been observed during carcinoma development in grafts of irradiated mammary cells in the absence of known hormonal feedback (unpublished data; Kamiya K. and Clifton, K.H.). This suggests that local cell-cell interactions may play a role in this process.

We reasoned that any cell-cell mediated suppression of promotion-progression would likely be effective among the closely associated cells of glands *in situ*. We therefore designed an experiment in which thyroid glands were irradiated and left *in situ* for one to 84 days after exposure before removal for transplantation (Table 1).

TABLE 1 Experimental Design: Cell-Cell Interactions During Progression				
<u>Group</u>	<u>Days of Experiment</u>			
	<u>day -1</u>	<u>day 0</u>	<u>day +7</u>	<u>day +84</u>
<u>A</u> : donors: recipients:	0 Gy	collect cells graft cells		thyrex, LID
<u>B</u> : donors: recipients:	5 Gy	collect cells graft cells		thyrex, LID
<u>C</u> : donors: recipients:	0 Gy		collect cells graft cells	thyrex, LID
<u>D</u> : donors: recipients:	5 Gy		collect cells graft cells	thyrex, LID
<u>E</u> : donors: recipients:	0 Gy			collect cells graft cells, thyrex, LID
<u>F</u> : donors: recipients:	5 Gy			collect cells graft cells, thyrex, LID

Donors and recipients were all 4-5 week old male (WF x F344) F1 rats on day 0 of the experiment. Sixty recipient rats were accumulated in each experimental group in 10 replicate runs. Each recipient rat received 5 surviving thyroid clonogens at each of two subcutaneous fat pad graft sites and 40 surviving thyroid clonogens at each of three other fat pad graft sites. Donor rats were fed laboratory rat chow with normal iodine levels and acidified water *ad libitum* until killed for thyroid cell collection. On day +84, all recipient rats were thyroidectomized and placed on low iodine diet (<50 ng I/g diet) and distilled water containing calcium; this will be continued until the experiment is terminated (Mu84, Wa88). Set up of the carcinogenesis experiment was preceded by a series of transplantation assays to determine surviving clonogen concentrations at the time of cell collection in each donor group, and the transplantation inocula were adjusted accordingly. Blood samples and fat pad graft sites were or will be taken on days +83, +91 and +168. TSH (thyrotropin), T4 (thyroxin) and T3 (triiodothyronine) serum levels will be determined at each interval, and the histology of sample graft sites will be examined. Animals will be inspected and their graft sites examined frequently (daily after masses begin to appear). When a palpable mass in a graft site reaches 10-14 mm in diameter, it will be surgically resected and the rat will be returned to the experiment unless the mass is the last of the graft sites remaining. Rats that are found moribund and those few in which all graft sites have been removed will be

killed. These rats and those found dead will be autopsied with special reference to the graft sites, the thyroidal area, the pituitary gland and general pathologies and causes of illness. All masses in the graft sites removed surgically or at autopsy will be fixed for histopathological diagnosis.

It should be noted that during the period from day -1 to day +84, the thyroid cells in question will be in a euthyroid milieu either in the donors *in situ* or in the recipients in graft sites. The difference between the two environments will thus be largely the level of association of the epithelial cells one with another. From day +84 until death they will all be graft sites in an iodine deficient hypothyroid environment exposed to elevated TSH levels. This experiment will yield data that will greatly aid in interpretation of the nature of the grafted cell number-dependent suppression of neoplastic promotion-progression.

Age dependency of mammary clonogen numbers, concentrations and radiosensitivity: In a series of experiments supported in part by other sources, we have investigated the radiation responses and numbers of mammary clonogens at ages from one to 12 weeks after birth. These studies were undertaken because of the now recognized high susceptibility of prepubertal and pubertal women to radiogenic initiation of breast cancer (To87, Hi89). Such quantitative information on aspects of the radiosensitivity of the presumptive mammary target cells during this critical period is necessary for the design of experiments on the age dependence of the susceptibility of mammary clonogens to radiogenic initiation. The findings are striking.

AD50 values (alveolar dose 50% values) are the mean numbers of monodispersed mammary cells per graft site required to produce at least one multicellular clonal alveolar structure in 50% of the sites, and are the most stable parameters of clonogen numbers from the transplantation assays. AD50s are inverse functions of the clonogenic fractions of mammary cells; e.g. the higher the AD50s, the lower the fractions of viable clonogens (Cl85). The AD50 values of cells from the mammary glands of untreated prepubertal rats fell rapidly from the first observation at one week of age and then stabilized about the start of puberty (Figure 1). Thereafter the AD50s remained essentially constant in cells from the glands of pubertal and postpubertal rats four weeks through 12 weeks of age (Figure 1).

The total numbers of recoverable clonogens increased with a doubling time of ~3.4 days during prepubertal weeks one to four. Thereafter the growth rate slowed markedly to a doubling time of ~ 31.4 days in the immediate postpubertal sixth to twelfth weeks of age (Figure 1). Throughout the period of observation, AD50s of monodispersed mammary cells from rats exposed to 5 Gy gamma rays and taken for assay soon thereafter were significantly higher, i.e. contained significantly lower clonogen concentrations, than those from untreated rats of the same ages respectively (Figure 2). Calculation of the surviving clonogen fractions measured immediately after 5 Gy showed they were relatively constant in rats exposed before the onset of puberty at one to four weeks of age, were higher during puberty at six weeks, and increased further immediately postpuberty to about the same values at eight and 12 weeks of age (Figure 3).

Multidose-clonogen survival response curves of mammary cells from two-week-old, four-week-old and eight-week-old irradiated rats were analyzed according to the α/β model:

$$S = e^{-(\alpha D + \beta D^2)}$$

The αD term dominated the relationships in analyses of the curves of clonogens from prepubertal two and four week old rats; i.e. in both cases clonogen survival decreased virtually exponentially from $D = 0$ Gy. In contrast the βD^2 term dominated the more radioresistant convex dose-response curve of the clonogens from irradiated postpubertal eight week old rats. This shift in the shape of the radiation dose-clonogen survival curve is similar to that seen when cells with previously short G_1 phases enter a state in which the G_1 phase is lengthy, and may reflect a lengthening of cell cycle concurrent with the increase in clonogen population doubling time.

The data thus show that the hormonal changes at puberty are accompanied by an increased capacity for intracellular protection against radiogenic damage and/or repair of such damage in the mammary clonogens. Insofar as susceptibility to neoplastic initiation parallels that of radiogenic cell killing, the greater radiosensitivity of mammary clonogens before puberty as compared to after puberty, if true in humans, may help to explain the high sensitivity of prepubertal girls to radiogenic breast cancer initiation. These data will aid in the design of experiments to further investigate age related effects of radiation exposure.

Concentration of thyroid clonogens: As noted in our last progress report, we have applied both improved cell separation procedures and especially the technology successfully used in a related project in this laboratory to concentrate the mammary clonogens by immunofluorescence-activated cell sorting (Ki93a and b) to the concentration of thyroid clonogens. In the mammary studies, fluor-labeled PNA (peanut agglutinin) and Thy-1 (antibody to the Thy 1.1 cell surface marker) were found to be good labels for the separation of mammary cell subpopulations by fluorescence-activated cell sorting (Ki93a and b). We have found that these reagents can be profitably applied to analysis of thyroid cell subpopulations.

Furthermore, we observed that the greatly expanded thyroid clonogen subpopulations of goiters are selectively conserved during the massive cell loss that occurs during goiter involution on withdrawal of goitrogen (Gr92). We reasoned that the clonogen subpopulations of the initially normal thyroid glands might be similarly conserved during thyroid atrophy secondary to inhibition of TSH secretion resulting from administration of exogenous T4 (thyroxin).

The addition of a filtration step to separate partially digested multicellular predominantly epithelial thyroid "organoids" from dispersed blood and stromal cells reduced the FD50 (follicle dose-50%; Cl85) values of dispersed thyroid cell suspensions from 128 ± 21 cells per graft site to 46 ± 12 cells, i.e. the clonogens were concentrated by a factor of ~ 2.8 . A dose of 10 mg T4 per 100 g body weight for two weeks caused thyroid atrophy, altered the sizes of the three immunologically tagged cell subpopulations, and decreased the FD50 values (Table 2).

TABLE 2
T4 Effects on FD50s and Thyroid Cell Subpopulations

<u>treatment</u>	<u>PNA+ cells</u>	<u>Thy 1+ cells</u>	<u>both neg. cells</u>	<u>FD50</u>
none	13±1%	11±3%	24±3%	46±12
T4	27±1%	7±2%	65±8%	20±6
P values	0.0005	0.049	0.017	0.011

The FD50 values in Table 2 are for unsorted whole cell populations. An FD50 value of 20 indicates that there was one thyroid clonogen per ~29 thyroid cells in the inoculum. We have subsequently shown that , as in comparable mammary cell preparations (Ki93a), the overwhelming majority of clonogenic cells are contained within the PNA+ cell subpopulation. In mammary cell suspensions, Thy 1+ cells are myoepithelial (Ki93a). We are not aware of myoepithelial cells in thyroid glands, and the Thy 1+ cells in thyroid cell suspensions are not strongly positive and have a distinctive morphology. Their nature is currently under investigation. These results encourage us to believe that we may ultimately be able to purify and more completely characterize the thyroid clonogens, the presumptive cancer progenitor cells, and further clarify the cellular economy of the thyroid gland.

Behavior of thyroid cells in culture: As noted in previous Progress Reports, we have been able to maintain and observe proliferation of thyroid epithelial cells in culture. In a related project we have observed the formation of multicellular structures by mammary cells in cultures in Matrigel, a preparation from biological material containing collagens, laminin and other basement membrane components (Ki93b). We are currently testing whether three dimensional structures will be formed by thyroid cells in such media with appropriate hormonal stimulation. In the best of all worlds, if formation of thyroid follicles could be reproducibly stimulated, both assays of clonogenicity and characterization of the early stages of radiogenic cancer induction might be greatly accelerated and simplified. We intend to stick to the transplantation assay as the prime standard in any event.

Genetic markers for testing clonogenicity : We have made very significant progress in application of molecular biological techniques to identifying the rat strain of origin of mammary alveolar and ductal structures that arise in grafts of mammary cells. SSRs ("Simple Sequence Repeats") are comprised of CA_n*GT_n and have been shown to be present at specific sites in chromosomal DNA (We90); they frequently differ in length in rats of differing inbred strains. SSRs that are known to differ between cells from F344 strain rats and WF strain rats have been chosen as markers of cell origin (M.N. Gould, unpublished). The cell suspension methodology has been adapted and transplantation experiments have been performed to determine the appropriate cell number to produce multicellular alveolar structures or ductal structures from inocula of mammary cells from WF or F344 donor rats in ~20% of the

graft sites in (WF x F344)F₁ hybrid recipients. This fraction of graft sites was chosen to reduce the number of positive graft sites that would be expected to have received more than one clonogenic cell to a practical minimum while allowing for a sufficient number of sites with glandular structures for statistically adequate testing. We have found that the alveolar and the ductal structures that develop from grafts (Ka91) of WF or F344 rats into F₁ hybrid recipients can be cleaned of host F₁ tissue. Their DNA can then be used with appropriate primers to amplify specific SSRs by the polymerization chain reaction (PCR). SSRs can then in turn be used in the positive identification of the strain source of the DNA, and hence the strain of origin of the structure in the graft site. Under appropriate conditions of grafted cell numbers and mixtures of cells from the two parent strains, a given mammary structure that developed in a graft site in an F₁ recipient would overwhelmingly be expected to contain the marker SSRs of one parent strain or the other, but not of both, if the structure was derived from a single clonogen. We believe the methodology can be directly applied to thyroid grafts to test the clonogenicity of FU, and will do so in the near future.

In summary, we are making significant progress in the major aims of this program, and believe continued support is fully justified. The methodology developed for these studies offers us a unique opportunity to characterize radiation carcinogenesis at the cellular level *in vivo*, and to gain information about the cancer progenitor cells that can not be otherwise obtained. The results will, we believe, be of importance in cancer risk assessment and cancer prevention, to thyroid physiology in general, and to our understanding of carcinogenesis.

Personnel and Effort 1/1/93-7/31/93

Dr. Clifton has continued to devote 25-30% of his effort to the overall direction of this project, and Dr. Gould has been available as needed. Mrs. Jane Barnes, project manager, is responsible for the day-to-day logistics and coordination of the laboratory personnel, runs the breeding program for the required hybrid and neonatal rats, and performs specialized technical work and development. She spends 50% of her time on the project. Mrs. Joan Mitchen, a highly skilled technician, spends 80% of her time on this project with Mr. Jinchuan Zhao, a graduate student. Mr. Zhao, spent 50% of his time on this project until June when he completed most of the requirements for the M.S. degree. He now spends 90-100% of his time on the project working on concentration of the thyroid clonogens by improved cell preparation, cell sorting and physiological treatment of the cell donors. He set up the large carcinogenesis study with Mrs. Mitchen's help, and is studying whether thyroid cells can be induced to form three dimensional structures in culture. Undergraduate aids Ms Brenda Robinson and, until summer recess, Ms Jill Schartner have spent 10 to 20% of their time helping with dishwashing, animal procedures, reagent preparation and general laboratory aid activities. Mr. Fernando Quintana, a graduate student in biostatistics, has spent about 5% of his time helping in data analyses and preparation of illustrations, and Mrs. Peggy Ziebarth has devoted 15% of her time to preparation of manuscripts and grant reports, as well as general office activities. It is of importance to note that Dr. Donna Williams-Hill has spent 100% of her time adapting and testing

the biological and molecular technologies for employment of SSRs in determination of the genotypes of multicellular structures in graft sites that will soon be applied to testing the clonogenicity of follicular units (FU) in thyroid grafts as well as of mammary structures in grafts. Furthermore, Dr. Yoshiya Shimada also returned to Madison from Japan for 45 days in November and December of 1992 during which he spent about 25% of his time in analyzing and collating the studies of age-dependent changes in clonogen numbers and radiosensitivity in immature rats. Drs. Hill and Shimada were largely supported by another grant during the course of these latter studies.

The principal investigator believes that we have complied with the requirements of the grant agreement.

Literature Cited

Note: Those titles with an asterisk are not from these laboratories.

- Cl85 Clifton, K.H. and Gould, M.N. Clonogen transplantation assay of mammary and thyroid epithelial cells. In: *Cell Clones: Manual of Mammalian Cell Techniques*, C.S. Potten, J.H. Hendry, eds., Edinburgh: Churchill Livingstone, pp. 128-138, 1985.
- Cl90 Clifton, K.H. The clonogenic cells of the rat mammary and thyroid glands: Their biology, frequency of initiation and promotion/promotion to cancer. In: *Scientific Issues in Quantitative Cancer Risk Assessment*. S.H. Moolgavkar, ed., Boston: Birkhauser, pp. 1-21, 1990.
- Co90 Committee on the Biological Effects of Ionizing Radiations. Health Effects of Exposure to Low Levels of Ionizing Radiation (BEIR V), Washington, DC: National Academy Press, 1990.
- Do93 Domann, F.E., Freitas, M.A., Gould, M.N. and Clifton, K.H. Quantifying the frequency of radiogenic thyroid cancer per clonogenic cell in vivo. *Radiat. Res.*, submitted, 1993.
- Gr92 Groch, K.M. and Clifton, K.H. The effects of goitrogenesis, involution, and goitrogenic rechallenge on the clonogenic cell content of the rat thyroid. *Acta Endocrinologica* 126:515-523, 1992.
- *Hi89 Hildreth, N.G., Shore, R.E. and Dvoretzky, P.M. The risk of breast cancer after irradiation of the thymus in infancy. *N. Engl. J. Med.* 321:1281-1284, 1989.
- Ka91 Kamiya, K., Gould, M.N. and Clifton, K.H. Differential control of alveolar and ductal development in grafts of monodispersed rat mammary epithelium. *Proc. Soc. Exper. Biol. Med.* 196:284-292, 1991.
- Ki93a Kim, N.D. and Clifton, K.H. Characterization of rat mammary epithelial cell subpopulations by peanut lectin and anti-Thy-1.1 antibody and study of flow sorted cells in vivo. *Exper. Cell Res.* 20:74-85, 1993.
- Ki93b Kim, N.D., Oberley, T.D. and Clifton, K.H. Primary culture of flow cytometry-sorted rat mammary epithelial cell (RMEC) subpopulations in a reconstituted basement membrane, Matrigel. *Exper. Cell Res.*, in press, 1993.
- Mu84 Mulcahy, R.T., Gould, M.N. and Clifton, K.H. Radiogenic initiation of thyroid cancer: A common cellular event. *Int. J. Radiat. Biol.* 45:419-426, 1984.

*To87 M. Tokunaga, M., Land, C.E., Yamamoto, T., Asano, M., Tokuoka, S., Ezaki, H. and Nishimori, I. Incidence of female breast cancer among atomic bomb survivors, Hiroshima and Nagasaki, 1950-1980. *Radiat. Res.* 112:243-272, 1987.

Wa88 Watanabe, H., Tanner, M.A., Domann, F.E., Gould, M.N. and Clifton, K.H. Inhibition of carcinoma formation and of vascular invasion in grafts of radiation-initiated thyroid clonogens by unirradiated thyroid cells. *Carcinogenesis* 9:1329-1335, 1988.

*We90 Weber, J.L. Human DNA polymorphisms based on length variations in simple-sequence tandem repeats. In: *Genetic and Physical Mapping, Vol. 1, Genome Analysis*, Davies, K.E., Tilghman, S.M., eds., Plainview, NY: Cold Spring Harbor Laboratory Press, pp. 159-181, 1990.

Figure 1. AD50 (alveolar dose-50%) values (open circles, upper graph) and total clonogens per six inguinal glands (closed circles, lower graph) of mammary glands of one to 12 week old untreated female rats.

Figure 3. Fraction of clonogens surviving following irradiation with 5 Gy at different ages. Calculated from data in Figure 2.

Figure 2. AD50 values of monodispersed cells from the mammary glands of untreated rats (lower closed circles) or 5 Gy g irradiated rats (upper open circles) at ages from one to 12 weeks.

Figure 4. Radiation dose-mammary clonogen survival response as a function of age at the time of irradiation *in vivo*. Rats were exposed when two weeks old (lower, squares), four weeks old (middle, diamonds), and eight weeks old (upper, circles)

DATE

FILMED

3 / 22 / 94

END