RADIOGENIC NEOPLASIA IN THYROID
AND MAMMARY CLONOGENS

Progress Report
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PROGRESS REPORT

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Abstract: We have developed rat thyroid and mammary clonogen transplantation systems for the study of radiogenic cancer induction at the target cell level in vivo. The epithelial cell populations of both glands contain small subpopulations of cells which are capable of giving rise to monoclonal glandular structures when transplanted and stimulated with appropriate hormones. Our results before the current grant term indicated that these putative clonogens are the precursor cells of radiogenic cancer, and that initiation, the first step in carcinogenesis, is a common event at the clonogenic cell level. Detailed information on the physiologic control of clonogen proliferation, differentiation, and total numbers is thus essential to an understanding of the carcinogenic process. Furthermore, we had found potentially significant difference between neutron and x-ray effects. X-ray exposure produced more mammary carcinomas when administered six weeks after the beginning of hormonally-induced proliferation and amplification of mammary clonogen numbers than the same dose given before hormone treatment; exposure to neutrons was equally and significantly carcinogenic when given at either time. Finally, knowledge of any endocrine interplay between follicles that develop from grafted thyroid clonogens and the hypothalamic-hypophyseal system is important in interpretation of neoplastic promotion-progression of radiation-initiated cells in such grafts. Since our last competitive renewal of support for this project, we have completed: a) investigations on the relationship between grafted thyroid cell number and the rapidity and degree of reestablishment of the thyroid-hypothalamus-pituitary feedback axis in thyroidectomized rats maintained on a normal diet or an iodine deficient diet; b) studies of the persistence of, and the differentiation potential and functional characteristics of, the TSH- (thyrotropin-) responsive sub-population of clonogens during goitrogenesis, the plateau-phase of goiter growth, and goiter involution; c) studies of changes in the size of the clonogen sub-population during goitrogenesis, goiter involution and the response to goitrogen rechallenge; and d) a large carcinogenesis experiment on the nature of the grafted thyroid cell number-dependent suppression of promotion/progression to neoplasia in grafts of radiation-initiated thyroid cells. We are currently testing new techniques for the culture, cytofluorescent analysis and characterization of mammary epithelial cells and clonogens in a parallel project, and have begun to apply similar technology to the thyroid epithelial cells and clonogen population. Data from these studies will be used in the design of future carcinogenesis experiments on neoplastic initiation by high and low LET radiations and on cell interactions during the neoplastic process.

Introduction: 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 effect 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. The ideal model for such studies would allow measurement of: a) the initial number of target cells at risk in vivo; b) the number of such cells which survive radiation exposure; and c) the fraction of surviving target cell; which

have suffered radiogenic neoplastic initiation (C180). The latter requires that each initiated cell give rise to an overt neoplasm in that initiated cells are not currently otherwise recognizable with certainty. Our model systems approximate these ideals.

Experiments with these systems completed before the current three year grant period indicated a) that both rat mammary and thyroid glands contain subpopulations of putative clonogenic epithelial cells which when transplanted and stimulated with appropriate hormones 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 when left in a tissue environment after irradiation before removal for transplantation assay, d) that at the target clonogen level, radiogenic initiation, the first step in the carcinogenic process, is a common event, and e) that the promotion-progression of initiated thyroid clonogens to overt cancer is suppressed by the addition of unirradiated thyroid cells to the transplant inoculum (C190, Appendix I). The thyroid clonogens have many of the characteristics of stem cells (C191, Appendix II).

Effort during the current grant year has been devoted to preparation for publication of the data from studies of the control of the thyroid clonogen sub-population and its role in thyroid physiology, hyperplasia and goitrogenesis, and in completion and analysis of the large carcinogenesis experiment on cell-cell interactions during neoplastic promotion-progression in grafts of radiation-initiated thyroid cells. With the aim of concentrating and further characterizing the clonogens, we have also begun new studies on the culture of clonogenic and non-clonogenic thyrocytes, and on the identification of thyrocyte sub-populations by cell surface antigens coupled with separation by fluorescence activated cell sorting techniques. Preparatory to further investigation to the difference in the carcinogenic response to neutrons as opposed to x-rays caused by hormonally-induced proliferation and expansion of the mammary clonogen sub-population (Ka89), we are investigating the concentration and proliferation rate of the mammary glands of rats of different ages. Progress in these areas is described below.

Dependence of reestablishment of the thyroid-hypothalamic-pituitary axis in thyroidectomized rats on the number of grafted thyroid cells, and on iodine intake(DO90; Appendix III): Groups of thyroidectomized rats were each sham grafted or grafted with 2.4×10^3 to 3.0×10^5 syngeneic rat thyroid cells. The grafted rats and intact ungrafted controls were then fed either a normal diet or a diet deficient in iodine. Blood samples were collected at weekly intervals for seven weeks, and the serum concentrations of T_3 (triiodothyronine), T_4 (thyroxine) and TSH were determined by radioimmunoassay. The results show the progressive restoration of hormone levels toward the norm in the thyroidectomized animals fed a normal diet and grafted with the largest cell numbers; those which received fewer grafted cells had progressively lesser and slower restoration. In intact ungrafted rats fed iodine deficient diets there was selective preservation of T_3 secretion. In thyroidectomized rats fed the low iodine diet and grafted with the highest thyroid cell doses, T_3 levels returned to near normal more rapidly than the other hormones. This selective hormone secretion pattern would have potential adaptive value under conditions of limited iodine availability.

Thyroid clonogen proliferative and differentiative potentials during goitrogenesis and goiter involution: We have measured the concentrations of clonogens within the cell populations of thyroid glands by their capacity to give rise to monoclonal multicellular structures (FU, follicular units) at the sites of transplantation of known numbers of monodispersed thyroid cells

in thyroidectomized recipient rats. The mean number of thyroid cells per graft site required to produce at least one FU in 50% of the sites (the FD50, follicle dose-50%) in such animals is an inverse function of the clonogenic fraction (Cl90, Appendix I); these two parameters are used to express clonogen concentrations. The formation of FU is, we believe, maximized by exposure of the clonogens to the elevated TSH levels consequent to thyroidectomy-induced thyroid hormone deficiency in such grafted rats. In similar grafts in intact euthyroid recipients, i.e. in the presence of normal TSH levels, about one fourth to one sixth as many of the grafted clonogens give rise to FU as in thyroidectomized rats.

The FD50 assay system has been used to quantitate changes in the clonogenic fractions of thyroid cells during goitrous growth, in "quiescent" goiters and during goiter involution (Gr92a, Appendix V). Goiters were induced either by aminotriazole treatment of rats given a normal diet (ATA/ND) or by perchlorate treatment of rats given a low iodine diet (KClO₄/LID). ATA is an inhibitor of thyroid peroxidase, thereby blocks the polymerization of tyrosine and organification of iodide, and hence prevents T₃ and T₄ synthesis. The perchlorate ion competitively inhibits the uptake of inorganic iodide at the plasma membrane level and thus also interferes with hormone production. Rapid goitrous growth occurred during the first six weeks of treatment with either goitrogen after which gland growth plateaued. TSH levels peaked at about the same level at six weeks of treatment with the two regimens and remained elevated as long as the goitrogens were administered. Interestingly, total clonogen numbers per gland did not increase during the period of rapid goitrous growth; clonogens then began to increase rapidly as total gland growth slowed to reach the growth plateau. Plateau-phase goiters induced by ATA/ND were more than twice as large as those induced by KClO₄/LID.

Importantly, suspensions of cells prepared from either goiter type contained significant numbers of clonogenic cells as measured by transplantation and FU formation. This was true despite the fact that the clonogenic fractions of goiter cell preparations were significantly less than those of untreated control glands. Furthermore, the clonogenic fractions of cells from ATA/ND goiters were significantly greater than those of KClO₄/LID goiters. When transplanted to recipient rats, the clonogens that persisted in both goiter types responded to TSH stimulation in a fashion indistinguishable from that of clonogens from untreated glands. For example, the efficiency of FU formation in grafts of suspensions of goiter cells and of normal thyrocytes in euthyroid recipients was reduced about the same extent from that in grafts of the same cell suspensions in thyroidectomized recipients. The FU formed in the graft sites of clonogens from goiters were not detectably different morphologically or functionally from untreated clonogens (Gr92a, Appendix V).

As discussed above, studies of thyroid hormones and TSH in the sera of thyroidectomized graft recipients showed that reestablishment of thyroid function depended on the number of thyroid cells from untreated rats transplanted per recipient (Do90, Appendix III). Although this dependence was clear when the donors were euthyroid rats, this appears to be due to the consistency of the clonogenic fractions of such rats. For example, groups of thyroidectomized rats were transplanted with series of thyroid cell concentrations prepared from goiters induced by one or the other of the two treatments or from normal glands. The cell concentrations in these suspensions were chosen to contain the same numbers of thyroid clonogens; the total thyroid cell concentrations in these suspensions thus varied according to the previous treatment of the donor rats. Virtually the same serum T₃, T₄ and TSH levels were observed eight weeks after grafting 10 or 80 or 640 clonogens, respectively, from either goiter type per recipient as after grafting the same clonogen numbers from normal euthyroid donor glands. The results unequivocally show that thyroid hormone secretion by such grafts is dependent on the numbers

of clonogens grafted, irrespective of the total numbers of thyroid cells per graft or the previous treatments of the donor rats (Gr92a, Appendix V).

Finally, FD50 assays of cell suspensions from involuting goiters show preferential preservation of the clonogen sub-population. Total clonogen numbers per gland were not significantly altered despite the massive cell loss that occurred during involution (Gr92a, Appendix V).

In sum, these data are highly supportive of the conclusion that goiters contain sub-populations of pre-existent stem cell-like clonogens which retain their responsiveness to TSH and their proliferative and differentiative potentials during goiter growth, the goiter growth plateau phase, and goiter involution. It is very likely that these cells are the progenitor sub-population from which tumors arise after long term exposure to goitrogens or after radiation exposure.

Clonogenic cell populations during goitrogenesis, goiter involution and goitrogen rechallenge: A series of experiments were performed to determine the fates of the sub-populations of clonogens during initial goitrogenesis with ATA/ND or KClO_4 /LID treatment, following withdrawal of goitrogens and during goitrogen rechallenge with the aim of gaining further insight into the pathogenesis of thyroid neoplasia (Gr92b, Appendix VI). The clonogenic fraction of cells from untreated age-matched control thyroid glands was 5.8×10^{-3} cells; that of ATA/ND-induced goiters was about 20-fold smaller (1.9×10^{-4} cells) and of KClO_4 /LID-induced goiters, about 60-fold smaller (9.8×10^{-5} cells) than the controls. With continued ATA/ND treatment after goitrous hyperplasia had ceased, the clonogenic fraction increased an order of magnitude to 2.0×10^{-3} cells; continued KClO_4 /LID treatment had little effect.

As noted above, the changes in clonogenic fractions induced by both goitrogenic regimens were reversed during involution. Rechallenge of the involuted glands with a second exposure to the goitrogens induced changes in the clonogenic fractions similar to those which occurred during the first treatment period. Clonogenic fractions and total clonogens per gland both decreased during the first six weeks of rechallenge. When gland size plateaued both parameters increased markedly in ATA/ND-induced goiters, and less markedly in KClO_4 /LID-induced goiters (Gr92b, Appendix VI).

It is important to note that the clonogenic fraction in the glands of ATA/ND-treated rats were greater than those of KClO_4 /LID-treated rats at all time points studied despite similar TSH values. The two goitrogenic regimens which act through different biochemical mechanisms thus affect the goitrogenic fractions in different ways. Despite this fact, the changes in the clonogen sub-populations induced by both goitrogens are reversed following withdrawal of treatment.

The behavior of the thyrocytes during goitrogen-induced growth, the growth plateau, goiter involution and goitrogenic rechallenge are consistent with the existence of three normal thyroid cell sub-populations: a) a small population stem-like clonogenic cells that retain the capacity to divide in response to TSH stimulation, b) a larger population of transit cells that can pass through two to five mitoses in response to TSH, and c) a larger population of terminally differentiated thyrocytes that can no longer respond to mitogenic stimulation by TSH (Gr92a, Gr92b; Appendices V, VI). The clonogenic cell population is preferentially preserved under periods of stress, and its component cells appear to have been unaltered by goitrogen exposure when they are placed in a common stimulatory environment.

Nature of cell-cell interactions in thyroid grafts during promotion/progression to neoplasia: In previous studies, we had observed that the incidence of carcinomas per irradiated clonogen in grafts of thyroid cells in thyroidectomized recipients maintained on a low iodine diet

was inversely related to the total number of cells grafted (Mu84). Furthermore, when unirradiated cells were added to irradiated cell grafts, final carcinoma incidence was further suppressed (Wa88). We reasoned that this suppressive effect could be mediated either: a) systemically by partial reestablishment of the thyroid-hypothalamic-pituitary axis by the grafted cells, the rapidity and degree of which is grafted cell number-dependent as demonstrated above (Do90; Appendix III); b) locally by direct suppressive thyroid cell:cell interactions within the grafts as reported in the tracheal epithelium by Terzaghi-Howe (Te87); or c) both. A large carcinogenesis experiment was thus designed to distinguish among these possibilities (Table 1) (Do92; Appendix IV).

The keys to this experimental design are: a) that each rat in control groups C, CE, and CM received the same total number of morphologically intact surviving thyroid clonogens as each rat in their respective paired irradiated cell recipient experimental groups R, RE and RM, and b) that within a given experimental group, every grafted clonogen number was transplanted in an animal that received the same total clonogen numbers. This design ruled out any differences in grafted cell number-dependent neoplastic promotion-progression by TSH which might have occurred in the previous experiments in which recipients of low cell numbers per graft had fewer total grafted cells than those with high cell numbers per graft. Groups CE and RE were designed to show any systemic tumor-suppressive effect. Groups CM and RM were included to investigate the possibility of promotion-progression suppressing local cell-cell interactions.

Table 1: Design of experiment to investigate hormonal and local inhibition of promotion/progression in radiation-initiated thyroid clonogens (Do92, Appendix IV).

Group	"Target" Clonogen Irradiation	<u>Transplantation Sites**</u>				
		First	Second	Third	Fourth	Fifth
		<u>Numbers of "Target" Clonogens/"Non-Target" Clonogens</u>				
C	0	90/0	30/0	10/0	3/0	0/0
R	5 Gy	90/0	30/0	10/0	3/0	0/0
CE	0	90/0	30/0	10/0	3/0	0/1400
RE	5 Gy	90/0	30/0	10/0	3/0	0/1400
CM	0	0/383	0/383	0/383	0/383	0/0
RM	5 Gy	90/350	30/350	10/350	3/350	0/0

*"Target" clonogens are irradiated clonogens and their paired unirradiated clonogen controls. "Non-target" clonogens are unirradiated clonogens added to test hormonal and local inhibition of promotion/progression in "target" clonogens.

**Transplantation sites were rotated during grafting such that, for example, the interscapular pad might be the first site in rat #1, the second in rat #2, the third in rat #3, etc. The experiment was set up in 20 replicate runs to accumulate 100 graft sites for each combination of transplanted clonogens.

Briefly, the results of this experiment are (Do92, Appendix IV):

- 1) As found previously, the incidence of radiogenic initiation is very high. Estimates based on the frequency of cancer in the graft sites that received three irradiated or control clonogens in groups R and C indicate that about one of five clonogens was initiated. As we have suggested (CI90, CI91; Appendices I,II), such frequencies strongly suggest that initiation is a generic term covering any event, genetic or epigenetic, which alters the probability that a cell or its progeny will give rise to cancer. Such events may include alterations in growth regulatory genes, but perhaps more frequently are radiation induced processes that are not necessarily mutational. Among the latter may be the induction of SOS-like error-prone repair, alterations in DNA methylation which influence gene function, and homologous chromatid exchanges.
- 2) As previously found (Mu84, Wa88), 5 Gy low LET radiation to the clonogens before transplantation significantly increased the carcinomas per 10^4 graft weeks at each cell dose in group R as compared to those in the unirradiated cells in group C, and similarly in group RE as compared to group CE (Do92, Appendix IV).
- 3) There were no detectable grafted cell number-related differences in carcinoma incidence or latency or in graft size in groups RM or CM due to the high cancer incidence from the large number of unirradiated clonogens in each graft site.
- 4) More carcinomas occurred per 10^4 graft weeks at each cell dose in group R than in group RE. Differences in tumor incidence per graft week between groups C and CE were not significant. Irrespective of histopathology, the diameters of the grafts per hundred graft weeks increased directly and significantly with grafted clonogen numbers in groups R, C, RE and CE. At each cell dose, graft diameters in R exceeded those in C, and those in RE those in CE. The serum TSH titers in groups RE and CE, though significantly greater than normal, were significantly less than in groups R and C.

The differences in tumor incidence and graft diameters among the above experimental groups are thus very likely due to less intense promotion-progression in groups RE and CE than in groups R and C as a result of thyroid hormone release from FU formed by the large number of unirradiated grafted cells in the fifth graft sites in RE and CE. We conclude that there was a grafted cell number-related suppression of neoplastic promotion-progression mediated by systemic inhibition of TSH secretion by thyroid hormone released from FU that developed in the grafts even in the presence of iodine deficiency (Do92, Appendix IV).

Suppression of promotion-progression by systemic inhibition of TSH secretion does not, however, account for the relatively lower incidence of carcinomas per graft-week per grafted cell in the high cell dose sites as compared to the low cell dose sites of groups R and RE, e.g. a four- to six-fold difference in carcinomas versus a 30-fold difference in grafted cell number. There are two inherent sources of uncertainty which we believe could account for these differences in observed cancer incidences (Do92, Appendix IV). First, during transplantation, the intact clonogens may be assumed to be randomly distributed in the inoculum. Hence, at a given mean grafted clonogen number per site, there will be a normal distribution of clonogen numbers per site surrounding the mean. At low cell numbers per site, this will include some sites with no clonogens, and hence no possibility for tumor formation. Conversely, there will be some sites with more than the mean number of clonogens and thus a slightly higher probability of cancer formation than the mean. Furthermore, the cancer incidence was conservatively derived; i.e. only one carcinoma was scored per graft site. In those sites with more than one grafted clonogen, more than one carcinoma might arise; indeed in those sites

grafted with many irradiated clonogens, the probability that more than one carcinoma arose or would have arisen is high.

A mathematical correction (Ha79) based on the assumption of normal distributions both of the clonogens in the inocula during transplantation and of the progression of initiated clonogens to cancer can be applied to the data to estimate the "true" number of carcinomas from the observed number. When the data are so corrected, the cell dose dependent differences in the cancer incidences per grafted clonogen within a given experimental group are no longer significant.

We conclude that our results do not support the hypothesis that local grafted cell number dependent cell-cell interactions within the graft suppress neoplastic promotion-progression in this system (Do92; Appendix IV). Our results do not, however, exclude the latter possibility. It should be noted that a grafted cell number dependent suppression in cancer development from irradiated mammary clonogens has recently been observed; this effect is not readily attributable either to cell distribution in the inocula or to hormonal feed-back to the hypothalamus-pituitary from the grafted cells (Kamiya and Clifton, unpublished).

Mammary clonogen concentrations, total numbers and proliferation rate as a function of age: As previously noted, we have found that when rats were irradiated with 40 cGy x-rays before beginning of treatment with high prolactin (Prl+) and low glucocorticoids (Glc-), mammary cancer incidence was insignificantly different from that in unirradiated controls. However, if irradiation was postponed until 48 days after beginning of Prl+/Glc- during which mammary cell proliferation resulted in about a seven fold increase in total cells and a fivefold increase in clonogens, carcinoma formation was significantly increased. In contrast, irradiation of rats with 10 cGy neutrons before Prl+/Glc- or 48 days after beginning Prl+/Glc- resulted in the similar significant increases in carcinomas above control (Ka89). This difference in the carcinogenic effect of low LET x-rays as opposed to high LET neutrons could result from a greater dependence of neoplastic initiation frequency of x-rays on target cell number and/or proliferation (Ka89). Hence, there may be differences in carcinogenic efficiency between radiation types related to age associated differences in clonogen numbers and/or proliferation rates. Preparatory to setting up long term high and low LET studies of the relationship of carcinogenesis to age at the time of exposure, we have determined the total recoverable mammary cell numbers (Fig. 1A) and estimated the AD50 values, i.e. the number of grafted cells required per transplant site to produce at least one AU in 50% of the sites (Fig. 1B), in the glands of rats at intervals from one week to twelve weeks of age (Shimada and Clifton, unpublished data). The clonogenic fraction, is inversely related to the AD50; a high AD50 indicates that the clonogenic fraction is small (Cl80, Cl90; Appendix I). We are currently investigating the proliferation rates and the radiosensitivity of the clonogens at intervals over the same age span.

Sorting and culture of sub-populations of thyrocytes; concentration of thyroid clonogens: In separately supported parallel studies of the characteristics of rat mammary clonogens and their neoplastic initiation by radiation, we have used fluorescence activated cell sorting to derive cell sub-populations that differ in their plasma membrane antigens. Four subpopulations have been routinely obtained: cells positive to FITC-labelled peanut lectin alone (PNA+), cells positive to phycoerythrin-labelled antibodies to the lymphocyte marker Thy 1.1 only (Thy 1.1+), those positive to both reagents (B+), and those unstained by either (B-). The PNA+ sub-population

Figure 1

1.A: AD50 values, i.e. the numbers of grafted cells required per site to produce at least one AU in 50% of the sites, of cells from glands of female F344 rats from one week through twelve weeks of age. Clonogenic fractions are inversely related to AD50 values (C180, C190; Appendix I). Vertical bars are standard errors of repeat determinations.

1.B: Millions of mammary cells recovered from six glands (two inguinal fat pads) from female F344 rats of different ages. Vertical bars as in Figure 1.A.

Figure 2

2.A: Total (left) and windowed (right) distribution of PNA+mammary epithelial cells (lower right quadrant, windowed), and PNA-cells (lower left quadrant, windowed).

U3:JZ04032004

Quad Stats

File: U3:JZ04032004 Sample: THYROID DIR 50 UL 012

Date: 4/ 3/92 Gate G1= R1

Parameters: FL1-H(LQG), FL2-H(LQG) Quad Location: 86.59,

Total= 5000 Gated= 1650

Quad	Events	% Gated	% Total	Xmean	Ymean
1 UL	0	0.00	0.00	1.00	1.00
2 UR	0	0.00	0.00	1.00	1.00
3 LL	858	52.00	17.16	29.18	3.72
4 LR	792	48.00	15.84	253.52	5.45

2.B: Total (left) and windowed (right) distribution of Thy 1.1+mammary epithelial cells (upper left quadrant, windowed) and Thy 1.1-cells (lower left quadrant, windowed).

U3:JZ04032007

Quad Stats

File: U3:JZ04032007 Sample: THYROID DIR 8 UL 015

Date: 4/ 3/92 Gate G1= R1

Parameters: FL1-H(LQG), FL2-H(LQG) Quad Location: 86.59,

Total= 5000 Gated= 1085

Quad	Events	% Gated	% Total	Xmean	Ymean
1 UL	431	39.72	8.62	4.95	539.75
2 UR	0	0.00	0.00	1.00	1.00
3 LL	654	60.29	13.08	9.82	25.57
4 LR	0	0.00	0.00	1.00	1.00

2.C: Total (left) and windowed (right) distribution of Thy 1.1+mammary epithelial cells (upper left quadrant, windowed), PNA+cells (lower right quadrant, windowed), B-cells (negative for both, lower left quadrant), and B+cells (positive for both, upper right quadrant).

U3:JZ04032010

Quad Stats

File: U3:JZ04032010 Sample: THYROID DIR 50UL/8UL 018

Date: 4/ 3/92 Gate G1= R1

Parameters: FL1-H(LQG), FL2-H(LQG) Quad Location: 86.59,

Total= 5000 Gated= 990

Quad	Events	% Gated	% Total	Xmean	Ymean
1 UL	367	37.07	7.34	22.00	520.58
2 UR	86	8.69	1.72	265.38	427.91
3 LL	403	40.71	8.06	16.65	32.48
4 LR	134	13.54	2.68	322.74	28.97

includes the clonogens as measured by their capacity to give rise to AU on transplantation. The Thy 1.1+ sub-population is thought to be predominantly comprised of myoepithelial cells, and has very low clonogenic capacity on transplantation. By using a differential filtration procedure during preparation of the monodispersed cell suspension followed by flow sorting, we have concentrated the mammary clonogens by twenty-thirty fold as compared to the first suspensions we prepared.

We are currently investigating the applicability of the same staining and sorting procedures to thyroid cell suspensions. The results of one such preliminary run show that ~13% of monodispersed thyrocytes were PNA+ (Fig. 2c: horizontal axis, lower right quadrant), ~37% were Thy 1.1+ (Fig. 2c: vertical axis, upper left quadrant), ~41% were B- (Fig. 2c: lower left quadrant) and ~9% were B+ (Fig. 2c: upper right quadrant). The clonogenic fraction of thyrocytes made directly from minced normal glands of young male rats is $1.0-1.5 \times 10^{-2}$. If the clonogens are confined to the PNA+ fraction and survive the manipulations, sorting with these reagents might achieve a tenfold concentration, i.e. a clonogenic fraction of 0.1. Such a step would render the further characterization of the clonogens far less difficult.

We successfully cultured thyroid cells in media containing small amounts of serum including 0.5% heat inactivated thyroidectomized rat serum for three or more weeks. Like most investigators, we have been unsuccessful at sub-passing such cells in culture, and their clonogenic capacity when transplanted also decreased with time. Again, in the companion mammary cell project, we have been able to culture mammary cells and cell sub-populations in a serum-free medium supplemented with hormones. This medium, KDM, was developed for the culture of keratinocytes. Our first attempt to culture thyrocytes in this medium resulted in sufficient success to encourage us to pursue this methodology. We have not yet tested the effects of addition of hormones or other growth factors. In both projects, we will further pursue the possibility of formation of three-dimensional glandular structures in matrix-containing media such as Matrigel and/or rat tail collagen.

Closing comments: We believe that the concepts that the thyroid contains TSH-responsive clonogens that are the ultimate source of new hormone-secreting cells as well as the progenitor cells in carcinogenesis, and that neoplastic initiation is a frequent event in these cells, will alter the general understanding of both normal thyroid physiology and of radiation carcinogenesis. As noted above, new cell culture and cell sorting methodologies have been adapted and found useful for the parallel mammary gland project. They are currently being tested and adapted for this project. We expect that they will play a significant role in the further characterization of the thyroid clonogens and their role in thyroid neoplasia. We believe that continuing short term studies of clonogen physiology in parallel with long-term carcinogenesis studies is an efficient way to proceed, and that the presence of the second parallel mammary clonogen system in which major observations can be cross-checked for broader applicability significantly strengthens the credibility of our findings. The techniques developed in or adapted to these laboratories represent a unique armamentarium for the study of thyroid cancer development in particular, and of radiogenic carcinogenesis in general.

Personnel and effort 1/1/91-5/31/91: During this period, the P.I. spent 25% of his time on this project, and Professor Gould has been available as needed. Mrs. Jane Barnes, laboratory manager, has spent 50-60% of her time in planning of the logistics of this project, in the day-to-

day coordination and in specialized technical work and in technique development. Mrs. Joan Mitchen, a highly skilled technician, has spent 80-85% of her time on the project helping with all aspects of the technology. She has learned the process of entering data into computer spread sheets for analysis, and has played an essential role in this project. Dr. Kevin Groch, who received his PhD in November 1990, is currently doing postdoctoral work in Professor Gould's laboratory, and has thus been available to complete the manuscripts on thyroid clonogen kinetics and function during goitrogenesis. Dr. Frederick Domann completed his PhD in May 1991. He has spent considerably more than the 50% of his time suggested by his stipend level on this project during this period. Since the end of August 1991, Dr. Domann has been a post-doctoral associate with Prof. Tim Bowden at the University of Arizona. Mr. Jinchuan Zhao, an excellent young student, joined our laboratory in September of 1990 to work toward the PhD, and is spending 50% of his time on the project. Yoshiya Shimada, Ph.D. Tokyo University and a Staff Member of the Institute for Radiological Sciences, Chiba, Japan, has spent about 50% of his time on this project since 1 September 1991. He will leave in mid August, but plans to return for at least one more year to continue his studies here. Dr. Shimada is an excellent young investigator.

The principal investigator believes that we have complied with the requirements of the grant in all respects.

References:

A. Papers Cited

Note: References marked with an asterisk are included as Appendices I-VI for reference by reviewers.

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B. Other Grant-Related Publications, 1990-92

- Cl91b Clifton, K.H., Groch, K.M. and Domann, F.E. Thyroid clonogen biology and carcinogenesis. In: *Chemically-induced Cell Proliferation: Implications for Risk Assessment*, B.E. Butterworth, T.J. Slaga, W. Farland, M. McClain, eds., Wiley-Liss, Inc., New York, pp. 173-183, 1991.
- Cl91c Clifton, K.H. Aspects of investigation of radiation carcinogenesis in human cells. *British Institute of Radiology Report* (in press).

Go91

Groch, K.M. and Clifton, K.H. The plateau phase rat goiter contains a sub-population of TSH-responsive epithelial cells capable of clonal proliferation following transplantation. Acta Endocrinologica 126:85-96, 1992.

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