Thyroid Cell Proliferation in Rats and Induction of Tumors by X-rays

Konstantin Christov

The National Center of Oncology, Academy of Medicine, Plovdivsko pole 6, Sofia-56 Darvenitza, Bulgaria

SUMMARY

There are very few proliferating cells in the thyroid gland of normal adult rats, as measured by the labeling and mitotic index. One-tenth \% 4-methyl-2-thiouracil in drinking water induced an exponential increase of thyroid weight after a lag phase of 2 days; the increase continued for 8 days and was followed by a plateau phase. The following sequence of events was found for the number of dividing follicular and stroma cells as well as for DNA synthesis: no significant changes during the 1st 2 days, a sharp increase between the 2nd and 8th days, a decrease between the 8th and 14th days, and an almost constant flow until the 24th day. Three-hundred rads of X-rays given to a nonproliferating thyroid gland induced tumor growth in 25\% of the animals 18 months after irradiation. The same dose of irradiation, applied to a proliferating thyroid gland, increased the tumor incidence to 30\% when administered in the lag phase, to 75\% when administered at the peak of the proliferating phase, and to 62.5\% when administered at the plateau phase. Subsequent treatment of irradiated animals with 4-methyl-2-thiouracil enhanced the number and the size of the thyroid tumors and lead to the occurrence of more carcinomas than appeared in animals treated with X-rays only or 4-methyl-2-thiouracil only.

INTRODUCTION

There is ample evidence that a prolonged increase in TSH\(^3\) circulation level leads to the development of thyroid tumors (9, 12). When carcinogens, radioactive iodine, or X-rays are applied before the onset of TSH stimulation, tumor incidence increases and latent period decreases (4, 14, 18). However, in some experiments a carcinogenic stimulus was applied to adult thyroids displaying minimal proliferative activity (7, 23, 24) and, 24 hr or more later, cell proliferation was stimulated in the gland. For several years we have undertaken studies to elucidate the effect of cell proliferation on the “initiating” phase of thyroid carcinogenesis. X-rays, AAF, and N-methylnitrosourea were used as carcinogens; they were applied either to thyroid cell systems that have a physiologically high level of cell division (e.g., fetuses and suckling animals; K. Christov, unpublished data) or to adult thyroids in which proliferation was stimulated by goitrogens (5).

The present study aims at determining the carcinogenic effect of X-rays on a thyroid cell system stimulated towards proliferation at the time of irradiation or afterwards. Treatment of the animals with MTU lead to an increase in the number of the proliferating cells in a typical manner: lag phase for 2 days, exponential phase for 6 days, decreasing phase for 4 to 6 days, and an almost constant value until the 24th day (5–7, 29). The animals were irradiated during the lag phase, at the peak of the proliferative wave, and after the decrease in the number of proliferative cells. Groups of animals were irradiated and subsequently maintained on MTU until the end of the experiment.

MATERIALS AND METHODS

Rats. A total of 286 male 3-month-old Wistar rats (Iwanovas, Kisslegg, West Germany) weighing 140 ± 23 g were used. The animals were housed in plastic cages in groups of 3 and fed on stock Altromin R diet (Altromin R, GmbH, West Germany). They were divided into 2 groups, one for studying the pattern of the thyroid cell proliferation and the other for testing the carcinogenic effect of X-rays. MTU. One-tenth \% of MTU (Fluka AG Chemische Fabrik, Buchs, Switzerland) was given in the drinking water ad libitum.

\([\text{methyl-}^3\text{H}]\text{Thymidine}\). [methyl-\(^3\text{H}\)]Thymidine (Radiochemical Centre, Amersham, England; specific activity, 6.7 Ci/mmmole) was injected i.p. in a dose of 1 \(\mu\)Ci/g body weight.

Irradiation. The animals were irradiated in the neck region with a single dose of 300 rads X-rays (150 keV, 20 ma, 80 rads/min) and protected by a 0.5-cm lead whole-body shield, which had a 1.5-cm diameter opening located over the thyroid gland. The exact position of the thyroid gland was determined in a preliminary study by dissection on several animals. X-Rays were administered 1 day before and 1, 8, and 20 days after the beginning of MTU treatment (Chart 2). In the animals irradiated and subsequently
treated with MTU, the goitrogen was started 2 hr after irradiation.

**Histological Examination.** Forty-five min before the animals were killed, they were given injections of \(^{[3}\text{H}]\text{thymidine (1 }\mu\text{Ci/g body weight). The thyroid gland was dissected from the surrounding tissues and weighed on an analytical balance. One lobe of the gland was placed in 10% neutral formalin for histological examination and autoradiography; the other lobe of the gland was stored in a refrigerator at -30° for estimation of DNA synthesis. For autoradiography the slides were dipped in a 50% solution of Ilford K5 emulsion. After exposure (up to 4 weeks as necessary) they were developed in a Kodak D19 developer and subsequently stained with hematoxylin and eosin. To study the number and structure of the thyroid tumors, the animals were killed between the 15th and 18th month after irradiation. The thyroids were cut in numerous parallel 5-µm sections. Small samples were also used for electron microscopy, cytospectrophotometry, and enzymecytochemistry (K. Christov et al., manuscript in preparation). Looking for metastases, we also examined specimens from the lung and the lymph nodules of the neck region, as well as from all other organs displaying pathological changes.

**Labeling Index.** The labeling index gives the number of DNA-synthesized cells in a cell population. The average values for the labeling index were obtained from the individual values of 3 glands examined after the counting of more than 100,000 cells/gland.

**Mitotic Index.** The mitotic rate was expressed as the number of meta- and anaphases per 10,000 cells.

**DNA Measurement.** The thyroid lobes stored in the refrigerator were homogenized and the acid-soluble fraction was extracted by cold perchloric acid. After digestion of the precipitate in boiling water for 30 min, 0.1 ml of supernatant was transferred to scintillation vials containing toluene-POPOP-PPO liquid scintillation system. From the same supernatant, 0.5 ml of the hydrolysate was also used for estimation of DNA by the diphenylamine method (2). The radioactivity was measured by a Packard machine. The specific activity of DNA was expressed as cpm/µg DNA.

**RESULTS**

**Pattern of Thyroid Cell Proliferation**

The thyroid cell proliferation was studied by changes in the thyroid weight, \(^{[3}\text{H}]\text{thymidine labeling index, mitotic index, and DNA synthesis.**

**Thyroid Weight.** As Chart 1 indicates, the thyroid weight underwent no increase during the 1st 2 days, an exponential growth between the 2nd and 10th days, followed by a plateau phase persisting until the 24th day (as long as the experiment lasts).

**Labeling Index.** In the thyroid gland of 90-day-old animals, only 0.11% of the follicular cells were found in the S phase of the cell cycle. After stimulation of thyroid cell proliferation by MTU, the number of dividing cells showed no changes during the 1st 2 days, an exponential growth between the 2nd and 8th days, a decrease between the 8th and 14th days, and an almost constant value until the 24th day. The number of labeled follicular and stroma cells on the top of the proliferative wave (8 days after starting MTU administration) was about 60 times higher than in the control animals. The stroma cells seemed to respond earlier to MTU stimulus and had a proliferative capacity higher \((p < 0.01)\) than that of the follicular cells (Chart 2).

**Mitotic Index.** The curve of the mitotic index was similar to that of the labeling index: no changes during the 1st 2 days, a sharp increase between the 2nd and 4th days, and a decrease between the 8th and 20th days (Chart 3).

**DNA Synthesis.** The changes in the specific radioactivity of the thyroids in MTU-treated animals were similar to the changes in the labeling index curve: no increase during the 1st 2 days, a sharp rise between the 2nd and 8th days, a decrease between the 10th and 14th days, and a small increase during the next 10 days (Chart 4).

**Carcinogenic Effect of X-rays**

**Mortality Rate of the Animals.** The control animals survived until the end of the experiment (18th month). The mortality rate among irradiated animals ranged between 4 and 25% (Table 1). Two of the animals died bearing large mediastinal lymphomas; the other developed inflammation of the lungs. The causes of death in the animals treated with MTU or irradiated and subsequently treated with MTU were pneumonia, large tumors of the thyroid gland, or lymphomas.

**Yield of the Tumors.** The number and structure of the thyroid tumors are shown in Table 2. It is evident that in the control animals no spontaneous tumors occur. When irradiation was used on nonproliferating thyroids or during the...
Chart 2. Labeled follicular and stroma cells after treatment of the animals with MTU. Points, mean value of the labeling indices of 3 animals.

Chart 3. Mitotic index gives the number of meta- and anaphases per 10,000 cells. Points, mean values of the individual values of 3 animals.

DISCUSSION

Pattern of Cell Proliferation. It is well known that MTU inhibition of thyroid hormone synthesis causes an increase in TSH level in the blood. TSH acts as a physiological stimulator on the thyroid gland (22). Our data indicate that, after rats are treated with MTU, the weight, number of proliferating epithelial and stroma cells, mitotic rate, and DNA synthesis in the thyroid gland all undergo a similar pattern of changes. Thus the growth curve of the thyroid weight consists of a lag, an exponential, and a plateau phase. The changes in thyroid weight during the 1st 2 phases correlate with the increase in the number of proliferating cells and DNA synthesis as well. However, during the plateau phase of the thyroid weight a decrease in the rate of proliferation and DNA synthesis occurs. Measurements on cell density (15) and cell volume (21) of the follicular cells revealed that the decline in the number of proliferating cells during the plateau phase of the thyroid weight is concurrent with an increase in the mean volume of the cells and a decrease of their density. The biphasic growth curve of the thyroid with a phase of cell hypertrophy lasting 2 weeks and a phase of cell proliferation also persisting for 2 weeks was described by Crooks et al. (11) but not confirmed in this study. The sharp increase in the labeling index between the 2nd and 8th days after the beginning of MTU administration might have resulted either from entrance of the nonproliferating cell population into the cell cycle or from shortening of the generation time, if we assume that all cells of the thyroid gland have undergone proliferation (6, 20, 23, 24, 29).

Carcinogenicity of X-rays. Our data indicate that irradiation of the thyroid gland is more effective in tumor induction when applied at the time of the highest number of dividing cells than at a time of nonproliferation (before or 1 day after start of MTU treatment). It is evident from the results in the different experimental groups that the higher the number of proliferating cells at the time of irradiation, the higher the incidence of thyroid tumors. Moreover, most
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Table 1
Tumor yield of experimental groups

Cell proliferation in the thyroid gland was induced by MTU treatment. X-Ray irradiation was given on nonproliferating thyroids or on thyroids stimulated by MTU to proliferate. In all groups the animals were killed between the 15th and 18th month after the beginning of the experiment.

<table>
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<th>Treatment group</th>
<th>No. of animals</th>
<th>No. of animals with tumors</th>
<th>%</th>
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<td>5 MTU (Day 1) + X-rays</td>
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<td>6 MTU (Day 8) + X-rays</td>
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Table 2
Number and histological structure of the thyroid tumors induced

The animals were killed between the 15th and 18th month after the onset of the experiment.

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* Foll., follicular; Pap., papillary; Trab., trabecular; Med., medullary.

malignant tumors appear in the animals irradiated at the top of the proliferative wave (Table 2). Our results also indicate that a subsequent treatment of irradiated animals with MTU increases tumor incidence. The analysis of data also shows that the combined effect of irradiation and MTU treatment on the nonproliferating thyroids is similar to the sum of tumors produced by MTU alone and by irradiation alone (Table 1). However, data show that in many cases it is not simply an additive effect, but that a synergism occurs, inasmuch as the number of tumors produced by the combination of both treatments is higher than the sum of their individual effects (13).

In a similar study we treated the animals with AAF, instead of irradiation, during the different phases of the thyroid proliferating wave (on the 1st, 8th, or 20th day after the start of MTU treatment). Most tumors occurred when the carcinogen was given at the top of the proliferative wave or when the animals received additional MTU (5).

The multistage hypothesis of carcinogenesis postulates a primary "initiating" process whereby normal cells are converted into "latent" tumor cells. Assuming that an alteration in DNA is necessary for carcinogenesis, then the increased oncogenicity of X-rays and AAF in the proliferating thyroids suggests that replicating DNA is more accessible than nonreplicating DNA to carcinogenic stimuli. In the latent tumor cells the alteration in DNA molecules must be replicated and thus fixed in a stable form before repair occurs (3, 17, 27). Subsequently, to evolve into neoplasia the latent tumor cells require a "promoting" action (1). On the basis of this hypothesis, it might be suggested that, in tumorigenesis of the thyroid gland, initiation can be effected by irradiation and promotion can be done by TSH. Since TSH alone (without any other apparent initiator) can induce tumors, it is possible that, besides being a promoter, TSH can also behave as an initiator. On the other hand, the mutagenic background irradiation or accidental errors in DNA replication should not be excluded as significant "spontaneous" initiators, in which case TSH would still act only as a promoter (13, 14).

Our data on the effect of cell proliferation in the initiating
Acknowledgments

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References


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Fig. 1. One trabecular and 6 follicular adenomas of the thyroid gland 12 months after irradiation and subsequent treatment of the animals with MTU. H & E, ×100.

Fig. 2. Follicular carcinoma with infiltrative growth into large vessels of the gland capsules in an animal irradiated at the top of the proliferative wave and subsequently treated for 16 months with MTU. H & E, ×100.

Fig. 3. Papillary carcinoma 18 months after irradiation of the thyroid gland during the plateau phase of the proliferative wave and subsequent treatment of the animal with MTU. H & E, ×400.

Fig. 4. Thyroid carcinoma growing into the soft tissues of the neck region. An animal irradiated at the top of the proliferative wave and subsequently treated for 18 months with MTU. H & E, ×250.
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