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Pregnancy Stimulates DNA Synthesis in R3230AC Mammary Adenocarcinoma*

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Abstract—*The transplantable R3230AC mammary adenocarcinoma was grown in virgin, pregnant and lactating rats and* in vivo *rates of* [^{3}H]*-thymidine incorporation into DNA and acid-soluble dTTP were compared between tumor and host mammary glands. The tumor differed from the host gland in that the rates of uptake and phosphorylation of the injected thymidine remained unchanged throughout the lactation cycle, but the dTTP pool increased greatly during pregnancy and declined during lactation. In both tumor and host gland, DNA labeling rates were higher during pregnancy than during lactation. Tumor DNA synthesis rates, in terms of incorporated dTMP, increased markedly during pregnancy and returned to pre-pregnant rates following parturition and during lactation. This pattern was similar to host mammary glands, but the change was of a greater magnitude. The data illustrate the usefulness of growing a transplantable mammary tumor in rats of varying physiological states. This way, similarities and differences between a mammary tumor and the host mammary gland regarding their responses to the hormonal milieu of pregnancy and lactation can be assessed.*

INTRODUCTION

THE TRANSPLANTABLE R3230AC Fischer rat mammary adenocarcinoma has been used extensively as an experimental model of breast cancer because of its similarities to normal mammary gland tissue. The tumor is chiefly composed of epithelia and has some differentiated function [1–5]. The tumor responds to administered estrogen by showing a decreased growth rate and to injected prolactin by further displaying lactation-like activity [2–4]. But, unlike normal mammary gland and many other rat mammary tumors, the growth of this tumor is independent of ovarian hormones [1, 2]. Most studies with this tumor, including studies of DNA synthesis [6, 7], have been done using either explants in culture or tissue transplanted into and grown in virgin rats. We recently observed that when R3230AC tumor was grown in females during different physiological states, the activity of RNAase H in the tumor changed in parallel with similar measurements in host mammary gland [8]. Since this enzyme is thought to be involved in cellular replication [9–11], the results suggested that DNA synthesis in the tumor might be regulated by the hormonal milieu of pregnancy and lactation in a manner similar to the normal mammary gland. In the present studies we compared DNA synthesis in the tumor and in mammary gland of the same pregnant and lactating hosts by measuring rates of thymidine incorporation into DNA.

MATERIALS AND METHODS

Materials

Poly d(A–T) was purchased from Miles Laboratories (Elkhart, IN, U.S.A.). [^{3}H]-dATP and [^{3}H]-dTTP were from International Chemical and Nuclear Corps (Irvine, CA, U.S.A.). [^{3}H]-Thymidine (methyl-^{3}H, 90 Ci/mmol) was from New England Nuclear Corp. (Boston, MA, U.S.A.). Non-labeled deoxyribonucleotides were from P–L Biochemicals (Milwaukee, WI, U.S.A.). *Escherichia coli* DNA polymerase and DNase I were from Worthington Biochemicals (Freehold, NJ, U.S.A.).

Animals and tumor

Fischer 344 rats (100–150 g, initially) were used throughout. Animal maintenance, mating, timed pregnancy and lactation, and trans-

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plantation of R3230AC mammary adenocarcinoma have been described previously [12]. The tumors were implanted on the dorsum so their presence did not affect normal suckling of pups. Transplantations were coordinated with mating schedules so that the tumor was 4–6 weeks old when the rats were in various stages of pregnancy and lactation. Lactating females were kept with their litters until killed. Only females with litters of six or more pups were used.

In vivo incorporation of thymidine into nucleotide pool and into DNA

The general protocol for assessing incorporation rates of dTMP into rat mammary gland and R3230AC tumor DNA is presented in Fig. 1. DNA synthesis rates were expressed as pmol dTMP incorporated per mg DNA, per mammary gland or per tumor. The normal mammary gland of the virgin host was not included because at this stage the gland contains primarily fat and connective tissue cells, in sharp contrast to the gland from pregnant and lactating rats, which are mainly composed of epithelia [13].

Each animal received [^{3}H]-thymidine (0.25 μCi per g body weight, i.p.). Fifty-five minutes later the animals were anesthetized (urethane 1 g/kg body weight i.p.). Abdominal and inguinal mammary glands were dissected free, while keeping the major artery intact. Mammary lymph nodes were removed. Tumors were separated from the skin and fascia, opened to check for necrotic tissue and accumulated fluid in the center. Exactly 60 min after [^{3}H]-thymidine injection, the gland and tumor were respectively clamped with precooled aluminum blocks at liquid nitrogen temperature. (Preliminary experiments showed that after [^{3}H]-thymidine injection, appearance of tritium label in the perchloric acid-soluble extract of mammary gland and tumor was linear for at least 80 min.) The frozen tissue was powdered in liquid nitrogen using a mortar and pestle, weighed and suspended in 10 vol. of ice-cold 5% perchloric acid. The content was homogenized with ten up-and-down strokes in a glass homogenizer attached with a teflon pestle in an ice-water bath. The homogenate was centrifuged at 10,000 *g* and 4°C for 10 min. The supernatant was neutralized with 2 M KOH, centrifuged at 5000 *g* for 10 min, then lyophilized.

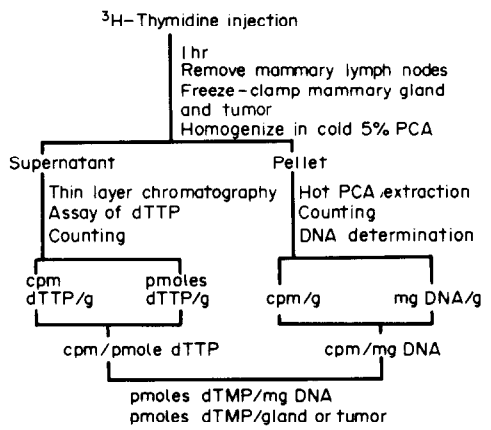

Fig. 1. General protocol for assessing rate of thymidine incorporation into DNA. Detailed conditions are described in the text.

The nucleotides and thymidine in lyophilized samples were separated using PEI-cellulose thin layer chromatography and their radioactivity determined [14]. To assess the recovery of dTTP during extraction, controls were treated in the same way by adding 1 μCi of [^{3}H]-dTTP prior to homogenization to tissue collected from uninjected animals. An average recovery of added [^{3}H]-dTTP, determined from 5 samples (3 glands and 2 tumors), was 56.9%. The amounts of dTTP were determined using a DNA polymerase reaction [15, 16]. DNA in the acid-precipitated pellets was first extracted by hot perchloric acid [17] and was determined by a diphenylamine reaction [18]. Radioactivity in DNA was determined by adding Aquasol (New England Nuclear, Boston, MA, U.S.A.) to aliquots of the hot perchloric acid extract and by counting in a liquid scintillation spectrometer.

RESULTS

Labeling of thymidine nucleotide pools at different physiological states

The chief concern in the measurement of thymidine incorporation *in vivo* is the state of the precursor pool which, by determining the specific activity of injected labeled thymidine, will affect the outcome and its interpretation. Table 1 shows the amount of tritium label in deoxynucleotides in normal mammary gland of hosts bearing the transplanted tumor. During pregnancy the amount of label in the acid-soluble extract was 2–3 times higher than during lactation. On the other hand, a greater fraction of the tritium counts was found in phosphorylated deoxynucleosides during the stages of lactation (59–100%) than during the stages of pregnancy (57–59%). During stages of pregnancy, greater portions of the tritium counts remained as thymidine (dThd) and dTMP, and only small fractions were phosphorylated to dTDP and dTTP. In contrast, during the stages of lactation, more tritium counts were distributed in dTDP and dTTP,

Table 1. Amount of tritium label in perchloric acid-soluble fraction of rat mammary glands from animals bearing 4 to 6 week-old R3230AC tumors

Physiological state	[^{3}H] Counts (10^{-3} × cpm/mg DNA) Total acid-soluble	dThd, dTMP dTDP, dTTP	dThd	dTMP	dTDP	dTTP	dTTP as % of total acid-soluble counts
P_{15}	78.2±11.0*	43.6±14.6	14.4±5.5†	20.3±7.5†	5.4±1.6	3.5±1.2	4
P_{20}	75.1±19.0*	44.1±10.8	24.1±7.1†	11.3±2.7	4.2±1.2	4.5±0.9	5
L_2	20.5±4.4	20.7±4.6	2.6±0.8	6.3±1.9	9.3±2.3‡	2.6±0.3	13
L_5	21.6±5.8	13.1±4.8	2.2±0.2	3.1±1.2	5.0±2.8	2.9±1.0	13
L_{12}	24.3±8.6	21.0±7.5	1.0±0.1	5.8±1.9	11.8±5.2‡	2.4±0.9	10

Conditions for [^{3}H]-thymidine administration and preparation of acid-soluble extracts are described in the text. Amounts of [^{3}H] counts in nucleosides and nucleotides were determined following separation by PEI cellulose thin layer chromatography. Values have been corrected for 56.9% recovery from acid-soluble extract. The abbreviations are: P_{15}, pregnancy day 15; P_{20}, pregnancy day 20; L_2, lactation day 2; L_5, lactation day 5; and L_{12}, lactation day 12.
The values are mean±S.E.M. (n=4–7).
*Significantly different (P<0.05) from that of L_2, L_5 or L_{12}.
†Significantly different (P<0.05) from L_2, L_5 and or L_{12}.
‡Significantly different (P<0.05) from P_{15} and P_{20}.

Table 2. Amount of tritium label in perchloric acid-soluble fraction of R3230AC tumor growing in rats of different physiological states

Physiological state	[^{3}H] Counts (10^{-3}×cpm/mg DNA) Total acid-soluble	dThd, dTMP dTDP, dTTP	dThd	dTMP	dTDP	dTTP	dTTP as % of total acid-soluble counts
V	10.5±2.0	9.6±2.5	1.6±0.2	4.2±1.1	3.2±1.8	0.6±0.2	6
P_{15}	24.5±3.3*	15.7±4.9	3.8±0.8	7.2±2.3	3.8±2.2	0.9±0.1	4
P_{20}	16.8±1.2*	9.9±1.6	4.7±1.5	1.2±0.6†	3.1±2.3	0.8±0.1	5
L_2	12.7±2.9	12.9±3.7	6.7±2.0*	4.3±1.6	1.0±0.2	1.0±0.2	8
L_5	14.5±5.9	8.0±3.4	3.4±1.3	3.2±1.6	0.6±0.3	0.7±0.3	5
L_{12}	20.1±8.6	14.6±4.9	3.7±1.8	7.3±2.0	2.2±0.8	1.2±0.5	6

Conditions are as described in the legend to Table 1. The abbreviations are: V, virgin; the remainders are the same as those in Table 1.
The values are mean±S.E.M. (n=4–7).
*Significantly different (P<0.05) from V.
†Significantly different (P<0.05) from V, P_{15} and L_{12}.

especially in dTDP, which contained approximately 40–60% of total precursor counts. By taking the dTTP counts as a percentage of total acid-soluble counts, the values were only 4–5% during pregnancy but increased to 10–13% during lactation. These results suggest that the metabolically more active lactating gland more efficiently phosphorylates the injected thymidine than the nonlactating gland.

Table 2 shows the results from the tumor growing in the same animals. In addition, we also included measurements from tumor grown in virgin rats. There was an increase in acid-soluble count from virgin state to pregnant state. But, the total acid-soluble tritium label in tumor tissue (Table 2) was consistently lower than that in the corresponding host mammary tissue from pregnant and lactating rats (Table 1). In tumor, the decrease in total acid-soluble counts from pregnant state to lactating state (Table 2) was not as great as that in normal host gland (Table 1).

Analysis of tritium counts distributed in individual precursors revealed that in the tumor, lactation did not result in increased phosphorylation of injected thymidine. In host mammary gland, thymidine and dTMP counts decreased and dTDP counts increased during lactation, suggesting an increase in phosphorylation of injected [^{3}H]-thymidine. Thymidine and dTMP counts in R3230AC tumor remained high and dTDP counts decreased

during lactation. The amounts of tritium found in dTTP in tumor from all stages were about 4–8% of total acid-soluble counts (Table 2), whereas the value in mammary gland increased to about 10–13% during lactation.

Endogenous dTTP concentrations and specific radioactivities of dTTP

Except for a decrease on day 5 of lactation which was not statistically significant, there was no change in pool size of dTTP in the mammary gland on a per mg DNA basis. Considerably more variations in endogenous dTTP pools were observed in the tumor, including a 16-fold increase from the tumor growing in the virgin animals to that growing in the 20-day pregnant animal. The lowest dTTP concentration was observed in tumor grown in the virgin rats in spite of continuing tumor growth in the absence of the hormonal milieu of pregnancy and lactation. Steadily increasing dTTP levels suggested that the tumors were able to respond to the hormonal environment of pregnancy. The dTTP level declined during lactation from its peak value of late pregnancy, but remained 4–7 times higher than the value of the tumor grown in virgin rats.

The specific radioactivity (cpm/pmol) of dTTP was calculated from the tritium counts of dTTP (Tables 1 and 2) and the dTTP concentration (Table 3). Except for an increase on the 5th day of lactation, in normal mammary gland the specific activity again did not change.

Table 3. Thymidine triphosphate levels and specific radioactivity in R3230AC mammary tumor and host mammary gland

	Mammary gland		R3230AC mammary tumor	
	Pool size (10^{-5}×pmol/mg DNA)	Specific activity (cpm/pmol)	Pool size (10^{-5}×pmol/mg DNA)	Specific activity (cpm/pmol)
V			0.23±0.07	3.61±0.85
P_{15}	1.37±0.65	3.81±0.73	0.61±0.28	2.43±0.80
P_{20}	1.64±0.73	4.96±1.56	3.29±0.48*	0.26±0.08‡
L_2	1.34±0.44	3.14±0.98	1.27±0.25†	1.02±0.35
L_5	0.81±0.37	8.12±2.35‡	1.40±0.84	2.00±1.90
L_{12}	1.73±0.40	2.60±0.45	0.82±0.32	1.92±0.70

The dTTP was separated by PEI-thin layer chromatography and then counted for tritium. The amount of dTTP was determined by a DNA polymerase assay. The physiological states of animals and the abbreviations are the same as those in Tables 1 and 2.

The values are mean ± S.E.M. ($n = 4$–7).
*Significantly different ($P<0.05$) from all other days.
†Significantly different ($P<0.05$) from V and P_{15}.
‡Significantly different ($P<0.05$) from V, P_{15}, L_2 and L_{12}.

The high value on the 5th day was due to the decreased dTTP level (Table 3).

Changes in dTTP-specific activities in tumors grown in pregnant and lactating rats were opposite to those in mammary glands. The activity decreased from the highest level in tumor grown in virgin rats to the lowest in late pregnant rats. The activities in tumors grown in lactating rats remained low. Since the total amount of radioactivity in tumor dTTP remained almost the same regardless of whether it was grown in virgin, pregnant or lactating rats (Table 2), the changes seen for dTTP-specific activities were primarily due to the changes occurring in endogenous dTTP pools (Table 3). Since dTTP, the primary precursor for DNA synthesis, has been shown to be exclusively localized in the nuclear compartment during S phase [19], the data on dTTP specific activity were used to calculate the amount of incorporated dTMP molecules in DNA.

Incorporation of tritium label and of dTMP into DNA

Incorporation of [^{3}H]-thymidine into rat mammary gland DNA on a DNA basis was high during pregnancy and decreased during lactation (Table 4). When cellular dTTP specific radioactivities from the data in Table 3 are combined with the DNA labeling data, it is possible to calculate amounts of dTMP incorporation into DNA. Amounts in pmol of dTMP incorporated into mammary gland DNA of pregnant rats was again higher than that of lactating rats. An increase during the later part of lactation was noted, but the interindividual variation was great and was not significant. Thus, both sets of data were generally in agreement with the concept of high proliferative activity of the gland during pregnancy [6, 20–24]. Since not all mammary cells in the gland are active in DNA synthesis at a given stage, the expression of the activity on a DNA basis may not accurately reflect the overall activity in the gland. Therefore, we also expressed the data on a per gland basis. For the purpose of obtaining a first approximation of total dTMP incorporation among varying gland sizes, only the average values of total mammary gland DNA were used in the calculation. The results showed again that glands of pregnant rats incorporated greater amounts of dTMP than those of lactating rats.

Despite lower rates of cellular uptake of tritiated [^{3}H]-thymidine in the tumor compared to the normal mammary gland (Tables 1 and 2), tumor DNA was labeled at rates roughly

Table 4. Amounts of tritium label and incorporated dTMP in mammary DNA per hour at different physiological states

	[^{3}H] Counts (10^3×cpm/mg DNA)	Incorporated dTMP (10^3×pmol/mg DNA)	Average DNA per gland (mg)	Total incorporated dTMP in gland (10^3×pmol/gland)
P_{15}	4.4±0.7*	1.2±0.3	5.2	6.2
P_{20}	3.6±1.4*	1.3±0.5	5.5	7.2
L_2	1.3±0.5	0.4±0.2	7.1	2.8
L_5	1.5±0.5	0.2±0.1	8.8	1.8
L_{12}	1.8±0.6	0.7±0.4	7.9	5.5

The value for incorporated dTMP is calculated from dividing the [^{3}H] count by the specific activity listed in Table 3. Physiological states of animals and abbreviations are the same as those described in Table 1.
The values are mean±S.E.M. (n=4–7).
*Significantly different ($P<0.05$) from L_2, L_5 or L_{12}.

Table 5. Amounts of tritium label and incorporated dTMP per hour in DNA of R3230AC mammary tumor growing in rats at different physiological states

	[^{3}H] Counts (10^{-3}×cpm/mg DNA)	Incorporated dTMP (10^{-3}×pmol/mg DNA)	Average tumor DNA (mg/tumor)	Total incorporated dTMP in tumor (10^{-3}×pmol/tumor)
V	5.5±1.0*	1.6±0.2	10.1	16.2
P_{15}	7.3±0.4*	4.6±1.7	17.1	78.7
P_{20}	6.8±0.6*	30.5±7.3†	11.3	345.0
L_2	2.3±0.9	3.4±1.4	13.3	45.2
L_5	3.0±1.6	1.9±1.0	15.0	28.5
L_{12}	2.4±1.3	2.0±0.9	24.9	49.8

The tumor-bearing animals are those of Table 4. Physiological states of animals and abbreviations are the same as those described in Tables 1 and 2.
The values are mean±S.E.M. (n=4–7).
*Significantly different ($P<0.05$) from L_2, L_5 and L_{12}.
†Significantly different ($P<0.05$) from all other days.

twice those of host mammary gland at every stage of development (Tables 4 and 5). The precipitous drop in DNA labeling after parturition, which was similar to that in normal gland of the same hosts, indicated that the R3230AC tumor retains biochemical characteristics of the normal mammary gland. When data on tumor DNA labeling at different physiological states of hosts are coupled with data of dTTP specific radioactivities seen in tumor (Table 3), vast differences in dTMP incorporation rates into tumor DNA resulted. As shown in Table 5, pregnancy resulted in a sharp increase in amounts of dTMP incorporation. At days 15 and 20 of pregnancy, rates were nearly 3- and 20-fold, respectively, above values for tumors grown in virgin rats. There was a sharp drop in dTMP incorporation *post partum* which was also similar to that of normal host mammary gland. A distinct difference between the tumor and the host gland was that the changes in tumor were much greater than the response seen in normal mammary gland. If the activity was expressed on a total tumor basis, the changes were the same despite some variation in tumor sizes (Table 5).

DISCUSSION

Comparison of tumor DNA synthesis with that of resting mammary gland, which is mainly composed of fat and connective tissue cells and is low in cellular proliferation, [13] is of little value. A more meaningful comparison is between rates of tumor DNA synthesis and mammary gland DNA synthesis of pregnant and early lactating rats when alveolar and ductal epithelia are proliferating. By growing the tumor in pregnant and lactating rats, both the tumor and host gland are exposed to the same hormonal milieux and information regarding the relative response of normal and tumor tissue to hormonal signals can be obtained. We found that, in addition to a high basal level of tumor DNA synthesis compared to normal

mammary gland (similar to previous observations using an explant system [6, 7]), the tumor and host mammary gland responded similarly to the hormonal milieux of pregnancy and lactation (Tables 4 and 5). However, the changes occurring in the tumor were greater than those in host gland. This could be explained, at least in part, by levels of dTTP pools which, in tumor, increased greatly during pregnancy and returned to pre-pregnant levels during lactation (Table 3). The high tumor DNA content [1, 8, 12], which distributes mainly in polyploid cells [25, 26], combined with a greater number of proliferating cells than in normal mammary gland [6] and a shortening in the duration of DNA replication during pregnancy [27], may all contribute to the greater changes in rates of DNA synthesis in tumor than in mammary gland.

The apparent growth rates of the tumor also reflect the changing rates of DNA synthesis. For instance, the growth rate averaged 0.08 g ($n = 10$), 0.12 g ($n = 22$) and 0.10 g ($n = 30$) per day when the tumor was grown respectively in virgin, pregnant and lactating hosts (unpublished results). It should be noted that all these tumors were grown for 30–45 days. Those grown in lactating hosts had gone through the stages of virgin and pregnancy, otherwise the real growth rate during lactation might have been lower than that shown above. By the same token, the growth rate of tumor in pregnant rats would be greater if the pre-pregnant period was excluded.

The increase in tumor DNA synthesis during pregnancy and subsequent decline during lactation are consistent with our previous data, showing increased DNA levels and RNAase H activity in tumors grown in pregnant hosts and decreased RNAase H activity in tumors grown in lactating rats [8]. RNAase H, which removes the RNA moiety of RNA–DNA hybrids, is thought to be a factor involved in regulation of cellular DNA replication [9–11].

Which hormone or hormones are responsible for the observed changes in rates of tumor DNA synthesis remains unknown. Estrogen has no effect on DNA synthesis in tumor explants [6], so the increase in DNA synthesis during late pregnancy must be due to stimuli other than estrogen alone. During pregnancy and lactation there are major changes in the levels of estrogen [28], progesterone [29, 30], prolactin [31, 32] and corticosteroids [32]. Except for low levels of estrogen receptor [33–36], the tumor contains receptors for all these hormones [33–40]. It should be noted also that the R3230AC tumor does not always respond to hormones in the same manner as the host gland. For example, the tumor is less sensitive to insulin for DNA synthesis and growth [6, 41].

In the pregnancy-dependent mammary tumors of mice, progesterone, estrogen, and prolactin all are necessary to stimulate DNA synthesis [42, 43]. Among them, progesterone has a greater effect on tumor than host glands [43]. The *post partum* fall in DNA synthesis in the R3230AC tumor (Table 5) is reminiscent of a glucocorticoid-induced regression of a hormonally responsive mammary tumor of the rat [44, 45]. It would be of interest to learn whether DNA synthesis in R3230AC tumor is similarly under the control of these hormones.

The low radioactive label of thymidine or its nucleotides in total acid-soluble counts (Tables 1 and 2) is consistent with recent observations showing large amounts of thymidine metabolites, β-amino isobutyric acid and β-ureido isobutyric acid, in rat liver and kidney following [^{3}H]-thymidine administration [46]. Although mammary gland had a more rapid uptake of injected [^{3}H]-thymidine during pregnancy than that of tumor grown in the same hosts (Tables 1 and 2), the tumor, at the same time, had a greater expanded dTTP pool than the host gland. These results suggest that R3230AC tumor grown in pregnant hosts may preferentially utilize dTTP synthesized *de novo*, a phenomenon compatible with the hypothesis that actively replicating cells prefer the *de novo* pathway [47, 48]. In normal mammary gland this preferential utilization of dTTP is less apparent.

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