Metabolic Activities of Murine Carcinoma 755 at Various Stages of Growth

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SUMMARY

The rates of synthesis of RNA and DNA decreased as murine Carcinoma 755 increased in size during a period of 26 days. The rates of synthesis were determined by measuring the incorporation of thymidine-methyl-3H into the acid-insoluble fractions in vivo, of thymidine-2-14C into the acid-insoluble fractions of minced tumors in vitro, and of formate-14C and adenine-8-14C into both the acid-insoluble fractions and the isolated adenine and guanine of RNA and DNA in vivo. These decreasing rates of synthesis in growing tumors correlated well with the decreasing proliferative fractions previously reported by others.

The quantities of soluble metabolites formed in vivo by the tumors following the administration of formate-14C or adenine-8-14C decreased as the tumors enlarged. The decreases in the formation of amino acids (primarily serine), carboxylic acids (primarily lactic acid), and purine-containing soluble compounds lagged behind decreases in RNA and DNA synthesis.

It is unlikely that the decline in incorporation of the substrate into soluble and insoluble components is due entirely to decreased vascularization of the larger tumors, because similar decreases of incorporation of thymidine-2-14C into acid-insoluble compounds occurred during in vitro incubation of minced tumors.

The DNA polymerase activity of crude, cell-free preparations from tumors increased to a maximum for 14-day-old tumors and decreased thereafter. The lower incorporation of radioactive substrate into acid-insoluble material by preparations of the older tumors was not due to deficiencies of thymidylate kinase or limited pools of dTTP. Information about the sizes of the generated pools of the other necessary deoxyribonucleoside triphosphates is not available, but a maximum of polymerase activity was observed for the 14-day-old tumor preparation, even when all 4 of the deoxyribonucleoside triphosphates were added to the incubation mixtures.

The rates of synthesis of RNA and DNA in the spleens and marrows of the host animals increased with increasing tumor size, while there was relatively little change in the rates of synthesis of RNA and DNA in the livers, kidneys, lungs, and brains.

INTRODUCTION

It is known that the rate of growth of a solid tumor usually decreases when the tumor gets large, as a result of altered growth kinetics (3, 5, 7, 10), and that larger, slowly growing tumors frequently respond less to chemotherapy than do smaller, more rapidly growing tumors (4, 5). It is also a clinically common occurrence that tumors reach the large, slowly growing stage before the patient presents himself for diagnosis and treatment. There is evidence (4, 5, 8) that knowledge of the interrelationships of the metabolism and kinetics of growth of cells and tissues can be exploited to attain "cures" in certain rapidly growing experimental neoplasms. Therefore, it is desirable to know what changes in the metabolic activities of the tumors accompany the changes in growth kinetics. Such information might be useful in designing therapy for large and/or slowly growing neoplasms.

Simpson-Herren and Lloyd (7) have reported the growth curve (for a period of 35 days) and the kinetic parameters (for a period of 19 days) of Carcinoma 755 in the mouse. A Gompertz curve fitted the growth data rather well, and the kinetic parameters changed as the rate of growth changed. These parameters are plotted in Chart 1 to facilitate correlation of kinetic parameters with the biochemical phenomena presented in this report. The pulse-labeled fraction (IT) and the proliferative fraction (PF) decreased greatly after the 6th day after implantation, and the lengths of the cell cycle, TC, and the S phase, TS, increased greatly between Days 14 and 19. The cell loss fraction (CLF) was relatively high on Day 14, but on Day 19 it was similar to that on Days 6 and 8. Because it possesses these properties, Carcinoma 755 was chosen as a suitable experimental tumor for the study of changes in metabolism during tumor enlargement, when the decrease in proliferative fraction accounts for a large portion of the decrease in growth rate.

MATERIALS AND METHODS

Animals and Tumors. Fragments of murine Carcinoma 755 were implanted s.c. into male and female BDF1 (C57BL X DBA/2) mice weighing 20 to 27 g. The weight of each fragment was approximately 25 mg. Beginning on the 4th day following implantation, the tumors were measured in 2 directions with calipers and the approximate weights of the tumors were calculated, with the assumption that each tumor was a prolate spheroid with a density of 1 g/cu cm. Measurements were made at intervals of 2 or 3 days. In each experiment, tumors were implanted into approximately 300 mice, and only tumors with calculated weights falling within
the previously determined range for that day were used for studies of metabolism. After the animals were killed, the excised tumors were weighed, and the mean weights for each day were plotted on semilog paper. The curves for the calculated weights (determined on the preceding day) and the actual weights were visually similar, but the actual weights were always less than the calculated ones. This discrepancy was partially due to the fact that the tumors were not actually prolate spheroids and that liquid surrounding the tumors in situ made measurement difficult.

Injection of Radioactive Substrates and Sacrifice and Dissection of the Animals. All of the radioactive materials used in this study were obtained from New England Nuclear, Boston, Mass.

On designated days following implantation of the tumors, the selected mice received i.p. injections of 1 of the following radioactive compounds in the dosage indicated: formate-14C (specific activity, 5 mCi/mnmole), 100 μCi/mouse; adenine-8-14C (specific activity, 6.6 mCi/mnmole), 10 μCi/mouse; thymidine-methyl-3H (specific activity, 75 mCi/mnmole), 1 μCi/g body weight. Two hr later, the mice were killed by asphyxiation with carbon dioxide; and the tumors, livers, spleens, marrow (forced from the femurs with saline solution), kidneys, lungs, and brains of the individual animals were weighed and pooled. The number of animals per group varied, because the number of animals required to yield the needed quantities of tumor tissue decreased as the tumors became greater. Whereas there were 40 to 60 animals in the groups bearing 4-day-old tumors, there were only 4 or 5 animals in the groups bearing 25- or 26-day-old tumors.

Preparation and Radioassay of Defatted, Acid-insoluble Material. The tissues other than marrow were minced with knives and forced through a tissue press, and portions were used for the preparation of defatted, acid-insoluble fractions and subsequent radioassay, as described previously (14).

Preparation, Chromatography, and Radioassay of Aqueous Ethanol Extracts. Other portions of the comminuted tissues mentioned above were extracted with hot ethanol:water mixture (5:1, by volume). The resulting extracts were chromatographed 2-dimensionally on paper, and the radioactive components were located by radioautography as described previously (13). The radioactive areas were cut from the chromatograms and assayed for radioactivity in a liquid scintillation spectrometer.

Isolation and Radioassay of the Purines of RNA and DNA. In certain experiments, the purines of RNA and DNA were isolated as follows. After the alcoholic extract was taken, the tissue was washed twice each with water, cold 5% trichloroacetic acid, water, absolute ethanol, and ether and was allowed to dry overnight at room temperature.

The dried, defatted tissue was subjected to a Schmidt-Thannhauser hydrolysis overnight at 37° with 0.3 N sodium hydroxide solution. After cooling in ice, the hydrolysate was made slightly acidic with 5 N sulfuric acid and allowed to stand in an ice bath for at least 15 min. Ice-cold 20% trichloroacetic acid was added to a final concentration of 3%, the suspension was centrifuged, and the supernatant containing the hydrolyzed RNA was decanted.

The precipitate containing the DNA and protein was given another 2-hr hydrolysis, and the procedure was repeated through the cold 3% trichloroacetic acid stage. The supernatant from this fraction was discarded, and the DNA:protein precipitate was heated (90–95°) with 5% trichloroacetic acid for 20 min with continuous stirring. The trichloroacetic acid extraction mixture was cooled and centrifuged, and the sedimented protein was discarded. The DNA-containing supernatant solution and the hydrolyzed RNA-containing supernatant solution previously mentioned were made 1 N with concentrated sulfuric acid and refluxed at 115–125° for 1 to 2 hr. After cooling, the pH was adjusted to 8 to 9 with ammonium hydroxide, and any precipitate that formed was separated by centrifugation and discarded. We precipitated silver purines by adding an excess of a 1:1 solution of 10% silver nitrate and concentrated ammonium hydroxide and allowing the mixture to stand cold overnight. The silver purines were sedimented by centrifugation, washed twice each with cold water, alcohol, and ether, and allowed to dry.

The dried samples were heated for 1 hr with 0.5 ml 1 N hydrochloric acid at 90–95°, after which the silver chloride was removed by centrifugation and washed with water, and the supernatant plus wash was used for 1-dimensional ascending chromatography on Whatman No. 1 paper with a methanol:hydrochloric acid:water (65:17:18) solvent.

The adenine and guanine bands thus separated were cut out and eluted with 3 N ammonium hydroxide, and the concentration and purity of the resulting solutions were determined with a Gilford spectrophotometer. The adenine and guanine were assayed for radioactivity by counting in a liquid scintillation spectrometer.

Incorporation in Vitro of 14C of Thymidine-2-14C into Defatted, Acid-insoluble Material of Minced Tumors. A portion (200 mg) of the minced tumors from the animals that had received injections of thymidine-methyl-3H was incubated in a Dubnoff shaking incubator at 37° for 2 hr with 4 ml of Krebs-Ringer phosphate buffer containing 4 μCi of
thymidine-2-\(^{14}\text{C}\) (specific activity, 43.7 mCi/m mole) under a stream of oxygen. The tissue was then separated by centrifugation and homogenized in cold 5% trichloroacetic acid. The remainder of the preparation and radioassay of the defatted, acid-insoluble material was the same as described above, except that double-label counting was used.

Cell-free Preparations of Ruptured Tumor Cells and Assays of These Preparations for DNA Polymerase and for Certain Enzymes Involved in Metabolism of Thymidine Phosphates. The methods used for obtaining cell-free preparations and for assaying them for DNA polymerase and other activities have been described (14).

RESULTS AND DISCUSSION

Weights of Tumors. The plotted points of Chart 2 show the mean weights of the tumors harvested on the indicated days after implantation of the tumor. The 3 sets of points show that the sizes of the tumors were comparable for the 3 sets of experiments in which the 3 radioactive substrates were used.

Metabolism of Formate-\(^{14}\text{C}\). Chart 3A shows the specific radioactivities of the defatted, acid-insoluble fractions of the various tissues for different times following implantation of the tumors when the substrate was formate-\(^{14}\text{C}\). The specific activity was maximum in the 8-day tumor, and it was progressively less as the size of the tumor increased. [It has been reported that under these conditions of tumor implantation and growth the median day of death of the animals was 21 to 24 days (4, 6).] There were relatively smaller decreases for the spleens and the marrow at the later times, but the specific activities for the liver, kidney, lungs, and brain were essentially the same for all times. Since the decreases in specific activity for the tumor might be partially explained by simple dilution resulting from growth of the tumor and administration of a constant dosage of the labeled compound, it was desirable to calculate the total quantities of radioactivity in the defatted, acid-insoluble fractions of the tissues for various days. These quantities are plotted in Chart 3B. The total quantities of radioactivity incorporated by the tumor on Days 19, 21, and 26 were lower than those on Days 11 and 14, while such relative decreases did not occur with the other tissues. These results suggest that the decreases in specific activity of the tumor are not altogether due to dilution. Since the \(^{14}\text{C}\) from formate-\(^{14}\text{C}\) that was present in the defatted, acid-insoluble material could be present in protein, RNA, and DNA, it was desirable to determine whether the changes in the specific activities of the purines of RNA and DNA are similar to those in the acid-insoluble fraction. The curves of Chart 4 show that the incorporation of \(^{14}\text{C}\) into the adenine and the guanine of both RNA and DNA are similar to those in the acid-insoluble fraction. The specific activities of the purines of RNA of the other tissues were much lower than those of tumors and spleen, and they changed relatively less with increasing time after implantation of the tumors. The specific activities of the purines of DNA of the tissues other than the tumor and spleen (purines were not isolated from the marrow) were too low on all days to be meaningfully included in the graphs.

If the growth fraction or proliferative fraction of a tumor decreases, it would be expected that the rates of synthesis of DNA, RNA, and protein would also decrease. It is more difficult to predict what changes in intermediary metabolism might accompany enlargement of the tumors.

Chart 5 shows that the quantity of \(^{14}\text{C}\) incorporated into the chromatographically separated, soluble purines and related compounds (formyl glycinamide ribonucleotide, 4-amino-5imidazolocarbamidoxime, hypoxanthine, inosine, IMP, adenylosuccinic acid, adenine, adenosine, AMP, ADP, ATP, guanine,
guanosine, GMP, GTP, xanthine, uric acid, and allantoin) per unit weight of tumor was maximum at 8 days after implantation of the tumor, and the quantities were progressively less for the succeeding days. The differences in the quantities of $^{14}$C incorporated into the free amino acids (alanine, glutamic acid, serine, glutamine, aspartic acid, and cysteine) and carboxylic acids (lactic, succinic, citric, and malic) per unit weight of tissue on the successive days were much less than those for purines and related compounds.

Chart 6 shows that the changes in the total quantity of $^{14}$C incorporated into the "soluble" compounds and the soluble purines and related compounds of the whole tumors are similar to the changes in the total quantity of $^{14}$C incorporated into the acid-insoluble fraction of the whole tumor. More $^{14}$C was incorporated into soluble amino acids and carboxylic acid on Day 19 than on Day 14, while less $^{14}$C was fixed into purines and related compounds on Day 19 than on Day 14. These apparently elevated rates of synthesis of amino and carboxylic acids may be partially due to the increased availability of formate-$^{14}$C which was used less extensively for the synthesis of purines in larger tumors. Serine was the most radioactive amino acid, and lactic acid was the most radioactive carboxylic acid. The high rate of synthesis of lactic acid is consistent with the often-observed elevated rate of anaerobic glycolysis in tumors (2), and the increase in the quantity of labeled carboxylic acids was chiefly due to the formation of more lactic acid. (It is important to realize that any free formic acid or other radioactive volatile compounds would be lost in these experiments during preparation of the

Chart 4. Specific activities of the purines of the RNA and DNA of tissues following i.p. administration of formate-$^{14}$C on the indicated days after implantation of the tumors. $T$, tumor; $S$, spleen; $K$, kidney; $Li$, liver; $Lu$, lung; $Ade$, adenine; $Gua$, guanine.

Chart 5. Quantities of radioactivity present in various components of extracts of 40 mg of wet tumor tissue 2 hr following the i.p. administration of formate-$^{14}$C on the indicated days after implantation of the tumors. See the text for the composition of the 3 fractions indicated.

Chart 6. Total quantities of radioactivity present in various forms in tumors 2 hr following the i.p. administration of formate-$^{14}$C on the indicated days after implantation of the tumors. Acid insol., acid insolubles; Tot. sol., total solubles; Sol. pu., soluble purines, etc., Sol. a.a.'s soluble amino acids; Sol. c.a.'s, soluble carboxylic acids. See the text for the composition of these fractions.
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Thus the biosynthesis of amino and carboxylic acids can occur extensively even in large tumors.

Metabolism of Adenine-8-\(^{14}\)C. Chart 7A shows the specific activities of the defatted, acid-insoluble fractions of the various tissues when the substrate was adenine-8-\(^{14}\)C. The curves are generally similar to those of Chart 3A. The total activity incorporated into the acid-insoluble material by the tumor was less at the later times (Chart 7B), but the meaning of these decreases is uncertain, because decreases also occurred for the liver and the spleen. Chart 8 shows that the specific activities of the RNA-purines and DNA-purines of the tumors decreased as the tumors got larger, but those of the other tissues in the same animals decreased much less or not at all. These patterns are generally similar to those obtained when the substrate was formate-\(^{14}\)C.

The quantity of total radioactivity (data not given) present as soluble (in aqueous ethanol) activity per unit weight of tumor decreased greatly after Day 11. The quantities of radioactive adenine and related compounds (adenine, adenosine, AMP, ADT, ATP, and adenylosuccinic acid) decreased similarly to the total radioactivity of the soluble fraction, while the quantities of radioactive hypoxanthine and related compounds (hypoxanthine, inosine, and IMP) and guanine and related compounds (guanine, GMP, and GTP)

Chart 7. A, specific activities of defatted, acid-insoluble material of tissues following i.p. administration of adenine-8-\(^{14}\)C on the indicated days after implantation of the tumors. T, tumor; M, marrow; S, spleen; Li, liver; K, kidney; Lu, lung.

Chart 8. Specific activities of the purines of the RNA and DNA of tissues 2 hr following the i.p. administration of adenine-8-\(^{14}\)C on the indicated days after implantation of the tumors. T, tumor; S, spleen; K, kidney; Li, liver; Lu, lung; Br, brain; Ade, adenine; Gua, guanine.
decreased somewhat earlier. The gross quantity of soluble radioactivity present in the whole tumor was maximum on Day 19, although the gross quantity of "acid-insoluble" radioactivity in the tumor decreased after Day 14. The gross quantity of radioactive adenine and related compounds decreased greatly after Day 19, but the quantities of radioactive hypoxanthine and related compounds and of radioactive guanine and related compounds increased throughout the period of observation. Thus, the compositions of the "solubles" were quite different at different times postimplant.

Incorporation of Thymidine-methyl-3H in Vivo and of Thymidine-2-14C in Vitro. Chart 9, Curve I, shows the values for the specific activities of the defatted, acid-insoluble fraction of tumor when the in vivo substrate was thymidine-methyl-3H. Both the specific activity and the total activity (not shown) decreased when the tumors got large, and these decreases evidently were not due to dilution, because in this experiment the substrate was given on the basis of the weight of the animals. The similarities of the curves for the tumors in Charts 3 and 7 to those of Chart 9 would make it unlikely that the decreases in incorporation of 14C of formate-14C and adenine-8-14C in the larger tumors were due primarily to dilution of substrate.

To determine whether the capacity for in vitro synthesis of DNA changed similarly to the capacity for in vivo synthesis of DNA by tumors as they became larger, portions of the tumors that were obtained in the experiment for the in vivo incorporation of thymidine-methyl-3H (Chart 9, Curve I) were minced finely and incubated with thymidine-2-14C. Following incubation, the tissue was homogenized with cold trichloroacetic acid for extraction of acid-soluble material and was subsequently defatted and dried. The dried material was assayed for both 3H (Chart 9, Curve II) and 14C (Chart 9, Curve III). Curve II is very much like Curve I, as it should be. Curve III shows that the capacity for in vitro synthesis is also less for the larger tumors.

The lower quantities of total radioactivity (soluble plus insoluble) present in the tumors after Day 19, when formate-14C (Chart 6) or adenine-14C (data not given) were administered in vivo, might be partially due to decreased vascularity of the large tumors, as decreased vascularity of other large experimental tumors has been observed (1, 9, 11). However, any decreased vascularity with the resultant decreased provision of labeled substrate to the tumor does not adequately account for the observed decrease in synthesis of DNA, because similar decreases were noted with thymidine-2-14C and minced tissues in vitro, in which the degree of vascularization would have no significance.

DNA Polymerase Activities and the Metabolism of Thymidine-2-14C Phosphates in Vitro. To determine whether the cell-free DNA polymerase activity of the tumors changed in a manner analogous to the changes in rates of DNA synthesis in vivo and in minces, we used portions of the same tumors for the preparation of cell-free supernatant fractions by sedimentation of homogenates in sucrose, and these fractions were assayed for DNA polymerase. The quantities of cell-free fractions used were normalized on the basis of protein content. The assays were conducted under the following 3 sets of conditions:

- (a) heat-denatured salmon sperm DNA was used as primer, and all 4 deoxyribonucleoside monophosphates were added, including TMP-2-14C;
- (b) heat-denatured salmon sperm DNA was used as primer, and all 4 deoxyribonucleoside triphosphates were added, including TTP-2-14C;
- (c) native salmon sperm DNA was used as primer, and all 4 deoxyribonucleoside triphosphates were added, including thymidine-2-14C triphosphate. Chart 10A (curves) shows the quantities of radioactivity incorporated into the acid-insoluble material during the incubation interval between 10 and 40 min after addition of the primer. During this interval, the rate of incorporation is essentially constant, so that the quantity incorporated is a measure of the enzyme activity of the preparation. With denatured primer and triphosphates as substrates (Curve I), which should avoid dependence on kinases, maximum polymerase activity was found for the 14-day tumors, and the activity decreased afterwards. Perhaps it is significant that maximum incorporation of thymidine-2-14C into minced tissues also occurred on Day 14 (Chart 9, Curve III). The activity measured with denatured primer and monophosphates as substrates (Curve II) was about the same for tumors between Days 6 and 25. The activity measured with native primer and triphosphates as substrates (Curve III) increased during the period between Days 4 and 25. The curves of Chart 10B show the quantities of radioactivity fixed by the same incubation mixture during the period between 10 and 240 min after the addition of primer to the mixtures. These quantities indicate the total capacities of the incubation mixtures to fix the labeled substrate, as the incorporation ceased prior to this time. The preparation from the 14-day tumors had the maximum capacity for incorporating the monophosphates and triphosphates, and the capacities were less for the larger tumors.

To see whether the apparent differences in DNA polymerase...
activity for tumors of different ages and sizes were due to different quantities of available radioactive substrates, we took samples of the incubation mixtures for chromatography and radioassay at the same times that samples were taken for determination of incorporation of $^{14}C$ into the acid-insoluble material. The graphs of Chart 11A show the distribution of radioactivity among the soluble components of the incubation mixtures during the course of the incubation when the deoxyribonucleoside monophosphates were the substrates and denatured primer was used. The quantities of the various components are represented by the areas between the lines and not by the lines themselves. The area identified as the origin represents the material that remained at the origin during the chromatography, and this material probably consists of high-molecular-weight substances, including the DNA plus some lower-molecular-weight substances that were trapped by the high-molecular-weight material. This area reflects the DNA polymerase activities shown in Chart 10. The values at 60 min show the distribution of radioactivity among the various components of the mixtures at the end of the 60-min preincubation which preceded the addition of the primer at 0 min. The values for tumors on Days 4, 14, and 27 are shown; similar data were obtained for tumors on Days 6, 8, 11, 19, and 21. The data show that the preparations from all of the tumors contained thymidylate kinases, and dTTP was present in the incubation mixtures during the first 60 min (or longer) of the incubation periods. Evidently, the availability of dTTP was not a limiting factor in the synthesis of the DNA, because dTTP was present throughout the incubation period for the 21-day tumor preparation (data not shown), although less synthesis of DNA occurred with this preparation than with the preparations from the 8-, 11-, 14-, and 19-day tumors (Chart 10B). The degradation of the thymine deoxyribonucleotides to thymidine and thymine was more extensive with the preparations from the younger tumors than those from the older tumors. With the preparations from the older tumors, the quantities of dTMP increased during the 3 hr of the incubation; this increase probably resulted from the dephosphorylation of the dTTP and dTDP that were formed during the 1st part of the incubation period.

The graphs of Chart 11B show the distributions of radioactivity in the incubation mixtures for 4-, 14-, and 27-day tumors when the substrates were the deoxyribonucleoside triphosphates including dTTP-$^{14}C$ and the primer was heat-denatured salmon sperm DNA. Similar data were obtained when the substrates were deoxyribonucleoside triphosphates and the primer was native salmon sperm DNA. Examination of these curves leads to the same conclusions that were stated in the preceding paragraph.

**General Comments.** These experiments show that Carcinoma 755 tumors in the asymptotic stage of growth carry out biosynthesis of low-molecular-weight compounds, although the biosynthesis of macromolecules is greatly decreased. It is likely that other types of assays would show...
that various other metabolic events are also occurring in these tumors. If certain of these metabolic events are necessary for the viable but nonproliferating cells to maintain the potential for reinitiating proliferation and if means can be used to interfere with these critical events, then improved therapy directed toward viable but nonproliferating cells might be possible.

The data presented here lead to 3 other conclusions that should be recognized by investigators of experimental (and probably human) cancers. First, since the metabolic activities of tumors may vary widely with the sizes of the tumors, the investigator must consider the stage of growth of the tumors with which he is working; he should beware of selecting for use the animals with the largest tumors simply to have larger quantities of tissue with which to work. In characterizing the metabolism of the tumor, he should state the size of the tumors. Second, the relative metabolic activities of tumors and the other tissues of the host may be dependent upon the size of the tumor; these relative activities might be reversed for tumors of different sizes (Charts 3, 5, 7, and 8). Third, since the characterization of the metabolism of a tumor depends upon its stage of growth, one must be very cautious in comparing the metabolic patterns and capacities of tumors of two or more types or lines, e.g., hepatomas of different rates of growth.

Experiments similar to, but less extensive than, those reported here for Carcinoma 755 have been performed with murine leukemia L1210, and similar conclusions were reached. Some of the results of these experiments have been presented and discussed elsewhere (12). Although the ascitic L1210 system has the advantage of having no problem of vascularization, it has the disadvantage of having a very short asymptotic stage of growth prior to death of the animal.

Tumors that decrease in growth rate primarily because of increase in the lengths of the phases of the cell cycle and/or because of increase in cell loss might have changes in metabolism that are different from those observed in this study.

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