Arrest of Synthesis of Specific Proteins at the Onset of Mammary Tumor Regression

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ABSTRACT

MTW9 mammary carcinoma regressing after removal of mammotropin stimulation was used as a model to test the hypothesis that the augmented activity of lysosomal enzymes observed during regression may be triggered by a modification of the protein composition of the cytoplasm. In support of the hypothesis, we observed that: (a) the pattern of leucine incorporation into cytosol proteins differs between growing and regressing tumors; (b) the difference is localized in three bands of the electrophoretic pattern; and (c) the change in pattern appears within 6 hr after hormone removal, about 4 hr after prolactin levels in blood were below the concentration needed by MTW9 to grow. These observations are in line with our previous finding of an increased susceptibility of cytosol proteins to proteolytic digestion during MTW9 regression.

INTRODUCTION

Lysosomes are often implicated in tissues undergoing physiological and pathological regressive changes, such as postpartum mammary gland (8, 11) and uterus (27, 30, 31) involution; muscle breakdown (18, 29) or remodeling (9, 28); and spontaneous (7, 13, 23), irradiation-induced (4, 22), or drug-induced (1, 20) regression. An increase in lysosomal enzyme activities has also been observed during the regression of hormone-dependent mammary tumors in rats, such as MTW9 adenocarcinoma (10, 15) and dimethylbenz(α)anthracene-induced primary carcinomas (27).

The published results, however, do not provide evidence that hydrolyses are involved in the initiation of tissue degradation (17) or tumor regression (6, 10, 15). Indeed, an increase of activity was observed for several lysosomal enzymes 48 to 60 hr after hormonal deprivation of the host when phagosomes were already evident (15).

We hypothesized that the increment of lysosomal enzyme activity could be an effect and not the cause of the regression process. In support of this hypothesis, we observed (25) that lability of cytosol proteins was augmented in MTW9 mammary carcinomas following hormone removal.

Since the rate of incorporation of labeled amino acids into proteins did not change during the first 24 hr after hormonal deprivation but susceptibility to proteolytic attack was already enhanced (25), regression could be triggered by a change in the protein pattern of cells being digested. The purpose of the work described here was to investigate any specific change in protein synthesis that may occur at the onset of regression induced by hormone removal. Beckingham Smith and Tata (2) have addressed themselves to a similar problem using as an experimental model the tadpole tail regressing after addition of thyronine to the culture medium. They showed that no significant changes in the pattern of synthesis of tail proteins were induced by the hormone during the first 3 to 4 days in culture.

In this paper we report that in MTW9 mammary carcinomas: (a) the pattern of leucine incorporation into cytosol proteins differs between growing and regressing tumors, (b) the difference is localized in 3 bands of the electrophoretic pattern, and (c) it is observed as early as 6 hr after hormonal deprivation.

MATERIALS AND METHODS

Tumor Model and Treatment. MTW9 mammary adenocarcinomas (2 to 5 g) were transplanted into the inguinal fat pads of inbred female Wistar/Furth rats that weighed about 150 g and were 2 months old. The high level of mammotropins needed for tumor growth was provided by a pituitary tumor, MTWT10, transplanted into the interscapular region of the MTW9-bearing animal. Removal of MTWT10 consistently produced regression of MTW9 to about 50% of the initial size within 3 to 5 days. The treatment of the animals and the handling of the tumors have been described previously (25). All experiments were performed in triplicate.

Incorporation of Radioactive Precursors in Tumors. The radioactive precursors used were: L-[4,5-3H]leucine (6 Ci/mmol) and L-[14C]leucine (309 mCi/mmol) (Schwarz/Mann, Orangeburg, N. Y.).

All radioactive precursors were injected i.p. at 9:00 a.m., and unless otherwise indicated the animals were killed 1 hr later. Tumors were homogenized with a 30-ml Teflon-to-glass homogenizer (Arthur Thomas, Inc., Philadelphia, Pa.) in 0.05 M phosphate buffer, pH 7.6, containing 0.15 M NaCl (1:3, w/v) and were centrifuged at 105,000 x g for 1 hr. The supernatants thus obtained are referred to as cytosols.

Nuclear pellets were prepared from tumor homogenates (1:5, w/v) in 0.25 M sucrose plus 5 mM MgCl2, centrifuged at 1500 x g for 20 min. The pellet was first resuspended in the same solution and then was washed twice with 0.2 M sucrose plus 1 mM MgCl2 and was centrifuged at 50,000 x g for 60 min over a cushion of 2 M sucrose placed in the bottom of the tube (5, 12). Histone and nonhistone fractions were separated from the nuclear...
pellet (3, 26), lyophilized, and dissolved for polyacrylamide gel electrophoresis.

**Polyacrylamide Gel Electrophoresis.** The cytosols and nuclear fractions were made 1% sodium dodecyl sulfate, 10% glycerol, and 3% mercaptoethanol, and then were boiled for 3 min and used for electrophoresis.

The 3-mm-thick gels were prepared as described previously (25). After being destained they were frozen by contact with a block of dry ice and sliced transversely into approximately 1-mm slices with a Model 190 gel slicer (Bio-Rad, Richmond, Calif.). Each slice was placed in a Packard Combusto-Cone, dried overnight at 55°, and combusted in the Model 360 sample oxidizer (Packard Instrument Co., Inc., Downers Grove, Ill.). The radioactivity in each scintillation vial was counted for 10 min in a Model 3380 liquid scintillation spectrometer (Packard Instrument Co., Inc.). Since the quenching, as determined by the automatic external standardization method, did not vary from sample to sample after oxidation, the results were reported as cpm/slice. The efficiency for $^3$H was 34%, and the efficiency for $^{14}$C was 68%.

Molecular weight estimations in the gels were obtained by the use of the following markers: human $\gamma$-globulins, bovine serum albumin, ovalbumin, and chymotrypsinogen A from bovine pancreas (Schwarz/Mann) and rat $\alpha$-lactalbumin and casein (P. Qasba).

**Assay of Serum Prolactin.** Blood was collected from the tails of rats, which were awake and in a restraining cage, before and after removal of MTTW10 and ovaries. The serum obtained after clot retraction was stored at -70°. Concentrations of serum prolactin were determined by radioimmunoassay with reagents supplied by the Rat Pituitary Hormone Distribution Program, National Institute of Arthritis, Metabolism, and Digestive Diseases, NIH, Bethesda, Md. The standard procedure supplied with the reagents was followed. Serum samples were analyzed in duplicate and, if possible, at 2 or more dilutions. The values were averaged and expressed as ng of NIAMDD-Rat Prolactin-RP-1 per ml of serum.

**RESULTS**

**Labeling Pattern of Cytosol Proteins.** Rats bearing a MTW9 carcinoma, either growing or regressing for 24 or 72 hr, were given i.p. injections of 0.5 mCi of $[^{14}]$Cleucine and were killed 1 hr later. The tumor was removed immediately, the cytosol was analyzed by polyacrylamide gel electrophoresis, and the incorporation of $[^{14}]$Cleucine was determined for each slice. The results in Chart 1 show that growing and regressing tumors have the same incorporation profile except in the areas corresponding to Slices 24 to 28, 40 to 45, and 60 to 65. A decrease of label uptake was observed in all 3 areas as regression of the tumor proceeded. When proteins of both growing and regressing tumors (24 and 48 hr) were labeled with $[^{3}]$Hleucine, the same results were obtained (Chart 1).

**Double-Isotope Labeling of Cytosol Proteins in Vivo.** A double-labelling experiment was performed to overcome the uncertainty in comparing 2 separate gels with 2 separate sets of slices. Preferential uptake was tested by simultaneous i.p. injection of $[^{3}]$Hleucine and $[^{14}]$Cleucine into 1 rat bearing a growing MTW9. The extent of labeling of the tumor proteins by the 2 isotopes was determined 1 hr later. In another experiment the difference between 2 supposedly identical tumors was tested by injecting i.p. $[^{3}]$Hleucine into 1 rat and $[^{14}]$Cleucine into another rat, both bearing a growing tumor. One hr later the 2 tumors were pooled and processed as a single tumor, and the extent of incorporation of $[^{3}]$H- and $[^{14}]$Cleucine into cytosol proteins was determined. The results of these 2 control experiments are presented in Chart 2, A and B. The absence of preferential uptake of 1 isotope over the other by the same tumor is revealed by the good correlation ($r = 0.993$) between the uptake of $^3$H and $^{14}$C in the cytosol proteins found in each of the 85 slices of the gel (Chart 2A). The similarity of label incorporation by 2 different growing tumors is illustrated in Chart 2B. The proportionality between $^3$H and $^{14}$C in each slice ($r = 0.983$) indicates that no difference in uptake existed when the tumors were under identical conditions.

**Double-Isotope Labeling of Cytosol Proteins during Growth and Regression.** In this experiment the rat bearing the growing tumor received an i.p. injection of $[^{3}]$Hleucine and the rat bearing the tumor deprived of hormone for 24 hr received an i.p. injection of $[^{14}]$Cleucine. One hr after the injections, the tumors were pooled and processed as described in "Materials and Methods."

The good correlation between the $^3$H and $^{14}$C uptake observed in the control experiments did not hold when a growing tumor was mixed with a regressing one. The correlation coefficient went from 0.99 or 0.98 down to 0.78 (Chart 2C). If the same proteins labeled with 2 different
isotopes were synthesized to the same extent by the 2 tumors, the $^3$H:$^{14}$C ratio would be constant throughout the gel. Such was the case in Chart 3A, where a growing tumor was labeled with $[^3]$H- and $[^{14}]$Cleucine. However, if 1 particular protein was not labeled with 1 of the 2 isotopes used, the $^3$H:$^{14}$C ratio would differ for this protein but remain constant for all the others. Such a case is illustrated in Chart 3B. The $^3$H:$^{14}$C ratio for a mixture of growing and regressing 24-hr tumors oscillated around 0.4 except at 3 different places, namely Slices 27 to 30, 43 to 45, and 55 to 58, corresponding to molecular weights of 68,000, 42,000 and 25,000, respectively. Results shown in Chart 1 indicated that the increase in the $^3$H:$^{14}$C ratios was due to a decreased incorporation by the regressing tumor.

The cytosol proteins prepared from the livers of the tumor-bearing animals showed a pattern different from that of the tumor preparations, but the $^3$H:$^{14}$C ratio did not vary significantly between animals with or without mammotropin. Thus, the difference in the ratios is peculiar to the tumor.

Time Course of the Effect of Hormone Removal. The time sequence between mammotropin deprivation and changes in $^3$H:$^{14}$C ratios was studied by mixing a growing tumor labeled with $[^3]$H-leucine with a tumor labeled with $[^{14}]$Cleucine and deprived of hormones for 4, 6, 18, 24, and 72 hr. Each regressing tumor was mixed with a different growing tumor so that the potential effect of diurnal variation in incorporation of amino acids into proteins (16) could be compensated for.

To simplify the comparison of different ratios of radioactivity, we normalized the ratio ($R$) for each slice by the average ratio ($\bar{R}$) for the entire gel. The plot of the normal-
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The normalized ratio ($R/\hat{R}$) versus slice number will hover around the line $R/\hat{R} = 1$ if the $^3$H and $^4$C distributions are the same and will diverge from the line $R/\hat{R} = 1$ if the distributions are different. The results of this experiment are shown in Chart 4. Up to 4 hr after hormone removal, no difference in the ratio of incorporation of labeled precursors could be detected. At 6 hr a difference appeared in the normalized ratio at 1 place along the gel corresponding to a molecular weight of about 25,000. From 18 hr on 2 more peaks were observed at molecular weights of 42,000 and 68,000, respectively.

Serum Prolactin Level after MTW10 Removal. Two MTW9-bearing rats were used to establish the time at which blood mammotropins would reach a level below that necessary for MTW9 growth (19). Before surgical excision of MTW10 and at different times thereafter (1, 2, 4, 7, 24, and 48 hr), a sample of tail blood was withdrawn, and prolactin content was determined (Chart 5). Within 1 hr after removal of the source of mammotropin, the level of circulating prolactin fell below 100 ng/ml, which is considered the critical

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**Chart 3. Electrophoretic distribution and ratio of radioactivity of cytosol proteins from MTW9 mammary tumors.** A, 1 hr before sacrifice a rat bearing a growing MTW9 received an i.p. injection of 0.5 mCi of $[^3]$H]leucine and 0.5 mCi of $[^4]$C]leucine. The tumor was homogenized and centrifuged at 105,000 × g, and cytosol was subjected to electrophoresis. The gel was cut into 1-mm slices, each slice was oxidized, and its content of $^3$H and $^4$C was determined and plotted (upper graph), as was the ratio of radioactivity for each slice (lower graph). B, 1 hr before sacrifice a rat bearing a growing tumor received an i.p. injection of 0.5 mCi of $[^4]$C]leucine and a rat bearing a tumor regressing after deprivation of mammotropins 24 hr earlier received an i.p. injection of 0.5 mCi of $[^3]$H]leucine. The 2 tumors were homogenized together and processed as described for A. Note the difference in the ratios presented in the graphs of A and B.

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**Chart 4. Normalized ratios of radioactivity in cytosol proteins from regressing (R) versus growing (G) tumors.** Experimental details were as described in the legend to Chart 3. The ratio of radioactivity of each slice (R) was normalized by the average ratio (R) for the entire gel. A normalized ratio larger than 1 indicates a decrease of incorporation of the labeled precursor injected into the rat bearing a regressing MTW9 tumor.
obtained, and the prolactin determination was carried out by radioimmu-

for instance in the liver of the same animal. No change was

were analyzed following the same procedure applied to the

tive of the work reported here was to examine whether

hormone removal altered the composition of tumor pro-

of the cytosol counts. Fig. 1 shows a gel where the Rf

tions reported here indicate that a change in the protein

labeling time of 20 hr and were unable to detect differences

between growing and regressing cytosol proteins; however,

regression system that, to our knowledge, is comparable to

MTW9 model. Beckingham Smith and Tata (2) used a

labeling time of 20 hr and were unable to detect differences in

the protein profile during tadpole tail regression. In our

system also, 20 hr of labeling failed to reveal any difference

between growing and regressing cytosol proteins; however,

a difference was detected with a 1-hr pulse.

Arrest of ovalbumin synthesis following estrogen with-
drawal has been explained by the rapid inactivation of

mRNA coding for that protein (21). The same kind of

interpretation has been used to explain the hormonal mod-

ulation of the synthesis of α 2u globulin in rat liver (14).

Qasba and Gullino (24) observed that α-lactalbumin is

present in growing MTW9 at about 10% of the level in the

lactating gland; casein is present in this tumor, although

the exact quantity is unknown (unpublished observation).

Since serum prolactin disappears very rapidly after removal

of MTW10 (Chart 5), a drop in α-lactalbumin and casein

mRNA synthesis could explain the rapid disappearance of

the cytosol counts. Fig. 1 shows a gel where the missing radioactivity in the cytosol bands are

compared with the positions of rat α-lactalbumin and casein. As a preliminary impression it appears that the missing counts might be, in part, in the bands where caseins migrate.

Our previous finding (25) of an increased susceptibility of

cytosol proteins to proteolytic digestion and the observations

reported here indicate that a change in the protein profile occurs at the onset of MTW9 regression. The modi-

fication of the protein profile supports, although it does not

prove, the hypothesis that the augmented activity of lyso-

somal enzymes observed during regression of several tis-

sues may be triggered by a change of the protein profile
determined by the hormonal deprivation. The relationship,

if any, between this change and the triggering of tumor

regression remains to be clarified.

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Fig. 1. Rf position of missing counts in the cytosol of regressing MTW9. Comparison with positions of rat casein and α-lactalbumin (αLA).
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