PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **186**, 60–63 (1987)

Milk-Specific RNase as a Marker of Differentiation of Rat Mammary Tumors (42584)

DAI KEE LIU AND GAIL F. OWENS

Department of Pharmacology, The Milton S. Hershey Medical Center, College of Medicine, Pennsylvania State University, Hershey, Pennsylvania 17033

Abstract. Various rat mammary tumors were analyzed for the presence of a milk-specific Ca^{2+}-stimulated RNase (Ca^{2+}-RNase). When crude extracts of some differentiated tumors—adenocarcinomas of MT/W9, MT/W9a, R3230AC, DMBA-1, DMBA-8, and DMBA-14 and 3MN squamous cell carcinoma—were assayed for RNase activity under various ionic conditions, it was always highest in the presence of Ca^{2+}/EDTA than under any other ionic condition. The opposite was true in invasive MT/W449a and 13762 adenocarcinomas, poorly differentiated SMT/2A carcinomas, $MAMF_2$/TC fibrosarcoma, and MT/A fibroadenoma. Sephacryl S-200 chromatography separation of tumor extracts confirmed the presence of Ca^{2+}-RNase in those differentiated tumors and absence of the enzyme from other tumors. Expressing the activity as a ratio of Ca^{2+}/EDTA to either Mg^{2+}/EDTA or EDTA alone to more clearly represent the relative level of Ca^{2+}-RNase activity further illustrates the distinct differences between tumor classes. Thus Ca^{2+}-RNase is a sensitive marker for use in the characterization of rat tumors with respect to differentiated mammary functions.

Rat mammary gland contains a Ca^{2+}-RNase which increases during lactation and which is absent from both nonmammary tissues and sera of either lactating or nonlactating rats (1). The complete dependence on Ca^{2+} for catalytic activity distinguishes this RNase from other RNases in sera and tissues of rats (1–3). High levels of this enzyme are present in rat milk rendering it a constitutive milk component (3). In a preliminary survey we observed that the Ca^{2+}-RNase was present in estrogen- and prolactin-sensitive R3230AC rat mammary adenocarcinomas and was absent from a rat hepatoma (1, 4), suggesting that the enzyme could be a marker for the characterization of rat tumors with respect to hormonal sensitivity and to differentiated mammary function, i.e., milk production and secretion. In this study we assayed a series of established rat mammary tumors for RNase activity and demonstrated that high Ca^{2+}-RNase activity is related to epithelial cell type and to hormone sensitivity.

Materials and Methods. All rat mammary tumors, except R3230AC mammary adenocarcinoma, were provided by Dr. A. E. Bogden of EG & G Mason Research Institute Tumor Bank, sponsored by the Division of Cancer Biology and Diagnosis, National Cancer Institute. These tumors included adenocarcinomas of MT/W9 (mammotropin-dependent), MT/W9a (growth retarded by ovariectomy), DMBA 1 (9,10-dimethyl-1,2-benzanthracene-induced, carried in lactating females), DMBA 8 (actively secreting), DMBA 14 (growth inhibited by ovariectomy), metastatic adenocarcinomas of MT/W449a (invasive) and 13762 (inhibited by estrogen, invasive), undifferentiated carcinoma SMT/2A (invasive) and mammary squamous carcinoma 3M2N (stimulated by estrogen), mammary fibrosarcoma $MAMF_2$/TC (inhibited by estrogen), and mammary fibroadenoma MT/A (5). Each mammary tumor was received in dry ice in a sealed vial containing small pieces of tumor suspended in tissue culture medium 199 fortified with 10% dimethyl sulfoxide and 15% rat serum. Tumors were kept at −70°C until used. The R32230AC mammary tumor (stimulated by estrogen and prolactin) (5, 6) was that transplanted and grown in F344 female rats for 3 to 4 weeks as previously described (7).

Membranous connective tissues were removed from freshly collected R3230AC tumor pieces (6) and excess suspending medium was drained from the frozen tumor pieces while on filter paper on ice. All subsequent steps were carried out at 4°C. The tumors were homogenized in 20 vol of buffer A (50 m*M* Tris/HCl, pH 7.9, 0.1 m*M* EDTA, 0.1 m*M* dithiothreitol, 1 mg/ml bovine serum albumin, 50 m*M* ammonium sulfate, 25% glycerol) using an Ultra-Turrax (Tekmar Co., Cincinnati,

0037-9727/87 $1.50

OH) for four 15-sec bursts at medium setting. The homogenates were centrifuged at 105,000*g* for 60 min and the supernatants were used for enzymic assay and Sephacryl S-200 separations.

Each sample was assayed for RNase activity using a combination of ionic conditions (1–3). Each condition was done in triplicate. Briefly, for routine assay, each tissue extract was incubated at 37°C for 30 min with [^{3}H]poly(U) (0.48 nmole phosphate, 19 μCi/μmole phosphate) under the same buffered conditions (1) simultaneously with three different ionic combinations: (a) 10 m*M* EDTA (EDTA), (b) 15 m*M* $CaCl_2$ plus 10 m*M* EDTA (Ca^{2+}/EDTA), and (c) 15 m*M* MgOAc plus 10 m*M* EDTA (Mg^{2+}/EDTA). The enzymatic activity was based on the rate of increase of ^{3}H-nucleotides soluble in 95% ethanol–10 m*M* MgOAc at −20°C, expressed as units (1 unit = 0.024 nmole phosphate) per milligram of tissue (1). The presence of Ca^{2+}-RNase was indicated when RNase activity was highest with Ca^{2+}/EDTA than with other ionic conditions. The ratio of RNase activity with (b) to (a) or to (c) was taken to indicate the relative level of Ca^{2+}-RNase activity.

For separation of Ca^{2+}-RNase from other tissue RNases, 300 μl of extract was placed onto a Sephacryl S-200 column (0.5 × 92 cm) equilibrated with buffer B (0.1 *M* Tris/HCl, pH 7.3, 1 m*M* dithiothreitol, 0.34 *M* sucrose, 0.2 *M* KCl) and the enzymes were eluted with the same buffer (3). Each fraction was assayed, in triplicate, for RNase activity as described above using 5 m*M* Ca^{2+}, 5 m*M* Mg^{2+}, and no added ion. Longer incubation time at room temperature was used in case of low enzymatic activity.

Results. Table I shows RNase activity in various tumors assayed under different ionic conditions and the ratio of RNase activity between that assayed with Ca^{2+} and without Ca^{2+} present. Similar values from normal lactating mammary gland are included for comparison (2). With the exception of the MT/W449a and 13762 adenocarcinomas, all the adenocarcinomas of differentiated tumors exhibited the highest RNase activity with Ca^2/EDTA, followed by those with Mg^{2+}/EDTA, and the lowest with EDTA. This order was the same in normal lactating mammary gland (2). The ratio of RNase activity between Ca^{2+}/EDTA and Mg^{2+}/EDTA among these tumors ranged from 5 to 13 and the ratio between Ca^{2+}/EDTA and EDTA ranged from 73 to 604, with the two ratios increasing essentially in the same order. The ratios from lactating mammary gland were respectively 52 and 81. In the MT/W449a and 13762 adenocarcinomas, both

TABLE I. RNase Activity in Various Rat Mammary Tumors Assayed under Different Ionic Conditions

Mammary tissue	RNase activity (units/mg tissue) in			Activity ratio of Ca^{2+}/EDTA and	
	EDTA	Ca^{2+}/EDTA	Mg^{2+}/EDTA	Mg^{2+}/EDTA	EDTA
Adenocarcinoma					
R3230AC	1.1	251.0	19.0	13	228
MT/W9	0.5	302.0	27.8	11	604
MT/W9a	1.3	94.0	18.0	5	73
DMBA 1	0.3	147.0	12.4	12	490
DMBA 8	0.2	106.0	10.0	10	530
DMBA 14	0.7	90.0	12.4	7	129
MT/W449a	27.1	22.0	25.8	0.9	0.8
13762	0.6	4.9	13.7	0.4	8.2
Carcinoma					
SMT/2A (undifferentiated)	2.0	2.5	12.9	0.2	1.3
3M2N squamous	1.5	116.0	19.3	6	77
Fibrosarcoma $MAMF_2$/TC	0.9	2.8	12.9	0.2	3.0
Fibroadenoma MT/A	0.2	0.7	2.5	0.3	3.5
Lactating mammary gland[a]	15.0 ± 9.7	1220 ± 24.0	23.4 ± 12.6	52	81

[a] Data from Ref. (2).

metastatic tumors, RNase activity in Ca^{2+}/EDTA was lower than that under other ionic conditions. Thus the ratio of RNase activity between Ca^{2+}/EDTA and Mg^{2+}/EDTA or EDTA was respectively 0.9 and 0.8 in MT/W449a and 0.4 and 8.3 in 13762. The 3M2N hormonal-sensitive squamous carcinoma was the only other tumor examined that had higher Ca^{2+}-RNase activities, with an RNase activity ratio between Ca^{2+}/EDTA and Mg^{2+}/EDTA or EDTA of 6 and 77, respectively (Table I). The undifferentiated SMT/2A carcinoma, $MAMF_2$/TC fibrosarcoma, and MT/A fibroadenoma were all higher with Mg^{2+}/EDTA than under other ionic conditions, with lower ratios of Ca^{2+}/EDTA to Mg^{2+}/EDTA or EDTA.

Figure 1 shows typical Sephacryl S-200 column profiles of RNase separation from several tumor extracts. Without exception, the column fractions obtained from R3230AC (Figs. 1A and 1C), 3M2N (Fig. 1B), DMBA 8 (Fig. 1D), and MT/W9a (Fig. 1E), all of which exhibited high ratios of Ca^{2+}/EDTA to Mg^{2+}/EDTA or EDTA, consistently showed higher

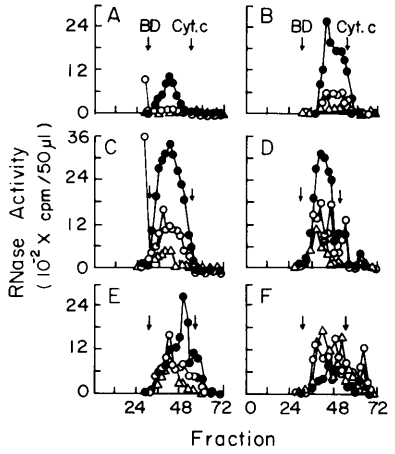

FIG. 1. Sephacryl S-200 gel filtration of mammary tumor extracts (300 µl) and assay for RNase in column fractions (0.4 ml/fraction) under different ionic conditions: ●, 5 m*M* Ca^{2+}; ○, 5 m*M* Mg^{2+}; and △, no added ion. (A) R3230AC tumor, (B) 3M2N tumor, (C) R3230AC tumor, (D) DMBA 8 tumor, (E) MT/W9a tumor, and (F) MT/449a tumor. BD indicates the elution volume of Blue Dextran 2000 and Cyt. c indicates the elution volume of cytochrome *c* (M_r 12,300). All enzymatic assays were carried out at room temperature for 16 hr, except those in (A), which were done at 37°C for 30 min.

peaks with Ca^{2+}/EDTA than with Mg^{2+}/EDTA or EDTA. The Ca^{2+}-RNase peaks distributed in a region ranging from M_r 35,000 to 55,000. This range suggested multiple forms of Ca^{2+}-RNase, as in similarly separated Ca^{2+}-RNase in rat milk (2). The Ca^{2+}-RNase peak was absent from the MT/W449a tumor (Fig. 1F), which was low in the RNase ratios between Ca^{2+}/EDTA and Mg^{2+}/EDTA or EDTA.

Discussion. Characterization of mammary tumors with respect to their responsiveness to hormones such as estrogen, progesterone, and prolactin would provide insights into growth and differentiated properties of the tumors. Clinically, determination of receptors for estrogen and progesterone aids in therapeutic design (8, 9) and in prognosis (9, 10). Still, some receptor-positive tumors do not always respond to endocrine therapy (9). Thus, assaying for the presence of unique milk components such as lactose, α-lactalbumin, or casein in mammary tumors would more directly reveal hormonal sensitivity or functional receptors. Unfortunately, the presence of a unique milk component like α-lactalbumin may also not correlate with hormone dependency (11). Other markers are therefore required. We now demonstrate that the milk-specific Ca^{2+}-RNase is a sensitive marker for mammary differentiation. Assaying for its presence is useful in the characterization of unknown tumors, present at least in rat tumors. Although most tumors that we have examined were a single pooled tumor specimen, the data clearly showed that without exception Ca^{2+}-RNase activity occurred only in adenocarcinomas and adenomas and only in those known to be sensitive to ovarian or pituitary hormones (5). Included among the Ca^{2+}-RNase-positive tumors was the low estrogen receptor and prolactin-sensitive R3230AC adenocarcinoma (12–14), which are also high in α-lactalbumin (15). The Ca^{2+}-RNase-positive 3M2N tumor, consistent with its sensitivity to estrogen (5), however, is low in α-lactalbumin content (14). Thus it is possible for nonparallel expression of different milk proteins in the same tumor. The mechanisms remain to be investigated.

The advantage of using the current RNase assay method is that the procedure is simple, and most of all, accurate and sensitive. Less

than 1 mg tumor tissue is necessary for carrying out the determination (for each set of enzymatic assay less than 15 μg tissue would suffice). The absolute requirement of Ca^{2+} for enzymatic activity makes the present method useful for crude samples without sacrificing specificity. It should be noted, however, that since other non-mammary-specific RNases are not totally inactive with Ca^{2+} present in the assay, short of total separation of the milk-specific Ca^{2+}-RNase from other enzymes it would be difficult to estimate precisely the activity attributable to the Ca^{2+}-RNase. By taking the ratio of enzymatic activity with Ca^{2+}/EDTA and Mg^{2+}/EDTA or EDTA, we were able to distinguish the Ca^{2+}-RNase-positive and -negative tumors. Mg^{2+} was included to maximally activate Mg^{2+}-requiring cellular RNases (16). EDTA was included to remove endogenous ions from the extract which might interfere with Ca^{2+}-RNase. Since EDTA alone has no direct inhibitory effect on cellular RNases (16), by using higher Ca^{2+} than EDTA concentrations in Ca^{2+}/EDTA we were able to accurately obtain Ca^{2+}-RNase activity (3). From the data shown in Table I, for practical application using crude tissue extract, an RNase activity ratio between Ca^{2+}/EDTA and Mg^{2+}/EDTA of greater than 5 or between Ca^{2+}/EDTA and EDTA of greater than 10 would be a useful index for judging the Ca^{2+}-RNase-positive and -negative tumors.

The presence of the Ca^{2+}-RNase activity can be equally well demonstrated by gel filtration. The Ca^{2+}-RNase-positive tumors contained various amounts of different size enzymes. The reason for the differences as well as the nature of heterogeneity is not known. The multiforms were, nevertheless, similar to that of milk enzyme, but different from the secretory RNase of pancreas or serum types (3).

This work was supported by American Cancer Society Grant BC374 and by National Cancer Institute Grant CA35916. We are grateful to Dr. Arthur E. Bogden for the rat tumor specimens.

1. Liu DK, Kulick D, Williams GH. Ca^{2+}-stimulated ribonuclease: A new marker enzyme of differentiated rat mammary tissue. Biochem J **178**:241–244, 1979.
2. Liu DK, Williams GH. Species differences in ribonuclease activity of milk and mammary gland. Comp Biochem Physiol B **71**:535–538, 1982.
3. Liu DK, Owens GF. Mammary origin of rat milk ribonuclease. Int J Biochem **15**:1273–1277, 1983.
4. Liu DK, Liao WSL, Fritz PJ. Ca^{2+}-stimulated ribonucleases from rat mammary gland and R3230AC mammary adenocarcinoma nuclei. Biochemistry **16**: 3361–3369, 1977.
5. Bogden AE. Bibliography pertinent to tumors maintained by the animal and human tumor bank. In: Breast Cancer Task Force Animal and Human Tumor Bank. EG & G Mason Res Inst, Worcester, MA, p8, 1981.
6. Hilf R. Biochemical studies of experimental mammary tumors as related to human breast cancer. Methods Cancer Res **7**:55–114, 1973.
7. Liu DK, Williams GH, Fritz PJ. Alkaline ribonuclease and ribonuclease inhibitor in mammary gland during the lactation cycle and in the R3230AC mammary tumor. Biochem J **148**:67–76, 1975.
8. Bloom N, Tobin E, Degenshein GA. Clinical correlation of endocrine ablation with estrogen and progesterone receptors in advanced breast cancer. In: McGuire WL, Raynaud JP, Baulieu EE, Eds. Progress in Cancer Research and Therapy. New York, Raven Press, Vol **4**:p25, 1977.
9. McGuire WL. Steroid hormone receptors in breast cancer treatment strategy. Recent Prog Horm Res **36**: 135–146, 1980.
10. Horwitz KB, McGuire WL. Estrogen and progesterone: Their relationship in hormone-dependent breast cancer. In: McGuire WL, Raynaud JP, Baulieu EE, Eds. Progress in Cancer Research and Therapy. New York, Raven Press, Vol **4**:p103, 1977.
11. Hall L, Craig RK, Ralphs DNL, Campbell PN. α-Lactalbumin is not a marker of human hormone-dependent breast cancer. Nature (London) **290**:602–604, 1981.
12. McGuire WL, Huff K. Mammary carcinoma: A specific biochemical defect in autonomous tumors. Science **175**:335–336, 1972.
13. Boylan ES, Wittliff JL. Specific estrogen binding in vivo in the R3230AC mammary adenocarcinoma of the rat. Cancer Res **33**:2903–2908, 1973.
14. Costlow ME, Buschow RA, McGuire WL. Prolactin receptors in an estrogen receptor-deficient mammary carcinoma. Science **184**:85–86, 1974.
15. Schultz GS, Ebner KE. Measurement of α-lactalbumin in serum and mammary tumors of rats by radioimmunoassay. Cancer Res **37**:4482–4488, 1977.
16. Roth, JS. Some observations on the assay and properties of ribonucleases in normal and tumor tissues. Methods Cancer Res **3**:153–242, 1967.

Received November 25, 1986. P.S.E.B.M. 1987, Vol. 186.
Accepted May 22, 1987.