Some Biochemical Characteristics of Human Breast Cancer and Nonmalignant Breast Lesions[1] (34271)

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(Introduced by A. Borman)

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A significant research effort is directed toward the development of experimental models of neoplasia. In the case of mammary cancer, the investigator can study spontaneous, transplantable, or viral and carcinogenic-induced neoplasms in rats or mice. Usually, the experimentalist evaluates the response of a tumor to various treatments by monitoring neoplastic growth and morphology.

For several years, our studies have been directed toward biochemical characterization of experimental mammary tumors. These investigations have entailed the measurement of several biochemical end points in various experimental rat carcinomas and the examination for correlations of these parameters with gross and microscopic morphologic changes as influenced by hormonal treatment of the host (1–5). Biochemical studies of cancer invariably consist of comparisons between normal and neoplastic tissues and our studies have been consistent with this approach. Thus, we have ascertained some of the biochemical characteristics of transplantable and primary mammary adenocarcinomas of the rat and compared them to those of normal mammary glands in the rodent. We have subsequently examined human breast tissue and compared various biochemical parameters of normal, fibrocystic disease, and cancer in this tissue in order to ascertain if any biochemical similarities exist between tumors in women and any of the neoplasms in the rodent, and, more importantly, are any parameters in the cancer significantly different from normals.

The data presented here are a summary of our studies of human infiltrating ductal carcinomas of the breast, selected for study because it is the most common malignant neoplasm of the human breast and as such, should represent the type of carcinoma for which an experimental model would be most useful. The problems represented in studies of human tissue such as homogeneity, preservation, stability, control tissue, etc., have been carefully outlined in the comprehensive paper by Shonk and Boxer (6), and concern with these difficulties may have been responsible for the relative paucity of biochemical data on human breast cancer (7–10). The most extensive report that we found is a recent paper by Smith *et al.* (11), containing data obtained on 13–17 human breast carcinomas and an equal number of normal breast tissue samples. These investigators examined 6–8 different enzymes, as well as nucleic acids and protein, and concluded that the neoplasms were characterized by an increased glucose metabolism and further, that some of these enzyme activities were correlated.

Materials and Methods. Tissues were obtained immediately from the operating room and were carefully trimmed of grossly normal, necrotic, and hemorrhagic contaminants. Samples for biochemical analyses were quick-frozen in vials immersed in an acetone–dry ice freezing mixture. The time from removal of the specimen from the patient to freezing was maximally 10–15 min. All tis-

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TABLE I. Some Biochemical Characteristics of Human Infiltrating Ductal Carcinomas, Fibrocystic Disease of the Breast and Normal Mammary Tissue.

End point	Normal breast tissue (10)[a]	Fibrocystic disease (8)	Infiltrating ductal carcinoma (34)
Nucleic acids (μg/mg tissue)			
RNA	1.49 ± 0.48[b]	1.86 ± 0.40	4.79 ± 0.29[e,f]
DNA	1.87 ± 0.56	2.91 ± 0.56	6.78 ± 0.49[e,f]
RNA/DNA ratio	0.95 ± 0.19	0.64 ± 0.04	0.77 ± 0.05
Enzyme activity (μmoles cofactor/min/100 mg of tissue)			
PYK[c]	0.070 ± 0.031	2.048 ± 0.832[d]	7.796 ± 0.719[e,f]
GPI	0.581 ± 0.168	1.274 ± 0.252[d]	4.080 ± 0.531[e,f]
AAT	0.046 ± 0.019	0.213 ± 0.052[e]	0.449 ± 0.054[e,f]
ICD	0.022 ± 0.011	0.092 ± 0.022[d]	0.318 ± 0.047[e,f]
G6PD	0.009 ± 0.003	0.035 ± 0.007[e]	0.138 ± 0.016[e,f]
PGM	0.002 ± 0.001	0.023 ± 0.008[d]	0.046 ± 0.004[e]
ME	0.003 ± 0.001	0.008 ± 0.002[d]	0.029 ± 0.004[e,f]
GDH	0.002 ± 0.001	0.003 ± 0.001	0.038 ± 0.008[e,f]
HK	0.002 ± 0.001	0.003 ± 0.002	0.032 ± 0.004[e,f]
GK	0.002 ± 0.001	0.001 ± 0.001	0.003 ± 0.001
αGPD	0.088 ± 0.018	0.081 ± 0.023	0.048 ± 0.007[d]
Lipids (μg/mg of tissue)			
CHOL	1.21 ± 0.26	0.90 ± 0.16	2.39 ± 0.23[e,f]
FFA	1.33 ± 0.11	1.06 ± 0.16	3.10 ± 0.29[e,f]
TG	244 ± 66	77.0 ± 25.0[d]	49.4 ± 14.4[e]
CHOL esters	0.43 ± 0.08	0.35 ± 0.05	1.02 ± 0.11[e,f]

[a] Number of tissues assayed given in parentheses.

[b] Mean ± standard error.

[c] Abbrev.: PYK, pyruvate kinase; GPI, glucose-phosphate isomerase; AAT, aspartate aminotransferase; ICD, isocitrate dehydrogenase, decarboxylating; G6PD, glucose-6-phosphate dehydrogenase; PGM, phosphoglucomutase; ME, malate dehydrogenase, decarboxylating; GDH, glutamate dehydrogenase; HK, hexokinase; GK, glucokinase; αGPD, α-glycerolphosphate dehydrogenase; CHOL, cholesterol; FFA, free fatty acids; TG, triglycerides; CHOL esters, cholesterol esters.

[d] Significantly different from normal breast tissue, ($p<0.05$); [e] ($p<0.01$).

[f] Significantly different ($p<0.01$) from fibrocystic disease tissue.

sues were stored in a deep freeze until packed in dry ice and shipped from one laboratory to another.

After thawing and prior to homogenization, aliquots were made from each sample wherever possible and each aliquot was then treated as a separate sample for analyses. In addition, a small sample of the particular tissue was obtained from each aliquot for microscopic examination, to confirm the original diagnosis on tissue immediately adjacent to the actual sample analyzed. Of the 34 infiltrating ductal carcinomas examined, 22 neoplasms were large enough for replicate assays such that 12 tumors were assayed in duplicate, 4 tumors were assayed in triplicate, 4 tumors were assayed in quadruplicate, 1 tumor was assayed as 6 aliquots and 1 neoplasm was large enough to allow for 8 separate assays. The mean values shown in Table I were calculated from the average values obtained for each different neoplasm.

The biochemical assays, some of which have been given in detail previously (4), were modified so that 200 mg of tissue were sufficient for all analyses. Tissues were thawed and homogenized in cold 0.05 *M* Tris buffer, pH 7.4, with the volume of diluent added to produce either a 10% or 5% homogenate (w/v). Aliquots of the homogenate were taken for determinations of nucleic acids and lipids, the remainder of the homogenate being used for enzyme assay.

Enzyme assays were performed on the su-

pernatant of the homogenate following centrifugation at 20,000*g* for 20 min. Each of the enzyme assays was performed under identical conditions by measurement of the absorbancy change at 340 mμ, due to the production of NADPH or oxidation of NADH. Under these conditions (zero order kinetics), the enzyme values are comparable and are expressed as micromoles of NADPH produced/minute/100 mg of tissue weight, or micromoles NADH oxidized/minute/100 mg of tissue weight. The enzymes were measured by the following procedures: glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate-NADP oxidoreductase, EC 1.1.1.49),[3] Glock and McLean (12); isocitrate dehydrogenase, decarboxylating (L-isocitrate:NADP oxidoreductase, decarboxylating, EC 1.1.1.42), Ochoa method (13); malate dehydrogenase, decarboxylating (L-malate: NADP oxidoreductase, decarboxylating, EC 1.1.1.40), Ochoa *et al.* (14); glucosephosphate isomerase (D-glucose-6-phosphate ketolisomerase, EC 5.3.1.9), Shonk and Boxer (15); phosphoglucomutase (α-D-glucose-1,6-diphosphate; α-D-glucose-1-phosphate phosphotransferase, EC 2.7.5.1), enzyme activation with $MgCl_2$ according to Harshman *et al.* (16) and assay as outlined by Shonk and Boxer (15); α-glycerolphosphate dehydrogenase (L-glycerol-3-phosphate: NAD oxidoreductase, EC 1.1.1.8), Beisenherz *et al.* (17), modified by using dihydroxyacetone phosphate as the substrate and measuring the oxidation of NADH; glutamate dehydrogenase (L-glutamate:NAD oxidoreductase, deaminating, EC 1.4.1.2), employing the reverse reaction and oxidation of NADH described by Hogeboom and Schneider (18); aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1), slightly modified from the assay procedure of Karmen (19); pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40), method of Susor and Rutter (20); hexokinase (ATP:D-hexose-6-phosphotransferase, EC 2.7.1.1.), method of Sharma *et al.* (21); and glucokinase (ATP:D-glucose-6-phosphotransferase, EC 2.7.1.2.), method of Sharma *et al.* (21).

Nucleic acid concentrations were measured by a modification of the Schneider method (22), using the orcinol reaction for the determination of ribose (23) and the diphenylamine reaction for deoxyribose determination (24).

Aliquots of the original tissue homogenate were extracted for lipids with diethyl ether and separation of cholesterol, cholesterol esters, free fatty acids, and triglycerides was accomplished by thin-layer chromatography. The isolated lipids were then localized, extracted from the gel and quantitated. Details of this procedure were presented previously (4).

The data are presented as the mean ± standard error. Significance was determined by the Student's *t* test and the probability (*p*) values are presented.

Results and Discussion. The study summarized in Table I represents the data obtained during our examination of nucleic acids, lipids, and 11 different enzymes selected on the basis of earlier considerations for their involvement in various aspects of carbohydrate, lipid, and amino acid metabolism. In general, good agreement was obtained amongst replicate samples of the same carcinoma. This was also true for the samples of normal human mammary tissue, 8 of the 10 being large enough to allow 2–6 aliquots to be made for assays.

Considering the randomness of the clinical sampling, *i.e.*, some patients were premenopausal and others postmenopausal, some patients showed evidence of metastatic lesions and others none, lesion size ranged from 1.2 to 8.0 cm, etc., the standard errors of the mean values of the carcinomas were quite low. Indeed, these standard errors represented sample to sample variations that were similar to our findings with carcinogen-induced mammary carcinomas of rats. The apparently greater variation from sample to sample (larger standard errors of the means) in normal and fibrocystic disease tissue samples may be a reflection of the smaller number of patients in these categories. Additional

[3] The enzyme nomenclature and numbering system are according to the Report of the Commission on Enzymes of the International Union of Biochemistry [Macmillan (Pergamon), New York (1961)].

analyses will be needed. We have found many highly significant correlations amongst several of the enzyme activities in the infiltrating ductal carcinomas, such as a correlation coefficient of 0.836 ($p < 0.01$) between the activity of glucose-6-phosphate dehydrogenase and malate dehydrogenase (decarboxylating). These results are similar to the type of correlations reported by Smith *et. al.* (11) in their series of patients.

The data in Table I clearly demonstrate the striking differences in most of the biochemical parameters in the carcinomas compared to the nonmalignant mammary tissue. Using the Student's t distribution test, the values given in column 4 of Table I (P vs. normal) indicate a very high degree of significance assigned to the differences between tumor and normal. It is interesting that the RNA/DNA ratios are similar in both normal and neoplastic tissue and that glucokinase activities are similar in both sets of tissues. In all cases, except α-glycerolphosphate dehydrogenase activity and triglyceride levels, the neoplasm demonstrates higher values. Previous studies on rodent neoplasms demonstrated that quick-freezing and storage at −20° maintained these enzyme activities for at least 2 months. The elevated enzyme activity demonstrated in the human tissue samples indicated adequate preservation of the neoplastic tissues with the procedures employed. If these data are recalculated on the basis of the DNA levels, *e.g.*, enzyme activity/mg of DNA, the same overall significant differences are obtained when comparing the infiltrating ductal carcinomas with nonmalignant tissue. Thus, the alterations in the biochemical parameters in the neoplasms are not due primarily to differences in cellularity. Also of concern is the contribution of adipose tissue to the biochemical measurements, particularly when examining normal breast tissue. The high levels of triglyceride and α-glycerolphosphate dehydrogenase activities are in agreement with the anticipated adipose tissue content of normal breast tissue and expressing enzyme activities in terms of triglycerides would make the differences between normal and cancerous tissues even greater. We are in the process of analyzing adipose tissue from other parts of the body to ascertain similarities and differences to quiescent breast tissue.

It was important to establish that the changes observed in the carcinomas were not a result of nonmalignant proliferative processes. Therefore, we examined samples of fibrocystic disease, a common proliferative disease of the breast, which may clinically simulate carcinomas. The results of biochemical assays on fibrocystic disease (Table I) indicate that all of the biochemical parameters studied are not elevated in fibrocystic disease compared to normal mammary tissue. Although 8 of 18 and points were elevated, it should be noted that these changes did not exhibit a very high degree of significance. The most important point of these studies is shown in the last column of Table I, where the biochemical characteristics of infiltrating ductal carcinoma are compared with those of fibrocystic disease. These values demonstrate highly significant differences between neoplastic tissue and this nonmalignant disease, further strengthening the conclusion that the results obtained in carcinomas are not solely due to increased proliferation or a nonmalignant disease process. It is certainly of interest that all of the biochemical parameters that were significantly different in fibrocystic disease, compared with normal tissue, had numerical values that fell between normal and carcinoma. Thus, depending on the particular end point, one could rank the tissues as follows: normal < fibrocystic disease < infiltrating ductal carcinoma.

Finally, these studies show that the biochemical profile of infiltrating ductal carcinoma in women resembles to a degree the biochemical profile obtained in rodent mammary tumors induced by a single intubation of 7,12-demithylbenz[a]anthracene (25). These data on human tumors should be most helpful in efforts to establish a biochemical basis for validation of an experimental tumor model.

Summary. Human infiltrating ductal carcinoma of the breast, fibrocystic disease of the breast, and normal human mammary tissues were assayed for their concentration of nucleic acid, lipids, and 11 selected enzymes involved in carbohydrate, lipid, and amino

acid metabolism. Comparing infiltrating ductal carcinomas to normal mammary tissue, striking elevations ($p < 0.001$) were seen in pyruvate kinase (100-fold), glucose-6-phosphate, isocitrate and malate dehydrogenases (10–15-fold), free fatty acids and cholesterol (2–3-fold). The RNA/DNA ratios in the carcinomas were unchanged but triglycerides and α-glycerolphosphate dehydrogenase were reduced. Also studied were samples from eight cases of fibrocystic disease, a common nonmalignant proliferative disease of the human breast. Ranking the levels of nucleic acids and lipids and the activities of the enzymes in the tissues studied clearly showed that normal $\leqq$ fibrocystic disease $\leqq$ infiltrating ductal carcinoma. No significant differences in the biochemical characteristics of the carcinomas were found relative to the size of the lesion or to the menopausal state of the patient. These data on infiltrating ductal carcinomas closely resemble the data obtained from single dose 7,12-dimethylbenz[a]anthracene-induced rat mammary carcinomas, suggesting that the latter experimental tumor may represent a valid metabolic model of human breast cancer.

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