Biochemical Characterization of Carcinogen-induced Mammary Hyperplastic Alveolar Nodule and Tumor in the Rat

Thomas L. Dao, Dilip Sinha, S. Christakos, and R. Varela

Department of Breast Surgery and Endocrine Research Laboratory, Roswell Park Memorial Institute, Buffalo, New York 14203

SUMMARY

Biochemical parameters characterizing growth, functional activity, and hormone dependence were compared in the normal mammary gland, the 7,12-dimethylbenz(a)anthracene-induced hyperplastic alveolar nodule (HAN), and the mammary tumor in the rat. The rate of DNA synthesis in HAN was significantly lower than the rates in either the normal mammary gland cells or the mammary tumor cells cultured in medium containing identical hormone supplement for the same duration of time. The rate of casein synthesis in the explants of pregnant rat mammary gland and HAN was greatly stimulated when cultured in medium containing insulin, cortisol, and prolactin. The mammary tumor, however, failed to produce an increase in casein synthesis under the same experimental conditions. The specific estradiol-binding proteins were either absent or present in very low concentrations in HAN. In contrast, mammary tumor cytosol contained high concentrations of specific estrogen-binding proteins. The normal mammary gland, in spite of its low cellularity, had twice as many estradiol-binding macromolecules as did HAN. Altogether, these results show that HAN cell populations differ from normal mammary gland and mammary tumor cells by their nonresponsiveness to hormonal stimulation for growth and by their lack of specific receptors for estradiol. They retain, however, the functional capacity to synthesize casein, a biochemical property that the mammary tumor does not possess.

INTRODUCTION

Previous studies on histogenesis and pathogenesis of mammary tumors have suggested that virus-, hormone-, and carcinogen-induced mammary hyperplasia and dysplasia in mice and rats are preneoplastic because they progress to form mammary tumors. DeOme et al. (5) were the first to demonstrate that virus-induced HAN in mice develop neoplastic outgrowth upon transplantation into the mammary gland free fat pad in the isologous host. Similar results have been reported by Beuving (2), who transplanted carcogen-induced HAN in the rat into the gland free mammary fat pad. These observations suggest that HAN's are preneoplastic because they are induced by a carcinogen and become neoplastic after transplantation. On the other hand, recent work from our laboratory strongly suggests that mammary tumor induction in the rat is a direct process (15). It is proposed that, when mammary cells are exposed to a carcinogen, they are transformed directly to neoplastic cells and require no intermediate steps.

If HAN's are indeed "preneoplastic," one would expect to find certain similarities in the biological characteristics of these lesions and the tumors. Since mammary adenocarcinomas induced by DMBA are highly hormone dependent, it would be reasonable to assume that their precursor cells are equally hormone sensitive. To test this hypothesis, we have studied the hormone responsiveness of the normal mammary gland, HAN, and the mammary tumor in vitro by measuring DNA and casein synthesis and the presence or absence of specific EBP's in these tissues. This paper reports the results of this study. In addition, a method for identification of HAN in vivo is described.

MATERIALS AND METHODS

Induction of HAN and Mammary Tumors. Fifty-five- to 60-day-old Sprague Dawley female rats (Holtzman Co., Madison, Wis.) were used for tumor induction. Mammary tumors were induced by a single i.v. injection of 5 mg DMBA in an emulsion prepared by Dr. Paul Schurr of the Upjohn Company, Kalamazoo, Mich. HAN's appear as early as 30 days after carcinogen administration (15). On Day 45 the rats were anesthesized with ether; HAN's were identified by a simple method developed in this laboratory (see "Results") and were excised for biochemical studies. Mammary tumors are usually palpable 60 days after carcinogen administration. The tumors, upon reaching the size of about 1.5 cm in diameter, were removed for similar biochemical studies. Both HAN's and mammary tumors are sectioned and routinely stained for histological studies. Biochemical studies were also carried out with mammary glands from pregnant rats because they bear a morphological resemblance to HAN.

Biochemical Studies. DNA synthesis in the normal mammary gland from virgin 60- to 66-day-old female rats, HAN, and mammary tumor was measured by autoradiography after labeling with [3H]thymidine. Explants of these tissues were cultured in Eagle's medium containing insulin,
Carcinogen-induced Rat Mammary HAN and Tumor

RESULTS

In Vivo Identification of HAN

It is not possible to identify HAN in a mammary gland by visual examination of the tissue in situ. A method for positive identification of these lesions in situ is a prerequisite for experiments involving in vitro cultivation of HAN and its biochemical analysis thereafter. We have successfully developed a "foolproof," simple technique for identification of these lesions. The technique is as follows. The mammary gland is dissected out carefully under aseptic conditions while the rat is under anesthesia. The gland is then laid flat over a 4-inch x 4-inch sterile glass plate. The mammary gland is kept moist with a few drops of Eagle's medium. Using a basic principle of dark-field illumination, the glass plate with the mammary gland on it is illuminated by a bright light source underneath. The specks of HAN can be recognized easily in the more or less transparent mammary gland under a dissecting microscope (Fig. 1). The microscopic tumor, if present, can easily be recognized as a solid speck with no visible lobular structure, which is typical of a HAN. To test the accuracy of this technique, HAN thus observed was dissected out under aseptic conditions and was sectioned, stained, and examined microscopically. This technique has proven 100% accurate in identifying these minute lesions in the living gland (Fig. 2).

Comparison of Hormone Responsiveness of Normal Mammary Gland, HAN, and Mammary Tumors

These 3 tissues were cultured for 3, 6, and 9 days in Eagle's medium containing the following hormones: insulin, 5 µg; 17β-estradiol, 0.001 µg; progesterone, 1 µg; and prolactin, 5 µg/ml medium. The [3H]estradiol labeling index of these tissues was determined. The results are shown in Table 1. The labeling index in HAN is significantly lower...
than the index in either the normal mammary cells or the mammary tumor cells. The data suggest that HAN contains cell populations that are not in proliferation, as compared with those from the normal mammary gland or the mammary tumor.

The duration of DNA synthesis in these 3 tissues was compared by the pulse-labeling technique and autoradiography following culture in the same hormone combination of insulin, prolactin, estradiol, and progesterone. Chart 1 shows that the S phase of the mammary gland is about 20 hr, and that of HAN is around 18 hr. Mammary tumors cultured under the same condition have an S phase of 10.5 hr. It seems that, in spite of the hyperplastic nature of HAN, it has the same DNA synthetic period as the normal mammary gland. This would mean that either the HAN cells proliferate no faster than the normal mammary gland cells in an in vitro system or that perhaps there is a shortening of the other phases of the cell cycle.

Casein Synthesis. The rate of casein synthesis was measured by incorporation of 32P into tissue explants of pregnant mammary gland, HAN, and mammary tumor. It has been demonstrated that the midpregnant mouse mammary gland requires insulin, a glucocorticoid, and prolactin for optimal casein synthesis in vitro (16). Our present experiments utilizing the midpregnant rat mammary gland in vitro gave similar results. We also found that mammary glands from virgin rats, like those in the mouse, are unresponsive to the hormonal stimulation for casein synthesis (17). Our present results also show that, although insulin alone can induce casein synthesis in the midpregnant mouse mammary gland, the presence of estrogen, prolactin, and a glucocorticoid (Compound F) further increases the rate of synthesis of casein. The data likewise disclose that HAN in explanted culture can be stimulated to synthesize casein like the pregnant mouse mammary gland (Chart 2). In contrast, mammary tumors fail to produce a significant increase in casein synthesis in vitro in the presence of insulin, estrogen, cortisol, and prolactin. This finding is in agreement with the earlier report of the failure of tumor from C3H mice to respond to insulin, hydrocortisone, and prolactin for casein synthesis (18).

Estrogen Receptors in Normal Mammary Gland, HAN, and Mammary Tumor

The presence or absence of estrogen receptors in these 3 tissues has been examined by the sucrose gradient method, and the specificity of estrogen binding was determined by inhibition with an antiserum of steroid compound nafoxidine. DMBA-induced HAN's were identified in situ by the method described in this paper, were excised from several mammary glands, and were pooled to obtain a total amount of at least 300 mg or more of tissue, to give enough protein concentration (15 to 20 mg/g tissue) for a meaningful experiment. A total of 3 experiments was done with HAN. In 2 experiments, specific estrogen receptor protein was not detected. Chart 3 shows the results of a 3rd experiment in which as much as 500 mg of HAN were used to prepare the cytosol for sucrose gradient analysis, which reveals the presence of a small peak of 8 S binding protein that can be partially inhibited by preincubation with nafoxidine. In contrast, the mammary tumor contains significantly greater
amounts of both 4 S and 8 S binding proteins, which are estrogen specific because they are inhibited by nafoxidine (Chart 4). The presence of specific estrogen binding receptors in a normal mammary gland was also demonstrated. The rather low concentration of EBP in the normal mammary gland is probably due to the extremely low cellularity of this tissue. To test this hypothesis, we carried out 1 experiment to compare the level of estrogen binding of these 3 tissues on the basis of EBP per mg of DNA. DNA content of these tissues was determined by the method of Burton (4). Scatchard analysis (14) was carried out to estimate the actual binding sites. Table 2 summarizes the results of this experiment. The data clearly show that the number of EBP binding sites in HAN is about one-half of that in the normal mammary gland and only one-third of that in the mammary tumor on the basis of fmoles/mg of DNA.

DISCUSSION

HAN’s developing in the mammary gland have been frequently associated with the appearance of neoplasia in this tissue. The question whether HAN may indeed progress into neoplasia is not yet settled. Earlier, Beuving et al. (3) reported that HAN’s did not give rise to tumors on transplantation into Fischer rats. However, in a later paper by Beuving (2), the observation was made that, in Lewis rats, about 20% of HAN progressed to form “tumors” on transplantation. These HAN’s were identified as “scattered yellow pigment granules” by direct visualization. There is no evidence that this method of identification can readily and accurately distinguish a microscopic tumor from a HAN. In the present method of identification, the lesions were visualized through direct illumination, which can distinguish HAN from a tumor in the mammary gland. Positive identification is imperative in experiments involving transplantation, particularly in view of the recent findings by Sinha and Dao (15) demonstrating the presence of microscopic tumors in mammary gland as early as 30 days after DMBA treatment. Conceivably, microscopic tumors instead of HAN could be transplanted if positive identification cannot be ascertained.

The results from studies of the labeling index and the S phase together suggest that the hormonal environment, while adequate to produce a significant stimulation of

<table>
<thead>
<tr>
<th></th>
<th>Total amount of tissue (mg)</th>
<th>Protein mg/g tissue</th>
<th>DNA mg/g tissue</th>
<th>dpm bound/mg protein</th>
<th>dpm bound/mg DNA</th>
<th>fmoles bound/mg protein</th>
<th>fmoles bound/mg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mammary gland</td>
<td>900</td>
<td>19.4</td>
<td>0.19</td>
<td>366 (8 S)</td>
<td>37,700 (8 S)</td>
<td>1.7 (8 S)</td>
<td>171 (8 S)</td>
</tr>
<tr>
<td>HAN</td>
<td>570</td>
<td>28.2</td>
<td>0.81</td>
<td>449 (8 S)</td>
<td>15,500 (8 S)</td>
<td>2.0 (8 S)</td>
<td>90.6 (8 S)</td>
</tr>
<tr>
<td>Tumor</td>
<td>1,190</td>
<td>29.8</td>
<td>3.86</td>
<td>7,980 (8 S)</td>
<td>61,400 (8 S)</td>
<td>36.2 (8 S)</td>
<td>279 (8 S)</td>
</tr>
</tbody>
</table>

*Normal mammary gland from virgin: 55- to 60-day-old female rats.
growth of the mammary gland and mammary tumor, is not sufficient to induce a similar effect on HAN's. In spite of their hyperplastic nature, HAN's had the same DNA synthetic period (about 20 hr) and a lower labeling index than did the normal mammary gland. It appears that the HAN cells are not in proliferation, and there is perhaps also a shortening of the G1 or G2 phases of the cell cycle. Banerjee and Walker (1) reported earlier that the duration of DNA synthesis in the cells of the outgrowths of HAN of C3H/He mouse mammary gland was around 13.7 hr. By estimation, this DNA synthetic period appeared to lie between those of the pregnant mouse mammary gland (21.5 hr) and the C3H mouse mammary tumor (11.6 hr). It should be noted, however, that these were in vivo experiments and those described in this paper were in vitro experiments.

It has been well established that DMBA-induced mammary cancers are highly hormone dependent and contain appreciable cytoplasmic EBP's (7, 9, 12). Results from the present study with this mammary adenocarcinoma are in agreement with these earlier findings. We have also noted the presence of a radioactive peak in the 4 S region. Since preincubation with nafoxidine markedly reduces this peak, it appears that the radioactive peak in the 4 S region in this rat tumor cytosol may also be the estradiol-binding macromolecule. The specificity of this 4 S peak is being investigated. In contrast, EBP was either undetectable in HAN (in 2 experiments) or present in very low concentration (in 1 experiment). When the number of specific binding sites was determined on the basis of DNA content of the tissue, HAN was found to contain only one-half of the amount of EBP in the normal mammary gland and one-third of that in the tumor. Thus, HAN appears to have lost the unique biological characteristics of an estrogen-sensitive target tissue, i.e., as determined by specific estrogen-binding capacity. If HAN cells are destined to become neoplastic, it would be reasonable to expect that the specific estrogen-binding capacity of the "precursor" lesion be at least between the normal mammary gland and the tumor. The data so far do not support this assumption.

Earlier studies by Stockdale and Topper (16) suggest that insulin and a glucocorticoid-induced cellular proliferation and prolactin stimulated casein synthesis in differentiated mammary gland cell. We now demonstrate that insulin, estrogen, cortisol, and prolactin greatly stimulate casein synthesis in both the pregnant rat mammary gland and HAN, suggesting that HAN cells are as differentiated as the pregnant rat mammary gland cells. The mammary tumor cells, in contrast, fail to synthesize casein in response to the same combination of hormones. This finding is in agreement with a similar observation with a C3H mouse mammary carcinoma (17). Thus, even though DMBA-induced mammary carcinoma responds to insulin and other hormones in vitro with an increase in DNA synthesis, there is essentially no increase in the synthesis of casein. The data suggest that the specialized function of synthesizing casein by the mammary cells is lost during the process of neoplastic transformation, in spite of the fact that the neoplastic cells are hormone dependent.

Although biochemical studies on HAN and mammary tumor in the mouse have been reported, these experiments were concerned mainly with the metabolic activities of these tissues. Kopelovich et al. (10) examined a broad spectrum of enzyme activities of the soluble Krebs cycle in the normal mammary gland, HAN, and the mammary tumor in the mouse. In general, the enzyme patterns of HAN and pregnant mouse mammary gland were similar, and some enzymes of the HAN outgrowth and the tumor arising from them were different. Of all the enzymes examined, the levels of malic enzymes, malate dehydrogenase, and citrate cleavage enzymes were different in HAN and the tumor, but aconitase and isocitrate dehydrogenase were essentially similar in these tissues. It appears that there is no clear-cut difference in the enzyme patterns of these tissues to delineate the biochemical and biological characteristics of different cell populations. The data reported in this paper clearly show that HAN cell populations differ from both the normal mammary gland and the mammary tumor cells by their nonresponsiveness to hormonal stimulation for growth and the loss of 1 characteristic of the estrogen target tissues, i.e., the capacity to bind estradiol. However, they retain the functional capacity to synthesize casein, a biochemical property that the mammary tumor does not possess.

REFERENCES

Carcinogen-induced Rat Mammary HAN and Tumor


Fig. 1. A whole mammary gland illuminated by a bright light source underneath. The HAN’s are clearly visible (arrows).

Fig. 2. a, HAN in a whole mount preparation of mammary gland excised from a rat 50 days after DMBA treatment. × 9. b, section of the HAN (shown in Fig. 2a) showing alveolar lobular growth. × 200.
Biochemical Characterization of Carcinogen-induced Mammary Hyperplastic Alveolar Nodule and Tumor in the Rat

Thomas L. Dao, Dilip Sinha, S. Christakos, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/35/5/1128

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/35/5/1128. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.