Stimulation of DNA Synthesis by Human Placental Lactogen or Insulin in Organ Cultures of Benign Human Breast Tumors

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SUMMARY

Twenty biopsy specimens of benign human breast tumors obtained from 20 patients were processed into small slices and individually cultured for 2 days in Medium 199. The medium was supplemented with bovine insulin (5.0 µg/ml), human placental lactogen (HPL) (10.0 µg/ml), or ovine prolactin (10.0 µg/ml). Four hr prior to termination, [3H]thymidine was added to the culture medium to determine DNA synthesis. The addition of insulin to the culture medium consistently increased: (a) the mean incorporation of [3H]thymidine into chemically extracted DNA (p < 0.05); (b) the mean number of [3H]thymidine-radiolabeled epithelial cells (p < 0.05), and (c) the mean number of epithelial cells bearing mitotic figures (p < 0.05). [3H]Thymidine incorporation into chemically extracted DNA was also increased when HPL was added to the medium, although this increase did not quite achieve the 5% level of significance. The addition of ovine prolactin to the culture medium did not have any significant effect on DNA synthesis. This study provides evidence that insulin and HPL are direct stimulants of DNA synthesis of the epithelium contained in benign human breast tumors.

INTRODUCTION

In recent years, there has been a surge of interest in pituitary prolactin as a hormone that may be important in human breast tumorigenesis. This renewed interest has evolved largely because of the recent availability of sensitive assays for this hormone (14) and its membrane receptor (13, 23). Results showing that pituitary prolactin is a key hormonal factor in murine mammary tumorigenesis have also markedly served to focus attention on this peptide as a potentially critical hormonal agent in oncogenesis of the human breast (for review, see Ref. 31).

Although prolactin has been shown to stimulate growth of normal and tumorous rodent mammary tissue, in vivo (19, 21, 24, 29) and in vitro (6, 8, 20, 32), it has not yet been established that this hormone is mitogenic to human breast tumors. Organ culture techniques provide one means whereby the direct effects of hormonal agents on growth and differentiation of human breast tissues can be determined. It is the purpose of this study, therefore, to determine whether or not prolactin is mitogenic to benign human breast tumors maintained in organ culture.

Two hormones known for their mammary effects in rodents, HPL (12, 33) [which is chemically similar to human pituitary prolactin] and growth hormone (3, 15), and OPR (8, 24), have been used as prolactin sources in these studies. The mitogenic effects of these hormones are compared with those of bovine insulin, a peptide previously reported to promote in vitro growth of both rodent (8, 11, 20, 28, 32) and human (5, 10, 28) mammary epithelium.

MATERIALS AND METHODS

Twenty human benign breast tumor biopsy specimens, obtained from 20 premenopausal patients, were placed in a chilled holding medium (Medium 199, Hanks’ base, containing 50.0 IU of penicillin G per ml) and returned to the laboratory within 30 min. The biopsy specimens were immediately and carefully trimmed of adipose tissue while immersed in the holding medium. All tissue preparations were performed in a laminar flow hood under aseptic conditions.

Preparation of Slices for Organ Culture. Slices of biopsy specimens were prepared with the aid of a Stadie-Riggs tissue slicer and a No. 10 Bard-Parker surgical blade. Each biopsy specimen provided 5 to 15 large slices ranging from 10 to 15 mm in diameter and 0.1 to 0.3 mm thick. Each slice was processed by a series of halvings with a surgical blade, whereby the direct effects of hormonal agents on growth and differentiation of human breast tissues can be determined. It is the purpose of this study, therefore, to determine whether or not prolactin is mitogenic to benign human breast tumors maintained in organ culture.

Each biopsy specimen was divided into 3 groups, i.e., a control and 2 experimental groups. Each group (controls and experimental) had 9 small Petri dishes containing a single larger slice (3 x 3 mm) placed in each dish. These dishes were then placed in a covered water-saturated larger Falcon disposable Petri dish, 10 slices/dish. In addition, a single larger slice (3 x 3 mm) was added to each small Petri dish. Each Petri dish contained 2.0 ml of the culture medium.

Each biopsy specimen was divided into 3 groups, i.e., a control and 2 experimental groups. Each group (controls and experimental) had 9 small Petri dishes containing a total of 90 small slices and 3 (for biopsy Specimens 11 to 20) or 9 (for biopsy Specimens 1 to 10) larger slices. The small Petri dishes were placed in a covered water-saturated larger Falcon disposable Petri dish (15 x 100 mm), 3 small dishes per larger dish. These Petri dishes were then placed in a small gassing chamber and housed in an incubator at 37°. The chambers were continuously infused with gas (95% O₂-

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2 The abbreviations used are: HPL, human placental lactogen; OPR, ovine prolactin.
were never combined. The large number of randomly selected small slices per group provides reasonable assurance that an equal quantity of epithelium is distributed among the 3 groups at the onset of culture.

The culture medium used in these studies was Medium 199, modified Earle's salts (1250 mg NaHCO₃ per liter) obtained from Grand Island Biological Co., Grand Island, N. Y. The hormones used in this study and their concentrations in the culture media were: OPR (NIH-S-9, 27.0 IU/mg) (10.0 µg/ml), human placental lactogen (Nutritional Biochemical Corp., Cleveland, Ohio) (10.0 µg/ml), and bovine pancreas insulin (California Biochemical Corp., La Jolla, Calif., 22.5 IU/mg) (5.0 µg/ml). All media contained penicillin G (Nutritional Biochemical Corp.) (50 IU/ml). After all additions, the media were passed through a Millipore filter (0.45 µm), added to the Petri dishes, and the entire culture assembly was frozen (−20°) until the biopsy specimens were brought to the laboratory (within 1 month).

The addition of HPL to culture medium containing slices of human breast tumors resulted in a mean increase in: (a) incorporation of [³H]thymidine into chemically extracted DNA (p < 0.05); (b) number of [³H]thymidine-radio-labeled epithelial cells (p < 0.05), and (c) number of epithelial cells bearing mitotic figures (Tables 1 and 2). Nineteen of the 20 breast biopsies showed an increase in the incorporation of [³H]thymidine into chemically extracted DNA when insulin was added to the culture medium. Of the 15 cultured breast biopsy specimens that were microscopically examined for [³H]thymidine-radio-labeled epithelial cells, all showed an increase in the number of labeled cells when insulin was added to the culture medium. Of the 18 cultured biopsy specimens examined for mitotic figures, 15 showed an increase in the total number of epithelial cells bearing mitotic figures after treatment with insulin; 3 showed no mitotic figures, regardless of whether insulin was added to the culture medium.

The addition of HPL to culture medium containing slices of human breast tumors resulted in a significant (p < 0.05) mean increase in the total number of: (a) [³H]thymidine-radiolabeled epithelial cells and (b) epithelial cells bearing mitotic figures (Table 1). [³H]Thymidine incorporation into chemically extracted DNA was also increased when HPL was added to the medium, although this increase did not quite achieve the 5% level of significance (p < 0.10) (Table 1). Seven of the 10 cultured breast biopsy specimens showed a substantial increase in the incorporation of [³H]thymidine into chemically extracted DNA and in the

Radioautographic [³H]Thymidine and Mitotic Figure Analyses of Cultured Slices. For radioautographic analyses, the fixed slices (9 slices/group for Biopsies 1 to 10, 3 slices/group for Biopsies 11 to 20) were embedded in paraffin. Each slice was processed into 6 paraffin sections (5 to 7 µm thick), and placed on microscopic slides. The slides were coated with a standard emulsion (Kodak NTB-2) and stored at 4° for 3 weeks. They were developed, fixed, and stained (hematoxylin and eosin) by a standard procedure (25). Only cells bearing >4 silver grains were scored. The total number of radiolabeled epithelial cells was determined in each section.

RESULTS

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For mitotic figure analyses, paraffin sections adjacent to those used for radioautographic analyses were stained with hematoxylin and eosin. Total number of mitotic figures were determined in each section. Significance of differences between mean number of mitotic figures of each group and mean number of radiolabeled cells of each group was analyzed by the t test for paired observations.

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Hormones and Breast Tumors in Vitro

Table 1

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Mean 118.7* 197.3* 158.1* 759* 2410* 2009* 8* 29 19*

* Ten benign breast biopsy specimens were obtained from 10 premenopausal patients. Biopsy Specimens 1, 2, 6, and 10 were evaluated histopathologically as fibroadenomas; Specimens 4, 5, and 7 to 9, as fibrocystic disease; and Specimen 3, as both fibroadenoma and fibrocystic disease.

Table 2

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Mean 31.5 69.0 40.1 377* 932* 336* 8 19 7

* Ten benign breast biopsy specimens were obtained from 10 premenopausal patients. Biopsy Specimens 11 to 17 were evaluated histopathologically as fibroadenomas; Specimens 12, 14, 16, and 20 as fibrocystic disease; Specimen 15 as both fibroadenoma and fibrocystic disease; and Specimen 13 as virginal hypertrophy (patient was 15 years old).

The addition of OPR to culture medium containing slices of human breast tumors did not significantly influence the mean incorporation of [3H]thymidine into DNA, the mean total number of [3H]thymidine-radiolabeled cells or the mean total number of cells bearing mitotic figures (Table 2).

The radioautograph and mitotic figure results in this study are presented as total number of cells rather than as the ratio of these cells to a given number of epithelial cells, the more customary procedure. Because of the heterogene-
ity of benign breast tumors, it is necessary to use a large sampling of the tumor slices, as we have, in order to obtain representative figures for \([^{3}H]\)thymidine-labeled cells. One cannot count selected areas as is done for more homogeneous tissues. It is therefore necessary to count all the epithelial cells in the tissue sections used for a given group, in order to present the data as a ratio of a given number of epithelial cells. This procedure is extremely time consuming. We randomly chose 2 of the cultured breast tumors (Table 1, Biopsy Specimens 2 and 4) and counted the total number of epithelial cells in the sections from which radioautographic counts were obtained. In the sections derived from Biopsy Specimen 2, a total of 22,250 cells were observed in the control group, 25,540 cells in the insulin group, and 20,338 cells in the HPL-treated group. The ratios of labeled epithelial cells per 1000 epithelial cells were: controls, 23.4; insulin-treated, 86.6; and HPL-treated, 63.6. In the sections of cultured slices from Biopsy Specimen 4, a total of 5298 cells were observed in the control group, 3462 in the insulin-treated group and 6170 in the HPL-treated group. The ratios of labeled epithelial cells per 1000 epithelial cells were: controls, 77.5; insulin-treated, 218.0; and HPL-treated, 195.0. Thus, whether the results are reported as total numbers of \([^{3}H]\)thymidine-labeled cells or as a ratio, \textit{i.e.}, labeled cells per 1000 nonlabeled cells, a marked stimulatory effect of insulin and HPL is observed. We therefore decided that our sampling was representative enough between groups to warrant use of total labeled cells as a criterion of response.

In general, there was an excellent correlation in this study between the 3 indices of DNA synthesis, \textit{i.e.}, specific activity of \([^{3}H]\)thymidine into chemically extracted DNA, number of \([^{3}H]\)thymidine-radiolabeled cells, and number of mitotic figures. When examining individual cultured biopsy specimens, an enhancement by either insulin or HPL of one of these indices almost invariably resulted in an increase in both of the other indices of DNA synthesis. The morphology of all 20 of the cultured human breast specimens was well preserved; areas of necrosis were rare. Consistent morphological changes of the cultured specimens, as a function of the hormonal milieu, however, were not readily apparent. Although, at times, numerous fibroblasts were observed in the cultured specimens, they were seldom radiolabeled. There was an insufficient quantity of biopsy material from Specimens 11 and 12 (Table 2) for radioautographic and histological analyses. A radioautographic examination was attempted on Specimens 6 to 8 (Table 1) but, for yet unexplained reasons, these tissues did not process effectively and therefore were not available for analysis.

**DISCUSSION**

DNA synthesis was increased in all 20 of the breast biopsies examined in this study when insulin was added to the culture medium. Only 1 of the 20 biopsy specimens failed to show a response to insulin of increased incorporation of \([^{3}H]\)thymidine into chemically extracted DNA. This particular biopsy specimen (No. 9) was histologically unique in that it contained a large quantity of adipose tissue. The adipose tissue may have interfered with the chemical extraction of DNA; \textit{i.e.}, a portion of the sample may have been lost during lipid extraction, causing erroneous results. Radioautographic analysis showed that insulin sharply increased the number of \([^{3}H]\)thymidine-labeled cells, not only in this particular specimen, but in all the cultured biopsy specimens examined by this procedure. The biopsy specimen in which insulin induced the greatest incorporation of \([^{3}H]\)thymidine into chemically extracted DNA (No. 18) was a specimen obtained from a patient during pregnancy; all other specimens were derived from nonpregnant patients. It has been previously reported, using strictly morphological criteria, that insulin enhances (5, 10) or has no effect (26) on growth of normal or dysplastic human breast tissues maintained in organ culture. The results of our study provide evidence that this peptide is consistently stimulatory to DNA synthesis of tumorous (benign) human breast tissues maintained in short-term organ culture. It is well recognized that these biopsy specimens contain varying proportions of diffusely infiltrated normal, hyperplastic, and cystic epithelial elements. Studies designed to determine which particular types of human breast epithelium (normal and dysplastic) respond to the mitotic stimulus of insulin \textit{in vitro} are currently in progress in our laboratory.

HPL also substantially increased DNA synthesis of the breast tumor explants, although to a lesser degree than did insulin; OPR was ineffective. These results are in accord with the data described in a recent brief communication by Dilley and Kister (7), indicating that human pituitary prolactin or HPL in combination with insulin increased the mitotic index of organ cultures of normal human breast tissues to a level higher than that in cultures containing no hormones or insulin alone; OPR was without a stimulatory effect. It is fairly well established that OPR is an effective mitogen in organ cultures of normal (6, 8) and carcinomatous (20, 28, 32) rat mammary tissues. There are conflicting reports as to whether OPR is stimulatory to cultures of human breast carcinomas (1, 2, 18, 22, 28). Rodent mammary tissues have membrane receptors for OPR (13, 23), whereas human breast tissues may be lacking receptors for OPR, as suggested by the recent report of Holdaway and Worsley (16). This would explain the lack of effect of OPR on human breast epithelium shown in our study and in that by Dilley and Kister (7).

HPL was used in our study in lieu of human pituitary prolactin because the purified human pituitary peptide has not been available in sufficient quantities for use in our organ culture studies. HPL is structurally slightly more similar to ovine or human growth hormone than it is to OPR or human prolactin, although the sequence homologies of the placental peptide and these pituitary peptides are sufficiently similar to suggest that all of these molecules evolved from a common ancestor (3, 15). Because of the closer structural similarity of HPL to growth hormone, HPL is frequently referred to as human choric somatomammotropin (3). Despite this close structural similarity to the growth hormones, the somatotrophic activity of HPL is considerably less than that of growth hormone, having 1.0% of the potency of the pituitary peptide (12, 17). On the other hand, HPL has marked mammotrophic-lactogenic activities in lower animals \textit{in vivo} and \textit{in vitro}. This placental peptide
reportedly (12, 33) stimulates pigeon crop sac activity, mammary gland development in pseudopregnant rabbits, mammary gland secretion in mice, and growth of precan- ceros mammary gland lesions in mice (12, 33). It is empha- sized that the word “lactogen” in HPL is derived from num- meros mammary gland lesions in mice (12, 33). It is emphasized that the word “lactogen” in HPL is derived from nu-
demonstrated that this hormone is lactogenic in primates (17). The results described in this paper and the earlier brief communications by Dilley and Kister (7) are the first direct evidence, to our knowledge, that HPL is mammotrophic to human breast epithelium.

HPL is secreted by the placental syncytiotrophoblast and has been detected as early as 12 to 18 days after concep- tion. The level of this hormone then rises progressively until late pregnancy and then declines slightly (17). The results of our study provide evidence suggesting that HPL may be a factor in the etiology of dysplastic breast lesions seen in multiparous women, although these lesions are frequently encountered in nulliparous women as well. Although little is known of the biological activity of human pituitary prolac- tin secretion in certain mammary tumor-susceptible mice it may possess mammotrophic activities similar to HPL (7) and therefore may also play a role in the etiology of dysplastic (benign and cancerous) breast diseases in nulliparous or nonpregnant women. Prolactin is mitogenic to the rat mammary epithelium (6, 8, 24, 29) and may be an essen-
tial hormonal factor in mammary tumorigenesis in this spe-
cies. Indeed, chronic drug-induced suppression of prolac-
tin secretion in certain mammary tumor-susceptible mice prevents the development and progression of hyperplastic and neoplastic mammary gland lesions in these animals (27, 30). If prolactin can be shown to influence the development and growth of human breast tissue, as it does in rodents, then prophyllaxis and/or chemotherapeutic control of hu-
man breast dysplasias may be possible by acute or chronic drug-induced prolactin suppression.

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