Autoradiographic Demonstration of Estrogen Binding in Human Breast Cancer after in Vitro Incubation

Richard H. Buell and Gilles Tremblay

Departments of Pathology of McGill University [R. H. B., G. T.] and the Royal Victoria Hospital [G. T.], Montreal, Quebec, Canada H3A 2B4

ABSTRACT

This report describes the localization of bound radioactivity as visualized by thaw-mount autoradiography in 35 cases of human breast carcinoma after in vitro incubation with [3H]estradiol. The findings have been compared qualitatively with results of biochemical assays. Twenty-six tumors were considered to be estrogen receptor positive by autoradiographic criteria. Of these, 23 were assessed biochemically; 21 were found to be positive. Specific uptake of radioactivity was observed primarily in neoplastic epithelial cells. The grains were localized mainly over the nuclear region of putative target cells. Within nests of infiltrating carcinoma, positive cells could be identified admixed with negative cells. There were no autoradiographic criteria established for borderline cases. The remaining nine cases were considered negative. In these cases the appearance of tissue exposed only to [3H]estradiol resembled that of tissue incubated with excess unlabelled estrogen. The few grains were randomly scattered with no evidence of nuclear localization. Of these negative cases, two were positive by biochemical assay, four borderline, and three negative. It is concluded that, as assessed in this investigation, estrogen receptor-positive mammary carcinomas may be composed of target and nontarget neoplastic cells. Thaw-mount autoradiography should prove useful in the laboratory investigation of steroid hormone responsiveness of human breast carcinoma.

INTRODUCTION

Knowledge that the biochemical assay of human breast cancers for steroid hormone receptors is useful for predicting patient response to hormonal therapy has stimulated interest in developing a morphological method for assessing tissue content of these receptors. To date the major emphasis of research in this field has been on immunocytochemical and histochemical methods (for review, see Ref. 1). While these methods offer promise for future routine application, their validity in demonstrating steroid hormone receptors as assayed biochemically remains to be established (1, 2). Thaw-mount and dry-mount autoradiography are also applicable to the problem and were among the first methods to be applied successfully for localization of bound radioactive steroids in tissue sections of experimental animals (3, 4). Autoradiographic techniques offer the advantage of utilizing the same ligands as biochemical assays and under appropriate conditions can be quantitated (5–7). Indeed, in early investigations of the biochemical parameters of the ER, dry-mount autoradiography was used to provide morphological evidence on the intracellular localization of these receptors (8). Despite recent questions concerning the interpretation of some of these data (9, 10), the methods described by Stumpf and Roth (11) remain established in their ability to demonstrate bound steroid hormones in target tissues, in part because these methods avoid procedures that might produce artifactual diisolation of the steroid.

The application of thaw-mount autoradiography to human tissue, however, has been more or less overlooked as a means of localizing specifically bound steroids in material like human breast cancers. Since autoradiographic methods with human tissue require in vitro incubation of fresh specimens, a number of potential artifacts must first be evaluated. It must also be ascertained that data derived from these methods do indeed correlate well with results of biochemical assays. In previous studies we assessed the accuracy of an in vitro incubation method using mouse uterus (12), a well-characterized target organ, and applied a similar method to localize putative target cells in benign human breast lesions (13) and five cases of ER-positive human breast cancer (14). In the present report we extend these findings in mammmary carcinoma with an additional 35 cases, including ER-positive and -negative cases, and correlate these data qualitatively with results of biochemical assays.

MATERIALS AND METHODS

Tissues. Tissues from primary, recurrent, and metastatic breast carcinomas were obtained at the time of frozen section, diced into sections 1 to 2 mm thick, and transported to the laboratory in Medium 199 with 25 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (GIBCO, Burlington, Ontario), pH 7.3. All patients were female and ranged in age from 23 to 74 with a mean of 54 yr.

Chemicals. [2,4,6,7-3H]Estradiol (specific activity, 102 or 111 Ci/mmol) was purchased from New England Nuclear, Lachine, Quebec, and purified by thin-layer chromatography prior to use. Unlabeled estradiol and diethylstilbestrol and bovine serum albumin were from Sigma Chemical Co., St. Louis, MO. Liquid Freon 12 was from Dupont Canada. Other miscellaneous reagents were purchased from Fisher Scientific, Ltd., Montreal, Quebec. For thaw-mount autoradiography, Kodak NTB 2 emulsion and developing reagents were from Eastman Kodak, Montreal, Quebec.

Incubation of Tissue. The methods used for the incubation of the tissue have previously been described (14). Briefly, to determine total and nonspecific binding of [3H]estradiol, blocks of tissue were incubated for 45 min at 30°C in Medium 199 containing 5 nm [3H]estradiol with and without a 100-fold excess of unlabelled estradiol or diethylstilbestrol. The tissues were then transferred to a medium containing bovine serum albumin (3.5 g/100 ml) and incubated, usually for 4 h. After these incubations the tissue was embedded in OCT compound, frozen with
liquid Freon 12, and stored at −76°C. Sections were cut for autoradiography, generally within 2 wk.

Thaw-Mount Autoradiography. The thaw-mount autoradiographic method used was similar to that described by Stumpf and Roth (11) and has already been described (12–14). Sections 4 μm thick were mounted on slides previously coated with photographic emulsion and exposed at −15°C. Since, during the course of these experiments, a single appropriate length of exposure could not be determined in advance, slides (demonstrating total and nonspecific binding) of a given case were developed after varying periods of exposure time, generally from 2 to 6 mo. The slides were then stained with hematoxylin-eosin. In addition to the 35 cases, in several other cases used as a control for negative chemography, the emulsion was fogged by light prior to mounting, and the sections were then mounted and exposed as usual (15).

Quantitation of Autoradiographs. Previous studies have shown that, as expected under the experimental conditions used, specific labeling of putative estrogen target cells was nuclear (12). Accordingly only nuclear labeling was evaluated. The amount of nonspecifically bound [3H]estradiol retained in the nuclei was first determined by examining slides of tissue incubated with excess unlabeled estradiol or diethylstilbestrol. The number of grains per 10-μm2 nuclear cross-sectional area was determined as previously described (14) for 50 cells in a selected area of the slide. Since the appearance of these slides was relatively uniform showing only randomly scattered grains, the area was selected to avoid obvious artifacts (e.g., micromote knife marks, tissue folds, etc.). The calculated average for these 50 cells was considered to represent the amount of labeling from nonspecifically bound [3H]estradiol. For tissue exposed only to [3H]estradiol, the number of grains per 10-μm2 nuclear cross-sectional area was also determined for 50 cells in a selected area of the slide. This area was chosen by examining the slide and subjectively identifying an area, if any, showing predominantly nuclear labeling over what appeared to be the greatest percentage of cells. Other areas were avoided because of possible technical artifacts related to poor diffusion of the steroid or to a variable efficiency of the albumin wash as previously discussed (12). If no such area was identified (as was found in cases with relatively little labeling), an area was selected as described above for tissue exposed to unlabeled steroid. Positive cells were arbitrarily defined as those demonstrating a 3-fold or greater number of grains per 10-μm2 nuclear cross-sectional area for tissue exposed only to [3H]estradiol than the average for tissue exposed to excess radioinert competitor. To compare qualitatively the autoradiographic data with results of biochemical assays, a case was considered to be positive by autoradiographic criteria if the mean number of grains per 10-μm2 nuclear cross-sectional area for tissue exposed only to [3H]estradiol was greater than the mean exposed to excess unlabeled competitor, and at least 25% of cells quantified were considered to be target cells. If a case did not meet these criteria it was classified as negative. Criteria were not established for borderline cases.

Biochemical Assays. Biochemical assays were performed in the clinical laboratories of the Royal Victoria Hospital using established methods (16). A case was considered biochemically positive if there were specific binding of greater than 12 fmol/mg of protein, borderline if there were more than 6 but less than 12 fmol/mg of protein, and negative if there were less than 6 fmol/mg of protein.

RESULTS

Twenty-six cases (74%) were considered to be estrogen receptor positive by autoradiographic criteria. These cases revealed that, in tissues exposed only to [3H]estradiol, the radioactivity was incorporated primarily by the neoplastic epithelial cells and could be suppressed by exposure to unlabeled estradiol. In some of these nests most cells were labeled, in some a heterogeneous population was observed, and in others most cells were unlabeled. Several foci of intraductal carcinoma were identified. In some of these a heterogeneous population of positive and negative cells could be seen (Fig. 3). In other places most of the intraductal component was unlabeled.

In nine cases (26%) considered to be negative by autoradiographic criteria, the appearance of the tissue exposed only to [3H]estradiol resembled that of the tissue incubated with excess unlabeled estradiol (Fig. 4). The grains were randomly scattered over the section. They were few in number, when compared with positive cases, and there was no evidence of a nuclear localization.

Biochemical assays were carried out on 32 of the 35 cases. Of these, 72% were estrogen receptor positive, 19% borderline, and 9% negative (Table 1). Of the 23 cases biochemically positive, 21 were positive by autoradiographic assessment, resulting in 91% agreement. In those cases where there was disagreement, the tissue contained 15 and 14 fmol/mg of cytosolic protein. Of the biochemically borderline cases, four were considered negative by autoradiography, while two were positive, including one (Case 27) which was markedly positive. The mean number of grains in the other cases tended to be lower than usually seen for positive cases. There was uniform agreement between the autoradiographic and biochemical evaluation of all three biochemically estrogen receptor-negative cases. Therefore, if one excludes borderline cases for which there were no autoradiographic criteria established in this investigation, the overall agreement between this method and biochemical assay in the 26 cases is 92%. If the borderline cases are included, the overall agreement drops to 75%.

DISCUSSION

This investigation has demonstrated that estrogen receptor positive human mammary carcinomas are often composed of a heterogeneous population of "target" and "nontarget" cells as assessed by the present autoradiographic criteria. It should be pointed out that the observed heterogeneity may be in part due to random cell death during the incubation, or to failure of exchange of the radiolabeled ligand for endogenously bound estradiol. Moreover, binding sites may be present in the unlabelled cells but in insufficient numbers to be detected by this method, possibly as a result of nuclear processing of ER (17). However, in a previous study on mouse uterus using a similar method where such inherent technical factors would also have been operative, no comparable cellular heterogeneity was found (12). It would therefore seem that an absence of specific labeling indicates in most instances a nontarget cell.

Cellular heterogeneity for ER in breast carcinoma has also been observed with immunohistochemical (18, 19) and histochemical methods (20–26), and the present report thus supports these earlier findings. This possible heterogeneity has also been suggested from biochemical (27, 28) and clinical (29, 30) studies. The significance of this tumor cell heterogeneity in relation to
patient response to hormonal therapy has not been clearly established. Clinical studies have shown that not all estrogen receptor-positive tumors respond to hormonal therapy (31). Since the binding of estrogen to its receptor represents only the first step in the biochemical pathway of estrogen action, measurements of progesterone receptors, which provide an assessment of the functional integrity of the estrogen receptor system, are done and have proved to correlate well with the clinical behavior of mammary carcinoma (31, 32). While failure of ER-positive tumors to respond to endocrine therapy may be the result of a defect in any step in the pathway subsequent to the initial binding, it has also been suggested that tumor cell heterogeneity for ER may also be a factor for lack of response or early recurrence (33–35).

In this report, we have demonstrated that the autoradiographic findings correlate well qualitatively with data from biochemical assays, but it is apparent that the autoradiographic method fails to detect the presence of specific estrogen binding in tumors with an ER content of less than about 15 fmol/mg of protein (see Table 1). It may be that longer exposure times would improve the sensitivity of the autoradiographic method, thereby allowing detection of these lower levels. It is also possible that this apparent loss of sensitivity is the result of potential artifacts of the autoradiographic method, including latent image fading or decreasing the nonspecific binding, thus permitting detection of estrogenic interactions between the tissue and emulsion resulting in loss of chemography, however, there was no evidence of significant

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**Table 1**

Comparison of autoradiographic and biochemical data

| Case | Age (yr) | Histological type | ER (fmol/mg cytosolic protein) | Exposure time (days) | [\(^3\)H]Estradiol + diethylstilbestrol | [\(^3\)H]Estradiol | Autoradiographic evaluation, % of positive cells
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**Note:** Autoradiographic evaluation indicating percentage of positive cells identified and assessment of case as ER positive or negative (see "Materials and Methods" for details).

**Note:** IDC, infiltrating duct carcinoma; ILC, infiltrating lobular carcinoma.

\[ ^\circ \]

Mean ± SD of number of nuclear grains per 10-\( ^6 \)m\(^2 \) nuclear cross-sectional area for 50 cells evaluated from tissue incubated in indicated medium (see "Materials and Methods" for details).
autoradiographic studies using a similar in vitro method, Tchernitchin et al. (37, 38) apparently did not note a decrease in total nuclear-bound [3H]estradiol in uterine glandular and stromal cells after perfusion with an albumin wash for 1 h. There was a decrease in label over the extracellular space (37). Strobl and coworkers (39, 40), however, have shown that, in the cell system they studied, an albumin wash, while being effective in decreasing nonspecific binding, also resulted in a loss of some specifically bound estradiol. Such a loss of specific binding, if it is occurring in the present method, might account for the relative lack of sensitivity.

The observation of one case (Case 27), which was markedly positive by autoradiography and only borderline by biochemical analysis, requires consideration. The biochemical assays measured cytosolic ER only, while the autoradiographic method assessed nuclear-bound radioactivity, and this difference might account for the discrepancy. Positive chemography in the autoradiographic method seems an unlikely explanation, since the labeling observed could be suppressed by radioinert estradiol and diethylstilbestrol. The label observed may be associated with labeling observed could be suppressed by radioinert estradiol and diethylstilbestrol. The label observed may be associated with

In these studies, as in our previously reported work, the presence of specific nuclear labeling was used to identify apparent target cells. We therefore assumed that the radioactive ligand has, as in classical theory (8, 42, 43), bound to the cytosolic receptor, and the resulting complex has undergone a temperature-dependent activation and translocated to the nucleus. In the cases studied, there was no subjective evidence of significant cytoplasmic labeling in the absence of nuclear labeling (i.e., failure of translocation) (44), although quantitation to support this impression has not been made. Moreover incubation for differing times and temperatures to demonstrate the temperature dependence of the presumed activation and translocation or to identify unbound nuclear receptor has not been done. It is therefore not possible from the present data to determine if unbound receptor is predominantly cytoplasmic or nuclear, as has recently been suggested (9, 10, 18, 45). The contribution to the labeling observed from nuclear type II sites has not been assessed, and in determining putative target cells, we assumed that, if any labeling were from such type II sites, these sites would have physiological significance, as may be true for other tissues (46, 47). Nuclear type II sites in human tumors can moreover be strongly correlated with the presence of progesterone receptor (41).

Since the present autoradiographic method assesses specific nuclear binding sites rather than cytoplasmic sites, the data from these experiments would be consistent with current theories on intracellular ER localization regardless of whether one accepts the classical theory (8, 42, 43) or more recently proposed hypotheses (9, 10, 18, 45). Other methods, although correlating well with biochemical assays, demonstrate often predominantly cytoplasmic binding sites. The nature of those sites may require further clarification if it is ultimately proven that the estrogen receptor is predominantly nuclear as suggested. The interpretation of data from the present autoradiographic method, however, would remain unchanged. The good qualitative correlation with biochemical assays as demonstrated in this investigation supports the concept that the specifically labeled cells identified by autoradiography do indeed represent potential estrogen target cells.

From these studies it can therefore be concluded that, on the basis of an autoradiographic method such as the present one, estrogen receptor-positive human mammary carcinomas appear to be composed of "target" and "nontarget" cells at the time of biopsy. Subject to the potential experimental artifacts and limitations in interpretation discussed above, an autoradiographic method could be feasible as an investigative tool in studying the biology of human breast cancer, as it has already proved to be in the study of the physiology of experimental animals. It is unlikely, however, that such a method would prove practical in the assessment of human tumors in a clinical setting, because of the need to incubate fresh tissue and the lengthy exposure times required. It is nevertheless an important method of localizing estrogen binding sites, and, since it uses the same radioactive ligand utilized in biochemical assays, direct comparative studies including "saturation" studies and demonstration of high affinity binding (5, 6) would be feasible and could provide important information in the study of the biology of human breast cancer.

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The authors wish to thank M. Volkov for expert technical assistance and Laura Florita and Eileen Mackay for secretarial assistance.

REFERENCES


Fig. 1. a, autoradiograph of estrogen receptor-positive human mammary carcinoma after in vitro incubation with [3H]estradiol only. Grains can be seen associated with many of the neoplastic epithelial cells, and, on closer examination, this labeling is located mainly over the nuclear region. Data from the biochemical assay of this lesion revealed it to be estrogen receptor positive with 348 fmol/mg of protein. H&E, × 400, 68 days of exposure. b, breast cancer tissue adjacent to that shown in Fig. 1a after in vitro incubation with [3H]estradiol plus a 100-fold excess of radioinert estradiol. The majority of labeling seen in Fig. 1a has been suppressed, and there is no evidence of a nuclear concentration of the few remaining grains. H&E, × 400, 68 days of exposure.

Fig. 2. High power view of an estrogen receptor-positive infiltrating duct carcinoma (containing 107 fmol/mg of protein) incubated in [3H]estradiol only. In this nest of tumor cells one can appreciate the predominantly nuclear localization of radioactivity in putative target cells admixed with unlabeled cells. The variability of degree of labeling in apparent target cells is also evident. H&E, × 800, 78 days of exposure.
Fig. 3. A focus of intraductal carcinoma with central necrosis from an infiltrating lesion. Some, but not all, neoplastic cells within this area reveal specific labeling. Biochemical assay was not done on this lesion. H&E, × 600, 66 days of exposure.

Fig. 4. Section from an estrogen receptor-negative carcinoma (5 fmol/mg of protein). The appearance of this autoradiograph is similar to that of adjacent tissue exposed to excess unlabeled estradiol. The grains are sparse and randomly scattered with no evidence of a nuclear localization. H&E, × 400, 203 days of exposure.
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