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4-Hydroxylation of estrogens as marker of human mammary tumors

(estrogen metabolism/catecholestrogen formation/2-hydroxyestradiol/4-hydroxyestradiol/human breast tumors)

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ABSTRACT **Estrogen is a known risk factor in human breast cancer. In rodent models, estradiol has been shown to induce tumors in those tissues in which this hormone is predominantly converted to the catechol metabolite 4-hydroxyestradiol by a specific 4-hydroxylase enzyme, whereas tumors fail to develop in organs in which 2-hydroxylation predominates. We have now found that microsomes prepared from human mammary adenocarcinoma and fibroadenoma predominantly catalyze the metabolic 4-hydroxylation of estradiol (ratios of 4-hydroxyestradiol/2-hydroxyestradiol formation in adenocarcinoma and fibroadenoma, 3.8 and 3.7, respectively). In contrast, microsomes from normal tissue obtained either from breast cancer patients or from reduction mammoplasty operations expressed comparable estradiol 2- and 4-hydroxylase activities (corresponding ratios, 1.3 and 0.7, respectively). An elevated ratio of 4-/2-hydroxyestradiol formation in neoplastic mammary tissue may therefore provide a useful marker of benign or malignant breast tumors and may indicate a mechanistic role of 4-hydroxyestradiol in tumor development.**

The prolonged exposure of women to high estrogen levels has been associated with an elevated incidence of breast cancer (1, 2). Thus, risk factors for this disease include high serum or urinary estrogen concentrations (3–5), the early onset of menstruation, and late menopause (1, 2). In rodent models, in which the natural hormone estradiol (E_2) induces tumors, there is a differential formation of the two catechol metabolites, 2- and 4-hydroxyestradiol (2- and 4-OH-E_2, respectively), which correlates with the organ's resistance or susceptibility to estrogen-induced carcinogenesis. Thus, in Syrian hamster kidney, CD-1 mouse uterus, and rat pituitary, all susceptible to tumor induction by E_2 (6–8), 4-OH-E_2 formation predominates, whereas in these rodents' livers, where tumors are not induced under these conditions, 2-hydroxylation of E_2 predominates (9–11). By analogy, in human uterine myoma, 4-hydroxylation of E_2 by a distinct and specific estrogen 4-hydroxylase activity predominates over that in surrounding myometrium and over 2-hydroxylation in either tissue (12). Moreover, in human MCF-7 breast cancer cells, the formation of 4-OH-E_2 is catalyzed by cytochrome P450 IB1 and is inducible by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, a common environmental pollutant (13). In contrast, microsomal preparations from human liver or placenta catalyze the oxidation of E_2 mainly to 2-OH-E_2 with $\leq$20% 4-OH-E_2 formation (12, 14–17). This hepatic formation of catecholestrogen metabolites is catalyzed mainly by the cytochrome P450 IIIA family of enzymes, which generates $\approx$85% 2-OH-E_2 and, in addition, $\approx$15% 4-OH-E_2 due to a lack of enzyme specificity (14–19). The localized occurrence of a specific estrogen 4-hydroxylase in human breast cancer cells, in uterine myoma, and in rodent target organs of estrogen-induced carcinogenesis has been taken as evidence that the elevated formation of 4-hydroxylated estrogens in estrogen target organs may increase the risk of hormone-induced oncogenesis and may also be a useful marker of hormone-induced tumors (9, 20). The specific estrogen 4-hydroxylase activity in breast cancer cells and its potential as a tumor marker prompted us to examine this enzymatic activity in microsomes prepared from human breast cancers, mammary fibroadenomas, and samples of normal tissue from breast cancer patients and other controls to see whether this 4-hydroxylase activity correlated with the presence of benign or malignant human mammary tumors and could be developed into a useful marker.

MATERIALS AND METHODS

Chemicals. E_2, NADPH, ascorbic acid, Hepes, Tris base, and Tris·HCl were obtained from Sigma; 2-OH-E_2 and 4-OH-E_2 from Steraloids (Wilton, NH); [6,7-^{3}H]estradiol (specific activity, 40–60 Ci/mmol; 1 Ci = 37 GBq) was from Amersham; neutral alumina, hydrochloric acid, hexane, and ethyl acetate (HPLC grade) were from Fisher Scientific.

Microsome Preparation. Samples of 12 human mammary fibroadenomas, 8 adenocarcinomas, normal tissue either from 24 breast cancer patients or from 7 reduction mammoplasty surgeries/excision biopsies of patients with fibrocystic changes were obtained fresh from Surgical Pathology of UTMB or from the M.D. Anderson Hospital and Cancer Center, Houston, or frozen from the National Cancer Institute Cooperative Human Tissue Network. Fresh tissue was immediately snap-frozen to permit the separation of parenchyma from adipose tissues. Frozen mammary parenchyma, separated from adipose tissues, was allowed to thaw to 4°C in homogenization buffer (1.14% KCl/10 mM EDTA, pH 7.5) and then freed of remaining adipose tissue and homogenized with a Tekmar (Cincinnati) Ultra-Turrax homogenizer. Microsomes were prepared by differential centrifugation according to the method of Dignam and Strobel (21). Microsomal pellets were resuspended in storage buffer (0.25 M sucrose/10 mM EDTA, pH 7.5) and frozen in aliquots at −80°C until used. Protein concentrations were determined by the method of Bradford using bovine serum albumin as standard (22).

Microsome-Mediated Catechol Estrogen Formation. A validated direct product isolation assay was used to determine rates of catechol estrogen formation. The assay method and its validation have been described in detail (23). Briefly, microsomal protein (750–1500 μg), 5 mM NADPH, and 1–100 μM [^{3}H]E_2 as substrate were incubated in 0.1 M Tris·HCl/Hepes buffer, pH 7.4, containing 5 mM ascorbic acid in a final vol of 500 μl at 30°C for 30 min. After termination of reactions by rapid freezing, trace amounts of ^{14}C-labeled catechol metab-

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Abbreviations: E_2, 17β-estradiol; 2- and 4-OH-E_2, 2- and 4-hydroxyestradiol.

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olites were added to correct for procedural losses. The catechols were then adsorbed onto neutral alumina, washed to remove residual substrate, eluted from the neutral alumina with 0.25 M HCl, and separated by thin-layer chromatography. Blank values, determined with heat-denatured microsomes or by omitting either enzyme or NADPH, were subtracted. Values > 125% of blanks were minimum criteria for acceptance. Product formation was proportional to incubation time for up to 20 min and to protein concentrations for up to 3 mg/ml.

Statistical significance was determined by Student's t test.

RESULTS

In microsomes prepared from normal human mammary tissue obtained from breast cancer patients or reduction mammoplasty surgeries, the rate of 2-hydroxylation of E_2 did not markedly differ from that of 4-hydroxylation as shown in Fig. 1 (ratio of 4-OH-E_2/2-OH-E_2 formation; 1.3 or 0.7, respectively). It is noteworthy that in microsomes from normal mammary tissue taken from breast cancer patients, 4-OH-E_2 formation was favored. However, in microsomes of 10 samples of mammary fibroadenoma, rates of formation of 4-OH-E_2 exceeded those of 2-OH-E_2 formation by almost 4-fold ($P < 0.002$). Similarly, in microsomes from 6 adenocarcinoma samples, rates of 4-hydroxylation of E_2 also predominated over 2-hydroxylation (ratio of 4-OH-E_2/2-OH-E_2 formation, 3.8; $P < 0.02$).

The concentration dependence of catechol estrogen formation was examined with microsomes of one mammary fibroadenoma, one adenocarcinoma, and one sample of normal breast tissue. With both neoplastic tissues, 4-hydroxylation of E_2 predominated over 2-hydroxylation (Fig. 2). 2-OH-E_2 formation was detectable in the fibroadenoma throughout the substrate concentration range tested, whereas this activity in the adenocarcinoma microsomes reached plateau values at 10 μM E_2 and then dropped to values below the sensitivity of the assay at >20 μM E_2 substrate concentrations. In contrast, 2-hydroxylation predominated over 4-hydroxylation in one microsomal sample of normal breast tissue taken from a breast cancer patient (Fig. 2). Rates of formation of 4-OH-E_2 dropped below the assay limits of detection at >20 μM E_2 substrate concentrations.

DISCUSSION

Our data demonstrate that 4-hydroxylation of estrogen is the predominant form of catechol formation in the human mammary fibroadenoma and adenocarcinoma samples tested. Ratios of 4-/2-hydroxylase activities are also higher in normal mammary tissue compared to human liver or placenta, where 4-hydroxylation of estrogens is <20% of total catechol estrogen metabolite formation (14–17). The form(s) of cytochrome P450 catalyzing 4-hydroxylation of E_2 in normal or neoplastic mammary tissue may be related to cytochrome P450 IB1, which has previously been identified in MCF-7 breast cancer cells (13) and in human uterine myoma (12), but not in liver (24, 25), and which has been shown to catalyze the specific 4-hydroxylation of E_2 (12, 13).

The increased expression of estrogen 4-hydroxylase in benign or malignant neoplastic tissue when compared to normal breast identify this enzyme activity as a potentially useful marker of neoplastic disease in biopsy material. In contrast, rates of 2-hydroxylation of E_2 were fairly constant among tissues. As a result, ratios of 4-OH-E_2/2-OH-E_2 formation in neoplastic tissue were also elevated over values in normal breast. The expression of estrogen 4-hydroxylase in normal and, at elevated levels, in neoplastic tissues is a requirement for the development of this enzyme activity as a tumor marker. The elevated expression of this activity in mammary fibroadenomas and adenocarcinomas, which do not appear to be related neoplasms, indicates the scope of this possible marker.

The product of metabolic 4-hydroxylation of E_2, 4-OH-E_2, is as carcinogenic as the parent hormone E_2 in the hamster kidney tumor model (26), whereas 2-hydroxylated estrogens do not induce tumors in this animal test system. The elevated expression of an estrogen 4-hydroxylase activity in organs of rodents in which estrogens induce tumors (9–11), in MCF-7 breast cancer cells (13), and in human uterine myoma (12) but not in livers of these species has been taken as evidence that this metabolite may mediate the induction of tumors by steroidal estrogens (9, 20). 4-Hydroxyestrogens may generate potentially mutagenic free radicals by metabolic redox cycling between the quinone, semiquinone, and hydroquinone (catechol estrogen) forms (20, 27, 28). Evidence for free radical damage by redox cycling of estrogens includes single-strand breaks of DNA in MCF-7 cells induced by estrone-3,4-quinone (29). Moreover, 4-OH-E_2 induces single-strand breaks of DNA in hamster kidney and hydroxy radical-mediated 8-hydroxylation of guanine bases of DNA *in vitro* and in hamsters *in vivo* (30–32). 2-Hydroxylated estrogens and other catechols have been shown to inhibit inactivation of 4-OH-E_2 by catechol *O*-methyltransferase-catalyzed methylation (33, 34). In addition to inducing free radical damage, 4-OH-E_2 is known to be

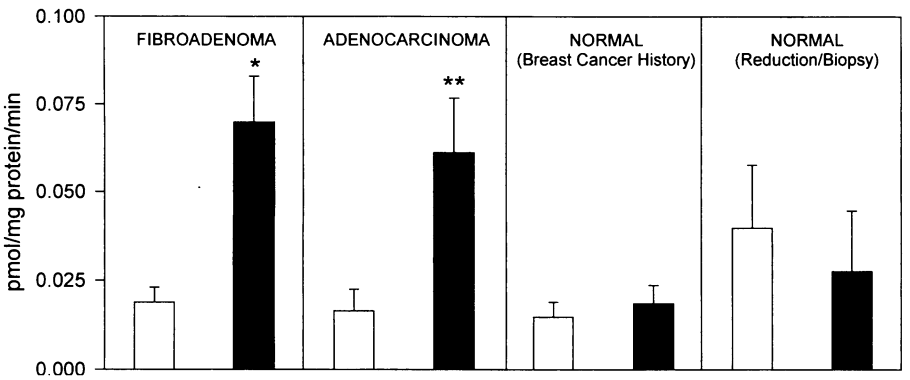

FIG. 1. Microsome-mediated formation of 2-OH-E_2 (open bars) and 4-OH-E_2 (solid bars) from 5 μM E_2 substrate. Samples were obtained fresh from 12 human mammary fibroadenomas, 8 adenocarcinomas, and normal tissue either from 24 breast cancer patients or from 7 reduction mammoplasty surgeries/excision biopsies of patients with fibrocystic changes. Microsomes were prepared by differential centrifugation (21). Catechol estrogen formation was determined by a validated product isolation assay (23) described in detail in a previous publication (12). Blank values, determined with heat-denatured microsomes or by omitting either enzyme or NADPH, were subtracted. Values of 10 fibroadenoma, 5 adenocarcinoma, 14 and 3 normal tissues, respectively, were higher than the limits of detection (125% of blank values) and are expressed as means ± SE. The rates of 2- and 4-hydroxylation by microsomes of one additional adenocarcinoma sample also showed the predominant 4-OH-E_2 formation from 5 μM E_2 substrate but were not included in the bar graph because values were 25-fold above mean rates (rates of 2- and 4-OH-E_2 formation, 0.433 and 1.570 pmol per mg of protein per min; ratio of 4-OH-E_2/2-OH-E_2 formation, 3.6). Data were analyzed by Student's t test.

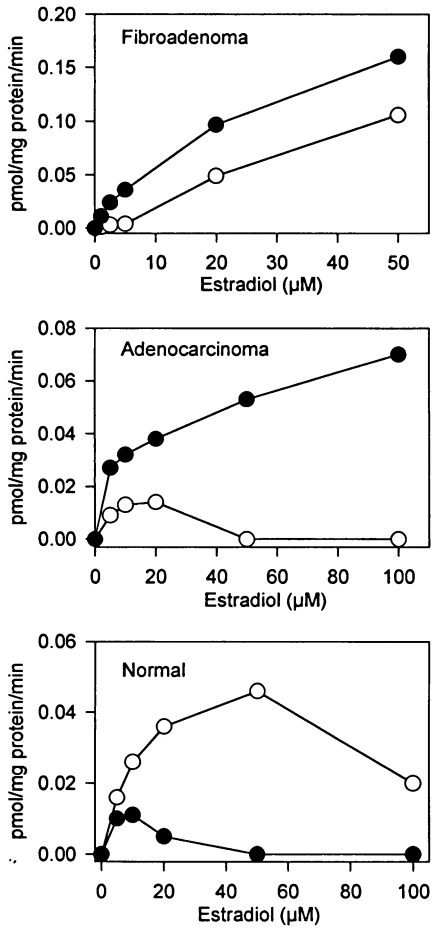

FIG. 2. Substrate concentration dependence of formation of 2-OH-E_2 (open circles) and 4-OH-E_2 (solid circles) catalyzed by microsomes of one sample of human mammary fibroadenoma, adenocarcinoma, and normal tissue of a breast cancer patient. The preparation of microsomes and the assay conditions are described in the legend to Fig. 1. Kinetic data for 4-OH-E_2 formation have been obtained from a Lineweaver–Burk plot (data not shown) and are as follows: V_{max} = 0.14 pmol per mg of protein per min and K_m = 12.0 μM for the fibroadenoma; V_{max} = 0.06 pmol per mg of protein per min and K_m = 6.4 μM for the adenocarcinoma. Kinetic parameters were not calculated for 4-OH-E_2 formation for the normal mammary tissue, but kinetic data for 2-OH-E_2 formation are as follows: V_{max} = 0.06 pmol per mg of protein per min and K_m = 13.8 μM. Values for 2-OH-E_2 formation by the adenocarcinoma microsomes and 4-OH-E_2 formation by the normal microsomes at 50 and 100 μM E_2 substrate concentrations were below the sensitivity of the assay and thus were not included in the Lineweaver–Burk plot (data not shown).

a long-acting estrogen (35). Thus, this estrogen metabolite may act as a complete carcinogen by a combination of damage to cellular macromolecules and stimulation of proliferation of damaged cells by receptor-mediated processes. We suggest that mammary tumors in humans are induced by a mechanism analogous to carcinogenic events observed in hamsters: DNA damage initiated by locally formed 4-hydroxyestrogen metabolites in combination with an estrogen receptor-mediated proliferative stimulus. Elevated levels of 8-hydroxyguanine bases of DNA in mammary tissue of breast cancer patients (36) is consistent with this suggestion.

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