Benzo(a)pyrene Binding to DNA in Organ Cultures of Human Endometrium

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ABSTRACT

Benzo(a)pyrene was found to bind to DNA in human endometrial tissue in vitro. Among specimens from 41 individuals examined, there was a 70-fold range in the observed specific activities of carcinogen binding to DNA. To determine whether this interindividual variability was correlated with the hormonally determined state of differentiation of the endometrial tissue, this population was subdivided to separate postmenopausal patients from premenopausal patients; among premenopausal patients, further division was made according to location within the menstrual cycle. Tissue obtained late in the proliferative phase or early in the secretory phase of the menstrual cycle had the highest mean specific activity of benzo(a)pyrene binding. In spite of the relatively small group sizes, the observed difference between this and the level of benzo(a)pyrene binding in the mid- and late secretory phases was statistically significant. The average binding level among the small number of patients studied who had entered a natural menopause was lower than the average binding for any of the subgroups of premenopausal patients and significantly lower than the mean for the whole population of premenopausal patients.

INTRODUCTION

Endometrial cancer is presently the most common invasive cancer of the female genital tract in the United States (5). Physiological abnormalities in which a disproportionate estrogen excess persists for prolonged periods has long been known to increase a woman's risk for endometrial cancer. During the past several years, prolonged exposure to exogenous estrogens has also been determined to be a risk factor (4, 16, 18), though some epidemiological evidence suggests its effect is akin to that of a promoter or cocarcinogen (8, 10, 17). With the exception of estrogens, however, epidemiological investigations have not identified exposures to exogenous chemicals which increase a woman's risk of endometrial cancer nor explained this cancer's rising incidence in the United States. It is possible that environmental factors are contributing to this increase. These factors, however, may be elusive to the usual approaches of epidemiological analysis because knowledge of the biology of this tissue and this patient population is limited. For example, the effects of estrogens in contributing to endometrial cancer may be so large that important but more subtle effects of environmental chemicals are masked.

The binding of carcinogens to DNA in target tissues of experimental animals appears to be an event associated with the transformation of these tissues by most, if not all, chemical carcinogens. Although the relationship between the binding of carcinogens to DNA in human tissues and the neoplastic transformation of these tissues is not known, the role of carcinogen binding to DNA is presumed to be similar to that in experimental animals. Recent studies have shown measurable levels of carcinogen binding to DNA of several human tissues in organ culture (2, 6, 7). BP6 is the carcinogen most commonly used in these studies, and their results show that these human tissues can metabolically activate BP to forms that bind to DNA. Studies of this type permit comparisons of metabolism and binding to be made between different individuals and between different human tissues. Experimental studies of this type are important for several reasons. Analysis of carcinogen binding in human tissue in vitro may suggest the potential importance of specific chemical carcinogens in human cancer. Comparisons of carcinogen binding to DNA in tissue from humans and experimental animals may indicate the extent to which extrapolations between species may be valid. Also, fresh human tissue maintained in vitro for brief periods may preserve many of the characteristics of the tissue in vivo, and this may allow studies to examine how the normal properties of the tissue contribute to the carcinogenic process.

In previous studies, we have shown that human endometrial tissue can be maintained in organ culture for prolonged periods of time with retention of many differentiated properties (11). We have also shown that organ cultures of human endometrial tissue can metabolize BP and that the proportions of various metabolic products is influenced by the estrogen status of this tissue (13). In this study, we show that [3H]BP binds to the DNA of human endometrial tissue in vitro. Although there is no epidemiological evidence either to prove or disprove BP as a relevant carcinogen for human endometrium, we chose to evaluate BP in this study because it is the carcinogen that has been most thoroughly investigated with regard to metabolism and binding to DNA in several human tissues. This permits comparison between observations concerning BP binding to DNA in other human tissues with those in this study of human endometrial tissue. Furthermore, since the previous study of metabolism of BP in human endometrial tissue found an influence of hormonal status on metabolism (13), it was desirable to determine whether it also influenced BP binding. Unlike other human tissues in which BP binding to DNA has been studied, endometrium undergoes profound physiological changes in differentiation in response to changes in circulating hormone levels. This is reflected in variations in physiological factors such as DNA replication (11), BP metabolism (13), and estrogen receptor levels (3). Alterations of differentiation may influence levels of BP binding and thus alter the potential carcinogenic effects on the tissue. Therefore, the relationship between binding levels and various biological states of the tissue appeared to be an important subject for further study to

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The abbreviation used is: BP, benzo(a)pyrene.
identify factors which might contribute to an increased risk for development of endometrial cancer.

MATERIALS AND METHODS

Tissue Obtention and Treatment. Human endometrial tissue without intrinsic pathology was obtained from hysterectomy specimens of patients offering informed consent as described previously (11). Tissue was transported from the operating suite under aseptic conditions in cold transport medium consisting of CMRL Medium 1066 supplemented with 10% heat-inactivated fetal bovine serum, 1000 units penicillin per ml, 1000 µg streptomycin per ml, 10 µm L-glutamine (all obtained from Grand Island Biological Co., Grand Island, N. Y.), 0.5 mg gentamicin per ml (Schering Corp., Kenilworth, N. J.), and buffered with 25 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4. Upon arrival in the tissue culture laboratory, the specimen was washed 3 times in the transport medium and minced into cubes 2 mm on a side. Approximately 8 pieces of tissue were incubated for 18 hr in a CO2 incubator at 37° in 5 ml of CMRL Medium 1066 supplemented with 10% heat-inactivated fetal bovine serum, 100 units penicillin per ml, 100 µg streptomycin per ml, 10 µm L-glutamine buffered with 28 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, and containing 1 µM [3H]BP (19 CI/mmol; Amersham Corp., Arlington Heights, Ill.). The concentrations of estrogen and progesterone measured in fetal bovine serum used in these studies were 5 pg/ml and 0.5 ng/ml, respectively. Following incubation, the endometrial explants were washed in the incubation medium, separated into duplicate samples, and frozen at —80°. The purity of [3H]BP was maintained by storage at —80° and by repurification at weekly intervals using column chromatography on silica gel (12). For each specimen of endometrial tissue, the day of the menstrual cycle at the time of hysterectomy was determined morphologically by 2 pathologists using standard morphological criteria (14).

DNA Isolation and Purification. Frozen tissue was thawed and homogenized using a TenBroeck tissue grinder in 0.5 ml of 0.1 M Tris-HCl, pH 7.4, containing 0.15 M NaCl, 5 mM trisodium EDTA, and 2% sodium dodecyl sulfate. A sample of the homogenate (25 µl) was removed for protein determination (15). The remainder of the homogenate was extracted with phenol 5 times at 37° to remove protein and unreacted [3H]BP. For each extraction, the phenol phase was separated from the aqueous phase by centrifugation for 2 min at 10,000 rpm and discarded. After the final phenol extraction, 2 volumes of absolute ethanol were added to the aqueous phase, and the samples were stored at —20° overnight. The resulting precipitate was sedimented by centrifugation at 1000 x g for 20 min, and the supernatant was discarded. The pellet was resuspended in 0.5 ml of 0.1 M Tris-HCl, pH 7.4, containing 0.15 M NaCl and 0.5 mM trisodium EDTA using a Dounce homogenizer. Traces of phenol were removed from this aqueous solution by twice extracting with ether, and residual ether was evaporated with N2. RNA was enzymatically removed by incubating the samples with 50 µl of a 1-µg/ml RNase solution ( Worthington Biochemical Corp., Freehold, N. J.; previously incubated at 90° for 1 hr to inactivate DNase) for 1 hr at 37°. Protein was hydrolyzed by adding 100 µg of proteinase K to the aqueous solution (E. M. Biochemical Corp., Darmstadt, Germany; final concentration, 200 µg/ml) for 1 hr at 37°. The enzymatic reaction was terminated by rapidly cooling samples to 0°. Unbound [3H]BP was removed from the samples by extensive ether extractions which continued until radioactivity in the ether phase reached background levels. Residual ether in the aqueous phase was evaporated with N2. Samples were dialyzed at 4° against 500 ml volumes of a buffer solution consisting of 0.1 M Tris-HCl, pH 7.4, containing 0.5 mM trisodium EDTA and 0.15 M NaCl; the dialyzing solution was changed at 30-min intervals during the 4-hr dialysis period. Dialyzed samples were diluted to 5 ml with the buffer solution and mixed with 6.2 g CsCl. DNA was isolated at equilibrium in density gradients generated centrifugally during 66 hr at 35,000 rpm and at 25° in a Beckman type 40 fixed-angle rotor. Gradients were fractionated from the bottom of the tubes into 0.5-ml aliquots to which 0.5 ml of the buffer solution was added. Absorbance of UV light at 260 and 280 nm was determined for each fraction. The DNA peak fractions were isolated, dialyzed as described above, and reband in a second CsCl gradient. Following rebanding, the DNA content of each gradient fraction was calculated from absorbance determinations at 260 and 280 nm. Radioactivity in each fraction was determined by liquid scintillation counting. After subtracting background radioactivity and correcting for variations in counting efficiency using the internal standard method, dpm were determined for each of the fractions. Specific activities of [3H]BP binding were calculated by dividing the dpm for each fraction by the DNA content.

RESULTS

When centrifuged to equilibrium in a second CsCl gradient, purified human endometrial DNA formed a well-defined band with UV absorbance coincident with radioactivity. Very low background levels of radioactivity and UV absorbance were present in other fractions indicating the thoroughness of removal of other cellular constituents or unbound [3H]BP. Protein contamination was negligible in the DNA peak fractions as evidenced by an average A260/A280 absorbance ratio of 1.9. The specific activities of [3H]BP binding to DNA for duplicate samples derived from individual specimens are plotted on

Chart 1. Least-squares regression analysis of variation of [3H]BP binding to DNA between duplicate samples derived from individual endometrial specimens. Human endometrium was incubated for 18 hr in organ culture in medium containing 1 µM [3H]BP. The explants were separated into duplicate samples for [3H]BP binding determination. Specific activities of duplicate samples were plotted on separate axes; for each specimen, the higher value was assigned to the ordinate. A regression analysis was performed to construct the least-squares line.
order to most clearly illustrate the range of binding of [3H]BP to DNA in endometrial tissue examined, specific activities of binding were determined. In organ culture in medium containing 1 µM [3H]BP. For each of the 41 specimens from these patients, this histogram has been constructed with specific activities of [3H]BP binding to DNA measured at 30 dpm/µg DNA or less. Specific activities among this population show no pattern of clustering at any binding level.

The variation of [3H]BP binding to the DNA of human endometrial specimens is shown in Chart 2. Specific activities of [3H]BP binding between specimens from different individuals varied from 1 to 70 dpm/µg DNA. Thus, even within a population including only 41 individuals, there was a variation of nearly 2 orders of magnitude in [3H]BP binding levels. Between these extremes, specimens display a smooth monophasic progressive increase in specific activities. Among the specimens, 88% have specific activities of [3H]BP binding to DNA measured at 30 dpm/µg DNA or less. Specific activities among this population show no pattern of clustering at any binding level.

The range of specific activities of [3H]BP binding to human endometrial DNA obtained from 41 individual human specimens is shown in Chart 2. Specific activities of [3H]BP binding between specimens from different individuals varied from 1 to 70 dpm/µg DNA. Thus, even within a population including only 41 individuals, there was a variation of nearly 2 orders of magnitude in [3H]BP binding levels. Between these extremes, specimens display a smooth monophasic progressive increase in specific activities. Among the specimens, 88% have specific activities of [3H]BP binding to DNA measured at 30 dpm/µg DNA or less. Specific activities among this population show no pattern of clustering at any binding level.

The variation of [3H]BP binding to the DNA of human endometrial specimens is shown as a function of the menstrual cycle in Table 1; this shows the mean specific activity ± S.E. for each of 3 portions of the menstrual cycle. Specimens were grouped among 3 categories according to their position in the menstrual cycle at the time of tissue obtainment and analysis: early and mid-proliferative, late proliferative and early secretory, or mid- and late secretory. The highest levels of [3H]BP binding appeared between Days 10 and 21 of the menstrual cycle, corresponding to the late proliferative and early secretory segments; the 16 specimens evaluated in this interval had an average specific activity of [3H]BP binding to DNA of 25 dpm/µg DNA. In contrast, the lowest levels of binding were observed in the mid- and late secretory segments of the menstrual cycle between Days 21 and 28. The 10 specimens analyzed had an average specific activity of 7 dpm/µg DNA. In the early and mid-proliferative phases, representing the initial segment of the menstrual cycle between Days 1 and 10, the average specific activity of [3H]BP binding calculated from 11 specimens was 15 dpm/µg DNA. Statistical analysis for equality of means with both parametric (ANOVA, p < 0.05) and nonparametric (Kruskal-Wallis, p < 0.001) methods showed that at least one of the population means differs from the others. The Fisher’s protected least significant difference procedure (α = 0.05) and Bonferroni’s procedure (α = 0.05) indicate that the mean binding level in the late proliferative and early secretory phases of the cycle is significantly higher than the mean binding level in the mid- and late secretory phases.

Endometrial specimens from 4 postmenopausal women were analyzed for [3H]BP binding to DNA. One of these patients had been maintained on estrogen replacement therapy prior to hysterectomy. Because this patient was biologically different from the other patients who had entered a natural menopause, she was excluded from this group. The range of specific activities of [3H]BP binding in the remaining 3 postmenopausal patients was 3 to 8 dpm/µg DNA with an average value of 5 dpm/µg DNA. This is less than one-third the level of the 17 dpm/µg DNA average specific activity of [3H]BP binding of the 37 premenopausal patients’ tissues. The mean specific activity ± S.E. for premenopausal and postmenopausal specimens is shown in Table 1. Statistical analysis for equality of means showed with a 2-sample t test that the mean binding level in premenopausal tissue is significantly higher (p = 0.01) than the mean binding level for postmenopausal tissue. The binding level observed in the postmenopausal specimen excluded is of considerable interest. The specific activity of [3H]BP binding in this specimen was 27 dpm/µg DNA, a value consistent with levels of binding observed in the late proliferative and early secretory phases of the normal menstrual cycle.

**DISCUSSION**

These results demonstrate the binding of a carcinogen, [3H]BP, to the DNA of human endometrial tissue in vitro. It is clear that the association between the carcinogen and DNA persists despite extensive purification and solvent extraction of the DNA. Other studies utilizing similar methodology for the extraction and purification of DNA after carcinogen binding in other human tissues have documented the covalent nature of this binding (2, 7, 9). The consistency between binding of [3H]BP to human endometrial DNA and DNA in other human tissues suggests that the binding of [3H]BP to human endometrial DNA is also covalent.
Among the 41 cases studied, there was a 70-fold range of specific activities of \[^{3}H\]BP binding to endometrial DNA. This wide interindividual variability parallels results of BP binding to DNA in other human tissues. The ranges of binding levels were 100-fold for 32 specimens of colon (1), 99-fold for 8 specimens of esophagus (7), and 75-fold for 37 specimens of bronchus (6). The statistical analysis of duplicate measurements of binding in this study showed that experimental variation for a given specimen was small (6% on average); thus, the overall variability was not the reflection of random error. Consequently, other specific biological properties that characterize the individual human subjects are presumed to contribute to the wide range of observed binding levels among specimens of endometrial tissue.

The state of differentiation of the endometrium during the menstrual cycle and after menopause reflects the hormonal status of the tissue. Since this affects BP metabolism in human endometrium and might also influence \[^{3}H\]BP binding to DNA, binding was evaluated first as a function of menstrual cycle location. Specimens were assigned to 3 groups according to location within the menstrual cycle. This was done because the number of specimens for any given day of the menstrual cycle was limited and because the morphological dating of the endometrial tissue more accurately reflected an approximate 3-day range than a specific day. Grouping specimens also increased the number of observations in statistical comparisons. The intervals selected appeared to enclose specific activities of similar magnitudes and also divided the menstrual cycle into biological groups that reflected in vivo differences in physiology. The results show significantly higher levels of \[^{3}H\]BP binding in the late proliferative and early secretory phase as compared to the mid- and late secretory phase. Binding of \[^{3}H\]BP was also compared between endometrial specimens from premenopausal and postmenopausal patients. Only 4 postmenopausal specimens were obtained, and one patient who was receiving exogenous estrogen replacement therapy was considered separately from the remaining 3 patients who had progressed through a natural menopause. Endometrial tissue from the 3 patients in natural menopause had a low mean level of \[^{3}H\]BP binding, 5 dpm/μg DNA, as compared to the average for premenopausal patients. Despite the small numbers of observations, this 3.7-fold lower binding level is significantly different from the mean binding level of all premenopausal specimens. This difference is even greater if the postmenopausal specimens are compared with premenopausal specimens obtained during the first two-thirds of the menstrual cycle. In contrast, the specific activities of binding in premenopausal specimens from the middle and late portions of the secretory phase are comparable to those for the group of 3 postmenopausal specimens. Endometrium from the fourth postmenopausal patient had a specific activity of \[^{3}H\]BP binding of 27 dpm/μg DNA. This is considerably higher than the other postmenopausal specimens and comparable to specimens in the late proliferative portion of the menstrual cycle, a time when tissue and blood levels of estrogen are high.

Only one patient has been studied to date who was maintained on exogenous estrogens after menopause. Therefore, conclusions should be drawn cautiously concerning the effects of exogenous estrogens on endometrium. Nonetheless, the results of this study, taken as a whole, do suggest a relationship between estrogen levels and \[^{3}H\]BP binding to DNA in this tissue. Among specimens from premenopausal women, the highest binding was observed in specimens collected during the periods of the menstrual cycle when estrogen levels are high. Binding was significantly reduced when specimens were obtained at times in the menstrual cycle when estrogen levels are low. Similarly low levels of \[^{3}H\]BP binding were observed among menopausal women who had entered a natural menopause with its associated reduction in natural estrogens. In the case where exogenous estrogens were administered to a postmenopausal patient, the level of \[^{3}H\]BP binding was high, comparable to that found among younger women at the peak of estrogen levels in their menstrual cycle. Although these results are consistent, it should be recognized that estrogen levels in the preceding discussion are inferred from normal physiology. They were not directly measured. Direct comparisons of estrogen levels and \[^{3}H\]BP binding levels in the same patients would be helpful in determining whether estrogen levels have a uniform and consistent relationship to binding levels. Studies of endometrial specimens from additional postmenopausal patients receiving exogenous estrogens are also needed to determine the generality of our isolated observation.

Estrogen has numerous and diverse effects on the endometrium. Even if the effects of estrogen suggested in this study are subsequently confirmed, the mechanism for the increased binding of \[^{3}H\]BP to DNA will remain obscure. However, studies have shown that BP metabolite profiles from human endometrial specimens obtained at various stages in the menstrual cycle vary with respect to cycle stage (13). Although there may not be a direct relationship between BP metabolism and binding, it is possible that variations in primary and secondary stages in BP metabolism are responsible for some of the variation observed. We have no information to indicate whether this effect is specific for BP or whether it applies to other carcinogens as well. However, if estrogen does have a measure of generality regarding its effect on the binding of many carcinogens, then this may account, at least in part, for the epidemiological effects concerning exogenous estrogens in relation to endometrial cancer. The increased risk of endometrial cancer is associated with prolonged use of exogenous estrogens, and the risk rapidly returns to normal levels following cessation of estrogen administration (8, 10, 17). These features have led to the impression that exogenous estrogen functions as a cocarcinogen rather than directly as an initiator (8). This might be the type of effect that would be predicted if exogenous estrogen facilitated the binding of carcinogens to endometrial DNA and thereby augmented the process of malignant transformation. The results of this study offer only a narrow base of evidence in support of this hypothesis. Many additional investigations are needed to support or refute it. This report, however, does demonstrate that carcinogenesis studies which utilize human tissues in vitro have the potential for contributing to our knowledge of important features in the development of human cancer.

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**References**

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