Peroxidase Content in Cell Subpopulations of 7,12-Dimethylbenz(a)anthracene-induced Mammary Tumors in Rats

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ABSTRACT

Peroxidase has been investigated as a potential marker protein for the prediction of response to hormonal therapy in tumors of steroid-sensitive tissues, but the cellular origin of the enzyme has been questioned. To localize the observed peroxidase activity, in this study cell subpopulations were isolated from mammary tumors induced by 7,12-dimethylbenz(a)anthracene and from mammary tissue of virgin and lactating female rats. Cells from each of the four cell bands, regularly obtained by isopyknic velocity sedimentation after mechanical and enzymatic dispersion of these tissues, were assayed biochemically and histochemically for peroxidase activity. In addition, cells from each subpopulation were examined at both the light and electron microscopic levels. Elevated enzyme levels were observed in each of the cell subpopulations of 7,12-dimethylbenz(a)anthracene-induced tumors when these were compared with tissue in either of the physiological states assayed. In each tissue type, the levels of peroxidase increased from the lighter cell bands to the heavier cell bands. Light and electron microscopic examination revealed the highest proportion of epithelial cells in the lighter bands and an increase in granulocytes and fibroblasts in the heavier bands, suggesting a nonepithelial contribution. Histochemical examination of intact tissue revealed most peroxidase activity in the stromal compartment with limited activity in parenchymal cells.

INTRODUCTION

Peroxidase has been proposed as an estrogen-inducible marker of hormone-dependent tumors arising in hormone-responsive tissues such as uterus and mammary gland (1, 3, 4, 11, 16). While estrogen-inducible peroxidase activity has been reported in the DMBA1-induced mammary tumor (4), a recent study failed to confirm this (18). In testing this hypothesis, investigators have generally used solid tumor samples (2, 4, 6, 17). This approach fails to take account of the cellular heterogeneity of solid tumors (5, 9) or of the contribution to the observed activity of myeloperoxidase granules in the granulocytes that frequently accumulate in tumors. Moreover, recent evidence suggests that much of the estrogen-inducible peroxidase activity of uterus can be accounted for by eosinophils that migrate into the tissue after estrogen administration (14).

Enzymatic dispersion of mammary tumors has been used to separate epithelium from the supporting stroma (15, 20), and the monodispersed cells can be further separated isopyknically on a density gradient to yield various cell subpopulations. The latter study also reported that more highly differentiated cells which predominate in the lighter cell bands contain more estrogen receptor.

In this study, the density-defined cell subpopulations from DMBA-induced mammary tumors were examined ultrastructurally, and peroxidase activity was assayed to determine the contribution of each subpopulation to the enzyme activity previously reported.

MATERIALS AND METHODS

Tumor Induction and Cell Separation. Mammary tumors were induced in 50-day-old female Sprague-Dawley rats by gastric intubation of DMBA (Upjohn Company, Kalamazoo, Mich.). Primary tumors arose in 4 to 8 weeks. To obtain isolated cell subpopulations, tissues were excised, freed of necrotic foci or capsular material, and sliced to a thickness of 1 mm on a Stadie-Riggs hand microtome. These slices were then chopped into 1-mm cubes on a McIlwain tissue chopper. These tissue bits were digested in Medium 199 supplemented with collagenase (0.1%), hyaluronidase (0.08%), and soybean trypsin inhibitor (0.001%) for 60 min at 37°C with continuous agitation. The resulting cell suspension was filtered through 110- and 40-μm Nitex filters, layered on a continuous Ficoll gradient (5 to 30%, w/v), and centrifuged for 45 min at 3000 rpm (IEC PR20) International Equipment Co. Four distinct cell bands were harvested for enzyme determinations. Mammary tissues from virgin and lactating rats were treated identically. Cells from each band were counted in a Coulter Electronics Model ZI cell counter. The viability of the dispersed cells was determined by dye exclusion.

Histochemical and Enzymatic Detection of Peroxidase Activity. Cells from each band were spun onto glass slides according to the method of DeSombre and Lyttle (4). The salt-extracted protein was assayed colorimetrically at 470 nm in a reaction mixture containing H2O2, guaiacol, and CaCl2 in Tris-HCl buffer. The reaction rate was expressed as a percentage of total cells counted. To determine the tissue localization of this peroxidase activity, 10-μm frozen sections were cut and stained with Kaplow’s reagent.

For enzymatic determinations, peroxidase was extracted according to the procedure of DeSombre and Lyttle (4). The salt-extracted protein was assayed colorimetrically at 470 nm in a reaction mixture containing H2O2, guaiacol, and CaCl2 in Tris-HCl buffer. The reaction rate was monitored for 5 min, and peroxidase activity was expressed as increase in absorbance/min/106 cells.

Electron Microscopy. Small pieces of tumor were fixed by immersion in 3% glutaraldehyde-paraformaldehyde, washed in 1% cacodylate buffer, and postfixed in 1% osmium tetroxide before dehydration in ascending concentrations of ethanol and embedding in Araldite 502. Isolated cell subpopulations were collected in 1.5-mL microcentrifuge tubes, fixed, and processed as described above. Between fixatives, buffers, and ethanol solutions, cells were sedimented by low-speed centrifugation. After polymerization, the tips of the tubes were cut off,
RESULTS

An inverse relationship exists between the proportion of vacuolated cells in different cell subpopulations of a single tumor and their location in the Ficoll gradient (Table 1). Electron microscopy studies revealed that vacuolated cells are structurally better differentiated. Nuclei of these cells are filled with well-dispersed chromatin and a prominent nucleolar complex. The cytoplasm, in addition to lipid droplets and vacuoles, contains a large Golgi complex (Fig. 1a). Contrasting with these are the nonvacuolated cells. Nuclear/cytoplasmic ratio is greater in these cells, and nuclear chromatin often are less well dispersed (Fig. 1b). Granulocytes and mast cells, due to a lack of vacuoles, are also included in this tabulation.

Levels of peroxidase activity in cell subpopulations of the virgin rat ranged from \(2 \times 10^{-4}\) to \(3.5 \times 10^{-3}\) absorbance unit/min/10^6 cells. The average peroxidase activity increased from Band 2 to Band 5 as shown in Chart 1; these levels of peroxidase activity were contrasted with those found in lactating mammary tissue, which ranged from \(7.0 \times 10^{-4}\) to \(6.8 \times 10^{-3}\) absorbance unit/min/10^6 cells. The average enzyme activity again increased from a minimum in Bands 2 and 3 to a maximum in Band 5 (Chart 1). This observed difference between virgin and lactating female mammary tissue, however, was not statistically significant.

In comparison to the normal glands in different physiological states, peroxidase levels in DMBA-induced mammary tumors were elevated to between \(8.0 \times 10^{-4}\) and \(1.67 \times 10^{-3}\) absorbance unit/min/10^6 cells. Although large variations were observed between tumors, the trend toward higher peroxidase activity in the cells of lower bands continued to be observed (Fig. 1, c and d). Enzyme levels in each band were significantly higher (\(p < 0.05\)) than in the corresponding subpopulations of either virgin or lactating normal mammary tissue.

Histochemical results correlated well with biochemical determinations (Table 2). In the virgin rat, only faintly staining cells were observed in each cell subpopulation, but their number increased from Band 2 to Band 5. Each subpopulation in the lactating rat showed a slight increase in peroxidase staining compared to the corresponding subpopulation from the virgin rat.

By contrast, numerous intensely stained cells were observed in each cell subpopulation of the DMBA-induced mammary tumor. Again, the presence of histochemically reactive cells rose from Band 2 to Band 5 (Fig. 1, c and d).

As illustrated in Figs. 2 and 3, 2 patterns of intracellular staining were observed. Some cells showed discrete granular staining and were found predominantly in the heavier bands (i.e., Bands 4 and 5) of each category of tissue. It was this cell type the distribution of which increased most dramatically in neoplasia. The second pattern was a less intense, diffuse staining. This pattern was observed in all cell subpopulations, but mainly in the lighter cell bands where the highly differentiated epithelial cells are found. Light microscopic examination of sections of intact tissue stained for peroxidase activity showed that most reactive cells were stromal (Fig. 2). The stained cells exhibited the granular pattern seen in the heaviest isolated cell bands. Some activity was detected in the epithelial cells of scattered acini (Fig. 3); their staining resembled that of the peroxidase-reactive cells of the lighter cell bands.

Although all tumors studied histologically were adenocarci nomatous, considerable variation in fine structure of the paren chymal cells and in the stromal/parenchymal cell ratio was seen. Mast cells retained their characteristic granulation pattern through the dispersion procedure and were readily discerned (Fig. 4). These cells were more frequently seen in the stroma of regressing tumors. In such specimens, groups of 2 to 5 fully granulated mast cells often were encountered near the bas al lamina (Fig. 5). Granulocytes were most frequent near vascular channels or necrotic foci.
DISCUSSION

Peroxidase activity was detected in each subpopulation of normal and neoplastic mammary tissues (Chart 1). This suggests that peroxidase activity is present both in more highly differentiated epithelial cells and in stromal components, since ultrastructural analysis revealed an enrichment of the former cell type in the upper bands and of the latter cell type in the lower, heavier bands. Histochemical evaluation of intact tissue sections verified this finding, revealing reactive cells in both stromal and epithelial compartments. Peroxidase activity in mammary tumors thus represents a summation of both components and is not merely a property of epithelial cells. This finding contrasts with the conclusions that previous investigators have drawn from observations of whole tumor peroxidase activity.

Our data reveal a trend toward increased peroxidase activity from the lighter, more differentiated to the denser, less well differentiated cell subpopulations. Since the more differentiated parenchymal components reside in the lighter bands, either the peroxidase activity found in the heavier bands must be due mainly to activity in nonparenchymal cells or, less likely, the poorly differentiated epithelial cells found in these bands must contain large amounts of enzyme activity. Indeed, our histochemical analysis corroborates the former hypothesis, since the most intensely staining cells in the lower bands were granulocytic.

Recent studies of another hormone-responsive tissue, the uterus, have shown that, in the human, peroxidase activity is preferentially localized in the stroma (10). In the rat, uterine peroxidase increases dramatically in response to estrogen administration, but it has been demonstrated that practically all of the increase can be attributed to the migration of eosinophils into the uterus (14). Our histochemical observations are consistent with these studies, indicating a nonparenchymal contribution to the peroxidase activity of hormone-responsive tissue.

Like ourselves, Strum et al. (19) have found mast cell infiltration in tumor parenchyma of N-nitrosomethylurea-induced mammary tumors. Administration of cis-hydroxyproline has induced regression of N-nitrosomethylurea-induced mammary tumors in rats with accumulation of mast cells in the residual tumor (19). Unlike our findings, in which mast cells seem to be excluded from the tumor parenchyma, these investigators reported the mast cell to be scattered among the epithelial cells. The difference in distribution of mast cells between the 2 studies may have resulted from the breakdown or lack of type IV collagen synthesis induced by cis-hydroxyproline treatment in the former study.

A recent study has demonstrated peroxidase activity in mast cell granules (8). Hence, the mast cell may also contribute to the increased peroxidase activity of neoplastic tissue, as was found in the membrane-bound peroxidase activity of mitochondria (13). The cell subpopulations with the highest peroxidase activity in our study did in fact contain the highest proportion of granulocytes and mast cells.

While the nonparenchymal contribution to the peroxidase pool is the most significant source of the increased peroxidase activity observed in the lower cell bands of the DMBA-induced mammary tumor, the increase in activity from cells in Band 2 of the virgin or lactating normal rat to those of the DMBA-induced mammary tumor should not be overlooked. This band consists mainly of highly differentiated epithelial cells. Thus, an increase in parenchymal peroxidase activity does appear to occur after neoplastic transformation. However, this increase is small in comparison to those in the heavier cell bands, which reflect the stromal contribution.

Our findings in this study suggest that the usefulness of peroxidase assay of solid tumor tissue extracts may be limited and that the isolation of subpopulations and subsequent histochemical analysis may provide an avenue to more appropriately address this issue.

REFERENCES

Fig. 1.  a, representative cells from Band 3. Note the presence of large vacuoles. \( \times 4500 \).  b, aggregate of cells from Band 5. Note the paucity of cytoplasm. \( \times 4200 \).  c, peroxidase activity in cells from Band 3. \( \times 400 \).  d, cells from Band 5 stained for peroxidase activity. \( \times 400 \).
Fig. 2. Peroxidase activity in DMBA-induced mammary tumor in a 10-μm frozen section. Arrows, peroxidase-positive cells. Note the granular nature of reaction products in stromal cells. × 400.

Fig. 3. Epithelial cells containing peroxidase activity (arrows). Note the diffuse staining pattern. × 400.

Fig. 4. Isolated mast cell typically found in Band 5. × 4800.

Fig. 5. A cluster of mast cells near the base of an acinus in a regressing DMBA-induced mammary tumor. × 4500.
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