Histochemical Studies on Hydrolytic Enzyme Activities in Spontaneous Mammary Cancer of C3H Mouse

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SUMMARY

Histochemical observations were carried out on various hydrolytic enzymes in the spontaneous mammary carcinoma of C3H-strain mice.

Alkaline phosphatase activity was observed in the luminal border of the tubular structure and in the periluminal surface, which showed a cystic dilatation in ducts and papillae. Capillaries in the stroma showed a high enzymatic activity. Acid phosphatase activity was moderate in the inner layer of large ductlike structure of neoplasms and rather slight in other parts. Esterase activity was variable in neoplastic epithelia. Activities for β-glucuronidase and aminopeptidase were low in tumor cells. There was a complete lack of alkaline phosphatase activity in a medullary type of mammary cancer. The distribution of other hydrolytic enzymes was almost the same in all types of mammary carcinoma of C3H mouse.

Many histopathological investigations have been reported on the mammary cancer in C3H mice, but very few of histochemical observations. In a series of the present experiment, a variety of hydrolytic enzymes in this tumor was studied.

MATERIALS AND METHODS

Materials

The materials consisted of 23 subcutaneous tumors, which developed spontaneously in female mice of C3H strain at 7–12 months of age. Fresh specimens were frozen in dry ice and cut 15–30 μ thick in a −20°C cryostat with a sliding microtome. The fresh frozen sections were employed for the histochemical demonstration of acid phosphatase, β-esterase, β-glucuronidase, and aminopeptidase. For the demonstration of alkaline phosphatase, specimens were fixed in cold alcohol at 4°C overnight and embedded in paraffin. A section of every specimen was stained with hematoxylin-eosin for histopathological diagnosis.

Histochemical Methods

Alkaline phosphatase.—Cold alcohol-fixed paraffin sections were stained for the demonstration of alkaline phosphatase by the simultaneous azo coupling method. These were incubated for 30 minutes at 20°C in the following mixture: 10 mg. of sodium α-naphthyl acid phosphate, 20 mg. of Clark and Lub's buffer at pH 9.2, and 20 mg. of Diazobue B. The positive portion of enzymatic activity showed purple against a yellowish brown background.

Acid phosphatase.—Sections, fixed in 10 per cent neutral formalin for 10 minutes, were incubated for 1 hour at 20°C in a substrate solution, which was prepared in the same manner as that for alkaline phosphatase except that a 0.1 M acetate buffer at pH 5.8 was used. A positive enzyme reaction resulted in a dark purple-colored area.

Non-specific esterase.—The cold, microtome sections, fixed in 10 per cent neutral formalin for 10 minutes, were placed in a solution consisting of 10 mg. of β-naphthyl acetate dissolved in 1 ml. of acetone, 20 ml. of 0.1 M Michaelis buffer at pH 7.2, and 20 mg. of Diazobue B, for 30 minutes at 20°C. The slides were mounted in glycerin. Enzymatic activity was shown by a red-purple azo dye color.

β-Glucuronidase.—For the demonstration of β-glucuronidase the post-azo coupling method (18) was used. Formalin-fixed sections were incubated for 6 hours at 37°C. In the following mixture: 15

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Alkaline phosphatase.—Enzymatic activity was variable in different glands (Figs. 3, 4), the periacinar part in tumor sections were rinsed in water and put in 50 ml. of 0.08 M phosphate buffer at pH 7.5 containing 50 mg. of Diaz blue B. The positive portion of enzymatic activity was revealed by a purple color.

Acid phosphatase.—Enzymatic activity was usually low and diffuse in tumor tissue. The enzymatic activity was rather frequently localized in the luminal border of the ductlike structure.

Non-specific esterase.—The tumor tissue was diffusely stained for β-esterase reaction, and the enzymatic activity was strong to moderate (Fig. 11). The neoplastic epithelium in dilated cysts and papillary structures had a large amount of the activity. The stroma stained slightly (Fig. 12).

β-Glucuronidase.—The reaction of β-glucuronidase in tumor cells was rather slight and usually localized in the inner side of the ductlike structure. The necrotic mass showed more intense activity than a proliferating neoplastic epithelium. The stroma tissue stained slightly.

Aminopeptidase.—When l-leucyl-β-naphthylamide was employed, only a trace reaction occurred in all specimens. However, when the substrate was DL-alanyl-β-naphthylamide, the neoplastic epithelium showed various degrees of staining. The necrotic foci showed a markedly intense coloration.

RESULTS

Histological Findings

The tumors examined in the present study were all adenocarcinoma of mammary origin, classifiable into three histological types. The first type was tubular adenocarcinoma, which was most common and showed an irregular tubular architecture (Figs. 9, 10). The second type was characterized by a papillary or pseudopapillary growth of columnar-shaped neoplastic cells (Fig. 5) and by occasional cyst formation. In the third or medullary type, the tumor cells were cuboidal or polygonal in shape and packed closely together without showing any definite histological arrangements. The necrotic foci were most common in the medullary type. In general, the scirrhous pattern was scarce, except in the peripheral areas of neoplastic foci (Fig. 7).

Histochemical Findings

Alkaline phosphatase.—Enzymatic activity was mostly confined to the luminal border of the tubular architecture (Figs. 1, 2). The intraluminal area of the papillary structure and the inner area of the portion of cystic dilatation also showed an intense enzymatic reaction (Fig. 6). In the medullary type, the enzymatic activity was present only in necrotic foci and not in tumor cells. Capillaries in the stroma contained an abundant enzymatic activity (Fig. 8). Contrary to an intense activity shown by the myoepithelium in normal mammary glands (Figs. 3, 4), the periacinar part in tumor tissue did not show any enzymatic activity. Activity of alkaline phosphatase was variable in different portions of the same specimens.
salivary gland tumors, alkaline phosphatase was present in reticular and solid structures, but not in the ductlike components (14). Therefore, the occurrence of alkaline phosphatase in both mammary carcinoma and salivary gland tumor showed contradictory effects. It is assumed from these findings that the development of the alkaline phosphatase activity in mammary tumor was characteristic.

Fanger and Barker (4) reported that alkaline phosphatase activity in normal mammary glands appeared under the influence of estrogen, but that acid phosphatase activity was not augmented. In the present experiment, acid phosphatase activity was usually low and was found preferentially in the inner layer of the duct epithelium. Contrary to the present findings, several authors have reported a rather intense activity of acid phosphatase in human breast cancer as well as in other malignant tumors (4, 17). They also described variations in intensity among the specimens.

That the localization of esterase is noncharacteristic, both in neoplastic and normal epithelia, has been accepted. Monis and Weinberg (11) stated that the esterase activity of normal and neoplastic epithelia was frequently high, whereas mesenchymal tumor and connective tissue showed little or no activity. It is reported that enzymatic activity is independent of malignancy (11). Similar to the above authors’ view, the intensity of esterase in the present results did not show any considerable difference between tumors and normal mammary glands.

An increase of β-glucuronidase activity in malignant tumors has been reported by Fishman and Anlyan (5). Cohen and Bittner (2) confirmed that mammary cancer in mice was more active for β-glucuronidase than was a nonmalignant mammary tissue. However, as in the present experiment, the β-glucuronidase activity of mammary cancer in C57 mice was not stronger than that of the normal matrix and rather low compared with that of other cancer. Apparently Fishman’s view of the increase is contradictory to the present results. Monis, Banks, and Rutenberg (9) described that in breast cancer there was a considerable variation in β-glucuronidase activity among different fields of the same material, as well as among different carcinomas. Such variation was not clearly correlated with the histological grade of differentiation. It has been reported that β-glucuronidase does not always lead to the same result with both the post-azo coupling method (19) and the 8-hydroquinoline glucuronide method (6).

Regarding biological significances in aminopeptidase, two main opinions are maintained: one is the proteolytic theory proposed by Burstone (1), and the other the fibroblastic one by Monis, Nachlas, and Seligman (10). The main difference of opinion is in interpretation of the finding of aminopeptidase activity in stroma around carcinoma cells. Both authors have seen aminopeptidase activity in cancer cells and in stroma. Burstone (1) believes that the aminopeptidase is elaborated by the cancer cell into the stroma as a means of tumor invasion and is an indicator for the grade of proteolysis. Monis, Nachlas, and Seligman (10) find aminopeptidase in all proliferating fibroblasts whether cancer cells are present or not, as in healing ulcers. Therefore, they do not believe that aminopeptidase in stroma has any special significance for growth of tumors.

Mottet (12) concluded that the aminopeptidase activity represented host reactions to an invading neoplasm and never any part of the mechanism by which malignant neoplasms invaded host tissue. Furthermore, it has already been reported that aminopeptidase activity developed simultaneously with alkaline phosphatase in inflamed and proliferating conditions of connective tissue (15, 16), and appeared in necrotic portions (15). The findings reported in the present paper do not elucidate the controversy. Aminopeptidase in necrotic tissue could come from tumor cells that became necrotic or from fibroblasts that may be invading the necrotic tissue. Perhaps the presence of both aminopeptidase and alkaline phosphatase in the same cells may show the proliferating sign or fibroblastic properties.

REFERENCES

9. Monis, B.; Banks, B. M.; and Rutenberg, A. M. β-o-


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**FIG. 1.**—Adenocarcinoma (tubular type) of C3H mouse. Alkaline phosphatase. Luminal border of the lobules shows an intense activity. ×100.

**FIG. 2.**—Higher magnification of Figure 5. ×400.

**FIG. 3.**—Normal mammary gland. Alkaline phosphatase. ×100.

**FIG. 4.**—Higher magnification of Figure 3. Alkaline phosphatase is present in the myoepithelia and duct cells. ×400.
Fig. 5.—Papillary type in mammary adenocarcinoma. Hematoxylin-eosin stain, X100.

Fig. 6.—Serial section from same block as in Figure 5. Alkaline phosphatase. Internal side of the papillary structures and cystic dilatation is showing an intense activity. X100.

Fig. 7.—Scirrhous type of CSH mouse mammary cancer. Hematoxylin-eosin stain, X100.

Fig. 8.—Serial section from same block as in Figure 7. Alkaline phosphatase. Enzymatic activity is observed in the luminal side of lobules, whereas there is little or none in the stromal connective tissue, X100.
FIG. 9.—Adenocarcinoma (small tubular type). Hematoxylin-eosin stain, ×100.

FIG. 10.—Higher magnification of Figure 9, ×400.

FIG. 11.—Esterase in small tubular type (Fig. 9). In neoplastic cells, the enzymatic activity is strong or moderate. ×100.

FIG. 12.—β-Esterase in scirrhouss type (Fig. 7). Tumor cells are showing an intense activity. ×100.
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