HISTOCHEMICAL OBSERVATIONS ON ENZYMES IN PREGNANCY-DEPENDENT MAMMARY TUMORS OF GRS/A MICE¹

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Groups of pregnancy-dependent mammary tumors in GRS/A females, composed of maximally grown, slightly and fully regressed tumors, were observed by enzyme-histochemical methods, as compared with normal mammary glands of the same hosts. In such dependent tumors, activities of lactate, succinate and glucose-6-phosphate dehydrogenases and of acid and alkaline phosphatases were weaker than those in parenchymal cells of neighbouring functioning normal mammary glands. ACP was prominently detected in the necrotic foci within regressing tumors after parturition, while other enzyme activity disappeared there. Intensive activity of LDH was detected in some of the lining cells of microcysts within regressed tumors, where other enzymes decreased. In pregnancy-independent mammary tumors of this strain, activities of dehydrogenases except for LDH were much less than adjacent resting normal mammary glands, which seemed to demonstrate similar activities to those of dependent tumors. LDH was quite variable in independent tumors, but its activity became stronger in cancer cells. ALP distributed irregularly in some independent tumors.

The concept of tumor progression was described in great detail for murine mammary tumors by Foulds (1). Such an "imperfect" neoplasia as found in the RIII strain was observed as so-called plaques, while preneoplastic features in the mammary tumorigenesis are usually hyperplastic alveolar nodules (HAN) in mice of American origin (2, 3). The pregnancy-dependent mammary tumors in the GR strain behaved biologically like the plaques. However, a small percentage of the mammary tumors in breeding GR females histologically resembled the plaques described by Foulds. The maximally grown features of pregnancy-dependent mammary tumors in GR mice were properly called mammary tumor type p (4, 5). These tumors were well confined, often cap-shaped, and light-red colored. Most of them were composed of ductal-like and alveolar-like elements within fibrous stroma, and they mimicked the architecture of normal mammary glands in a grotesque manner (6). The smaller-sized alveolar elements consisted of one or a few epithelial layers often surrounding a central lumen. The larger-sized duct-like elements were filled with many epithelial cells, sometimes leaving a cleft-shaped

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lumen, in which few, if any, myoepithelial cells were present. Such a papillomatous proliferation of ductal-like elements could never be detected in the plaques, and many type p tumors could regress substantially with severe necrosis in the papillomatous proliferations after parturition (7). The pregnancy-independent mammary tumors began to appear in later pregnancies, and they could be also harvested in old virgin GR mice. Although mammary tumorigenesis in the GR mouse has been studied very extensively, virtually no information is available on sensitive markers to express the function and growth of mammary glands in the tumor development. The mammary tumor type p has been observed by the authors (7) to reveal quite few myoepithelial cells, a low number of fat granules and poor expression of murine mammary tumor virus antigens as compared with the surrounding normal functionally active mammary parenchymas. This communication describes histochemical findings of some enzymic activities in the various types of neoplasia of this most unusual mouse strain.

MATERIALS AND METHODS

GRS/A mice originated from the Netherlands Cancer Institute, and some of the 79 inbred generation were donated to our department. They are now maintained by brother-sister matings. Experimental animals in the present observation were kept in natural reproductive states until the 3rd pregnancy. Some retired and virgin mice were observed at more than 40 weeks of age.

Fresh frozen cryostat sections from 48 palpable mammary tumors in 17 pregnant and 31 lactating GR females revealing various rates of tumor growth and from 17 big pregnancy-independent mammary tumors in old GR females were used to detect the histochemical activities of lactate dehydrogenase (LDH), succinate dehydrogenase (SDH) and glucose-6-phosphate dehydrogenase (G6PDH) by means of nitro-BT method (8). The formol-calcium fixed, frozen sections from the above-mentioned mammary tumors were applied to Barka and Anderson's method (9) for acid phosphatase (ACP), and to Stutte's method (10) for alkaline phosphatase (ALP).

RESULTS

Maximally grown mammary tumors were derived from 17 breeding GR females killed at the end of pregnancy. They were solid and sized from 5 to 20 mm in diameter. The reddish tumors were removed together with the surrounding yellowish pregnant mammary fatty tissues. Most of them in the present observation were the localized overgrowths of alveolar-like elements, in which some papillomatous proliferations of ductal-like elements were present. These tumor cells revealed quite few secretory pictures, while the surrounding normal pregnant mammary glands showed intensively secretory pictures. The activities of the examined enzymes became stronger in the functionally active mammary tubules, and those of type p tumor cells were much weaker than the neighbouring pregnant mammary parenchymas (Figs. 1-7). These enzymic activities were demonstrated almost uniformly throughout type p tumor tissues, except for some duct-like elements. In the cytoplasm of some proliferating epithelial cells of ductal-like elements,

- FIG. 1. LDH in the type p tumor. The activity was somewhat less than the neighbouring pregnant mammary parenchymas (arrows). $\times 50$
- FIG. 2. LDH in the type p tumor. The activity of larger ductal-like element ($\uparrow$) was stronger than those of the surrounding alveolar-like elements within the tumor, and it was almost the same as those of the prelactating mammary tubules (arrow). $\times 100$
- FIG. 3. SDH in the type p tumor. The activity was much less than those of the prelactating mammary parenchymas (arrows). $\times 50$
- FIG. 4. G6PDH in the type p tumor. The activity of larger duct-like element ($\downarrow$) was much weaker than those of the neighbouring prelactating mammary parenchymas (arrows). $\times 100$
- FIG. 5. ACP in the type p tumor. ACP activity was proved more in the macrophages and the neighboring pregnant mammary tubule (arrow) than those of type p tumor cells. $\times 100$
- FIG. 6. ACP in the type p tumor. The activity was much less than that of the surrounding prelactating mammary tubules (arrow). $\times 100$

LDH activity was similar to the neighbouring normal pregnant mammary glandular epithelia (Fig. 2), but the activity of SDH or G6PDH (Fig. 4) was much weaker in these epithelial cells. ACP was detected in the cytoplasm of type p tumor cells, but its activity was weaker than those of the macrophages in and around the tumors (Fig. 5) and of the neighbouring normal pregnant mammary parenchyma (Fig. 6). ALP was localized in the peripheral zones of mammary tubules, and its activity was demonstrated stronger in the myoepithelial cells than the glandular epithelial cells. Activity of ALP was observed less in the whole marginal zones of hyperplastic mammary tubules within type p tumors than the neighbouring normal prelactating mammary glands (Fig. 7). Especially, it was done less and spottedly in the peripheral portions of papillomatous changes within type p tumors.

In 31 regressing mammary tumors after parturition, various rates of regression were found. The tumors, in which particular necrosis occurred in many places, regressed enormously in a week. Macrophages migrated into and around the necrotic foci. ACP exhibited much in the migrating macrophages and necrotizing tumor cells (Figs. 8, 9), while the other enzymes disappeared in the necrotic foci (Figs. 10–16). Other tumors regressed slowly and in these many degenerating and atrophic tumor cells were enveloped by collagenous fibers. The enzymic activities in the regressing tumor cells within type p tumors were weaker than the surrounding normal lactating mammary glands (Figs. 10, 12, 14, 15, 17). In the late lactating stage, some tumor remnants consisted of groups of small atrophic tumor cells surrounded by thick hyalinous materials, and others appeared to be a group of microcysts in fibrous changes within type p tumors. The cysts were lined by atonic epithelial cells. It should be noted that the activity of one enzyme, that was LDH, became stronger in some of the lining epithelial cells of microcysts (Figs. 15, 16), while the activities of the other enzymes decreased here (Figs. 9, 12, 13, 14).

The histological appearances of 17 pregnancy-independent mammary tumors were diagnosed as adenocarcinoma type B in the present observation. Some less regressing mammary tumors, moreover, had small foci of adenocarcinoma type B rarely. LDH was quite variable in the independent mammary tumors (Figs. 18–20), but generally it became stronger in the malignant cells. The activity of SDH (Fig. 21) or G6PDH (Fig. 22) was much weaker in the independent mammary tumors, as compared with the surrounding normal resting mammary glands. The activities of both phosphatases were also variable in the independent mammary tumors, but ACP was stronger than the adjacent normal resting mammary glands, in general, otherwise, ALP was demonstrated much less in the independent mammary tumors than the normal resting mammary tubules. Moreover, an irregular distribution of ALP was sometimes recognized in the cancer tissues (Figs. 23–26). In some portions of cancer alveoli, even these borders revealed no ALP. Otherwise, in the other places, even luminal surfaces of cysts within adenocarcinoma type B, ALP

FIG. 7. ALP in the type p tumor. The activity was less than those of the neighbouring pregnant mammary tubules (arrows) and blood vessel (right lower corner). $\times 70$

FIG. 8. ACP in the regressing type p tumor. ACP activity was detected more in and around the necrotic focus ($\uparrow$). $\times 70$

FIG. 9. ACP in the regressed type p tumor. ACP activity was still detected in the macrophages and necrotic tumor cells ($\uparrow$). $\times 100$

FIGS. 10 and 11. ALP in the regressing type p tumor. ALP activity decreased or disappeared in the necrotizing foci ($\uparrow$), as compared with ALP in the lactating mammary tubules (arrow in Fig. 10). $\times 100$ and 40

FIGS. 12 and 13. ALP in the regressed type p tumor. ALP activity disappeared in the microcysts ($\uparrow$) within the tumor, as compared with ALP in the surrounding lactating mammary tubules (arrows in Fig. 12). $\times 40$ and 100

FIG. 14. SDH in the regressed type p tumor. The activity was less than those of the neighbouring lactating mammary glands (arrows), and it disappeared in the microcyst ($\uparrow$). $\times 40$

FIGS. 15 and 16. LDH in the regressed type p tumor. The activity was less than that of the neighbouring lactating mammary gland (arrow in Fig. 15), and it became stronger in the atonic epithelial cells ($\uparrow$) of microcyst within type p tumor. $\times 40$ and 100

FIG. 17. SDH in the regressed type p tumor. The activity of the atrophic tumor cells was weaker than those of the neighbouring lactating mammary glands (arrows). $\times 100$

FIGS. 18-20. LDH in the mammary adenocarcinoma type B. LDH activities were more (Fig. 18 and ↑ in Fig. 20) or less (Fig. 19 and ⬆ in Fig. 20) than those of the surrounding resting normal mammary glands (arrows). $\times 40$, 40 and 100

FIG. 21. SDH in the mammary adenocarcinoma type B. The activity was much less than that of the surrounding resting normal mammary gland (arrow). $\times 50$

FIG. 22. G6PDH in the mammary adenocarcinoma type B. The activity decreased in many places within the tumor, compared with normal resting mammary tubules (arrow). $\times 100$

FIGS. 23-26. ALP in the mammary adenocarcinoma type B. The activity was much less than those of the surrounding normal resting mammary glands (arrows). ALP disappeared in the borders of cancer, alveoli (⬆) or it increased in some apical portions of lining tumor cells of lumen within the carcinoma (↑). $\times 40$, 40, 100 and 100

TABLE 1. *Histochemical activities of dehydrogenases and phosphatases in mammary tumors of GR female mice*

State of animals Site observed	No. of tumors	Lactate dehydro- genase	Succinate dehydro- genase	G6P dehydro- genase	Acid phosphatase	Alkaline phosphatase
Late pregnant	17					
MG (late pregnant)		+ ~ ++	+ ~ ++	+ ~ ++	++	++
Field of adenosis ¹		+	+	+	+	+
Epitheliotic area ²		+ ~ ++	+	+	+	+
Early lactating	25					
MG (early lactating)		+ ~ ++	+ ~ ++	+ ~ ++	+ ~ ++	+ ~ ++
Degenerating tumor		+	+	+	+ ~ ++	+
Necrotic focus		- ~ +	- ~ +	- ~ +	- ~ ###	- ~ +
Small carcinoma		++	-	-	+	+
Late lactating	6					
MG (late lactating)		+	+	+	+ ~ ++	+
Fibrous ³		- ~ +	- ~ +	- ~ +	- ~ +	- ~ +
Cystic ⁴		+ ~ ++	- ~ +	- ~ +	- ~ +	- ~ +
Old retired or virgin	17					
MG (resting)		+	+	+	+	+
Adenocarcinoma (B)		- ~ ###	- ~ +	- ~ +	+ ~ ++	- ~ ###

MG: Normal mammary glandular epithelial cells, -: Negative, +: Moderately positive, ++: Strongly positive, ###: Prominently strong or diffuse positive, ¹: Overgrowth of alveolar-like elements within type p tumors, ²: Papillomatous hyperplasia of ductal-like elements within type p tumors, ³: Fibrous areas within type p tumors, resembling sclerosing adenosis, ⁴: Cystic degeneration or microcysts within type p tumors.

was proved strongly.

Histochemical activities of the enzymes seemed to be considerably variable from tumor to tumor in case of the pregnancy-independent mammary tumors, while they were much more constant in cases of the pregnancy-dependent mammary tumors. The results in the present observation are summarized in Table 1.

DISCUSSION

In most of the smaller pregnancy-dependent mammary tumors, tubular elements which protruded into the surrounding fat tissue were recognizable. The tubular elements had club-like endbuds. Although these endbuds had similarities with the club-like endbuds in normal growing mammary glands, they were often more irregular. The majority of larger pregnancy-dependent mammary tumors in breeding GR females were characterized by the existence of a papillomatous proliferation of ductal-like elements, in which quite few myoepithelial cells were present (7). The mammary tumor type p was somewhat different from the plaques described originally by Foulds (11), and it was completely different from HAN, which was sometimes observed in non-breeding GR mice. HAN is a focus of hyperplastic lobuloalveolar development with secretory activity within the non-stimulated

mammary tissues. The cytomorphology of HAN was indistinguishable from normal prelactating mammary parenchymal cells (12). The mammary tumor type p revealed quite few secretory pictures. The growth of type p tumors might be responsive to the pregnant states of hosts, but the secretory function of tumor cells themselves would be refractory to pregnancy. In the present observation, the activities of examined enzymes were demonstrated stronger in the functionally active mammary glandular cells than the resting mammary glands. They might be sensitive markers to express the growth and/or function of mammary glands. The lower enzymic activities in type p tumor cells might be related to their quite low secretory activities.

The activity of LDH in some large duct-like elements within type p tumors was almost similar to that of neighbouring normal pregnant mammary parenchymas, although the other enzymes became much less in these areas. LDH would be essential to some papillomatous proliferations of duct-like elements within type p tumors. ALP was detected to be less and more spotted in the peripheral portions of papillomatous foci than the marginal zones of alveolar-like elements within type p tumors. The papillomatous proliferations of duct-like elements within type p tumors were different from the adenocarcinomas, small foci of which could be found rarely within type p tumors in later pregnancies, because they revealed less cytological atypia and no focal staining of expression for MuMTV antigens (7). The histochemical findings of enzymes in larger duct-like elements within type p tumors deviated less than those of adenocarcinomas. Some lining epithelial cells of microcysts within the regressed tumors exhibited strong activity of LDH. Such cells might proliferate again in following pregnancies, as such a focal progression was often observed in the developing tumors of the successive pregnant mice. Recently, Sass and Dunn (13) distinguished mammary tumor type p from plaques as a true neoplastic lesion with pregnancy-responsiveness, as described originally by Van Nie and Thung (14). One could call the hyperplasia of alveolar-like elements "preneoplastic", the papillomatous proliferation of duct-like elements "neoplastic" and the focus of adenocarcinoma cell "malignant".

In the present observation, SDH and G6PDH were detected less in the foci of adenocarcinoma cells within type p tumors than the areas of papillomatous proliferation of duct-like elements within the same tumors. On G6PDH determined biochemically in GR mice, it increased insignificantly in the dependent tumors and decreased significantly in the independent tumors (15). G6PDH in BR6 mice decreased already at the dependent tumors and remained in the independent tumors (16). SDH was weaker in the independent tumors of GR mice, but C3H mammary cancers revealed more SDH than the normal parenchymas (17). Various human cancers decreased SDH compared to the homologous tissues (18). LDH was quite variable in C3H mammary tumors investigated histochemically (17), as well as the present observation. In general, LDH became stronger in many malignant tumor cells in GR mammary tumorigenesis. The deviation of LDH isoenzyme patterns should be studied in the tumor progression of GR mice, as well as human and rat mammary tumors (19).

In human cancer, there was sometimes intense activity of ACP (20). ACP was less in type p tumor cells than the neighbouring functionally active normal mammary parenchymas, but ACP of the independent mammary tumors was some-

what more conspicuous than the adjacent resting normal mammary glands. ACP as lysosomal enzymes may play a role in the regression of type p tumors.

ALP could be detected in the basal plasma membrane of resting mammary glandular cells touched directly with basal lamina, although it was much less than the whole plasma membranes of myoepithelial cells (21, 22). In lactating mammary glands, ALP increased and also became detectable in some lateral plasma membranes of glandular cells, but it was never proved in the apical plasma membranes (20). Such a distribution of ALP in plasma membrane of mammary glandular cells would indicate the direction of secretory process, namely functional polarity, of mammary tubules. Intensive ALP was always observed in the plasma membrane with pinocytotic vesicles of myoepithelial cells, especially in the inner plasma membrane close to the glandular cells. ALP in type p tumors was less than the neighbouring functionally active normal mammary tubules, because these tumors had quite few secretory pictures and small number of myoepithelial cells. The pregnancy-independent mammary tumors of GR mice demonstrated ALP activity much less than the adjacent normal mammary glands. Human scirrhous carcinoma of mammary glands with strong ATPase activity would be of myoepithelial origin (23). Some DMBA-induced mammary tumors in rats were ALP-positive strongly and diffusely, suggesting myoepithelial proliferation (24). GR mammary tumorigenesis would progress toward the proliferation of epithelial cells without the differentiation to myoepithelial cells. ALP rarely was observed in the luminal surfaces of cysts within adenocarcinoma type B. Such an irregularity in distribution and intensity of ALP would not suggest the myoepithelial origin of tumor cells, but it might symbolize the cellular atypia, namely loss of so-called functional polarity.

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