IMMUNOHISTOCHEMICAL LOCALIZATIONS OF ALPHA-LACTALBUMIN IN HUMAN BREAST LESIONS IN RELATION TO SECRETORY ACTIVITY

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Alpha-lactalbumin (LA) was isolated and purified from fresh human milk and injected into rabbits for antiserum production. By a PAP technique, LA was demonstrated in the cytoplasm of secretory (26 positive cases out of 26 examined cases) and resting (11/43) normal mammary glandular cells from operated human breasts. Some epithelial cells in the cases of mammary dysplasia (53/229), fibroadenoma (15/94) and carcinoma (10/183), and of gynecomastia (1/12) showed positivity. Histochemical staining features, reticular, granular and compact, corresponded to the secretory state of the cells. The diffuse and sporadic distribution of LA-positive cells within the mammary lobules and foci would suggest the character of biological alterations. Moreover, LA secretion may be affected by the menstrual status of patients. LA has never been detected in normal parenchymal cells of the skin, stomach, colon, liver, pancreas, lung, testis, ovary, uterus, placenta, umbilical cord, thyroid or salivary gland. The immunohistochemical examination for LA, therefore, has no significant role as a tissue marker in histopathologic diagnosis of breast cancer, but it could serve as a specific marker for the mammary gland, especially its differentiation.

The synthesis of lactose by the breast requires the presence of LA, which is synthesized almost exclusively by the mammary gland. LA has been detected recently by radioimmunoassay method in the cytosol of breast cancers (5, 12, 16), and immunohistochemistry with use of immunoperoxidase (2, 4, 6, 8, 9, 15, 17) and immunofluorescence (1, 3) methods in clinical and experimental tumors of the mammary gland. Some authors claim that LA could serve as a specific tissue marker for malignant neoplasms and their metastasis. But, positivity has been reported to differ widely in human breast cancers. The aim of this study was to observe the positivity and distribution of LA in humans, with reference to histopathologic diagnosis of the breast tissues and clinical status of the patients.

MATERIALS AND METHODS

LA was isolated from the milk whey of a lactating woman. After removal of

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casein by acid precipitation at pH 4.6, the absorbed protein was purified by dia-phoresis. The purified LA was further purified on Sephadex G-75, the LA eluting from column at a specific position. The band corresponding to LA was excised from the gel. The repurified LA gave a single band on polyacrylamide gel electrophoresis. Antiserum to human LA was raised in rabbits, and the primary antiserum was not crossreacted with any other purified proteins in Ouchterony double diffusion test and immunoelectrophoretic method on ADS-polyacrylamide gel disc (9).

Fresh samples for the mammary gland were obtained at the Kansai Medical University Hospital. Many of the breasts, which were selected from the files of the Department of Pathology, Kansai Medical University, could be used retrospectively, because LA antigen had been proven by our PAP technique to be stable against routine histological methods (9). Two cases of secretory carcinoma of the breast were given by Dr. Sakamoto of the Cancer Institute in Tokyo and Dr. Tokunaga of Kagoshima University. The examined samples are listed in Table 1. The following information was checked as far as possible from the patients' notes; age and date of the last menstruation, parity, and whether there was lactation or administration of special drugs. The dated cases were classified into 5 groups by days after the onset of last menses according to Vogel *et al.* (14), although in some cases the regularity of their menstrual cycles was not reconfirmed. Additionally, some biopsy materials of the skin, stomach, colon, liver, pancreas, lung, testis, ovary, uterus, placenta, umbilical cord, thyroid, and salivary gland were applied to our PAP technique for LA.

Formalin-fixed, paraffin-embedded sections were dewaxed and hydrated. The sections were placed in 0.3% H_2O_2 in ethanol for 30 min to block endogenous peroxidase. The slides were incubated for 10 min in normal swine serum diluted 1/20. The primary antiserum for human LA was placed on the sections at a concentration of 1 in 1,000, and the slides were incubated in a humidifier for 1 hr. This concentration gave negligible background staining and a two-fold increase in the concentration did not result in an increase in the grade of positivity. The antiserum was removed, and the slides were rinsed thoroughly in Tris HCl buffer containing saline, pH 7.6, for 15 min. Swine anti-rabbit IgG was applied for 30 min. Washing in Tris saline buffer was followed by incubation with a 1/100 dilution of a soluble complex of horseradish peroxidase and rabbit anti-horseradish peroxidase (PAP) for 30 min. Diaminobenzidine was used as the chromogenic substrate. All steps were

TABLE 1. *Number of examined samples (Dated Cases*)*

Focally proliferating and secretory mammary glands, neighbouring the foci of 8 dysplasias and 18 carcinomas, or a few probably later lactating glands	26
Resting mammary glands, neighbouring the foci of 20 dysplasias and 23 carcinomas	43 (42)
Mammary dysplasia	229 (93)
Mammary fibroadenoma	94 (27)
Gynecomastia	12
Intraductal mammary carcinoma	4
Infiltrating mammary carcinoma	179 (40)
(Including secretory carcinoma)	2 (2)

* The date of last menses could be checked only in the dated cases.

TABLE 2. Relationship between immunohistochemical positivity of α -lactalbumin in mammary glands and menstrual status of hosts

Histological diagnosis	Positivity rate		Pre-menopausal	Menstrual cycle					Post-menopausal
	Total cases	Dated cases		I	II	III	IV	V	
Resting gland	26	$\frac{26}{11}$ 42%	11/33		2/8	2/8	6/10	1/7	0/9
Dysplasia	23	$\frac{47}{44}$ 93%	43/86	0/1	8/11	15/25	12/34	8/15	1/7
Fibroadenoma	16	$\frac{26}{7}$ 27%	7/26		0/3	2/8	4/10	1/5	0/1
Carcinoma*	4	$\frac{10}{4}$ 38%	2/19		0/5	0/7	2/5	0/2	2/19
Secretory carcinoma	100	$\frac{100}{2}$ 2%	2/2			1/1	1/1		

* Excluding secretory carcinoma.

Five phases of menstrual cycle (proliferative, follicular, luteal, secretory and menstrual) were classified by dating from the last menses and histological appearances of each case according to Vogel *et al.* (14).

carried out at room temperature and were followed by rinsing and washing with saline buffer. Sections of lactating mammary tissue were applied as positive controls. Swine anti-rabbit IgG and soluble complex of PAP were purchased from Dakopatts Co. in Copenhagen. The sections were finally counterstained with Gill's hematoxylin.

RESULTS

In the operated breast tissues from 26 women, who were suffering from either dysplasia or carcinoma of the breast and not lactating at the time of biopsy, focally proliferating and secretory mammary glands, including a few possibly later lactating glands, could be observed. In all of the cases, LA secretion was the focal phenomenon involving some secretory lobules and not other resting lobules. Within such secretory lobules, however, the majority of glandular cells was positive. Otherwise, in the lactating gland-like mammary tissues, almost all the lobules showed strong LA secretion, which was observed in the cytoplasm of the glandular cells and the lumen of alveoli (Fig. 1). Abundance of reaction products against LA antigen appeared as reticular features at the periphery of cytoplasm (Fig. 2); focally intracytoplasmic granular features (Fig. 3); or weakly and diffusely intracytoplasmic compact features.

In the operated breast tissues of 20 mammary dysplasias and 23 mammary cancers, some normal resting mammary lobules could be seen by chance. The localization of LA was detected in such resting mammary glands of 11 out of 43 patients. The staining features were mostly granular, and the LA-positive cells were found sporadically in the lobules (Fig. 5). In 42 dated cases of this category, the higher positivity rate was noted in the secretory phase of the menstrual cycle, and all 9 postmenopausal cases showed no positivity.

LA was detected in some glandular cells of 15 out of 94 cases of mammary

TABLE 3. *Immunohistochemical detection of α -lactalbumin in various clinical states of human breasts*

Histological diagnosis	Walker PAP 1979 ⁽¹⁵⁾	Bahu IIF 1980 ⁽¹⁾	Bailey IIF 1982 ⁽²⁾	Clayton PAP 1982 ⁽⁴⁾	Morii PAP 1982*
Secretory gland			19/19**	2/2	26/26
Resting gland	6/80			9/13	11/43
Dysplasia		9/18	0/113	8/11	53/229
Fibroadenoma		3/6	5/20	5/5	15/94
Gynecomastia					1/12
Carcinoma					
Total percentage	51	32	0	62	6
Intraductal		0/8	0/6	4/7	0/4
Infiltrating					
duct	50/94	18/49	0/28	27/36	8/165
lobular	1/6		0/17	3/11	
mucinous			0/4	2/4	0/12
secretory				1/1	2/2
Paget				1/4	

* Presented at the annual meeting of The Japan Cancer Association in 1982 at Osaka.

** Additional 3 cases in early pregnancy, 8th, 12th and 14th weeks of pregnancy, showed no evidence of this protein secretion.

fibroadenoma. Moderate amounts of the reaction products against LA antigen could be seen diffusely in the cytoplasm of the glandular cells (Fig. 4) and in almost all the glandular cells of some adenomatous foci (compact feature and diffuse distribution). The other adenomatous foci showed no LA antigen, and few mammary ducts revealed positivity. No LA was detected in the proliferating fibromatous tissues. In 27 dated cases of fibroadenoma, 7 exhibited LA antigen, and the highest rate of positivity was noted in the secretory phase of the menstrual cycle. Only 1 case showed LA secretion out of 12 cases of gynecomastia. Out of 229 cases of mammary dysplasia, LA was focally found in some dysplastic foci of 53 cases. Relatively weak staining picture was either compact or granular in the

FIG. 1. Secretory mammary lobules, PAP stain for LA. LA secretion can be seen strongly in the majority of glandular cells, mainly reticular features, and in some dilated lumen within the mammary lobulus (diffuse type of distribution). $\times 40$

FIG. 2. Reticular feature, PAP stain for LA. Abundant reaction products can be seen at the periphery of cytoplasm of glandular cells of the possibly later lactating mammary gland. $\times 400$

FIG. 3. Granular feature, PAP stain for LA. Reaction products can be seen focally in the cytoplasm of glandular cells of the focally proliferating, secretory mammary lobulus. $\times 400$

FIG. 4. Compact feature, PAP stain for LA. Reaction products can be seen weakly and diffusely in the cytoplasm of the adenomatous cells within the fibroadenomatous foci, in which neither vacuolated myoepithelial cells nor proliferating fibromatous cells show any reaction products. $\times 400$

FIG. 5. Resting mammary lobulus at phase IV of menstrual cycle, PAP stain for LA. Either granular or compact feature can be seen in some resting normal glandular cells (arrows) (sporadic type of distribution). $\times 400$

FIG. 6. Mammary dysplasia, PAP stain for LA. LA antigen can be detected in almost all the glandular cells within a dysplastic focus (arrow), but it can be hardly seen in the other foci. $\times 40$

TABLE 4. Positivity rates and immunohistochemical appearances for α -lactalbumin in various clinical states of human breasts

Histological diagnosis	Positivity rate of LA		Histological appearance*
Secretory mammary gland	26/26	100 %	reticular/diffuse
Resting mammary gland	11/43	26	granular/sporadic
Mammary dysplasia	55/229	23	compact/diffuse or sporadic
Mammary fibroadenoma	15/94	16	compact/diffuse
Gynecomastia	1/12	8	compact/diffuse
Mammary carcinoma			
intraductal	0/4	0	
infiltrating**	8/177	4.5	compact/sporadic
secretory	2/2	100	reticular/diffuse

* Staining feature in the cytoplasm/distribution type of LA-positive cells in the mammary labulus or focus.

** Excluding secretory carcinoma.

cytoplasm of the dysplastic parenchymal cells, and LA-positive cells were diffusely or sporadically distributed in the dysplastic foci (Fig. 6). In 93 dated cases of mammary dysplasia, 44 exhibited positivity, which was not definitely related to the menstrual status of the patients.

No LA antigen was proven in any of 4 intraductal mammary carcinomas, otherwise LA secretion was intensively positive in both of 2 secretory carcinomas of the breast. The histochemical appearance of the latter two was similar to normal lactating mammary glands. Out of 169 cases of infiltrating mammary carcinoma, 8 showed LA antigen in some cancer cells. The compact feature of reaction products against LA antigen in some cancer cells was distributed sporadically in some cancer alveoli (compact feature and sporadic distribution). In 38 dated cases of malignant category, 4 cases comprising 2 in the secretory phase of menstrual cycle and 2 post-menopause exhibited positivity. All 12 mucinous carcinomas of the breast showed no positivity.

Neither secretion nor antigen of LA was observed immunohistochemically in normal parenchymal cells of the other 15 examined organs and tissues.

DISCUSSION

LA production was stimulated specifically by insulin, hydrocortizone, and prolactin in organ cultures of mouse mammary glands (13). In rodents LA levels increase only on initiation of lactation, which suggests that LA secretion reflects a high degree of mammary epithelial cell differentiation. In humans, however, an elevation of LA in the plasma can be detected by at least the 20th week of pregnancy. Bailey *et al.* (2) reported that 3 cases in the 8th, 12th and 14th weeks of pregnancy showed no evidence of LA secretion, but that 11 samples from pregnancies in the 18th week or longer showed LA in the cytoplasm and lumina of the acini. All samples of lactating breast were strongly positive. Even in advanced pregnancy and lactation, LA was not invariably detected in the lobules, which may reflect the different phases of secretory activity, whereas almost all the glandular cells in a lobulus were

positive. In other words, the LA-positive cells were distributed diffusely in almost all the mammary alveoli. In the cytoplasm of the glandular cells of such mammary alveoli, reaction products against LA antigen showed reticular, granular and compact features, depending on the localization of the other secretory materials, e.g. casein and fat. Occasionally, LA secretion, granular feature, could be seen in the non-pregnant breast as focal phenomenon involving some proliferating, secretory lobules not other resting lobules. However, LA-positive cells were found diffusely in these secretory lobules. This could be the result of variation in local concentration of hormones and growth factors or some other intrinsic factors common to the majority of cells in a lobulus. Such a focal pregnancy-like change in the breast has been reported in non-pregnant and non-lactating women, even in postmenopause. Such changes have been detected in one or more mammary lobules in 3% of cases of breast surgery (7). In our series, the pregnancy-like changes showing LA secretion were seen in 26 out of all the examined 586 cases (4.4%). Moreover, in some resting mammary lobules of 11 out of 43 cases LA antigen could be detected sporadically in many resting lobules. Such LA-positive cells usually showed granular features. The positivity of LA in normal resting mammary glands has been reported in 6/80 or 9/13 (4, 15). In the dated cases of this category, LA-positive cases were all premenopausal, and an especially high positivity rate was noted in the secretory phase of the menstrual cycle, as shown in Table 2. Morphological changes in the mammary stromal and epithelial components were identified as they related to specific pituitary ovarian events. Even resting mammary glands were affected by the menstrual cycle of the hosts. Histological criteria were identified that allowed reproducible morphological categorization of the resting normal mammary glands into 5 specific phases: (I) proliferative, (II) follicular, (III) luteal, (IV) secretory and (V) menstrual. Phase IV was classified as being 21–27 days after the onset of the last menses, and in this phase both active apocrine secretion from the luminal cells of the mammary acini and their large lumen filled with the secretion could be observed histologically within the loose, edematous intralobular stroma.

Variable positivities of the immunohistochemical detection for LA have been described in benign and malignant epithelial tumors of the human breast. A radioimmunoassay for LA indicated that LA was synthesized in human breast cancer cells cultivated as a continuous cell line *in vitro*, and that the ability to synthesize LA by these cells appeared to be independent of addition of prolactin in culture medium (11, 12). LA was found in the cytosol fraction of 20 out of 53 human breast carcinomas by a radioimmunoassay method, which was considered to be useful in identifying tumors responsive to endocrine therapy (16). LA in chemically induced primary mammary carcinoma in rats was observed by radioimmunoassay in quantities equal to or less than 10% of amounts found in the parenchyma of lactating gland at the peak of production (10). Specific staining for LA secretion was observed in the lactating rat alveolar epithelial cells, and in unstimulated transplantable rat mammary tumors some cells were shown to contain LA. This immunohistochemical method may be useful to determine the functional heterogeneity of mammary tumors directly (6). Recently, Clayton *et al.* (4) have described that LA was present in 39 out of 69 breast carcinomas of different types and in 55% of metastatic breast carcinomas, and that LA could serve as a specific tissue marker for malignant neoplasms of the breast. On the other hand, a wide variety of benign and malignant breast

lesions were studied by Bailey *et al.* (2), who reported the absence of LA antigen in all 44 breast cancers. As shown in Table 3, our data on mammary tumors was relatively similar to Bailey *et al.*'s results. It remains to be investigated whether our antiserum is less sensitive than those used by others, or the antisera of other workers contain impurity of antibodies which detect some components other than LA in the breast tissues. LA secretion is a highly specialized function which would be easily lost in the course of neoplastic transformation. Therefore, PAP technique for LA cannot serve as a tissue marker in the histopathologic diagnosis or clinical monitoring of breast cancers.

LA secretion may be a good marker for secretory activity of the mammary glands. Immunohistochemical appearances of LA secretion in some clinical states of the breast are summarized in Table 4. The cytological staining feature were related to the secretory states of the mammary glandular cells. A reticular feature indicates a strong secretory activity, and a granular feature a moderate one. A compact feature suggests a well-differentiated state in secretory function of LA. The distribution of LA-positive cells in the mammary lobules or foci was classified into 2 types in the present observation. A diffuse type was observed mainly in the lactating and proliferating, secretory lobules of normal mammary glands, and in some dysplasias, fibroadenomas, gynecomastias and secretory carcinomas of the breast. The sporadic type was detected in some resting normal mammary lobules, and in some dysplasias and carcinomas of the breast. The malignant neoplasms except secretory carcinoma showed a sporadic type, which suggests functional heterogeneity and polymorphism of malignancy. Mammary dysplasia showed both types of distribution in our series. These data indicate the complicated definition of mammary dysplasia. In brief, the malignant neoplasia of the breast would reveal a compact feature of immunohistochemical reaction products against LA antigen in some cancer cells appearing sporadically within the cancer alveoli, although the positivity rate may be low.

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