DISTRIBUTION AND SUBCELLULAR LOCALIZATION OF LACTOFERRIN IN HUMAN TISSUES WITH SPECIAL REFERENCE TO THE SUBMANDIBULAR GLAND

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Immunohistochemical localization of lactoferrin (LF) in human organs was studied light microscopically by means of PAP (peroxidase-antiperoxidase) method. Focusing on the submandibular gland, the localization of LF was compared with that of lysozyme (LZ) using double staining method and serial mirror-image sections, and subcellular localization of LF was studied with preembedding direct immunoperoxidase method. LF was distributed in the following sites: the salivary gland, the esophageal gland, the cardiac and pyloric glands, chief cells in the gastric gland proper, the gallbladder, the nasal and paranasal glands, the bronchial gland, neutrophilic granulocytes, thymic epithelium (Hassal's corpuscle), distal tubules and collecting tubules of the kidney, the seminal vesicles, the prostatic gland, the uterine cervical gland, intermediate lobe of the pituitary gland, the mammary gland in lactating phase, and the lacrimal gland. In the submandibular gland, LZ-positive serous cells were always less numerous than LF-positive cells, and intermingled among LF-positive acini. LZ-containing cells also occasionally contained LF. Electron microscopically, LF was observed in the serous granules of acinar cells in the submandibular gland. Central core of the granules stained intensely whereas peripheral rim stained weakly. LF was not observed in other cell organelles.

Lactoferrin (LF) was initially described by Sørensen and Sørensen (24) in 1939 as “red protein” fraction in bovine milk. Subsequent studies (9, 19) revealed that LF was also contained in milk from various species including man. Masson and Heremans (16) demonstrated that LF is contained not only in milk but also in many other human exocrine secretions, and Masson et al. (18) further observed LF in human and guinea pig neutrophilic polymorphonuclear leukocytes.

LF is a glycoprotein with iron-chelating property, which is considered to be responsible to its biological activities such as bacteriostatic (16) as well as bactericidal effect (1) and iron transporting capacity (2). In the recent hematological field, attention has also been paid to LF as a marker for specific granules of neutrophilic granulocytes (25) and as a negative feedback regulator of in vitro granulopoiesis (3).

Recent technical advances in immunohistochemical methods made it possible

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to detect LF morphologically, and immunohistochemical localization of LF in human tissues has been reported. These include the bronchial gland (15, 28), gastrointestinal tract (8, 15, 17), the kidney (17, 28), the uterus (15, 17), the salivary gland (22, 28), the lacrimal gland (5), the mammary gland (15) and the pancreas (4). However, different results have been reported about some organs, probably due to the differing method used, and moreover, ultrastructural localization of LF has been reported only in a few cell types.

In the present study, distribution of LF in various human tissues was systematically investigated by means of immunoperoxidase method. The observation was centered on the submandibular gland, and the localization of LF was compared with that of lysozyme (LZ), which has been demonstrated to have a similar distribution pattern (14) and close functional reciprocity with LF, and subcellular localization of LF was studied with preembedding direct immunoperoxidase method.

MATERIALS AND METHODS

(1) Materials

Specimens were obtained from autopsy, biopsy and resected human subjects. Thirty-three organs from 57 autopsy cases, 10 organs from 44 resected cases and 4 organs from 20 biopsy cases were examined to investigate the localization of LF light microscopically. Resected fresh materials from the submandibular gland were examined with ultrastructural immunocytochemistry for LF. Rabbit antisera to human LF and LZ, anti-rabbit immunoglobulins swine antisera and PAP (peroxidase-antiperoxidase) complex were obtained from DAKO-immunoglobulins, Ltd., Copenhagen, Denmark. The source of the purified LF used for preparation of the antibody was human milk. The antisera to human milk LF has been reported to react with LF in various exocrine secretions (16) as well as cell extracts (8). The antisera gave a single line when tested in immunoelectrophoresis against human milk, and Ouchterlony-double diffusion showed no interactions of precipitation lines formed by antibodies to human LF, LZ, IgA (α-chain) and secretory component reacted with human milk. For the ultrastructural study, Fab' fragments purified from pepsin-digested specific antisera (IgG) to human LF were conjugated with horseradish peroxidase (HRP) according to the method of Wilson and Nakane (29). With the same technique, HRP-labeled Fab' fragments were also prepared from non-immunized normal rabbit IgG.

(2) Immunohistochemical procedures

1) Light microscopic demonstration of LF

For light microscopic examination, PAP method of Sternberger et al. (26) was applied for formalin-fixed 1 μm thick paraffin sections. Endogenous peroxidase was blocked by immersing samples in methanol containing 0.3% hydrogen peroxide for 30 min at room temperature. Sections were treated with normal swine serum for 30 min prior to incubation with the first specific antisera. Dilution of the first and second antiseras and PAP complex were 1 : 200–300, 1 : 20, and 1 : 20 respectively. After peroxidase reaction in neutral diaminobenzidine (DAB)-H₂O₂ solution for about 10 min at room temperature, short counterstaining was performed by
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hematoxylin.

2) Double staining for LF and LZ

Localization of LF and LZ in the submandibular gland was observed simultaneously in a section with double staining method of Nakane (20). After peroxidase reaction for LF in DAB-H₂O₂ solution, antibodies and PAP complex were removed by immersing samples in 0.1 M glycine-HCl (pH 2.2) for 1 hr, and then LZ was likewise localized in the same section using 4-Cl-1-naphthol as a substrate for peroxidase reaction. The samples were mounted in glycerol: PBS (9:1) without nuclear counterstaining.

3) Direct immunoperoxidase method for LF

For electron microscopic study, the preembedding direct immunoperoxidase method was applied. Samples were fixed in periodate-lysine-4% paraformaldehyde (4% PLP) solution (13) at 4°C for overnight, and washed in phosphate buffered saline (PBS) with increasing concentration of sucrose (10%-15%-20%) and finally with 25% sucrose solution containing 5% glycerin. Six μm frozen sections of these samples were prepared and endogenous peroxidase activity was blocked by 25 mM HIO₄ at room temperature for 10 min. The sections were incubated with HRP-labeled antisera at 4°C for overnight and further fixed in 1% glutaraldehyde. Peroxidase reaction was performed in DAB-H₂O₂ solution for 2-5 min and subsequently the samples were postfixed in 1% OsO₄ for 1 hr, dehydrated in graded alcohols and embedded in Epon. Ultrathin sections were examined in JEOL 100-C electron microscope after counterstaining with lead citrate for about 5 min.

4) Control tests

For light microscopic study, control tests were carried out by substituting the specific antisera to human LF with non-immunized normal rabbit serum with equal dilution as the specific antisera. For electron microscopic study, HRP-labeled Fab’ fragments prepared from non-immunized rabbit IgG were used instead of the specific antisera.

RESULTS

Distribution of LF in human organs is summarized in Table 1. Serous acinar cells of the exocrine glands around respiratory tract, such as the nasal, paranasal and bronchial glands (Fig. 1), were strongly positive for LF, whereas mucous cells were negative. In the salivary gland, LF was also localized in serous acinar cells (Fig. 2). A part of acini, however, remained negative. LF was also localized in a few numbers of ductal epithelium (Fig. 13). In digestive tract, LF was occasionally found in some glandular and ductal epithelium of the esophageal gland (Fig. 3). LF-positive cells were preferentially found in inflamed glands. In the stomach, the cardiac and pyloric glands stained weakly for LF, whereas chief cells in the gastric gland proper stained intensely for LF (Fig. 4). Intestinal mucosa from the duodenum to the rectum showed no reaction products for LF. The liver and the pancreas were negative for LF, whereas small foci of gallbladder mucosa rarely showed weak reaction. In genitourinary tract, LF was observed in the epithelium of a portion of distal tubules and collecting tubules of the kidney (Fig. 5),
Table 1. Distribution of lactoferrin in human tissues

1. Digestive system
   (1) salivary gland (submandibular and minor salivary gland)
       ......serous acinar epithelium  + ~##/−
       ......ductal epithelium        −/+#
   (2) esophagus
       ......glandular and ductal epithelium  −(/(+~++))
   (3) stomach
       1) cardiac and pyloric gland
           ......glandular epithelium      −/(+)  
       2) gastric gland proper
           ......chief cell              −/(##)  
   (4) small intestine (duodenum, jejunum and ileum)  −
   (5) large intestine (colon and rectum)    −
   (6) liver                               −
   (7) gallbladder                        −/(+)  
   (8) pancreas                          −

2. Respiratory system
   (1) nasal, paranasal and bronchial gland
       ......serous acinar epithelium  ##
       ......ductal epithelium        −/+~++
   (2) ciliated epithelium          −
   (3) alveolar epithelium         −

3. Hematopoietic system
   (1) bone marrow
       ......neutrophilic granulocytes  ##
   (2) spleen                        −
   (3) lymph nodes                  −
   (4) thymus
       ......thymic epithelium (Hassal’s corpuscle)  −/(+~++)

4. Urinary system
   (1) kidney
       ......epithelium of distal tubules and
       collecting tubules          −/+~##
   (2) ureter                      −
   (3) urinary bladder            −

5. Reproductive system
   (1) testis                      −
   (2) seminal vesicles           ++/−
   (3) prostate                   ++~##/−
   (4) ovary                      −
   (5) uterus
       ......endocervical glandular epithelium  −/(+)
       ......endometrium                  −

6. Cardiovascular system
   (1) heart                      −
   (2) blood and lymphatic vessels −

7. Endocrine system
   (1) thyroid and parathyroid gland  −
   (2) adrenal gland                −
   (3) pancreatic island            −
In the submandibular gland, localization of LF and LZ was observed simultaneously in a section by double staining method as described above (Fig. 13). LZ was localized in serous acinar cells intermingled among LF-containing acini, and LF-positive cells always surpassed LZ-positive cells in number. Serial mirror-image sections, stained for LF and LZ separately, revealed that LZ-positive cells were also occasionally positive for LF (Figs. 14 a, b). Serous cells which contain neither LF nor LZ were also found in a few numbers.

In electron microscopic study, LF was demonstrated in the serous granules of the submandibular gland (Fig. 15a). Their central core stained strongly for LF, whereas the peripheral rim stained weakly. Reaction products were also observed on the membrane structures and the cytoplasm around these granules in a small amount. Rough endoplasmic reticulum, perinuclear space and Golgi apparatus were negative for LF. Negative control studies showed no reaction products in any portion of cells (Fig. 15b). Lipid droplets with peripheral electron density were found in both tested and control materials.

DISCUSSION

As shown in Table 1, the results were largely in agreement with the previous
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Data concerning the distribution of LF in human tissues (4, 5, 8, 15-19, 21, 22, 28). Roughly speaking, LF is distributed in the exocrine cells, locating relatively near the body surface. This localization pattern matches the data showing that LF has a defensive role against bacterial infections (1, 16).

In this study, LF was demonstrated in several organs, which have not been reported previously or which have been suggested but not demonstrated histochemically to contain LF. These sites were as follows: the esophageal gland, chief cells in the gastric gland proper, the prostatic gland, the seminal vesicles, intermediate lobe of the pituitary gland, thymic epithelium forming Hassal’s corpuscles, and small foci of gallbladder epithelium. On the other hand, the endometrium, the pancreas

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**FIG. 13.** Double staining for lactoferrin (LF) and lysozyme (LZ) in the submandibular gland. The grey reaction products indicate LF, and the brown LZ, respectively. LZ was localized in serous acinar cells intermingled among LF-containing acini, and LF-positive cells surpassed LZ-positive cells in number. ×50

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**Figs. 1-12.** Light microscopic demonstration of lactoferrin in human tissues.

**Fig. 1.** The bronchial gland. ×80
**Fig. 2.** The submandibular gland. ×200
**Fig. 3.** The esophageal gland. ×200
**Fig. 4.** The gastric gland proper. ×200
**Fig. 5.** The kidney (medulla). ×80
**Fig. 6.** The prostate. ×200
**Fig. 7.** The seminal vesicles. ×100
**Fig. 8.** The endocervical gland. ×150
**Fig. 9.** The mammary gland in lactating phase. ×80
**Fig. 10.** The lacrimal gland. ×120
**Fig. 11.** Intermediate lobe of the pituitary gland. ×160
**Fig. 12.** The thymus. ×300
and intestinal tract from the duodenum to the rectum showed no reaction products for LF on the contrary to some positive data by others (4, 17) using acetone- or methanol-fixed frozen sections. Although there could be the possibility that very small amount of LF could not be detected by the present method using formalin-fixed paraffin sections, the precise reason for this discrepancy is not clear.

Tourville et al. (28) described LF-positive site in the kidney as “tubules”, and Masson et al. (17) as “thin limb” respectively with direct immunofluorescent method. On the other hand, with immunoperoxidase method, Mason and Taylor (15) reported that the kidney is negative for LF. In the present study, LF was observed in the epithelium of distal tubules and collecting tubules, though the number of positive cells varied widely from individual to individual. LZ, which is a bacteriolytic enzyme and the function of which is considered to be reciprocal with LF, is known to be distributed in very similar sites as LF (14). In the kidney, LZ is localized in the epithelium of proximal tubules in contrast to the distal localization of LF. These localization patterns of LF and LZ also seem to imply their functional
relationship in antibacterial defense mechanism. However, the possibility that the presence of LZ in the kidney is as the result of reabsorption by tubular epithelium has been postulated (6). The same mechanism may be applied for LF, though this is less likely because serum LF level is only a trace amount (11). Ultrastructural observation of LF and LZ in the tubular epithelium would be of great value to clarify the functional significance of these proteins in the kidney.

In the submandibular gland, LF was localized in serous acinar cells as has been described in detail by Reitamo et al. (22). In the present study, localization of LF was compared with that of LZ by means of double staining method and serial mirror-image sections. Although LZ has been reported to localize in very similar sites as LF in the salivary gland (14), the present study showed that the distribution of LZ was not identical with that of LF. However, as LZ-positive cells occasionally contained LF, the populations of LZ-positive cells could not be distinguished clearly from that of LF-positive cells.

Ultrastructural localization of LF has been studied only in limited cells such as neutrophilic granulocytes (21) and gastric glandular epithelium (8), and no subcellular localization of LF has been reported about the salivary gland. The present study revealed that LF was localized in the serous granules of acinar cells in the submandibular gland. In human salivary gland, secretory granules of serous acinar cells have at least two structural units, that is, electron dense central core and peripheral rim of lower density (7, 27). While this bipartite structure of the serous granules of salivary glandular cells may be seen as an artifact due to imperfect fixation in some animal species (23), human salivary glandular cells are known to have this structure constantly regardless of fixation and staining methods (7, 27). Therefore, the bipartite structure seen in the present study seems not to be fixation artifact. Ultrastructurally, immunostaining for LF was intense in the core and weak in the periphery of the secretory granules. As the central core has been suggested to contain proteinaceous material and the peripheral rim mucosubstances respectively (7), this staining pattern of the serous granules may account for the strong reaction for LF in other serous cells such as bronchial glandular cells, chief cells in the gastric gland proper, etc., and weak reaction in mucous cells such as cardiac or pyloric glandular cells, endocervical glandular cells, etc.

In addition to the granules, reaction products were observed slightly on the membrane structures and the cytoplasm around the granules, possibly due to diffusion of reaction products. Limiting membrane of the secretory granules in salivary serous cells are known to be labile and apt to be lost with ordinary fixation method (12, 17). In the present study, inadequate preservation of limiting membrane of the granules is probably the cause of this artifact. Other fixatives, especially that contain glutaraldehyde even with low concentration, however, greatly reduce antigenicity of LF (10). Therefore, PLP solution was applied in this study.

LF was not observed in the organelles that participate in protein synthesis and

Figs. 15 a, b. Ultrastructural demonstration of lactoferrin in the submandibular gland. (a) Reaction products were observed in the secretory granules (SG). Central core of the granules (arrowhead) stained intensely, whereas periperal rim (asterisk) stained weakly. (b) A section from negative control studies. Reaction products were not found in any portions of cells. Some lipid droplets (L) with peripheral electron density were observed. N: nucleus. a, ×8,700  b, ×5,000
secretion, such as rough endoplasmic reticulum, perinuclear space and Golgi apparatus. The following possibilities may be considered for this: 1) antigenicity of LF is not expressed until being packaged into the secretory granules, so that the presence of LF in such organelles is not detectable by this method, 2) amount of LF in such organelles is too small to demonstrate for the present technique of sensitivity, and 3) the acinar cells observed were not in LF-producing phase in their secretory cycle. Further studies are needed to know which possibility is applicable in this case and what is the exact mechanism of LF production and secretion.

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