Differential Expression of Ca^{2+} -Binding Proteins on Follicular Dendritic Cells in Non-Neoplastic and Neoplastic Lymphoid Follicles

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We studied the Ca^{2+} -capture ability of follicular dendritic cells (FDCs) in tonsillar secondary lymphoid follicles (LFs) and the expression of six Ca^{2+} -binding proteins (CBPs), caldesmon, S-100 protein, calcineurin, calbindin-D, calmodulin, and annexin VI in LFs of various lymphoid tissues and caldesmon and S-100 protein in neoplastic follicles of follicular lymphomas. First, Ca^{2+} -capture cytochemistry revealed extensive Ca^{2+} capture in the nuclei and cytoplasm of FDCs, but little or none in follicular lymphocytes. All six CBPs were localized immunohistochemically in the LFs and were always present in the basal light zone. Immunoelectron microscopic staining of FDCs was classified into two patterns: caldesmon was distributed in the peripheral cytoplasm like a belt; S-100 protein, calcineurin, calbindin-D, and calmodulin were distributed diffusely in the cytosol. Annexin VI was, however, negative on FDCs. Immunocytochemistry also demonstrated CBP-positive FDCs within FDC-associated clusters isolated from germinal centers. *In situ* hybridization revealed diffuse calmodulin mRNA expression throughout the secondary LFs. These data indicate that the CBPs examined may regulate Ca^{2+} in the different subcellular sites of FDCs, and the roles of CBPs may be heterogeneous. We also investigated the distribution of caldesmon and S-100 protein in follicular lymphomas on paraffin-embedded tissue sections. FDCs within grades I and II neoplastic follicles clearly expressed caldesmon, but not S-100 protein, except a part of grade II neoplastic follicles. FDCs within grade III follicles showed no caldesmon, but frequently expressed S-100 protein. These results demonstrate that the caldesmon and S-100 protein staining patterns of grade I follicular lymphomas are different from those of grade III follicular lymphomas and suggest that FDC networks in grade I neoplastic follicles may be similar to those in the light zone within non-neoplastic follicles, FDC networks in grade III neoplastic follicles may be similar to those in dark and basal light zones within

non-neoplastic follicles, and grade II follicles may be intermediate between grade I and grade III follicles. (Am J Pathol 1999, 155:805–814)

The human secondary lymphoid follicle (LF) is composed of a germinal center (GC) and a mantle zone (MZ). The former is the site of antigen-driven oligoclonal growth and differentiation of memory B cells and plasmablasts,^{1–3} and it consists of outer (OZ), dark (DZ), apical light (ALZ), and basal light (BLZ) zones.^{4–6} In general, the DZ, BLZ, ALZ, and OZ are believed to be the sites of proliferation, selection, and differentiation and the pathway of the B cell, respectively. Some B cell proliferation, selection, and differentiation events are actually regulated by follicular dendritic cells (FDCs), nonlymphoid cells which functions in building up and maintaining the three-dimensional framework in the LFs, trapping and retaining the immune complex for a long time, presenting antigen to lymphocytes, forming the FDC-lymphocyte cluster, regulating the apoptotic death of lymphocytes, and others.

GCs and FDCs in the secondary LFs have been reported to express some Ca^{2+} -binding proteins (CBPs), including an acetic CBP, S-100 protein, a vitamin-D-dependent CBP, calbindin-D, and a Ca^{2+} -dependent phospholipid binding protein, annexin VI.^{7–10} Annexin VI has a single high-affinity Ca^{2+} -binding site and lacks the classic EF-hand Ca^{2+} -binding sites. An EF-hand family of CBPs, calmodulin, plays roles in diverse events, including cell proliferation, smooth muscle contraction, ion channel control, and microtubular assembly.¹¹ A calmodulin-dependent (type 2B) serine/threonine protein phosphatase, calcineurin, is also categorized as an EF-hand CBP.¹² Caldesmon is a major calmodulin- and actin-binding protein, which is essential for smooth muscle and nonmuscle contraction.¹³ These findings suggest that CBPs may be indispensable to the correct functioning of every cell by regulating the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). However, very few papers described details of the follicular localization of CBPs in lymphatic tissues and the inclusive CBP localizations in the secondary LFs remain to be clarified. It is well known that follic-

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Table 1. Antibodies Used in This Study

Antibody (clone)	Immunoglobulin subclass	Tissue section	Source
<i>Calcium binding protein</i>			
Caldesmon	Mouse IgG1	F	Chemicon International, Temecula, CA
Caldesmon (TD107)	Mouse IgG1	P	Novocastra, Newcastle upon Tyne, UK
S-100 protein	Rabbit	P	DAKO, Glostrup, Denmark
Calcineurin	Mouse IgG	P, F	Upstate Biotechnology, Lake Placid, NY
Calbindin-D (CL-300)	Mouse IgG1	P, F	Sigma, St. Louis, MO
Calmodulin	Rabbit	F	IgG Corp., Nashville, TN
Annexin VI (Z017)	Mouse IgG1	P, F	Zymed, San Francisco, CA
Calretinin	Rabbit	P, F	Chemicon
<i>Follicular dendritic cell-associated</i>			
R4/23	Mouse IgM	F	DAKO
Ki-M4p	Mouse IgG	P	Prof. Lennert K (Kiel, Germany)

F, cryostat sections of periodate-lysine-paraformaldehyde-fixed tissues; P, paraffin sections of formalin-fixed tissues.

ular lymphomas have FDC meshworks, and in some neoplastic lymphomas there is still a functional relationship between FDCs and neoplastic lymphoma cells similar to that observed in non-neoplastic LF. ^{14,15} Some authors investigated S-100 protein localization in malignant lymphoma, ^{16–19} but there is still a mystery about follicular distribution of CBPs and functions of FDCs in follicular lymphoma. ⁹

The aim of this study was to investigate the precise localization of six different CBPs, caldesmon, S-100 protein, calcineurin, calbindin D, calmodulin, and annexin VI, in the five zones of secondary LF, with special reference to FDCs. Furthermore, the distribution patterns of caldesmon and S-100 protein in neoplastic follicles of follicular lymphomas were determined and compared with that of the FDC marker Ki-M4p to investigate the characteristics of FDCs in neoplastic follicles.

Materials and Methods

Tissue Samples

Fifteen palatine tonsils from patients suffering from chronic tonsillitis, five lymph nodes showing reactive follicular hyperplasia, three appendices, and three terminal ileal specimens (including Peyer's patches) were studied. Palatine tonsils were obtained during therapeutic tonsillectomy, lymph nodes and terminal ileal tissue were obtained during right hemicolectomy for colonic cancer, and appendices were obtained during surgery for ectopic pregnancy. Tissues from 19 patients with follicular lymphoma (7 in superficial lymph nodes, 6 in stomach, and 6 in thyroid gland) were obtained during diagnostic or therapeutic surgery. Follicular lymphomas were classified and graded according to the Revised European-American Lymphoma Classification. ²⁰ Six, eight, and five cases with grades I, II, and III lymphomas, respectively, were investigated.

Ca²⁺-Capture Cytochemistry

Ca²⁺-capture cytochemistry was carried out with the method described previously by Menon et al. ²¹ Fresh

tonsils were cut into 500- μ m-thick slices using a DTK-1000 microslicer (Dosaka EM, Osaka, Japan) and immersed in ice-cold 90 mmol/L potassium oxalate containing 2% glutaraldehyde and 2% formaldehyde overnight for Ca²⁺ localization. The samples were post-fixed with 1% osmium tetroxide containing 2% pyroantimonate for 2 hours at 4°C, rinsed in ice-cold distilled water (pH 10, adjusted with 0.1 N KOH), dehydrated in a graded series of ethanols, and routinely embedded in a low-viscosity epoxy resin. ²² Thin sections containing GCs were observed using a Zeiss EM109 electron microscope (Zeiss, Oberkochen, Germany).

Antibodies

Heterologous antisera or monoclonal antibodies against the seven CBPs were used (Table 1). The five secondary LF zones were identified after hematoxylin and eosin staining and immunostaining of CD23 (H107, mouse IgG; DAKO, Glostrup, Denmark).

Immunohistochemical Staining

A portion of each lymphatic tissue was fixed with periodate-lysine-paraformaldehyde (PLP) ²³ for 6 hours at 4°C, washed with 0.01 mol/L phosphate-buffered saline (PBS, pH 7.4) containing graded concentrations of sucrose, embedded in OCT compound (Miles Laboratories, Elkhart, IN), and stored at –80°C until cryostat sectioning. Another portion of each lymphatic and follicular lymphoma tissue was fixed with 10% formalin at room temperature for 12 hours and processed routinely, and 3- μ m-thick paraffin sections were cut out. Both cryostat and paraffin sections were stained immunohistochemically using the avidin-biotin-peroxidase complex (ABC) technique ²⁴ with 3–3' diaminobenzidine (DAB; Dojin Chemicals, Kumamoto, Japan) and then counterstained with methyl green or hematoxylin. Antigen retrieval on paraffin sections was carried out in a 0.1% trypsin solution (37°C) for 20 minutes or a 1 mmol/L EDTA/NaOH solution (pH 8.0) at 90°C for 15 minutes. Control sections were treated with PBS or nonimmune serum instead of specific antibodies. The immunostaining intensities of the CBPs in each of the five zones (MZ, OZ, ALZ,

BLZ, and DZ) within the secondary LF and those of caldesmon, S-100 protein, and Ki-M4p in neoplastic follicles of follicular lymphomas were classified into four categories: –, negative; +, weakly positive; ++, moderately positive; +++, strongly positive.

Immunoelectron Microscopy

For immunoelectron microscopy of the CBPs, tonsillar specimens were fixed with PLP for 6 hours at 4°C, cut into 60- μ m-thick slices using a DTK-1000 microslicer, and subjected to indirect immunoperoxidase staining.²⁵ The tissues used for S-100 protein immunostaining were re-fixed with 10% formalin for 10 minutes at room temperature. After immunostaining, the specimens were fixed with 1.25% glutaraldehyde and 2% osmic acid, dehydrated in a graded series of ethanols, and embedded in Epon resin, and ultrathin sections were observed using a Zeiss EM109 electron microscope.

Isolation of FDC-Associated Clusters from GCs and Immunocytochemical Staining

After tonsillar specimens were fixed with PLP for 6 hours at 4°C, 300- μ m-thick slices were cut using a DTK-1000 microslicer, and the GCs were enucleated from the microslices under a stereoscope.²⁶ To obtain FDC-associated clusters, enucleated GCs were digested with 0.05% collagenase (Sigma Chemical Co., St. Louis, MO) for 1 hour at 37°C using the method described previously²⁶ with modifications.²⁷ The extracts were then filtered through 90- μ m nylon monofilament mesh, and cytopsin slides were prepared using a Cytospin centrifuge (Shandon, Pittsburgh, PA).

FDC-associated clusters mounted on cytopsin slides were subjected to double immunocytochemical staining. First, R4/23 immunostaining was carried out using the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method²⁸ with 5-bromo-4-chloro-3-indoxyl phosphatase and nitro blue tetrazolin chloride (BCIP/NBT; DAKO), which gave rise to a royal purple color, and then staining for each of the CBPs was performed using the ABC method, which yielded a brown color. The preparation subjected to S-100 protein immunostaining was re-fixed with 10% formalin for 10 minutes at room temperature before the ABC procedure. All of the tissues were counterstained with methyl green. Negative control cytopsin preparations were incubated with PBS or nonimmune serum instead of an anti-CBP antibody.

In Situ Hybridization (ISH)

A 1700-base cDNA probe complementary to human calmodulin mRNA was obtained from the American Type Culture Collection (Rockville, MD)²⁹ and labeled with biotinylated 11d-UTP using a nick translation kit (BRL, Gaithersburg, MD). For ISH, tonsillar specimens were fixed with 4% paraformaldehyde for 6 hours at 4°C, and cryostat sections were prepared, as described above,

Figure 1. A: Low-power electron micrograph demonstrating Ca²⁺ capture in the germinal center. Note the high levels of captured Ca²⁺ in the extracellular spaces (arrowhead). A positively labeled binucleated follicular dendritic cell (FDC) is located around a blood vessel (V). Magnification, $\times 6160$; bar, 2 μ m. B: High-power electron micrograph of a labeled binucleated FDC. Diffuse cytoplasmic and nuclear labeling is evident and peripheral cytoplasm is fairly intensely stained (arrows). Magnification, $\times 18,600$; scale bar, 0.5 μ m.

subjected to proteolytic digestion with 0.2 N HCl for 10 minutes at room temperature, followed by proteinase K (5 mg/ml) for 10 minutes at 37°C,³⁰ and hybridized with the calmodulin probe at 42°C overnight. They were then incubated with avidin-fluorescein isothiocyanate (FITC; Boehringer Mannheim, Indianapolis, IN) for 1 hour at room temperature, and the location of the bound probe was observed under a fluorescence microscope. Positive control preparations were incubated with FITC-labeled β -actin (DAKO) and negative controls with PBS instead of the hybridization probe for the hybridization and RNase digestion (200 mg/ml for 1 hour at 37°C).

Results

Ca²⁺ Capture in GCs

Extensive Ca²⁺ capture was observed in the extracellular spaces. Some follicular lymphocytes captured small amounts of Ca²⁺ in their cytoplasm, whereas the majority of FDCs exhibited marked Ca²⁺ capture in both their cytoplasm and nuclei (Figure 1, A and B), revealing that FDCs have a relatively high Ca²⁺-binding capacity. Cap-

Table 2. Localization of Ca²⁺-Binding Proteins in Secondary Lymphoid Follicles

Antibody	Mantle zone	Outer zone	Apical light zone	Basal light zone	Dark zone
Caldesmon	+	+	++	++	+
S-100 protein	—	—	—	+++	++
Calcineurin	+++	—	+++	++	++
Calbindin-D	—	—	+++	+++	—
Calmodulin	+	—	+++	+++	+
Annexin VI	+++	—	+	+	+
Calretinin	—	—	—	—	—

—, negative; +, weakly positive; ++, moderately positive; +++, strongly positive.

Table 3. Immunoelectron Microscopic Distribution Patterns of Ca²⁺-Binding Proteins on Follicular Dendritic Cells in Secondary Lymphoid Follicles

Antibody	Distribution pattern
Caldesmon	Belt-like peripheral cytoplasmic staining (positive only at the periphery of the cytoplasm under tension)
S-100 protein	Diffuse cytosolic staining
Calcineurin	Diffuse cytosolic staining
Calbindin-D	Diffuse cytosolic staining
Calmodulin	Diffuse cytosolic staining

Table 4. Lacy Pattern Immunostaining of Ki-M4p, Caldesmon, and S-100 Protein in Neoplastic Follicles of Follicular Lymphomas

Antibody	Follicular lymphoma		
	Grade I	Grade II	Grade III
Ki-M4p	+++	++	+
Caldesmon	+++	+/++	—
S-100 protein	—	—/+	+++

—, negative; +, weakly positive; ++, moderately positive; +++, strongly positive.

ture was fairly extensive at the FDC cytoplasmic peripheries, and vascular endothelial cells captured large amounts of Ca²⁺ in their cytoplasm and nuclei.

Immunohistochemistry of CBPs in Secondary LFs

The CBP immunohistochemical staining results for the LFs of various lymphatic tissues, including tonsils, lymph nodes, appendices, and Peyer's patches, are summarized in Table 2. Caldesmon, S-100 protein, calcineurin, calbindin-D, calmodulin, and annexin VI were positive in the LFs of lymphatic tissues. In general, immunostaining was more intense in the light zone (LZ) than the DZ (Figure 2, A and C–F). The BLZ contained all six CBPs, and S-100 protein was localized in the BLZ and DZ but not in the ALZ (Figure 2B). The MZ showed intense calcineurin (Figure 2C) and annexin VI immunostaining and weak reactions with caldesmon and calmodulin (Figure 2, E and F). Calretinin was negative in the LFs. There were no marked differences among the four different lymphatic tissues with respect to the immunoreactions in each secondary LF zone (Figure 2G).

Immunoelectron Microscopy of CBPs in FDCs

The immunoelectron microscopic results are summarized in Table 3. Five CBPs were observed in FDCs. Caldesmon was limited to the peripheries of the FDC cell bodies and long cytoplasmic processes, and it accumulated particularly in the cytoplasm, twining around extracellular thick fibers (Figure 3A). The peripheral cytoplasm of FDCs often contained stress fibers. In contrast, caldesmon was not detected in the sagging peripheral FDC cytoplasm in contact with lymphocytes, and the inner FDC cytoplasm either showed no or faint immunostaining. S-100 protein was distributed evenly in the FDC cytosol (Figure 3B). The FDC cytoplasm also showed diffuse calcineurin, calbindin-D (Figure 3C), and calmodulin immunostaining. Annexin VI was localized on the cell membranes of GC lymphocytes but not FDCs.

CBPs in FDCs within Isolated FDC-Associated Clusters

To verify the immunohistochemical results, the expression of the five CBPs except annexin VI in FDCs within isolated FDC-associated clusters was examined. FDCs expressing the FDC marker R4/23 also expressed caldesmon (Figure 4A), S-100 protein, calcineurin, calbindin-D, and calmodulin (Figure 4B) simultaneously.

Detection of Calmodulin mRNA in Secondary LFs

ISH detected calmodulin mRNA in the cytoplasm of the vast majority of follicular cells (Figure 5), whereas the LFs in negative control preparations showed no specific reactions.

Localization of Caldesmon, S-100 Protein, and Ki-M4p in Neoplastic Follicles of Follicular Lymphomas

Neoplastic follicles in grades I and II lymphomas were positive in a lacy pattern for Ki-M4p and caldesmon (Figure 6A), but never contained S-100 protein-positive FDCs (Figure 6B), except a part of grade II neoplastic follicles that contained only a few, small conglomerate positive patterns (Table 4). In contrast, those in grade III lymphomas were positive for Ki-M4p and S-100 protein

Figure 2. Tonsillar secondary lymphoid follicles (LFs) immunostained with anti-caldesmon (A), S-100 protein (B), calcineurin (C), calbindin-D (D), calmodulin (E and F), and annexin VI (G) antibodies. A: Note the lacy pattern of caldesmon throughout the LF. Cryostat sections; magnification, $\times 50$. B: S-100 protein immunostaining is strongly positive in the BLZ and DZ, but negative in the other zones. A small secondary LF (asterisk) is completely negative. Paraffin section; magnification, $\times 60$. C: Calcineurin is immunostained in a lacy pattern in the germinal center. The mantle zone lymphocytes are also immunostained. Cryostat section; magnification, $\times 50$. D: Calbindin-D immunostaining confined to the light zone. Paraffin section; magnification, $\times 60$. E and F: Calmodulin locates throughout the LF, showing particularly intense immunostaining in the light zone. Cryostat sections; magnification, $\times 50$ (E) and $\times 200$ (F). G: Strong positive annexin VI immunostaining of the mantle zone lymphocytes and weak positive immunostaining in the other zones. Cryostat section; magnification, $\times 60$; counterstained with methyl green. MZ, mantle zone; OZ, outer zone; ALZ, apical light zone; BLZ, basal light zone; DZ, dark zone.

demonstrating a diffuse pattern often intermingled with the large conglomerate pattern (Figure 6D, but not for caldesmon (Figure 6C).

Discussion

When T-cell-dependent antigens are recognized in an indefinite pathway, MZ B cells may migrate through the OZ to the DZ. Centroblasts in the DZ divide to exhibit clonal expansion and somatic mutation in the variable region of their immunoglobulin gene and then migrate into the BLZ,⁶ where antigens are entrapped on the FDC surfaces, accompanied by complement activation.³¹

Centrocytes expressing membrane immunoglobulin with high affinities for their antigens escape apoptosis and migrate into the ALZ, where they differentiate into memory B cells and plasmablasts.⁴ FDCs regulate some B-cell proliferation, selection, and differentiation events through mediators such as CD23, adhesion molecules, and cytokines. Recently, Yamada et al³² demonstrated that FDCs in the LZ express some cytokine receptors, which suggests that these FDCs may transmit information about their cytokines to GC B cells.

The vast majority of physiological phenomena depend extensively on $[Ca^{2+}]_i$ changes in response to various extracellular and intracellular stimuli, some of which lead to increases in $[Ca^{2+}]_i$, and cytosolic CBPs bind to Ca^{2+}

Figure 3. Immunoelectron micrographs of caldesmon (A), S-100 protein (B), and calbindin-D (C) in tonsillar germinal centers. **A:** Note the distinct caldesmon reactivity at the follicular dendritic cell (FDC) cytoplasmic periphery, where extension has been generated, but no reactivity in the flagging cytoplasm encircling two lymphocytes (Ls). The **arrow** indicates twinning of a short caldesmon-positive FDC cytoplasmic process around an extracellular thick fiber (Fs). Magnification, $\times 9300$; bar, $1\ \mu\text{m}$. **B:** Note the homogeneous cytosolic immunostaining of S-100 protein in the cytoplasm of a binucleated FDC. Magnification, $\times 9300$; bar, $1\ \mu\text{m}$. **C:** Note the diffuse cytosolic and nuclear staining of calbindin-D on a binucleated FDC. Magnification, $\times 12,000$; bar, $1\ \mu\text{m}$.

at concentrations in the range of 10^{-5} to 10^{-8} mol/L.³³ $[\text{Ca}^{2+}]_i$ changes are also important in the immunological phenomena that occur in LFs. For example, the Ca^{2+} -cAMP-dependent second messenger system appears to regulate apoptosis of GC B cells.³⁴ Although FDCs and GCs in the secondary LFs have been found to express some CBPs, including S-100 protein, calbindin-D, and annexin VI,^{7–10} precise evaluation of the CBPs in their LFs, the follicular localization of other CBPs, such as

caldesmon, calcineurin, and calmodulin, and Ca^{2+} itself in FDCs has not been reported until now.

We examined Ca^{2+} localization in tonsillar secondary LFs using Ca^{2+} -capture cytochemistry and observed extensive Ca^{2+} capture in the extracellular spaces. Some GC lymphocytes captured relatively little Ca^{2+} , whereas FDCs and endothelial cells captured strikingly large amounts of Ca^{2+} in both their cytoplasm and nuclei, suggesting that $[\text{Ca}^{2+}]_i$ may be regulated by CBPs.

Figure 4. Immunocytochemical staining of caldesmon (A) and calmodulin (B) on FDCs within a FDC-associated cluster. R4/23-positive cells, which stained a royal purple color after APAAP immunostaining, are simultaneously stained caldesmon and calmodulin by ABC immunostaining, which yielded a brown color. Counterstained with methyl green; magnification, $\times 320$.

Next, the follicular localizations of six CBPs were examined immunohistochemically. Caldesmon was found throughout the LF, and its localization on FDCs was confirmed by immunocytochemical examination of isolated FDC clusters. Immunoelectron microscopy revealed that it was present characteristically on the cytoplasmic processes of FDCs that twined around extracellular thick fibers and in the peripheral cytoplasm, presumably creating a high extension. FDCs twine around the extracellular fibers and connect to neighboring FDCs via desmosome-like junctions to build up a three-dimensional meshwork structure in the secondary LFs.⁶ Ogata et al³⁵ demonstrated that FDCs express fibronectin and laminin receptors that bind to the reticulin and laminin fibers in secondary LFs. Furthermore, FDCs possess actin filaments.³⁶ These data, including our results, suggested that caldesmon on actin filaments and extracellular matrix adhesion receptors on FDCs may be the principal ways whereby FDCs twine around extracellular fibers and generate tension in the peripheral cytoplasm, contributing to the formation and maintenance of the meshwork structure.

Our immunohistochemical experiment demonstrated other CBPs, such as S-100 protein, calcineurin, calbindin-D, and calmodulin in LFs. The BLZ showed intense immunostaining of six CBPs. S-100 protein was not detected in

Figure 5. Calmodulin mRNA expression in a tonsillar lymphoid follicle. Calmodulin mRNA is distributed diffusely throughout the lymphoid follicle. Magnification, $\times 85$. MZ, mantle zone; GC, germinal center.

the ALZ, but large amounts were present in the DZ. The presence of these proteins in FDCs was confirmed by immunocytochemistry. Immunoelectron microscopy revealed diffuse immunostaining of S-100 protein, calcineurin, calbindin-D, and calmodulin in the FDC cytoplasm. Furthermore, in our ISH study, we detected calmodulin mRNA expression throughout secondary LFs, although we were unable to identify where exactly it was localized in FDCs. S-100 protein inhibits the phosphorylation of various proteins, such as tau protein and p87 protein,^{37,38} calcineurin is a protein phosphatase regulated by the Ca²⁺-calmodulin complex,³⁹ calbindin-D modulate [Ca²⁺]_i,⁴⁰ and calmodulin regulates [Ca²⁺]_i by activating Ca²⁺,Mg²⁺-ATPase¹¹ and inositol 1,4,5-trisphosphate (InsP3) 3-kinase.⁴¹ These CBPs may regulate [Ca²⁺]_i in FDCs and exhibit diverse functions, such as protein phosphorylation in FDCs, although, at the moment, their precise roles in FDCs and follicular lymphocytes are not clear.

Annexin VI was detected immunohistochemically throughout the LF, but the highest levels were present in the MZ. Immunoelectron microscopy revealed that it was localized on the surfaces of GC lymphocytes but not FDCs. Lin et al⁴² suggested that annexin VI is required for budding of clathrin-coated pits, the initial step of endocytosis.

Figure 6. Immunostaining of caldesmon and S-100 protein in follicular lymphoma tissues. **A** and **B**: Immunostaining of caldesmon (**A**) and S-100 protein (**B**), respectively, in neoplastic follicles of a grade I follicular lymphoma arising from the lymph node. Caldesmon is strongly positive in the neoplastic follicles, whereas S-100 protein is completely negative. **C** and **D**: Immunostaining of caldesmon (**C**) and S-100 protein (**D**), respectively, in neoplastic follicles of a grade III follicular lymphoma arising from the thyroid gland. Caldesmon is negative in the neoplastic follicles, and S-100 protein is clearly positive in a neoplastic follicle. Paraffin sections (**A** to **D**), counterstained with methyl green (**A** to **D**); magnification, $\times 70$ (**B** and **C**) and $\times 80$ (**A** and **D**).

Finally, we investigated the localization patterns of caldesmon and S-100 protein and compared them with that of Ki-M4p in follicular lymphomas. All of the neoplastic follicles examined contained Ki-M4p-positive FDCs, although this was weaker in grade III follicles than in grades I and II follicles. Caldesmon was expressed clearly in a lacy pattern in grades I and II neoplastic follicles but never in grade III follicles. In contrast, S-100 protein was expressed frequently in cells with dendritic morphology (presumably FDCs) in grade III neoplastic follicles and in a part of grade II follicles, but never in grade I follicles. In the GCs of non-neoplastic follicles, S-100 protein was confined to the DZ and BLZ, whereas the LZ was richer in Ki-M4p and caldesmon than the DZ. These data suggest that the caldesmon and S-100 protein expression patterns of grades I and III follicular lymphomas differ, and furthermore, it is likely that FDC distribution pattern in the LZ within non-neoplastic follicles may be similar to that in grade I neoplastic follicles, FDC distribution pattern in the DZ and BLZ within non-neoplastic follicles may be similar to that in grade III follicles, and grade II neoplastic follicles may be intermediate between grade I and grade III follicles.

In conclusion, we have demonstrated here that 1) [Ca²⁺]_i is considerably higher in FDCs than in their surrounding follicular lymphocytes, 2) the BLZ shows intense immunostaining of CBPs in comparison with the other four zones in LFs, 3) FDCs express five CBPs in two different patterns, 4) caldesmon expression in FDCs is related to twining around extracellular thick fibers, 5) FDC meshworks in the non-neoplastic LZ are similar to those in grade I neoplastic follicles, and FDC meshworks in the non-neoplastic DZ and BLZ are similar to those in grade III follicles.

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