Enhanced Autophagy and Reduced Expression of Cathepsin D Are Related to Autophagic Cell Death in Epstein-Barr Virus-Associated Nasal Natural Killer/T-Cell Lymphomas: An Immunohistochemical Analysis of Beclin-1, LC3, Mitochondria (AE-1), and Cathepsin D in Nasopharyngeal Lymphomas

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This study investigated autophagy in 37 cases of nasopharyngeal lymphomas including 23 nasal natural killer (NK)/T-cell lymphomas (NKTCL), 3 cytotoxic T-cell lymphomas (cytotoxic-TML) and 9 B-cell lymphomas (BML) by means of antigen-retrieval immunohistochemistry of beclin-1, LC3, mitochondria (AE-1) and cathepsin D. Peculiar necrosis was noted in EBV\textsuperscript{+} lymphomas comprising 21 NKTCL, 2 cytotoxic-TML and 1 BML. Lymphomas without peculiar necrosis showed high expression of beclin-1, macrogranular cytoplasmal stain of LC3 with sporadic nuclear stain, a hallmark of autophagic cell death (ACD), some aggregated mitochondria and high expression of cathepsin D, suggesting a state of growth with enhanced autophagy with sporadic ACD. EBV\textsuperscript{+} NKTCL with the peculiar necrosis, showed significantly low level of macrogranular staining of LC3, aggregated mitochondria and low expression of cathepsin D in the cellular areas when degenerative lymphoma cells showed decreased beclin-1, significantly advanced LC3-labeled autophagy, residual aggregated mitochondria and significantly reduced expression of cathepsin D, suggesting advanced autophagy with regional ACD. Consequently it was suggested that enhanced autophagy and reduced expression of lysosomal enzymes induced regional ACD under EBV infection in NKTCL.

Key words: nasopharyngeal lymphoma, NKT-cell lymphoma nasal type, Epstein-Barr virus (EBV), autophagy, autophagic cell death, antigen retrieval immunohistochemistry (beclin-1, LC3, mitochondria (AE-1) and cathepsin D)

I. Introduction

Nasal natural killer (NK)/T-cell lymphoma (NKTCL), is the dominant type of nasopharyngeal lymphoma in Asia and South America, especially in China but rarely in Japan. NKTCL cells show diffuse proliferation and an immunophenotypic profile of either CD3\textsuperscript{+} TIA1\textsuperscript{+} CD56\textsuperscript{−} NK/T cells or CD3\textsuperscript{−} TIA1\textsuperscript{+} CD56\textsuperscript{+} cytotoxic T-cells [14]. NKTCL is usually Epstein-Barr virus (EBV)-associated; shows signals of in-situ hybridization (ISH) of EBV-encoded small RNA-1 (EBER-1) in their nuclei [9]; and associates with CD204\textsuperscript{+} macrophages to form a meshwork of their cytoplasmal processes [32]. Besides, NKTCL shows also peculiar necro-
sis that was first named rhinitis gangrenosa [20]. Peculiar necrosis has been suggested to be due to autophagy under EBV infection when neoplastic expression of survivin [1, 11, 31] suppressed apoptosis as judged by labeling with anti-cleaved caspase-3 antibody [32].

Autophagic cell death (ACD) [17] is a lysosome-related programmed cell death (PCD). Autophagy is modulated by target of rapamycin (Tor) that initiates autophagy through Atg1-related complex formation probably at the endoplasmic reticulum [12] under homeostatic mass control, physiological stimuli such as starvation and proliferation, pharmacological agonists such as rapamycin, and immunological stimuli [3, 4, 27]. Next, beclin-1 forms a complex with type III phosphatidylinositol 3-kinase (PI3K), Vps34 and UV-irradiation-resistance-associated gene (UVRAG) [25], and begins the vesicle nucleation forming the autophagophore. Atg5-Atg12 system including Atg7, Atg3, Atg10, and Atg16L lipidates LC3-I to LC3-II (membrane-bound form of LC3), forming autophagophore with LC3-II that engulfs altered organella such as mitochondria. Next, GTPase, Rab7-Rab5 system, matures the autophagophore into an autophagosome. The UVRAG-HOPS complex then induces fusion of the autophagosome with endosomes and lysosomes [25] including cathepsin D and also ATP-dependent H+ pump. The engulfed organella and molecules are finally degraded in the auto(phago)lysosome, where molecules, beclin-1 and LC3, and aggregated mitochondria as rapamycin, and immunological stimuli [3, 4, 27]. The contents of the autolysosome are then either excreted or remain as a residual body.

However, the relationship between autophagy and ACD has yet to be elucidated clearly [24]. Uchiyama [30] reported a close relationship between the activation of cathepsin D and the ACD of neurons. NK/TCL often shows cellular, degenerative and necrotic areas in its lesions and its peculiar necrosis shows aggregated and densely LC3-labeled nucleus-like cell debris (a hallmark of ACD) [32]. Mitochondria are representative target organella of the macroautophagy that is involved with ACD and are found in cytoplasm, autophagosomes and autolysosomes. Cathepsin D is a lysosomal enzyme digesting molecules and organella in autolysosomes. Therefore, their immunohistochemical detection with beclin-1 in the nucleus of autophagophore and LC3 in the autophagosome and autolysome was expected to be informative for the processes of the enhanced autophagy leading to ACD in NK/TCL cells.

In this study we mainly investigated autophagy-related molecules, beclin-1 and LC3, and aggregated mitochondria and cathepsin D in nasopharyngeal lymphomas [32] by means of immunohistochemistry (IHC). It was elucidated that EBV-induced enhanced autophagy and reduced expression of cathepsin D could explain the peculiar necrosis in EBV-associated NK/TCL.

II. Materials and Methods

Cases examined

Archival paraffin specimens of 134 cases of nasopharyngeal lymphoma from northeast China were re-examined [32] and among them 37 cases showing areas of lymphoma tissue free from obvious degenerative changes were selected for this study. In the clinicopathological information of patients, only age, sex and biopsy site were available. This study was performed with the approval of the Ethics Committee for epidemiological studies in Kagoshima University Graduate School of Medical and Dental Sciences and of the Ethics Committee for international co-operative studies in China Medical University.

Based on the typing of lymphomas and the detection of EBV latent infection by means of ISH of EBER-1 given below, these lymphomas comprised 28 cases of T/NK-cell neoplasm (TML) and 9 cases of B-cell neoplasm (BML). The 28 TML comprised 23 NK/T-cell lymphoma (cytotoxic-TML) and 2 polymorphous reticulosis (PR)/early NK/TCL (E-NKTL) (Table 1). These cases of TML were included in so-called nasal NK/TCL in WHO classification [14]. As shown in Table 1, there were two cases of EBV− NK/TCL when the other NK/TCLs were EBV+ cases and three of 9 cases of BML were EBV+ cases. TML and NK/TCL dominated in males and in the nasal

Table 1. Clinicopathological information and status of Epstein-Barr virus infection in the cases examined

| Case                        | No. | M/F | Mean | Range | Age (yrs) | Site (EBV infection status; EBV−: EBV−/−: EBV+) |
|-----------------------------|-----|-----|------|-------|-----------|-----------------------------------------------|
| T/NK-cell neoplasm (TML)    | 28  | 19:9| 44.5 | 18–70 | 19 (2:0.17) | 6 (0:2:5) 3 (0:0:3) |
| NK/T-cell lymphoma (NKTL)   | 23  | 17:6| 43.0 | 20–56 | 17 (2:0.15) | 3 (0:0:3) 3 (0:0:3) |
| Cytotoxic T-cell lymphoma   | 24  | 17:7| 43.2 | 20–56 | 17 (2:0.15) | 3 (0:0:3) 3 (0:0:3) |
| Polymorphous reticulosis   | 23  | 17:6| 43.0 | 20–56 | 17 (2:0.15) | 3 (0:0:3) 3 (0:0:3) |
| B-cell neoplasm (BML)       | 9   | 3:6 | 52.4 | 13–84 | 3 (0:0:3) 5 (3:2:0) 1 (0:1:0) |

So-called nasal NK/TCL comprises NK/TCL, Cytotoxic TML and PR/E-NKTL.
EBV infection status: EBV−: no infection in lymphoma cells; EBV−/−: incomplete neoplastic expansion of EBV latent infection in lymphoma cells; EBV+: complete neoplastic expansion of EBV latent infection in lymphoma cells.
cavity and BML in the pharynx. EBV-associated lymphomas were frequently seen in the nasal cavity, as reported previously [32].

**Typing of lymphomas**

The lymphoma cases were mostly re-categorized according to the WHO classification [14] based on the immunological phenotype of lymphoma cells determined by means of IHC. Antibodies (CD3ε, CD5, CD79a, TIA1 and CD56) listed in Table 2 were employed for determining the phenotype of lymphoma cells. After deparaffinization, endogenous peroxidase inactivation by incubating sections in 0.3% H2O2 methanol solution for 30 min was followed by three rinses in phosphate buffer saline (PBS), pH 7.2. Antigen retrieval (AR) was performed by heating sections in citrate buffer (Target retrieval solution, S1699, Dako), pH 6, for 5 min at 121°C in an autoclave. Enzymatic antigen retrieval was performed at 37°C for 5 min in an autoclave (KTS-2322, ALP Co. Ltd., Tokyo, Japan), followed by three rinses in PBS. After being treated with blocking of non-specific stain (Protein Ltd., Tokyo, Japan), the reacted primary antibody was labeled with a polymer reagent (ChemMate Envision, K5027, Dako) for 10 min, and was visualized by means of H2O2-diaminobenzidine (DAB) reaction (DAB+, Liquid, K3468, Dako) for 10 min (the polymer method in Table 2). These procedures were performed by an autostainer (Autostainer, Dako) with rinsing buffer warmed to 35°C. The sections removed from the autostainer were dehydrated and were mounted in a plastic medium.

Based on IHC of CD3ε, CD5, CD79a, TIA1 and CD56, the lymphoma with CD3ε+ CD5−/− CD79a− TIA1+/− CD56−/− phenotype was TML when that with CD3ε+ CD5+/− CD79a− TIA1− CD56− phenotype was BML. In TML the case with CD3ε+ CD5+/− CD79a− TIA1+ CD56− phenotype was cytotoxic TML and that with CD3ε− CD5+/− CD79a− TIA1+ CD56− phenotype was NKTCCL. Further categorization of lymphomas was performed according to the WHO classification [14].

**ISH of EBER-1**

Association of EBV infection was examined by ISH of EBER-1 according to the method reported previously [29]. Briefly, after deparaffinization, sections were digested with proteinase K at 37°C for 30 min, dehydrated, and dried. Hybridization with digoxigenin-labeled probes was then performed at 37°C more than three hr. The hybridized probes were visualized by means of alkaline phosphatase-labeled anti-digoxigenin antibody and a colorimetric alkaline phosphatase-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt activity reaction (DIG-nucleic acid detection kit, 1175041, Roche, Mannheim, Germany). After nuclear counterstaining with methyl green, sections were dehydrated and mounted in plastic medium. An EBV-positive gastric adenocarcinoma, simultaneously stained by the same method, was used as positive control.

EBV latent infection detected by EBER-1 ISH was evaluated as no EBV latent infection in lymphoma cells (EBV−) with or without rare probably small memory B cells infected latently by EBV, incomplete neoplastic expansion EBV (+), and complete neoplastic expansion (EBV+), as shown in Figure 1.

**Immunohistochemistry (IHC) of apoptosis and autophagy-related molecules**

Cleaved caspase-3 is key molecule in irreversible apoptosis when survivin [1, 11, 31] suppresses cleaved caspase-3 and these two molecules can be detected in

### Table 2. Antibodies used and antigen retrieval/detection methods

| Antibody          | Specificity or function               | Clone/Source                        | Dilution | Antigen retrieval | Detection method |
|-------------------|--------------------------------------|-------------------------------------|----------|-------------------|------------------|
| CD3ε              | T cells, NK/T cells                   | NCL-CD3-PS1/Vision Biosystems       | 1:100    | Heat*             | Polymer          |
| CD5               | T cells                               | NCL-CD5-4C7/Vision Biosystems       | 1:50     | Heat*             | Polymer          |
| CD79a             | B cells                               | M7050/Dako                          | 1:200    | Heat*             | Polymer          |
| TIA1              | T cells, NK/T cells (Cytotoxic granules) | TIA1/Coulter Immunology             | 1:500    | Heat*             | Polymer          |
| CD56              | NK/T cells, plasma cells              | NCL-CD56-IB6/Vision Biosystems      | 1:50     | Heat*             | Polymer          |
| Cleaved caspase-3 | Irreversible apoptosis                | 5A1 Asp175/Cell Signaling Co.       | 1:200    | Heat**            | Polymer          |
| Survivin          | Inhibitor of apoptosis                | ab469/Abcam                         | 1:500    | Heat**            | Polymer          |
| Beclin-1          | Autophagic vesicle nucleation         | Sc-11427/Santa Cruz                 | 1:50     | Enzyme            | Supersensitive   |
| LC-3              | Autophagic vesicle elongation         | PM036/MBL                           | 1:1000   | Heat**            | Polymer          |
| AE-1              | Mitochondria                          | AE-1/Lenieco Technologie Inc.       | 1:50     | Heat**            | Polymer          |
| Cathepsin D       | Lysosomal enzyme                      | NCL-CDm(C5)/Vision Biosystems       | 1:100    | Heat**            | Polymer          |

Antigen retrieval methods: Heat*; Sections were heated in citrate buffer pH 6 (Target retrieval solution, S1699, Dako) for 5 min at 121°C in an autoclave. Heat**; Sections in the citrate buffer (Diva Decloaker, Biocare Medical) for 5 min at 121°C in an autoclave. Enzymatic antigen retrieval, Sections were treated with 200 mg/ml proteinase K Trits buffer saline solution for 10 min at room temperature.

Detection methods: Polymer; ChemMate Envision system (K5027, Dako). Supersensitive; The polymer method (K5027, Dako) with blocking of non-specific reactions, the catalyzed reporter deposition (CARD) reaction and its detection in a catalyzed signal amplification (CSA) system with blocking diffused deposition in CARD reaction.
archival paraffin sections by IHC [7, 8]. Cleaved caspase-3 and survivin were retrieved by heating sections in the solution (Diva Decloaker, Biocare Medical, Concord, CA, USA) independently from pH of the solution and were detected by the antibody and polymer method listed in Table 2.

Beclin-1 is one of the autophagy-related molecules in the autophagic vesicle nucleation complex that was detected by means of enzymatic AR and supersensitive IHC [10, 15, 32] when beclin-1 in a complex with Bcl-2, Bcl-X 
L and H3-only proteins in cytoplasm would be digested in the enzymatic AR. After deparaffinization and the first endogenous peroxidase inactivation and rinse in PBS, enzymatic AR incubating sections in proteinase K (200 mg/ml, No. 9033, Takara Bio Co., Otsu, Shiga, Japan) -0.05 M Tris buffer saline, pH 7.2, solution for 10 min was followed by the second endogenous peroxidase inactivation in 3% H$_2$O$_2$ PBS for 5 min. Reacted primary antibody in the reaction for 15 min was labeled with a polymer reagent (ChemMate Envision, K5027, Dako) for 15 min followed by catalyzed reporter deposition amplification reaction (K1500, Dako), with pretreatment against the non-specific reaction (Protein blocking, X0909, Dako) before each process, and was visualized by means of H$_2$O$_2$-diaminobenzidine (DAB) reaction (DAB+, Liquid, K3468, Dako) for 5 min (The supersensitive method in Table 2). These procedures were performed by an autostainer with rinsing buffer warmed to 35°C [10]. The sections removed from the autostainer were dehydrated and were mounted in a plastic medium.

LC3-I and II in the elongation of autophagosome were detected in the western blot analysis of the lyophilized cell lysate from serum starved Neuro 2A cells (supplied with 0231s0104 from Nanotools) with anti-LC3 antibody, PM036 from MBL (Table 2) [32] and LC3-II (the membrane-bound form of LC3) was retrieved by heating sections in the solution independently from pH of the solution and were detected by the polymer method of antibodies listed in Table 2.

Mitochondria are the representative organelle engulfed in the autophagosome and are digested in the autolysosome. Cathepsin D is a digestion enzyme in the lysosome and autolysosome and its activation requires ATP-dependent H$^+$_pump [2, 33]. Mitochondria and cathepsin D were retrieved by heating sections in the solution independently from pH of the solution and were detected by the polymer method of antibodies listed in Table 2.

The positive and negative control staining in each IHC of cleaved caspase-3, survivin, beclin-1, LC3, mitochondria (AE-1) and cathepsin D is summarized in Table 3.

The representative immunostaining of the specimens was recorded at ×40 magnification by a digital microscopic camera (Fuji Digital Camera HC-300, Fujifilm, Tokyo, Japan) at cellular, degenerative and necrotic areas of the lymphoma tissue. The cellular areas of lymphoma tissue showed diffuse and adhesive growth of lymphoma cells. The peculiar necrotic areas were determined easily, showing a small number of residual cells and debris in the background of acidophilic necrotic tissue when a lesion of circulatory disturbance indicated a microthrombus in dilated blood

Fig. 1. Detection of EBV latent infection by means of ISH of EBER-1. The long axis of all microphotographs was 215 μm long at ×40 magnification. Lymphomas without EBV latent infection (EBV$^-$, a, d) show no or rare probably small memory B-cells infected latently by EBV. Lymphomas with EBV latent infection (EBV$^+$, c, f) show EBER-1 signals in the most lymphoma cells, suggesting complete neoplastic expansion of EBV latent infection. Lymphomas show EBER-1 signals in a few to some lymphoma cells (EBV$^{+/−}$, b, e), suggesting incomplete neoplastic expansion of EBV latent infection.
vessels (Fig. 2). The degenerative areas were located next to the necrotic areas.

Evaluation of the immunostaining was carried out on the recorded images for reproducibility of the evaluation by Hasui K. and Wang J. according to the graded score system of beclin-1, LC3, Mitochondria (AE-1) and cathepsin D in Table 4.

The evaluated scores of the lymphomas grouped in subtypes, EBV infection and degenerative tendency were tested by Kruskal-Wallis test for more than 3 groups, Mann-Whitney’s U test for not-corresponding data in two groups or Wilcoxon signed-ranks test for corresponding data in 2 groups by each antibody (beclin-1, LC-3, AE-1 and cathepsin D).

### III. Results

The representative case of NKTCL showed diffuse proliferation of lymphoma cells (Fig. 3a) positive for CD3ε, TIA1 (Fig. 3b), CD56 (Fig. 3c) and signals of EBER-1 (Fig. 3d), and revealed rare cleaved caspase-3 positive cells (Fig. 3e) and many cells expressing survivin densely (Fig. 3f). In the degenerative and necrotic areas of NKTCL, there were rare cleaved caspase-3-positive cells (Fig. 3h) and residual expression of survivin was noted (Fig. 3i). The other subtypes of TML and BML also showed rare cleaved caspase-3-positive lymphoma cells when most lymphoma cells expressed survivin [32], suggesting that the PCD of lymphoma cells was not apoptosis (PCD I type) [32].

**Autophagy in lymphoma cells in the cellular areas (representative areas)**

TML indicated higher expression of beclin-1, lower expression of LC3, less aggregation of mitochondria and lower expression of cathepsin D than BML (Table 5).

Higher expression of beclin-1 was noted in EBV− NKTCL than in EBV+ NKTCL (Table 5, Fig. 4a1, 4a2) whereas EBV− BML revealed lower expression of beclin-1 than EBV− BML (Table 5, Fig. 5a1, 5a3). In spite of lower expression of LC3 in TML than in BML (Table 5), macrogranular dense stain of LC3 with the microgranular background stain was noted in EBV− and EBV+ NKTCL (Fig. 4b1−4b3) and BMLs (Fig. 5b1, 5b3). Low aggregated mitochondria in EBV− NKTCL (Fig. 4c1) and E-NKTCL...
reduced the mean score in TML (Table 5). Aggregated mitochondria were obvious in EBV+ NKTCL (Fig. 4c2, 4c3) and BML (Fig. 5c1, 5c3). EBV infection effects were observed in the early stages of autophagy such as autophagic vesicle nucleation and elongation differently in NKTCL and BML.

Lower expression of cathepsin D in lymphoma cells was indicated in TML than in BML (Table 5). Significant lower synthesis of cathepsin D was noted in EBV+ NKTCL than in cytotoxic-TML (Table 5, #1 p=0.0387, #2 p=0.0396). The expression level of cathepsin D decreased in the order of EBV− BML (Fig. 5d1), cytotoxic-TML, EBV−/− BML, E-NKTCL, EBV− NKTCL (Fig. 4d1), EBV+ BML (Fig. 5d3) and EBV+ NKTCL (Fig. 4d3) (Table 5), suggesting a different level of cathepsin D expression in each subtype of lymphomas.

**Autophagy in lymphoma cells in the cases revealing degeneration and necrosis**

EBV+ NKTCL comprised four cases free from degeneration, six cases with degeneration and 11 cases with degeneration and peculiar necrosis (Table 6). Peculiar necrosis was recognized also in two cases of Cytotoxic-TML and one case of EBV+ BML (Table 6).

EBV+ NKTCL showed no difference in the expression of beclin-1 in the cellular areas among the cases free from degeneration, those with degeneration and those with degeneration and necrosis (Table 6). However, in the EBV+ NKTCL cases with degeneration and necrosis significantly lower expression of beclin-1 was noted in the degenerative lymphoma cells (Table 6, #7 p=0.0041, #8 p=0.0030, Fig. 4a4, 4a5), as well in the cases of EBV+ BML (Fig. 5a3, 5a4) and of cytotoxic-TML with necrosis (Table 6) suggesting decelerated autophagic vesicle nucleation.

EBV+ NKTCL cases with degeneration and necrosis showed significantly lower LC3 score in the cellular areas than those in the cellular areas of the cases free from degeneration (Fig. 4b2) and those with degeneration (Fig. 4b3, Table 6, #1 p=0.0036, #2 p=0.00103, #3 p=0.0062). In the cases with degeneration and necrosis the LC3 score ascended significantly in the degenerative areas (Table 6, #9 p=0.0035) and in the necrotic areas (Table 6, #10 p=0.0022, #11 p=0.0041), revealing nuclear stain of LC3, a hallmark of ACD [32], in the necrotic area (Fig. 4b5).
Autophagy and ACD in Nasopharyngeal Lymphomas

Table 4. Scoring system in IHC of beclin-1, LC3, mitochondria (AE-1), and cathepsin D

| Score | Definition | Sample image* |
|-------|------------|---------------|
| 0     | No staining | Fig. 4 a2, a5 |
| 1     | Weak staining | Fig. 4 a4, a9 |
| 2     | Moderate staining | Fig. 4 a3, a7, a8 |
| 3     | Strong staining | Fig. 4 a1 |
| 4     | Very strong staining | — |

Beclin-1

| Score | Definition | Sample image* |
|-------|------------|---------------|
| 0     | No staining | — |
| 1     | Microgranular staining in cytoplasm | — |
| 2     | Macrogranular staining in background of strong microgranular staining in cytoplasm | Fig. 4 b1, b3 |
| 3     | Macrogranular staining in background of decreased microgranular staining in cytoplasm | Fig. 4 b2, b4 |
| 4     | Nuclear or perinuclear dense staining in background of Score 2 or 3 | Fig. 4 b5, b7 |
| 5     | Nuclear or perinuclear dense staining without the background of Score 2 or 3 | — |

LC3

| Score | Definition | Sample image* |
|-------|------------|---------------|
| 0     | No aggregated mitochondria | — |
| 1     | Aggregated mitochondria in a few neoplastic cells | Fig. 4 c1, c2, c3 |
| 2     | Aggregated mitochondria in some neoplastic cells | Fig. 4 c5, c6, c7 |
| 3     | Aggregated mitochondria in many neoplastic cells | Fig. 4 c3, c4 |

Mitochondria (AE-1)

| Score | Definition | Sample image* |
|-------|------------|---------------|
| 0     | No staining in neoplastic cells | Fig. 4 d5, d6 |
| 1     | Weak staining in neoplastic cells | Fig. 4 d2, d3, d4 |
| 2     | Moderate staining in neoplastic cells | Fig. 4 d1, d4 |
| 3     | Strong staining in neoplastic cells | Fig. 4 d2 |

Cathepsin D

| Score | Definition | Sample image* |
|-------|------------|---------------|
| 0     | No staining | Fig. 4 a2, a5 |
| 1     | Weak staining | Fig. 4 a4, a9 |
| 2     | Moderate staining | Fig. 4 a3, a7, a8 |
| 3     | Strong staining | Fig. 4 a1 |
| 4     | Very strong staining | — |

Sample image*: Sample images (×100 oil magnification) of each score of each staining in Figures 4 and 5 were indicated when evaluation of the immunostaining was carried out on the recorded images at ×40 magnification for reproducibility. Score 4 immunostaining of beclin-1 was possibly detected also by the polymer method. Scores 4 and 5 immunostaining of LC3 were of ACD when the background staining diminished as residual LC3 molecules in acidophilic cell debris were gradually depleted. IHC of mitochondria (AE-1) employed detected aggregated mitochondria but not scattered mitochondria in cytoplasm such as those in small lymphocytes. Immunostaining of cathepsin D in lymphoma cells was graded when intermingling macrophages showed very strong staining.

peculiar necrosis in EBV+ NKTCL, cytotoxic-TML and EBV+ BML (Fig. 5b4) was grouped ACD when the case free from degeneration showed sporadic distribution of ACD (Fig. 4b2).

EBV+ NKTCL cells in the cellular area of the cases with degeneration and necrosis showed significantly more aggregated mitochondria (Fig. 4c3) than those in the cases free from degeneration (Fig. 4c2, Table 6, #4 p=0.0103). Labeled aggregated mitochondria decreased significantly in the degenerative areas of the cases with degeneration (Table 6, #6 p=0.0384) but not in the degenerative areas of the cases with degeneration and necrosis (Fig. 4c4), suggesting delayed degradation of mitochondria in the autolysosome with degeneration and necrosis. Faint stain of aggregated mitochondria remained in residual and degenerative EBV+ NKTCL cells in the necrotic area (Fig. 4c5), as in the cases of EBV+ BML with necrosis (Fig. 5c4), suggesting incomplete degradation of aggregated mitochondria in the autolysosome.

EBV+ NKTCL cells in the cellular area of the cases with degeneration and necrosis indicated lower expression cathepsin D (Fig. 4d3) than those in the cellular area of the cases free from degeneration (Fig. 4d2) and those with degeneration (Table 6), as did EBV+ BML cells in the cellular area of the cases with necrosis (Table 6, Fig. 5d3) than EBV+ BML cells (Fig. 5d1). In EBV+ NKTCL free from degeneration, engulfed aggregative mitochondria would be completely digested (Fig. 4c2) by a sufficient amount of cathepsin D (Fig. 4d2) under enhanced autophagy that induced sporadic ACD in the neoplastic growth (Fig. 4b2). On the other hand, in EBV+ NKTCL with degeneration and necrosis, enhanced autophagy and reduced expression of cathepsin D (Fig. 4d3) induced incomplete degradation of engulfed aggregated mitochondria (Fig. 4c3) and grouped ACD in the necrosis (Fig. 4b5). One of three cases of EBV+ BML indicated no expression of cathepsin D (Table 6, Fig. 5d3) and was associated with tiny ill-defined foci of necrosis with grouped ACD (Fig. 5b4).

One case of EBV+ BML formed an intraepithelial microabscess filled with degenerative lymphoma cells. The EBV+ BML cells in the microabscess showed faint immunostaining of beclin-1 (Fig. 5a2), some traces of ACD labeled by LC3 (Fig. 5b2), some aggregated mitochondria (Fig. 5c2) and no expression of cathepsin D (Fig. 5d2), reflecting an end state of autophagy with ACD in starvation when autophagy was active in the lymphoma cells in the cellular area (Table 6, Fig. 5a1, 5b1, 5c1 and 5d1).

IV. Discussion

This study analyzed PCD in nasopharyngeal lymphomas by means of IHC. IHC of anti-cleaved caspase-3 antibody signaled a new age in detecting apoptosis in human pathology specimens [8]. However, nasopharyngeal lymphoma cells express survivin (inhibitor of apoptosis-1) and do not show cleaved caspase-3-positive apoptotic cells, suggesting that survivin suppresses apoptosis [32], as shown in Figure 3. This is the first immunohistochemical study to analyze autophagy on sections of archival pathology specimens of human malignant neoplastic cells that show no apoptosis. As shown in Figure 6, the targets of IHC in this study are the indicators of autophagy between the autophagy initiating stage and the lysosomal enzymes activating stage. It is well known that the PI3K—Tor path-
The cellular areas (a to f) 
H.E. (a), TIA1 (b) and CD56 (d) 

ISH of EBER-1 (d), cleaved caspase-3 (e) and survivin (f) 

The degenerative and necrotic areas (g to i) 
H.E. (g), cleaved caspase-3 (h) and survivin (i) 

Fig. 3. A representative nasal NK/T-cell lymphoma (NKTCL). The long axis of all the microphotographs was 215 μm long at ×40 magnification. a to f cellular areas. g to i degenerative and necrotic areas. a and g: hematoxylin-eosin staining. NKTCL cells in the cellular areas (a) are positive for TIA1 (b) and CD56 (e) and show signals of EBER-1 ISH (d). Rare cleaved caspase-3-positive lymphoma cells were observed in cellular areas (e) and in degenerative and necrotic areas (h). The NKTCL cells expressed survivin (f and i). Necrosis in NKTCL was not due to apoptosis suppressed by neoplastic expression of survivin.

Table 5. Autophagy-related molecules and organelle in nasopharyngeal lymphomas in representative cellular areas free from degeneration and necrosis

| EBV         | N  | Beclin-1 | LC3 | Mitochondria (AE-1) | Cathepsin D |
|-------------|----|----------|-----|---------------------|-------------|
| TML         | 28 | 2.6 (1.0) | 2.3 (0.9) | 1.1 (0.9) |             |
| NKTCL       | –  | 2        | 2.2 (1.4) | 1.0 (1.4) | 1.0 (1.4) |
| NKTCL       | +  | 21       | 2.1 (1.2) | 1.0 (0.9) | 0.9 (1.0) |
| Cytotoxic-TML| +/− | 3     | 1.0 (1.0) | 2.0 (1.4) | 2.0 (1.4) |
| E-NKTCL     | +/− | 2     | 1.7 (0.4) | 1.3 (1.5) | 1.2 (0.0) |
| BML         | 9  | 2.9 (0.9) | 1.6 (0.9) |             |             |
| B-ML        | –  | 3        | 1.3 (0.6) | 2.3 (1.2) | 2.0 (1.0) |
| B-ML        | +/−| 3        | 1.7 (0.6) | 3.0 (1.0) | 1.7 (1.2) |
| B-ML        | +  | 3        | 2.3 (0.6) | 3.3 (1.2) | 1.0 (1.0) |
| B-ML        | +  | 3        | 2.3 (0.6) | 3.3 (1.2) | 1.0 (1.0) |

TML, T/NK-cell neoplasms; BML, B-cell neoplasm; NKTCL, NK/T-cell lymphoma; Cytotoxic-TML, Cytotoxic T-cell lymphoma; E-NKTCL, Early NK/T-cell lymphoma; EBV+, Complete neoplastic expansion of EBV latent infection (Fig. 1); EBV−; Incomplete neoplastic expansion of EBV latent infection (Fig. 1); EBV−, No EBV infection in lymphoma cells (Fig. 1). Statistical evaluation: Evaluated scores in IHC of each antibody were tested in all combinations of two entities of lymphomas by Mann-Whitney’s U test and in all combinations of more than three entities by Kruskal-Wallis test. In IHC of cathepsin D, only EBV+ NKTCL indicated significantly lower values than cytotoxic TML (#1: Mann-Whitney’s U test, p=0.0387). Significantly lower values were also seen in the order of EBV+ BML, cytotoxic TML and EBV+ NKTCL (2: Kruskal-Wallis test, p=0.0396), in that order.
nizing microgranular, macrogranular and dense perinuclear or nuclear immunostaining of anti-LC3 antibody in NKTL cells employing heat AR independent from the pH of the solution [32] when ordinary AR-IHC of anti-LC3 antibody was reported to label LC3-II in the human pathology specimens [5, 26]. These three patterns in the immunostaining of LC3 seemed to correspond to the cellular images of baseline autophagy, enhanced autophagy and ACD under a fluorescence microscope and an electron microscope. This study tried to detect mitochondria as macroautophagic target-organella and to see first the engulfed and degraded mitochondria in autophagosome and autolysosome by means of IHC of AE-1. Detecting beclin-1, LC3, aggregated mitochondria and cathepsin D by IHC, it became finally possible to monitor the condition of autophagy on sections of the archival pathology specimens.

We elucidated that NKTL cells in the case free from degeneration showed sporadic distribution of ACD, as shown in Figure 4b2. The proliferation with sporadic ACD of the NKTL cells in the case free from degeneration can be regarded to be that of the steady state of growth maintaining a mass of NKTL cells with certain ratios of

![Fig. 4. Immunostaining of beclin-1, LC3, mitochondria (AE-1) and cathepsin D in NKTL. 1: EBV- NKTL. 2: EBV+ NKTL free from degeneration. 3 to 5: EBV+ NKTL revealing cellular (3), degenerative (4) and necrotic areas (5). a to d: Immunostaining of beclin-1 (a), LC3 (b), AE-1 (c) and cathepsin D (d). The long axis of all the microphotographs was 86 μm long at ×100 magnification. EBV- and EBV+ NKTL cells in the cellular areas (1–3) showed expression of beclin-1 (a1 and a3), macrogranular staining with background microgranular staining (b1 and b3) or sporadic nuclear stain of LC3 (b2), aggregated mitochondria in many cells (c2 and c3) and expression of cathepsin D in lymphoma cells (d1 and d2). EBV+ NKTL with degeneration and necrosis showed gradually decreased expression of beclin-1 (a3 to a5), transition from macrogranular (b3 and b4) to nuclear stain of LC3 (b5), aggregated mitochondria labeled by AE-1 (c2, c3 and c4) and reduced expression of Cathepsin D (d3, d4 and d5). These findings indicated a baseline of autophagy with complete degradation of engulfed mitochondria in EBV- NKTL cells (1), enhanced autophagy with sporadic ACD and highly engulfed mitochondria in EBV+ NKTL cells in the case free from degeneration (2), and enhanced autophagy with incomplete degradation of engulfed mitochondria ending in regional ACD caused by reduced expression of cathepsin D in EBV+ NKTL cells in the case with degeneration and necrosis (3 to 5).]
proliferating cells and dying cells because of PCD [18] and possibly that of the steady state of growth of pancreatic beta cells in pancreas duodenal homeobox-1 (pdx1) deficient mouse maintaining a smaller mass and a smaller number of Ki67 antigen positive cells than in the normal mouse and beclin-1 knock-out mouse or pdx1 and beclin-1 double knock-out mouse [6].

Autophagy is enhanced in starvation. EBV− BML cells showed weak expression of beclin-1 (a1), macrogranular immunostaining of LC3 (b1), aggregated mitochondria (c1) and expression of cathepsin D (d1) when the lymphoma cells in the microabscess revealed little expression of beclin-1 (a2), nucleus-like staining of LC3 (b2), aggregated mitochondria (c2) and no expression of cathepsin D (d2), suggesting a baseline of autophagy in EBV− BML and ACD with incomplete degradation of mitochondria and no expression of cathepsin D in EBV− BML cells in starvation in the microabscess.

EBV+ DLBCL with peculiar necrosis

Cellular area (3)

Peculiar necrosis (4)

We found that the peculiar necrosis in NKTCL was of grouped LC3-densely labeled cells (ACD) in the ill-defined areas [32] (regional ACD). The peculiar necrosis was noted in EBV+ NKTCL, EBV+ Cytotoxic-TML and EBV− BML (Table 6), suggesting that the regional ACD was induced under EBV infection [32] when the lymphoma cells showed complete neoplastic expansion of EBV latent infection (Fig. 1) [32].

The EBV infection in EBV+ NKTCL is of Latency II [28] expressing a quite large numbered copies of EBER-1, small numbered copies of BamH1-A rightward transcripts (BARTs), EBV nuclear antigen-1 (EBNA-1) and Latent membrane proteins (LMPs). Viral products, including EBNA-1, are processed by autophagy and are presented on cell surface with HLA class II molecules [23]. EBER-1 originated from EBV+ cells is recognized as double-stranded DNA.
Autophagy and ACD in Nasopharyngeal Lymphomas

Table 6. Autophagy-related molecules and organelia in nasopharyngeal lymphomas in areas with degeneration and necrosis

|                      | Score value or Mean score value (S.D.) of IHC |
|----------------------|-----------------------------------------------|
|                      | Beclin-1 | LC3     | Mitochondria (AE-1) | Cathepsin D |
| EBV-BML              |          |         |                     |             |
| +microabscess        | n C D N | C D N   | C D N               | C D N       |
| EBV+BML              | 2 2.5 (0.7) | 4 (—)   | 3 (—)               | 1.5 (0.7)   |
| +necrosis            | 1 2 1 1 | 2 2 4   | 3 1 1               | 0 0 0       |
| Cytotoxic-TML        | 1* 2     | 4       | 2                   | 2           |
| +necrosis            | 2** 0.5 (0.7) | 3 (1.4) | 2.0 (1.4)           | 2 (0.7)     |
| EBV+ NKTCL cellular areas | 4 2.0 (1.8) | 3.3 (0.6) | 2.0 (0.8) | 1.5 (1.3) |
| +degeneration        | 6 1.8 (0.8) | 3.0 (0.6) | 2.5 (0.8) | 1.2 (0.8) |
| +degeneration and necrosis | 11 2.4 (1.1) | 3.1 (0.7) | 2.8 (0.8) | 0.5 (0.7) |

n, Number of cases; C, Cellular area; D, Degenerative area; N, Necrotic area; EBV- BML+microabscess, EBV infection-free BML revealing intraepithelial microabscess; +necrosis, lymphoma revealing foci of necrosis; +degeneration and necrosis, lymphoma revealing degeneration and necrosis; Cytotoxic-TML 1*, one EBV- case; Cytotoxic-TML+necrosis 2**, two EBV- cases.

Statistical evaluation: NKTCL cells in the cellular area indicated significant gradual decrease in LC3 score in the groups with cellular, degenerative and necrotic areas (**: Kruskal-Wallis test, p=0.0036, #: Mann-Whitney’s U test, #p=0.0103, #3 p=0.0062) and significant increase of Mitochondria (AE-1) score (#1, 2 p=0.0103, #3 p=0.0062) and significant decrease in Mitochondria (AE-1) score (#12, 13 p=0.0041, #14 p=0.0030). NKTCL cells in the group with degenerative and necrotic areas indicated significant decrease of beclin-1 score from groups with the cellular to necrotic areas (#7, 8 p=0.0076, #13 p=0.005) and significant increase in LC3 score (#9–11 p=0.0041). NKTCL cells in the group with degenerative and necrotic areas indicated significant decrease of beclin-1 score from groups with the cellular to necrotic areas (#12, 13 p=0.0076, #13 p=0.005) and significant increase in LC3 score (#9–11 p=0.0041, #10 p=0.0022, #11 p=0.0041), significant decrease in Mitochondria (AE-1) score (#10, 11 p=0.0041) and significant decrease in cathepsin D score (#11, Wilcoxon signed-ranks test, p=0.0455).

RNA by Toll-like receptor 3 and retinoic acid inducible gene-1 in the endosome (Fig. 6) and induces cellular anti-viral innate immunity responses such as production of type 1 interferon and secretion of proinflammatory cytokines in infectious mononucleosis, chronic active EBV infection and EBV-associated hemophagocytic lymphohistiocytosis [13]. However, innate immunity has not yet been reported to play a role in histogenesis of EBV-associated hemophagocytic lymphohistiocytosis [13]. In EBV- lymphoblastoid cell line and EBV+ Burkitt’s lymphoma cell line, low level of expression of LMP-1 maintained B-cell growth and enhanced autophagy when high expression level of LMP-1 induced cytostasis and transient inhibition of protein synthesis [19]. The stimuli from a low expression level of LMP-1 [16] with suppressed expression of Bcl-2 [32] has an indirect effect on beclin-1 to form a vesicle nucleation complex (Fig. 6) and enhance autophagy in EBV+ NKTCL free from degeneration and necrosis. Autophagy suppressed hypersensitive response inducing bystander tissue necrosis in tobacco mosaic virus infection [19].

EBV+ NKTCL free from the degeneration showed enhanced autophagy in Table 6, although the middle score of beclin-1 (Fig. 4a2, Table 6) suggested possibly directly enhanced elongation of autophagophore labeled by LC3 rather than the effect of beclin-1 under LMP-1 mentioned above, indicated by significantly high LC3 score (Fig. 4b2), significantly low aggregated mitochondria score (Fig. 4c2) and high cathepsin D score (Fig. 4d2). EBV+ NKTCL cells in the cellular areas in the cases with degeneration and necrosis showed suppressed autophagy as indicated by the likely compensatory high beclin-1 score (Fig. 4a3), significantly low LC3 score (Fig. 4b3), significantly high aggregated mitochondria score (Fig. 4c3) and significantly low cathepsin D score (Fig. 4d3) in Table 6.

However, the low synthesis of lysosomal enzymes cannot be explained by the transient low synthesis of proteins under the high level of LMP-1 expression. Protein kinase RNA regulated (PKR), induced by interferon-α under viral infection but usually suppressed by binding with EBER-1 [21, 22], can suppress protein synthesis. Depletion of a small amount of EBER-1 in cytoplasm under enhanced autophagy may reanimate PKR and induce low synthesis of proteins such as that of cathepsin D in the EBV+ NKTCL.
Hasui et al.

Cells in the cellular areas in the cases with degeneration and necrosis (Fig. 4d3, Table 6) as well as in the EBV + BML cells in the cellular areas (Fig. 5d3, Table 6). EBV + NKTCL with degeneration and necrosis suggested that advanced autophagy with low protein synthesis induces ACD of lymphoma cells in the regional areas (Table 6) probably by the destruction of symbiotic growth with CD204 + macrophages [32]. Tiny foci of ACD in EBV + BML unassociating with symbiotic CD204 + macrophages were also characteristic of which would be categorized as lymphomatoid granulomatosis in the WHO classification [14].

This study indicated that the IHC of beclin-1, LC3, mitochondria and cathepsin D could monitor the status of autophagy in nasopharyngeal lymphomas. It was also shown that ACD is caused by enhanced autophagy with failures in lysosomes and autolysosomes when apoptosis is suppressed and is not the result of molecular signaling in autophagy. Finally, in EBV + NKTCL, enhanced autophagy and reduced expression of cathepsin D induced peculiar necrosis of the regional ACD.

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