The role of eosinophils in necrosis mainly in single-system Langerhans cell histiocytosis

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Abstract

Background: Single-system Langerhans cell histiocytosis (LCH) shows massive infiltration of eosinophils and occasional necrosis and cyst/cavity formation, but the cause of the necrosis remains unclear. The role of eosinophils in necrosis in single-system LCH has not been examined yet, and therefore, we investigated their role pathologically.

Methods: Biopsy/lobectomy specimens were collected in 49 cases (50 lesions, 30 males, 19 females; mean age 31 years) satisfying the pathological criteria of LCH from 22 institutions from the beginning of 1998 to the end of 2011. Sources of organs were 33 lungs and 17 other organs. Forty-seven cases showed single-system LCH and 2 cases showed multiple-system LCH without involvement of "Risk Organs". The interrelationship between eosinophils and necrosis, various kinds of tissue destruction, and subsequent findings were investigated. Cellular features of the earliest LCH lesion were also examined in detail. Ordinal histopathological and immunohistochemical examinations that included anti-eosinophilic antibodies were used.

Results: Destructive features and subsequent findings of pulmonary LCH from 30 cases were as follows: vascular destruction, 6; cyst/cavity formations, 22; filling of tissue defect, 15; and elastic tissue, 30, and reticulin fiber destruction, 17. Tiny necroses accompanying degranulation of dead eosinophils without fibrosis were present in 2 cases. Large numbers of Langerhans cells and eosinophils were observed in the cellular-stage lesions and were associated with each other. Destructive features and subsequent findings of non-pulmonary LCH from 17 cases were as follows: coagulative necrosis of various extent without fibrosis, 8; cavity formation and cell shedding, 5; and reticulin fiber network destruction, 15. Large numbers of dead eosinophils accompanying degranulation were noted in most of the necrotic lesions along with a few Charcot-Leyden crystals. Degranulation of neutrophils in the necrotic lesions was limited to live cells.

Conclusions: These findings suggest an intimate relationship between preceding degranulation of eosinophils in necrosis and various kinds of tissue destruction following necrosis in LCH.

Keywords: Degranulation of eosinophils, degranulation of neutrophils, Langerhans cells, pathological examination

Introduction

Langerhans cell histiocytosis (LCH) integrates eosinophilic granuloma, Hand-Schüller-Christian disease, and Letterer-Siwe disease due to the proliferation of histiocyte-like cells, which have now been proved to be Langerhans cells [1,2]. The *BRAF* V600E mutation is an important genetic factor in LCH [3], and new terminology (inflammatory myeloid neoplasia) was introduced [4,5].

Nodular-shaped, single-system LCH is mainly composed of Langerhans cells and eosinophils histologically, and hence it was first named eosinophilic granuloma [6]. Serial computed tomography scans have suggested that radiological pulmonary LCH progresses over time from nodules to cavitated nodules and then to thick-walled cysts [7,8]. Intralesional cytokines released from Langerhans cells and T cells are thought to play a role including in necrosis, especially in bone and other organs [9-11], but the cause of this necrosis, which is followed by a destructive process, remains unclear, and until now, the role of eosinophils has also remained unclear. We have attempted to disclose the interrelationship of necrosis in LCH with eosinophilic degranulation from the pathologic aspect and have achieved this aim.

Materials and methods

Patients and tissue collection

Biopsy/lobectomy specimens in 49 cases of LCH (50 lesions) were collected from 22 institutions in Japan from the beginning of 1998 to the end of 2011. The patients comprised 30 males and 19 females whose ages ranged from 1 month to 62 years (mean age, 31 years). No patient was treated before a diagnosis was made. Sources of organs were lungs, 33; bones, 14; lymph nodes, 2; and soft tissue, 1. Histopathological methods used for diagnosis were surgical lung biopsy (31 cases), transbronchial lung biopsy (1 case), lobectomy for lung cancer (1 case), and excisional biopsy or curettage at the time of procedure for other organs (17 cases). Histologically, all materials were diagnosed as LCH because of the aggregation of characteristic Langerhans cells having a convoluted nucleus with a groove, and this was confirmed by immunostaining using antibody for CD1a [5] (Figures 1A-1C) in the lesion by YK and NT. Forty-seven patients presented with single-system one organ LCH, and 2 patients presented with multiple-system LCH without involvement of "Risk Organs" (2 organs and 5 organs, respectively). Among the 33 patients with pulmonary LCH, 28 were smokers, 2 were non-smokers, and smoking

Figure 1. Histology of pulmonary Langerhans cell histiocytosis.

A. Panoramic view of many cysts/cavities (Elastica van Gieson, EvG). **B.** Characteristic features of LCH at the edge of a cavity (hematoxylin & eosin, H&E, ×400). **C.** Langerhans cells (anti-CD1a antibody, ×200). **D.** Artery (EvG, ×40). **E.** Shedding of Langerhans cells (reticular fiber staining, ×100).

status in 3 was unknown.

This study was an interdisciplinary, multi-institutional, retrospective study approved by the Ethics Committee of Saitama Prefectural Cardiovascular and Respiratory Center (no. 2015013; Dec. 28, 2015) and each participating institution.

Lung

A representative single slide containing the earliest-stage lesion was stained with hematoxylin and eosin (H&E), elastica van Gieson (EvG), and reticular fiber staining. Immunostaining was performed using EG2 (mouse monoclonal antibody to eosinophilic cationic protein; Nichirei Biosciences, Inc., Tokyo, Japan), anti-hNE antibody (mouse monoclonal antibody to neutrophilic elastase; Kyowa Pharma Chemical Co., Ltd., Toyama, Japan), and anti-CD4 antibody, anti-CD8 antibody, and anti-CD1a antibody (all mouse monoclonal antibodies from Nichirei Biosciences, Inc.).

Check points were the number of lesions, the presence of vascular destruction, the presence and numbers of cysts or cavities (tissue defects related to discharge of necrotic material), the presence of degenerated cell shedding into the cavity, filling of tissue defects by granulation tissue such as a filled cavity [12] or organized hematoma after lung laceration [13], the presence of disruption of elastic fibers and reticulin fibers, and the presence of necrosis per 1 slide. The extent of infiltration of eosinophils and neutrophils and their degranulation in necrosis were also checked. Cysts and cavities were evaluated as different categories. However, intermediate histological findings were noted between cysts and cavities, and the term cyst/cavity is used when differentiation was difficult.

Next, the numbers of inflammatory cells in one earliest-stage, non-necrotic lesion per high-power field (HPF, $\times 400$) were counted with the help of immunostaining. The presence of ≥ 100 cells/HPF was semi-quantitatively expressed as density 3, 20–100 cells as density 2, and < 20 cells as density 1. The degree of cell shedding into the cavity was defined as follows: ≥ 100 cells as density 2 and < 100 cells as 1. The presence and numbers of granulocytic degranulation including necrotic areas were also checked, and the presence of ≥ 20 cells/HPF was classified as density 2 and < 20 was classified as density 1.

Bone, lymph node, and soft tissue

One slide was checked in 15 patients, and 3 slides were checked in 2 patients. Staining methods were the same as with the lung tissue. Check points were the presence of necrosis (including that of fibrosis and lytic or destructive change of the reticulin fiber network in necrosis), cavities, shedding of inflammatory cells into the cavity, and disruption of reticulin fibers in lesions per the entire slide. The degree of necrosis was defined as follows: > 1 cm² was classified as grade 3, intermediate as 2, and < 4 mm² as 1 per 1 slide. Numbers of inflammatory cells were counted in one representative earliest-stage lesion. The characteristics of the necrosis, necrotic cell structure, nuclear staining, and Charcot-Leyden crystals per the entire areas

of necrosis were also checked. The numbers of eosinophils and neutrophils and their degranulation were counted in the representative necrotic area.

Statistical analysis

Demographic data are expressed as the mean $\pm$ standard deviation (SD). The association between Langerhans cell density and that of other inflammatory cells was analyzed by Spearman's rank test. The Mann-Whitney U test was used to analyze comparisons in density between Langerhans cells and eosinophils and also between eosinophils and neutrophils, and the comparison of the density of eosinophils, neutrophils, and their degranulation between non-necrotic lesion and necrotic areas in non-pulmonary LCH. A *P* value of < 0.05 was considered statistically significant.

Results

Lung

Pathological characteristics of the 33 patients (22 men and 11 women, mean age 37 years) with pulmonary LCH are summarized in **Tables 1** and **2**. The mean number of lesions per 1 slide was 7.8. The lesions were observed at various stages (cellular, cellular and fibrous, and fibrous).

The general feature of the lesions was that of a tissue-destructive process and subsequent findings that were present in 6 cases of unilateral, muscular pulmonary arterial destruction (on the same side of the respiratory bronchiole, **Figure 1D**) at the edge of the cavity wall, 22 cases with a mean of 3.1 cyst/cavity formations (**Figure 1A-1E**), 20 cases of shedding of inflammatory cells into a cellular-stage cavity except for one cellular and fibrous-stage cyst/cavity (**Figure 1C** and **1E**), 15 cases of filling of a tissue defect by granulation tissue

Table 1. Patient sex and age and pathological features of one representative slide from the 33 pulmonary LCH cases.

	Number (%)	Mean $\pm$ SD
Sex (male/female)	22/11	--
Age (years)	--	37 $\pm$ 15
Number of lesions	--	7.8 $\pm$ 7.0
Vascular destruction	6 (18)	--
Cyst/cavity formation	22 (67)	3.1 $\pm$ 3.7
Shedding of inflammatory cells into cyst/cavity	20 (61)	1.7 $\pm$ 0.5
Filling of tissue defect	15 (45)	0.8 $\pm$ 1.0
Destruction of elastic fibers	30 (91)	--
Destruction of reticulin fibers	17 (52)	--
Necrosis of lesion	2 (6)	E 2.5, D/E 2, N 1.5, D/N 1

D/E: Degranulation of eosinophils; D/N: Degranulation of neutrophils; E: Eosinophils; LCH: Langerhans cell histiocytosis; N: Neutrophils; SD: Standard deviation

(**Figure 2A**), and in 30 and 17 cases of structural framework destruction (elastic tissue and reticuline fibers, respectively, **Figure 2B** and **2C**). Tiny necroses of up to 1 mm in size (**Figure 2D**) with reticuline framework destruction were noted in 2 cases, and the density of eosinophils and their degranulation in these necroses were 2.5 and 2 including live, degenerative, and dead eosinophils (cells with an unstained or ghost-like nucleus) (**Figure 2D**, upper and lower insets), and those of neutrophils (only live cells) were 1.5 and 1, respectively.

The density of the inflammatory cells is noted in **Table 2**. The proliferation of Langerhans cells and infiltration by various inflammatory cells were variously distributed (focal to diffuse) in even one lesion. In the earliest stage of the lesion, the density of Langerhans cells was 2.6, eosinophils (**Figures 1B** and **2E**) was 2.1, and neutrophils was 0.7. The difference in density was not statistically significant between Langerhans cells and eosinophils, but it was statistically significant between eosinophils and neutrophils. An association with the density of Langerhans cells was noted between that of eosinophils, degranulation of eosinophils (**Figure 2E** inset, black arrow) with uptake by macrophages (**Figure 2E** inset, red arrow), and degranulation of neutrophils.

Bone, lymph node, and soft tissue

Pathological characteristics of the 17 non-pulmonary LCH cases (10 males and 7 females, mean age 20 years) are summarized in **Tables 3** and **4**. The lesions were at various stages, and the general feature of the lesions was that of a tissue-destructive process and necrosis. Histological findings were as follows: necrosis of various extents at the cellular stage except for one lesion in 8 cases (microscopic to extensive, **Figure 3A**), no fibrosis but destruction of the reticuline network in necrosis (**3B**), cavity formation and cell shedding in 5 cases (mean 1.8 among 5 cavities, **Figure 3C**), and reticuline fiber network destruction of various degrees, including in every necrotic area, in 15 cases (**Figure 3B** and **3C**).

The distribution of eosinophils and neutrophils in the lesions varied from area to area. In the earliest cellular-stage lesion, LCH was mainly composed of Langerhans cells (density 3 in all) and eosinophils, with no significant difference, but the difference between the density of eosinophils and neutrophils was statistically significant.

Characteristics of the necrosis are noted in **Table 4**. Mean size was grade 2.3, and 4 cases showed multiple necroses. All cases showed coagulation necrosis without lytic necrosis. The

Figure 2. Destructive features of pulmonary LCH.

A. Filled cavity. (EvG, ×100). **B.** Destruction of elastic fiber networks with 1 tiny cavitation and cellular filling. (EvG, ×100). **C.** (reticulin fiber staining, ×200). **D.** A necrosis (H&E, ×100). Upper inset: (H&E, ×800). Lower inset: (EG2, ×800). **E.** Eosinophils around and in the cavity (EG2, ×100). Lower inset: (EG2, ×800).

Table 2. Density of inflammatory cells of the earliest lesion in the 33 pulmonary LCH cases (cellular [n=24], cellular and fibrous stage [n=9]).

	N	Positive	Density	Association
Langerhans cells	33	--	2.6±0.6	--
Eosinophils	33	--	2.1±0.9 NS	r ² =0.2187 P=0.0070
Degranulation of eosinophils	33	55%	0.9±0.9	r ² =0.1614 P=0.0227
Neutrophils	33	--	0.7±0.8 P<0.0001	r ² =0.1512 P=0.0278
Degranulation of neutrophils	33	18%	0.3±0.5	NS
CD4+ T cells	28	--	1.2±0.6	NS
CD8+ T cells	25	--	1.3±0.7	NS

Association: association with Langerhans cells;
LCH: Langerhans cell histiocytosis;
N: examined number; NS: Not significant.

Table 3. Patient sex and age and pathological features of slides and degree of inflammatory cells of the earliest lesion in the 17 non-pulmonary Langerhans cell histiocytosis cases.

	Number (%)	Mean±SD and significance
Sex (male/female)	10/7	--
Age	--	20±20 (1 M–58 Y)
Necrosis of lesion	8 (47)	0.5±1.0
Cavity formation	5 (30)	1.8±0.1*
Shedding of inflammatory cells	5 (30)	--
Destruction of reticulin fibers	15 (88)	--
Representative lesion of the earliest cellular stage		
Langerhans cells	--	3±0
Eosinophils	--	2.2±0.9, NS
Degranulation of eosinophils (n=16)	9 (56)	0.8±0.8
Neutrophils	--	1.1±0.9, P<0.001
Degranulation of neutrophils (n=14)	3 (21)	0.2±0.4

M: month; Y: years; NS: not significant; SD: standard deviation.
*Among 5 cavities.

cell structure was preserved in 6 cases. Nuclear staining was either ghost-like or showed no staining (**Figure 3A**, inset). A few nuclear fragmentations were observed in 3 cases, and a few Charcot-Leyden crystals were noted in 2 cases (**Figure 3D**, inset arrow).

Infiltration of eosinophils and neutrophils into necrosis varied from area to area, and in 3 cases, both eosinophils and neutrophils (**Figure 3D** and **3E**) had predominantly infiltrated into the necrotic area. The densities of the eosinophils and neutrophils were counted in the most infiltrated area and were 2.9 and 2.8, respectively, with no statistical difference. Most of eosinophils in the necrotic areas were either dead or degenerated eosinophils (**Figure 3D**), but the neutrophils were all alive with a clear nucleus (**Figure 3F**). The densities of eosinophils and neutrophils with degranulation were 1.9 and 2.0, respectively (**Figure 3D** and **3F**, black arrow), and were

Table 4. Characteristics of the necrosis in 8 non-pulmonary Langerhans cell histiocytosis cases.

	Value	P-value
Size (mean±SD)	Grade 2.3±0.9	--
Numbers of necroses	4 with multiple, 1 with two, 3 with one necrosis	--
Character	8, coagulation necrosis	--
Necrotic cell structure	6 preserved, 2 not preserved	--
Nuclear staining and shape of necrotic cells	6 ghost-like with preserved shape, 6 with no staining, 3 fragmented	--
Charcot-Leyden crystals	2 (a few among grade 3 necrosis)	--

Density of cellular infiltration

Eosinophils (mean±SD)	2.9±0.6	NS*
Degranulation of eosinophils(mean ± SD)	1.9±0.4	0.0030*
Neutrophils (6) (mean ± SD)	2.8±0.4, NS†	0.0007*
Degranulation of neutrophils (4) (mean ± SD)	2.0±0.0	0.0008*

ND: not determined; NS: not significant; SD: standard deviation.

*Density of cellular infiltration into the necrosis and degranulation and statistical comparison between the 17 lesions in Table 3 and the 8 necrotic lesions in Table 4.

†NS compared with eosinophils

statistically significantly denser than that in the 17 non-necrotic lesions. In one slide that contained multiple necroses, one area showed no eosinophils or neutrophils.

Discussion

In this study, we confirmed that a) pulmonary LCH showed a destructive process, including cyst/cavity formation and the destruction of arteries and of elastic and reticulin fiber networks; b) non-pulmonary LCH showed necrosis and a destructive process including cavity formation and reticulin fiber network destruction; c) dead eosinophils with degranulation were present to a significant degree in non-fibrotic, necrotic areas especially in non-pulmonary LCH; and d) an early feature of the lesion was its composition of Langerhans cells and eosinophils. Focusing on necrosis and cavity formation, we found the frequency and degree of necrosis to be clearly different between lung and other organs and tried to develop a unified explanation for this difference.

LCH of bone is characterized by the destruction of bony trabeculae, infiltration of eosinophils, and frequently focal but occasionally extensive coagulation necrosis [14-17]. Degranulation of eosinophils (with the release of three cationic proteins) and subsequent uptake of the proteins in macrophages was reported but no mention was made of tissue necrosis [16]. LCH of lymph nodes also shows various degrees and frequencies of necrosis [18,19]. The presence of Charcot-Leyden crystals was noted in coagulation necrosis in 3 of 18 cases [18]. Overall,

LCH of bone and lymph nodes showed a high frequency of coagulation necrosis and eosinophilic abscess [14-19]. We speculated that a) the earliest cellular lesion begins with the proliferation of Langerhans cells and infiltration of eosinophils, and b) by an unknown stimulus, eosinophilic degranulation in the cellular stage without fibrosis causes tissue necrosis, based on the presence of degranulated, dead eosinophils and some Charcot-Leyden crystals remaining in necrotic area with reticulin network destruction as a preceding phenomenon. Massive infiltration of neutrophils with degranulation might be a secondary phenomenon following necrosis as most of the cellular staged lesions contained limited numbers of neutrophils. Necrosis and subsequent destruction of lung structure and cavitation due to degranulation of eosinophils in parasitic pulmonary infections were reported because of the presence of dead eosinophils with degranulation and the escape of worms from the disrupted artery in necrosis [20,21]. Meanwhile, it was reported that the number of neutrophils equaled or exceeded the number of eosinophils in 21% of cases [14]. In the present study, detection of neutrophils was based on enzyme activity (elastase), and degranulation of neutrophils was limited to live cells. When neutrophils die, no enzyme activity can be detected by this method. One

area of necrosis showed no dead eosinophils, so it is possible that neutrophilic degranulation caused coagulation necrosis followed by death of the neutrophils and the complete disappearance of enzyme activity. However, we could not confirm the role of neutrophils in necrosis at this time.

How can the high frequency of cyst/cavity formation of pulmonary LCH [22-28] and various forms of tissue destruction including vascular destruction be explained? There are different reports concerning the nature and the mechanism of cyst/cavity formation. As for its nature, it is separated between cavities [23-27] and cysts [22,28], and as for the mechanism, both necrosis [23,26] and bronchiolar epithelial damage progressing to dilatation (cysts) [22,28] are reported. A small percentage of necrosis is reported, but progression of necrosis to cavity formation is not mentioned [23,26]. One of the present authors (YK) observed coagulation necrosis of 2 mm in size in the center of the lesion of an unreported case (personal experience). Although the frequency and degree of necrosis are mild, we think necrosis can explain subsequent phenomena including cavitation and filling of tissue defects because pulmonary LCH and non-pulmonary LCH are the same disease, and cavitation always follows necrosis. We speculated that necrosis would take place at the cellular stage

of LCH like non-pulmonary LCH as a) necrotic area showed destruction of the reticulin network without fibrosis, and b) almost all cell shedding with/without reticulin fiber network destruction into cavities was observed at this stage. The initial size of the necrosis might not be extensive, but soon, the size of the cavity enlarges due to the mechanism of an early-staged tension cavity seen in pulmonary tuberculous [29] or to traction from surrounding normal lung such as laceration of the lung seen in vascular Ehlers-Danlos syndrome [13] or in trauma. Cellular and degenerated tissue shedding into this cavity might result in continuous enlargement of the cavity. The necrotic material might soon be discharged from the cavity, and this might be the main reason why the histological specimens did not contain necrotic materials in the cavity in contrast with non-pulmonary LCH. An increase of Langerhans cells from bronchoalveolar lavage fluid [30-32] might also reflect discharge of Langerhans cells from the cavity. When the cavity is filled with granulation tissue as for repair, it may result in a nodule as seen in Figure 2A. Pulmonary LCH is also thought to be incited by cigarette smoking in certain predisposed individuals [8,33].

Patients with involvement of single-system LCH in one organ have an excellent prognosis [34]. When patients show a protracted course, therapy to suppress eosinophilic activity, such as stopping smoking, might be a one choice in single-system LCH, including non-pulmonary LCH, as smoking activates eosinophils as in acute eosinophilic pneumonia [35].

Limitations of this study include its retrospective design, and thus we could not stain cytokines to induce and activate eosinophils and neutrophils in LCH. We also could not examine the role of Langerhans cells, T helper cells, and macrophages in tissue damage and necrosis.

Conclusion

The present findings suggest an intimate relationship between the preceding degranulation of dead eosinophils in necrosis and various kinds of tissue destruction both in non-pulmonary and pulmonary LCH. Further investigation to prevent necrosis is the next important step in the control of single-system LCH.

List of Abbreviations

EvG: Elastic van Gieson
H&E: Hematoxylin and eosin
HPF: High-power field
LCH: Langerhans cell histiocytosis
SD: Standard deviation

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Authors' contributions	YK	NT	MAT	TO	MK	YF	KK	YS
Research concept and design	✓	✓	✓	✓	✓	✓	✓	✓
Collection and/or assembly of data	✓	✓	✓	✓	✓	✓	✓	✓
Data analysis and interpretation	✓	✓	✓	✓	✓	✓	✓	✓
Writing the article	✓	--	--	--	--	--	--	--
Critical revision of the article	--	✓	--	--	--	--	--	✓
Final approval of article	✓	✓	✓	✓	✓	✓	✓	✓
Statistical analysis	--	--	--	--	--	--	✓	--

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