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Overexpression of LC3A autophagy protein in follicular and diffuse large B-cell lymphomas

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BACKGROUND AND OBJECTIVES: Autophagy is a self-degradation mechanism induced under stress conditions in all eukaryotic cells. Its activity in human lymphomas has not been studied as yet.

METHODS: In this study, the autophagic activity of lymphoid cells was investigated in follicular lymphomas (FL; 48 cases), diffuse large B-cell lymphomas (DLBCL; 78 cases), and in reactive follicular hyperplasias (41 cases), using the light chain 3A (LC3A) antibody and a standard immunohistochemical technique.

RESULTS: In all cases, the pattern of LC3A reactivity was uniformly diffuse cytoplasmic, but expressed more frequently in FLs (68.8%) than in DLBCLs (41%) ($p = 0.02$), and much more commonly in DLBCLs than in reactive lymph nodes (24.3%) ($p < 0.006$). Interestingly, FLs expressing LC3A in >10% of lymphoid cells (high reactivity) were associated with the hypoxia-related protein HIF1α and the enzyme of anaerobic metabolism lactate dehydrogenase LDH5 ($p = 0.004$ and $p = 0.003$, respectively). Such associations, however, were not a feature in DLBCLs of increased LC3A activity.

CONCLUSIONS: LC3A expression in FLs is hypoxia-induced, whereas its expression in DLBCLs may be regulated by other molecular mechanisms. The current study provides a tool for further assessment of autophagic activity in translational and autophagy targeting therapy studies.

Follicular lymphoma (FL) is a relatively common B-cell neoplasm (about 22% of the total)[1] with an indolent clinical course which, however, is interrupted by frequent relapses.[2] It has a tendency for histological transformation to diffuse large B-cell lymphoma (DLBCL),[3] a much more severe form of the disease. DLBCL remains the most common type of non-Hodgkin lymphoma, accounting for 30–40% of all human lymphoid neoplasias.[3,4] It is a rather aggressive, clinically heterogeneous neoplasm[5–8] and an important cause of disease-specific death.[2]

Autophagy is a self-degradation mechanism by which cells recycle their own cytoplasmic constituents to survive suboptimal micro-environmental conditions, such as starvation and oxygen deprivation.[9–11] This is accomplished by disposing of excess or defective organelles and long-lived proteins into the lysosomes, forming the so-called autophagic vacuoles. Although autophagy is a major cell survival pathway, its excessive activation may lead to massive degradation of cellular components shifting the balance to self destruction – the so-called autophagic cell death. The role of autophagy in the growth and progression of human malignancies has only recently emerged, mainly as a marker of aggressive behaviour for epithelial tumours,[12–16] but its significance in haematological/lymphoid neoplasms remains completely obscure.

In this study, we investigated the autophagic activity in a series of FL, DLBCL, and reactive follicular hyperplasias, using the light chain 3A (LC3A) antibody recognizing both the soluble (LC3A-I) and the membrane-bound form (LC3A-II) of the protein.[17,18] The results were related with intratumoral hypoxia, as

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assessed by the expression of hypoxia inducible factors 1α and 2α (HIF1α and HIF2α) and their downstream proteins vascular endothelial growth factor (VEGF) and lactate dehydrogenase 5 (LDH5).[19,20]

MATERIALS AND METHODS

Tissue microarrays (TMA) prepared from paraffin blocks of hyperplastic and neoplastic lymph nodes were obtained from patients with follicular lymphomas (FL; 48 cases), diffuse large B-cell lymphomas (DLBCL; 78 cases), and reactive lymph nodes (41 cases). The material was collected from the archives of the Department of Cellular Pathology, John Radcliffe Hospital, Oxford, UK, and ethical approval was obtained from appropriate Institutional Boards for this study.

The tissues were primarily stained for LC3A to assess autophagic reactivity in lymphoid tissues. The results were correlated with the hypoxia inducible factors 1α and 2α (HIF1α, HIF2α), and their downstream proteins lactate dehydrogenase 5 (LDH5) and vascular endothelial growth factor (VEGF). A standard immunohistochemical technique was used. Table 1 shows details of the primary antibodies, the working dilutions and the antigen retrieval methods used in this study.[12,21–23]

Immunohistochemistry for LC3A and other markers. The primary antibody used was a purified rabbit polyclonal antibody (AP1805a, Abgent, San Diego CA) capable of detecting both the LC3A-I and LC3A-II forms. The antibody does not recognize the LC3B form (data not shown). A standard immunohistochemical technique was employed as previously described.[12] In brief, 3 μm thick sections were de-waxed and rehydrated in graded alcohol solutions. For heat-induced epitope retrieval, the sections were placed in citrate buffer (1:10 dilution, pH 7.2) and heated at 120 °C for 3 × 5 min. Endogenous peroxidase activity was neutralized using Peroxidase Block for 5 min. The non-specific binding was blocked by pre-incubation with Protein Block for 5 min at room temperature (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK). Slides were then incubated overnight at 4 °C with LC3A-II primary antibody diluted 1:20 (Abgent, San Diego, CA). The slides were washed with PBS (2 × 5 min) and then incubated with Post Primary Block for 30 min at room temperature (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK). They were subsequently washed in PBS for 2 × 5 min and incubated with NovoLink™ polymer for 30 min at room temperature (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK). After a thorough washing in BS (2 × 5 min), the colour

reaction was developed in 3,3′-diaminobenzidine (DBS) for 5 min. The sections were then counterstained with hematoxylin, dehydrated and mounted.

Controls
Normal rabbit immunoglobulin-G was substituted for the primary antibody as negative control. Staining with omission of the primary antibody was also performed as negative control.

Evaluation of LC3A reactivity, and other immunohistochemical markers
After immunohistochemical staining for LC3A, normal and malignant lymphoid cells showing a diffuse brown reactivity of the cytoplasm were recorded as positive. Other patterns of LC3A reactivity, such as a distinct linear condensation of stain around the nucleus (cytoplasmic/perinuclear pattern) or compact, amorphous, spheroidal structures within cytoplasmic vacuoles, the so-called "stone-like" structures (SLS), were not detected in malignant lymphomas or reactive lymph nodes.

The proportion of lymphoid cells with a diffuse cytoplasmic staining was determined in all optical fields at 200× magnification, and expressed as the mean of all counts. The cases under investigation were divided into groups of low and high LC3A reactivity, as follows.

Tumours with an LC3A staining in more than 10% of neoplastic lymphoid cells were considered as being of high LC3A reactivity.

The reactivity of HIF1α, HIF2α and LDH5 was mixed cytoplasmic and nuclear. Lymphoid tumours with nuclear HIF1α expression in more than 10% of neoplastic cells and/or a strong cytoplasmic expression in more than 50% of such cells were considered to be of high reactivity. The HIF2α and LDH5 reactivity was evaluated in a similar manner.

The reactivity of VEGF was purely cytoplasmic. The proportion of lymphoid cells with cytoplasmic staining per case was estimated. The median value was used as a cut-off point to define lymphomas of high and low VEGF reactivity.

The assessment was performed blindly by two independent observers (A.G. and E.S.).

Normal PBMCs
To assess the expression of LC3A and response to stress signals on normal peripheral blood mononuclear cells (PBMCs) with western blot, blood samples in EDTA were obtained from three healthy donors and PBMCs were isolated using standard procedures. The study was approved by the local Ethics and Scientific Committees. PBMCs were cul-

Table 1. Details of the antibodies, dilutions, and antigen retrieval methods used in this study.

Primary Antibody	Dilution/incubation Time[*]	Antigen Retrieval	Specificity	Source	Reference
AP1805a	1:20	MW[**]	LC3A	Abgent, CA	12
ESEE 122	1:20 (overnight)	MW	HIF-1α	Oxford University	21
EP 190b	Neat (overnight)	MW	HIF-2α	Oxford University	21
VG1	1:4 (overnight)	MW	VEGF	Oxford University	22
ab9002	1:200	MW	LDH5	Abcam, UK	23

[*]At room temperature.
[**]Microwave.

tured in RPMI-1640 medium that contained 10% fetal bovine serum, 2 mM L-glutamine, 1% penicillin, 1% streptomycin, at 37 °C, 5% CO_2 in air in Petri dishes. PBMCs were collected after four and 24 h following incubation with 100 nM Bafilomycin (an agent that blocks autophagosome/lysosome fusion) and with 150 mM of the hypoxia mimetic cobalt chloride ($CoCl_2$). Cultures were thereafter centrifuged for 30 min at 4000 rpm and stored in a sample buffer (0.5 M Tris [pH 6.8], 10% sodium dodecyl sulfate, 0.05% bromophenol blue, 10% β-mercaptoethanol, 10% glycerol) at −20 °C until analysis. Total protein concentrations were estimated according to the method of Lowry et al.[24] Protein extracts (40 μg) were separated on 12.5% SDS–PAGE gels and transferred to membranes. The anti-LC3A AP1805a primary antibody was added at 1:2000 dilution and incubated overnight at 4 °C, incubated for 2 h at room temperature with appropriate secondary antibody, rinsed, and developed in TMB (KPL Laboratories, Gaithersburg, MD). A molecular weight marker and protein extracted from normal human brain, and a tissue known to have a high concentration of LC3A, were used to verify the level where the protein bands appear.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 5.0 package (GraphPad, San Diego CA, www.graphpad.com). The Fisher's exact test (two-tailed) was used for testing relationships between categorical variables. The unpaired two-tailed *t*-test or linear regression analysis was used to compare groups of continuous variables. A *p*-value less or equal to 0.05 was taken as significant.

RESULTS

The LC3A expression

All positive lymphoid cells in the series, whether reactive or malignant, showed a diffuse cytoplasmic pattern of LC3A staining (Figure 1). The two other patterns of LC3A expression, the cytoplasmic/perinuclear and the so-called "stone-like" structures (SLS), previously described in malignant epithelial tumours,[12–16] were not observed in malignant lymphomas or reactive follicular hyperplasias. In western blot analysis (Figure 1f) normal PBMCs had very low levels of LC3A expression that were increased, mainly 4 h, after incubation with bafilomycin (accumulation of autophagosomes due to disrupted lysosomal fusion) and with Cocl2 (hypoxia mimetic agent).

LC3A reactivity was high in 33/48 (68.8%) FL, but only in 32/78 (41%) DLBCLs. This difference was statistically significant (*p* = 0.02). The mean ± SD percentage of lymphoid cells with LC3A expression was 38 ± 32% vs. 24 ± 34%, respectively (*p* = 0.02; Figure 2). With regard to the large cells (centroblasts) in follicular lymphomas, these expressed LC3A in a similar proportion to that of the centrocytes.

Cytoplasmic LC3A reactivity in more than 10% of lymphoid cells was also seen in reactive lymph nodes, but such an expression was present in 10/41 (24.3%) cases and restricted to the germinal centers. The mantle zone and the interfollicular area remained unreactive. It is obvious that both types of lymphoma had a significantly higher cytoplasmic LC3A reactivity than normal germinal centers (8 ± 17%) (*p* < 0.006).

Association of LC3A expression with hypoxia molecular variables. The association of LC3A expression with various markers upregulated by hy-
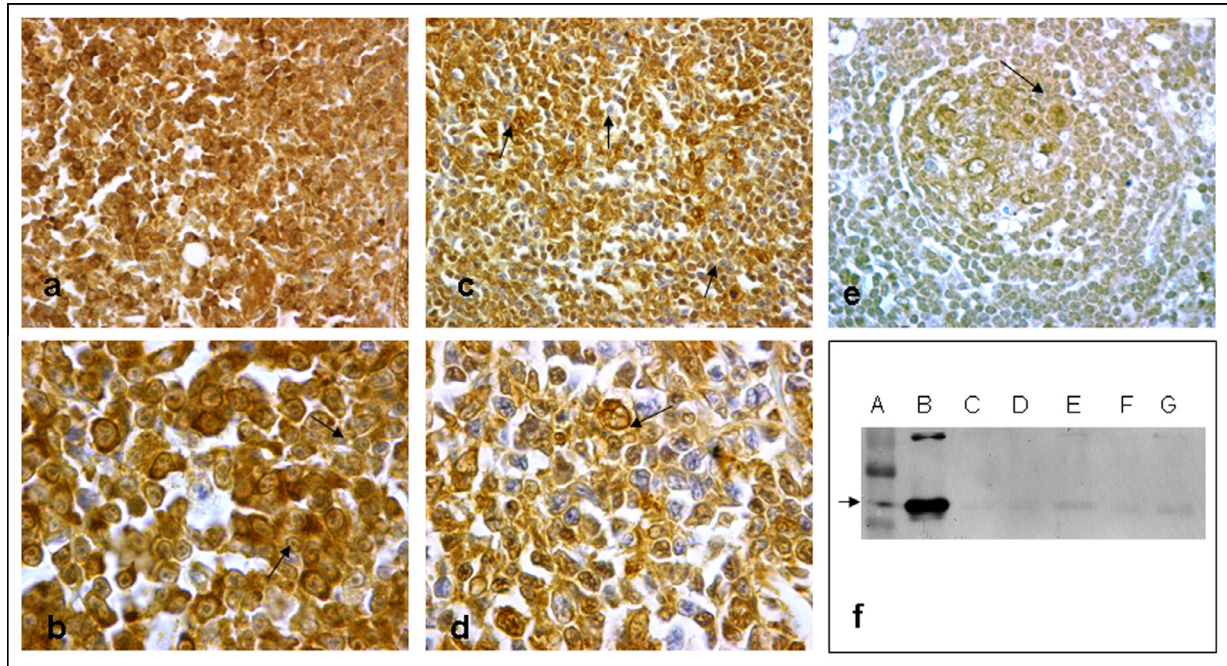
Figure 1. Diffuse cytoplasmic LC3A expression in FL (1a: x20, 1b: x40), DLBCL (1c: x20, 1d: x40) and in a reactive germinal center (1c, x20). Western blot analysis (1f) of normal PBMCs under various stess: A = molecular weight marker, B = normal brain with high LC3A expression, C and F = PBMCs in culture medium show low levels of LC3A, D and E = 100 nM of Bafilomycin induce slightly LC3A after 4 h and 24 h incubation respectively, G = PBMCs exposed to 150 μM Cocl2 for 4 h show slight increase of LC3A levels.
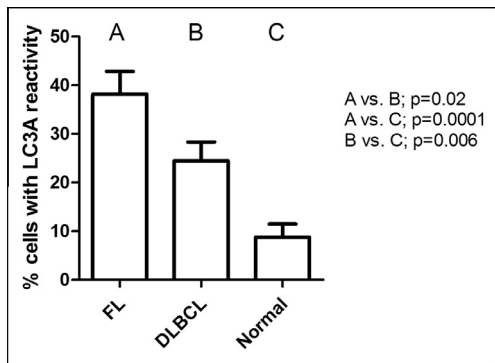

Figure 2. Schematic representation of cytoplasmic reactivity of LC3A in FL, DLBCL, and reactive germinal centers expressed as percentage of positive lymphocytes.

poxia and anaerobic metabolism are shown in Table 2.

FLs expressing high LC3A reactivity were associated with the hypoxia-related protein HIF1α ($p = 0.004$), and the enzyme of anaerobic metabolism LDH5 ($p = 0.003$). Such associations were not observed in DLBCLs expressing LC3A in more than 10% of lymphoid cells. No other significant associations were observed.

DISCUSSION

Autophagy is a common biological process activated under stress conditions in all eukaryotic cells including normal and neoplastic lymphocytes.[9–11] For, indeed, normal lymphoid cells exploit the phenomenon of autophagy to ensure lymphocyte maturation, proliferation, and survival; the redundant or faulty cellular constituents (long-lived proteins and dysfunctional organelles) may be selectively sequestered into autophagosomes and delivered to lysosomes for degradation and production of energy.[25–28] Furthermore, the neoplastic lymphoid cells may ensure their own survival by upregulating the same autophagic machinery.[11]

In this study, the state of autophagic activity was evaluated in FL and DLBCL after immunohistochemical staining for the LC3A antibody. In all cases, the reaction was diffuse cytoplasmic, corresponding presumably to the soluble form LC3A-I. It was more common in FL than in DLBCL, but it also occurred within reactive germinal centers, albeit in scanty lymphocytes. These findings reaffirm the upregulated state of autophagy in neoplastic tissues and reinforced its role in intra-nodal lymphocyte maturation and/or proliferation. Other patterns of LC3A expression, namely the cytoplasmic/perinuclear and the so-called

Table 2. Association of LC3 expression with hypoxia molecular variables in FL and DLBCL

| | | FL | | | DLBCL | |
	Low	High	*p*-value	Low	High	*p*-value
HIF1α						
Low	12	11	0.004	17	9	0.47
High	3	22		29	23	
HIF2α						
Low	12	19	0.19	32	19	0.46
High	3	14		14	13	
VEGF						
Low	11	21	0.74	23	14	0.64
High	4	12		23	18	
LDH5						
Low	10	7	0.003	9	4	0.54
High	5	26		37	28	

"stone-like" structures (SLS) which have been described previously in malignant epithelial tumors,[12–16] were completely absent from lymphoid tissues, both normal and malignant.

That FLs with an increased cytoplasmic LC3A expression are related to HIF1α over-expression and with its downstream protein LDH5 conforms with the contention that autophagy (as deduced from the increased expression of LC3A, and from cell cultures)[12] is induced by hypoxia. The latter triggers the release of hypoxia inducible factors 1α and 2α (HIF1α and HIF2α) which are key transcription factors regulating the expression of angiogenic factors, such as the vascular endothelial growth factor (VEGF), and enzymes, such as lactate dehydrogenase 5 (LDH5), that shift oxygen phosphorylation to anaerobic glycolysis.[19] More recently, it has been shown that under severe and prolonged hypoxia, an autophagic response may be facilitated further by the activating transcription factor 4 (ATF4) which plays a major role in the regulation of autophagic machinery, particularly in response to endoplasmic reticulum (ER) stress.[29]

An analogous association between LC3A and HIF1α has not been established for DLBCLs, and it is reasonable to infer that in this particular neoplasm, autophagic activity may operate through a HIF-independent pathway. It has been shown, for example, that autophagy is specifically induced by infection with intracellular pathogens,[30] and apoptotic signaling through B-cell antigen receptor (BCR) stimulation – BCR-mediated autophagy.[26] Such apoptotic stimuli were most commonly found in neoplastic cells.[31]

This study provides evidence that increased autophagic activity occurs in both types of lymphomas under investigation, and with a higher frequency in FLs than in DLBCLs. FLs expressing LC3A are closely linked with hypoxia and anaerobic metabolism whilst DLBCLs are not, indicating another non-hypoxia induced mechanism of autophagic process. As autophagy seems to be a major mechanism involved in lymphoma response to various cytotoxic and targeted agents, a tool is herein provided for further assessment in translational studies.[32]

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original research report

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