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ORIGINAL ARTICLE

Evaluation of apoptosis in classical Hodgkin's lymphoma comparing different methods

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Summary

Purpose: *Apoptosis is a type of programmed cell death (PCD) with specific morphologic changes in the dying cell. Since classical Hodgkin's lymphoma (cHL) is characterised by abnormalities in the apoptotic pathways, apoptosis may play a central role in its pathogenesis. Our purpose was to estimate the apoptotic process in cases of cHL using 3 different, widely accepted methods, comparing their results as well as with those found in the literature.*

Methods: *Detection of apoptosis was performed in 76 cases of cHL, using morphological criteria, TUNEL assay (TUNEL apoptotic index; T-AI) and immunohistochemical detection of active caspase 3 (casp3-AI) on paraffin embedded sections.*

Results: *When both apoptotic (MA) and mummified (mummi-I) cells were evaluated by morphological apoptotic index (morph-AI), the median value was 10.3%, while for MA and mummi-I the results were 3.4% and 6%, respectively. T-AI and casp3-AI values were 10.9% and 1.9%, respectively. Morph-AI was significantly higher in the mixed cellularity (MC) subtype (p=0.04), while MA was significantly higher in the male subgroup (p=0.03). MA was strongly correlated with casp3-AI (p=0.01).*

Conclusion: *Detection of apoptosis has become an important parameter in understanding tumor pathology and in designing antitumor treatment. A combination of methods is proposed in order to estimate accurately this form of cell death.*

Key words: apoptosis, caspases, classical Hodgkin's lymphoma, mummified cell

Introduction

Apoptosis is a form of PCD that involves a series of biochemical events leading to characteristic cell morphology and death. It was first described by Kerr et al. and was considered as a distinct form of controlled cell death with an opposite role to mitosis, that is implicated in cell turnover in both physiological and pathological situations, including neoplasia [1]. The Nomenclature Committee on Cell Death (NCCD) recently clarified that PCD and apoptosis should not be considered synonyms and proposed unified criteria for the better definition of cell death, including apoptosis [2]. Briefly, the latter is accompanied by rounding up, shrinkage and reduction of the cell volume (pyknosis), chromatin condensation, nuclear fragmentation (karyorrhexis), and severe blebbing of the plasma membrane. Conventional morphological evaluation is based on the specific features cells acquire while undergoing apoptosis that are rather distinct from cell death induced by other mechanisms, i.e. necrosis, as presented above [2]. Morphological evaluation remains the most widely used method for evaluating apoptosis in tissue sections, as it can be easily performed on routinely stained tissue sections without extra cost [3]. However, this method may underestimate the rate of apoptosis when used alone [4,5]. The **T**erminal deoxynucleotidyl transferase (Tdt) mediated d**U**TP **N**ick **E**nd **L**abelling (TUNEL) assay identifies DNA strand breaks occurring during apoptosis, and corresponds to a biochemical, more accurate approach

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of apoptotic evaluation [6]. However, it can often produce false positive results, or high background staining that makes evaluation difficult and demands interpretation with caution [7-9]. Caspases are central molecules in the apoptosis execution pathway that are triggered by a variety of stimuli resulting in the initiation of the apoptotic cascade [10,11]. However, various forms of caspase-independent cell death have been proposed, suggesting that, if used isolated, caspases cannot fully evaluate the phenomenon of apoptosis [12].

Hodgkin's lymphoma comprises two discrete entities, cHL and nodular lymphocyte predominance Hodgkin's lymphoma (NLPHL), according to the last WHO classification [13]. It is characterized by the presence of rare neoplastic multinucleated Reed-Sternberg and mononuclear Hodgkin's cells (HRS cells) within an inflammatory environment, consisting of T and B lymphocytes, admixed with plasma cells, macrophages, eosinophils and mast cells. Apoptotic cells are often observed in cHL, frequently bearing the characteristic morphology of "mummified" cells that lack crescent-shaped condensation of chromatin and fragmentation of the nucleus [14,15]. Only within the last three decades the cellular origin of the HRS cells was clarified in the vast majority of cases, corresponding to a population of germinal center-derived B cells, carrying "crippling" mutations that should normally lead them to apoptotic cell death [16]. A defective regulation of the apoptotic pathway has been suggested for the HRS cells [17], explaining partially their resistance to apoptosis [18-20], thus making the apoptotic process particularly interesting for the pathogenesis of the disease.

Published data reporting apoptosis in cHL are somehow controversial. Apoptotic indices derived from the same detection method present variable results [21,22], while different statistical approaches used to interpret the results make them non comparable, at least in a direct way [23,24].

The primary aim of this study was to investigate the apoptotic profile of cHL, comparing the results of the most widely used detection methods and with those reported in the literature.

Methods

Materials

Seventy-six cases of cHL were included in our study. Tissue samples of each case were selected from the files of the Laboratory of Histology and Embryology, National and Kapodistrian University of Athens Medical School, Greece. Only cases with sufficient material were included. All tissue samples were formalin-fixed and paraffin-embedded.

Histological sections were reviewed and subclassified according to the recent WHO classification [13] by two of the authors: 62 cases corresponded to the criteria of nodular sclerosis (NS), 11 cases to mixed cellularity (MC), 2 cases to lymphocyte rich (LR) and 1 case to lymphocyte depleted (LD) subtype of cHL. All experiments using human specimens were carried out according to the ethical guidelines of the Institutional Tumor Board (registration number 566, 8/12/2010).

Apoptotic index (AI) by morphological criteria

Standard hematoxylin and eosin stained slides were evaluated in order to determine morphological apoptosis. Both apoptotic and "mummified" cells were included in the determination of this index. Apoptotic cells were identified by their typical morphology. In brief, the presence of crescent -shaped condensation of nuclear chromatin, the rounding up and disintegration of the nucleus and the appearance of apoptotic bodies, composed of nuclear fragments and organelles, were the basic characteristics used for the identification of an apoptotic cell, as previously described [1]. "Mummified" cells were characterized by a condensed basophilic nucleus with homogeneous nuclear chromatin, tortuous nuclear contour, clumping at the nuclear membrane and increased stain intensity of both cytoplasm and nucleus [15]. Only cells of large size, exceeding that of a normal histiocyte, with the above characteristics were counted as neoplastic apoptotic or "mummified" cells. All results were evaluated by two of the authors (ECG and IAD-A). At least 100 HRS cells were counted in each case, using ×40 objective lens. Morph-AI was defined as the percentage of apoptotic and "mummified" cells in the total number of counted HRS cells. A second index was derived by evaluating only large cells with apoptotic morphology and characterized as morphological apoptosis (MA). Finally, when "mummified" cells were calculated alone, a third index was derived, named "mummification" index (mummi-I).

AI by TUNEL assay

The TUNEL method was performed in 74 cases of cHL, as previously described [25], modified by substituting dUTP for dATP. At first, sections were dewaxed in xylene, dehydrated in a graded series of alcohols and pretreated with proteinase K (20 μg/ml, Sigma), an enzyme that breaks the peptidic bonds among proteins, for 15 min at room temperature. Slides were incubated for 10 min in 3% H_2O_2 solution to block endogenous peroxidase activity, followed by application of Tdt (15 U/slide), an enzyme that catalyzes the addition of deoxynucleotides to the 3' hydroxyl terminus of DNA molecules, for 1 h at 37° C in 10XNEBIV, 10xCoCl2 (2.5 mM), biotin-dATP (Invitrogen) and dH20. Finally, for the identification of the incorporated nucleotides, streptavidin-biotin-peroxidase complex (AB complex, DAKO, Kalifronas, Greece) and 3,3´-diaminobenzidine tetrahydrochloride (DAB) were used, according to manufacturer's instructions. Hematoxylin was used as counterstain.

Sections from reactive lymph nodes served as positive control, after incubation with the enzyme DNAse I (1 U/slide, Invitrogen) for 10 min at room temperature. Reactive lymph nodes were also used as negative control, omitting enzyme incubation.

Nuclei from about 100 HRS cells were evaluated in each case. All nuclear staining in large cells was counted as positive, while staining in small cells was ignored. The percentage of positively stained nuclei corresponded to the AI based on TUNEL assay (T-AI).

AI by immunohistochemical expression of active caspase 3 (casp3-AI)

The immunohistochemical expression of active caspase 3 was

evaluated using a rabbit monoclonal antibody (mAb), directed against active caspase 3 (Anti-Caspase-3 Active, Millipore, dilution 1:40).

Immunohistochemistry was performed on formalin fixed and paraffin embedded 5 μm tissue sections, as previously described [26]. Briefly, sections were deparaffinised and dehydrated in a series of graded ethanol solutions. Antigen retrieval was performed in a 1 mM EDTA solution (pH=8), heated for 5 cycles of 5 min each in a household microwave at 700 W power. Then, slides were incubated in 3% hydrogen peroxidase for 10 min to quench endogenous peroxidase activity and then primary antibody was applied to each case for 40 min at room temperature. Next stage comprised 40 min incubation with Envision solution (DAKO, Kalifronas, Greece) at 37° C. For colour development and counterstain, DAB and hematoxylin were used respectively.

Sections of a reactive lymph node were used as positive control, while occasionally positive non neoplastic lymphocytes from the background of the studied cases served as internal positive control. For the determination of the AI based on the immunohistochemical expression of active caspase 3 (casp3-AI), 60 to 100 HRS cells were evaluated regardless of the intensity of the stain. Results were based on the percentage of positive cells in the total number of the counted HRS cells.

Statistical analysis

Statistical evaluation was carried out with the use of SPSS software package, version 17.0 (SPSS Inc; Chicago, Illinois), using nonparametric methods. Numeric variables were studied using Spearman's and Pearson's rank correlation coefficient and presented as medians. Categorical and numeric variables were analysed together, when possible, using the Kruskal-Wallis one-way analysis of variance test. Statistical significance was assumed for $p < 0.05$.

Results

Patient characteristics

Thirty-five of the 76 patients (46%) were male and the age, when available, ranged from 14 to 82 years. Details of patients characteristics are presented in Table 1.

Morphological determination of the AI (morph-AI, MA, mummi-I)

Neoplastic HRS cells with morphologic features of apoptosis (Figure 1a) and "mummified" HRS cells (Figure 1b) were observed in 72 (95%) cases, while in the remaining 4 cases, no cells with apoptotic morphology were detected. The morph-AI ranged from 2 to 37%, with median value of 10.3%, while MA, including only apoptotic cells, ranged from 0 to 24%, with a median of 3.4%. The third index, (mummi-I), evaluating only "mummified" cells, ranged from 0 to 30% with a median of 6%. The value of mummi-I was generally higher than MA ($p<0.001$, Wilcoxon's signed rank test).

AI defined by TUNEL (T-AI)

The TUNEL assay was evaluated in 74 cases. Only large cells with regular round nuclei were counted as positive (Figure 1c), while some variability in the intensity of staining was accepted. In some cases typical apoptotic bodies were detected. Reactivity was also found in a subset of non-neoplastic cells in the background of all cases of cHL. Interestingly, "mummified" cells were not labelled by the TUNEL technique. The T-AI had a median value of 10.9%, ranging from 3 to 33%. Although, the number of labelled HRS cells for the TUNEL method was higher than morph-AI, the difference was not statistically significant. In the small number of cases in which no cells with apoptotic morphology were detected, cells with a positive TUNEL reaction were identified.

Table 1. Patient and disease characteristics and expression of the apoptotic indices

Characteristics	*Patients N (%)*	*Morph-AI Median (range)*	*Mummi-I Median (range)*	*MA Median (range)*	*T-AI Median (range)*	*Casp3-AI Median (range)*
Age (years, n=76)						
<45	63 (83)	10.4 (2-37)	6 (0-30)	3.3 (0-24)	11.2 (3-33)	1.9 (0-5.5)
≥45	13 (17)	9.8 (5-32)	6.4 (0-28)	3.4 (1-6)	10.1 (5-28)	2.2 (0-2.9)
Gender (n=76)						
Male	35 (46)	10.7 (2-31)	6.4 (0-18)	3.9 (1-24)	11.4 (5-26)	2 (0-5.5)
Female	41 (54)	9 (3-37)	5.9 (1-30)	2.5 (0-8)	9.8 (3-33)	1.9 (0-3.5)
Histology (n=76)						
NS	62 (82)	10 (2-37)	5.9 (0-30)	2.8 (0-9)	10.8 (3-33)	2 (0-5)
MC	11 (14)	11.4 (7-32)	9.3 (3-28)	3.6 (1-24)	10.3 (5-26)	1.5 (0-5.5)
LR	2 (3)	9.3 (5-14)	1.1 (0-2)	8.2 (5-12)	n.a*	n.a*
LD	1 (1)	n.a*	n.a*	n.a*	n.a*	n.a*
Total	76 (100)	10.3 (2-37)	6 (0-30)	3.4 (0-24)	10.9 (3-33)	(0-5.5)

*non applicable, Morph-AI: morphological apoptotic index, Mummi-I: mummification index, MA: morphological apoptosis, T-AI: TUNNEL apoptotic index, Casp3-AI: caspase 3 apoptotic index. For other abbreviations see text (Methods)

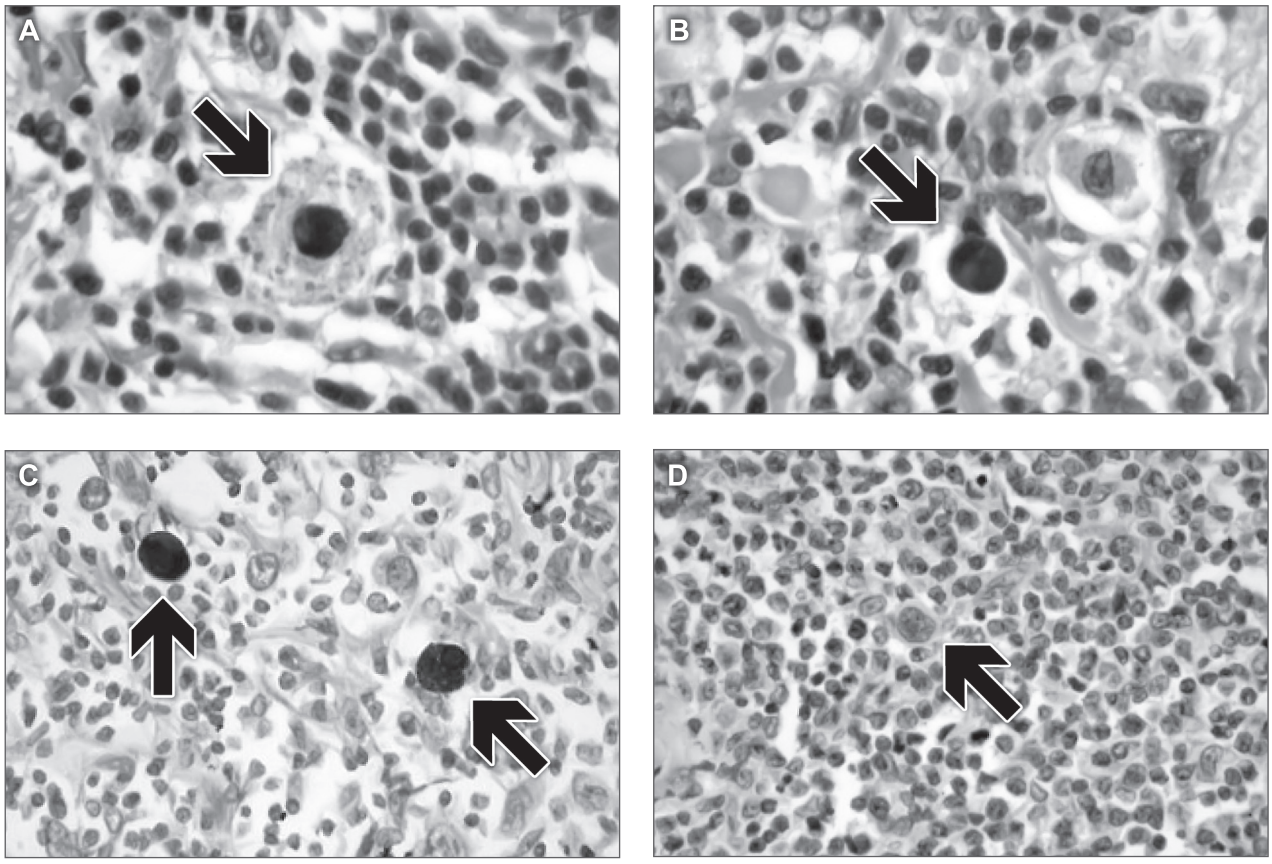

Figure 1. A: Neoplastic HRS cell with morphologic features of apoptosis (arrow; ×40). **B:** Mummified HRS cell with increased stain intensity of both cytoplasm and nucleus (arrow; ×40). **C:** Apoptotic neoplastic HRS cell positive for TUNEL (arrows; ×40). **D:** Neoplastic HRS cell positive for active caspase 3 (arrow; ×40).

AI defined by active caspase 3 expression (casp3-AI)

Immunohistochemical evaluation of active caspase 3 was performed in a subset of 41 cases. Expression of the active form of this enzyme was detected in 36 of the 41 cases (88%). The expression was present in both nuclei of morphologically identifiable apoptotic cells and of neoplastic HRS cells without morphologic signs of apoptosis (Figure 1d). A number of positive reactive small lymphocytes and larger cells consistent with histiocytes within the environment served as an internal control. The staining observed was diffuse nuclear, with accompanying cytoplasmic reaction being exceptional. The percentage of positive HRS cells ranged from 0.8 to 5.5%, with a median value of 1.9%.

Comparison of index results

The values of the above-mentioned indexes based on subgroups defined by age, sex and histologic diagnosis of the patients are presented in Table 1. We noticed that morph-AI was significantly higher in the MC subtype of cHL when compared to NS subtype (p=0.04), while mummi-I was also higher, although at a marginal level of significance (p=0.07). MA was significantly higher in the male subgroup, when compared to the female subgroup (p=0.03). Generally, our results showed variability as far as patient characteristics were concerned, however, there were no statistically significant correlations between apoptotic indices and any of the patient characteristics. We also didn't notice a significant correlation between apoptotic values and clinical/laboratory findings (data not shown).

Spearman's and Pearson's Rho correlation coefficient test was used for the assessment of correlation between the expression levels of any two methods taken as continuous variables. Interestingly, MA was significantly correlated with casp3-AI (Pearson's, p=0.01).

Discussion

In recent years, there has been a huge scientific interest in tumor apoptosis, which can either occur spontaneously or as a result of therapy-induced neoplastic regression. According to the literature, HRS cells in

cHL harbor the ability of escaping apoptosis through a major transforming event [14]. However, the exact mechanism of apoptotic evasion by the HRS cells still remains unanswered.

Since apoptosis has become an interesting area of anticancer treatments, accurate methods to quantify this type of cell death are needed [27]. Many different techniques are currently used to evaluate apoptosis [28]. Recently, the NCCD recommended that the method used to evaluate apoptosis should be clearly mentioned /defined and cell death should be quantified with more than one assays, as different approaches identify different events [2]. Furthermore, a combined approach would help to reduce the probability of artefacts.

Following that recommendation, three of the most common and popular methods were applied in the present study in a series of cHL cases, in order to evaluate thoroughly the apoptotic profile of the neoplastic cells. A degree of variability among the apoptotic indices was obtained when different methods were used. Our results also presented differences with those reported in the literature, where there is great divergence in the apoptotic index reported from different authors using the same method.

Using conventional morphological criteria, we found a median morph-AI of 10.3%, while MA and mummi-I assessing exclusively apoptotic or mummified cells showed median values of 3.4 and 6%, respectively. Based on morphology, Kolar et al. showed a median value of 0.4% for apoptosis, including apoptotic and "mummified" cells, while Lorenzen et al. found a mean value of 27.4%, evaluating only "mummified" cells [15,23]. Moreover, Macac et al. reported results closer to ours, with a 10.4% value of the mummification index in EBV positive and 8.2% in EBV negative HL cases [29]. We also noticed that mummi-I was higher than MA in all cases studied, an observation also made in the study of Lorenzen et al. [15].

When our series was assessed using the TUNEL method, the results ranged from 3 to 33%, with a median of 10.9%. Similarly, Kim et al. showed an AI higher or around 10% for the 43.2% (11/26) of their cases studied with TUNEL [30], while Smolewski et al. assessed TUNEL assay in 100 out of 110 patients included in their study, and found positive reaction in 43 patients, ranging between 10 and 60%, while in the remaining 57 patients the AI of the HRS cells was below 10% [21]. Lorenzen et al. reported higher apoptotic rates (24.7%) by TUNEL [15], while the results of both Bai and coworkers [22] and Kolar et al. [23] showed much lower rates with median values of 2.85 and 1.2%, respectively. Benharroch et al., in an expanded cohort of cHL cases (217 patients) that was first published in 1996 (50 patients), reported mean value of 19.1% for AI based on TUNEL assay [31].

Based on the immunohistochemical expression of active caspase 3, we noticed that staining was present in neoplastic cells with apoptotic morphology, as well as in HRS cells without apoptotic features. This could probably reflect an early presentation of active caspase 3 in the apoptotic cascade, thus making caspase activation an early marker for apoptosis detection in tumor tissues [32]. Using this method, we found an AI ranging from 0 to 5.5%, with a median value of 1.9%. Our results are in accordance with those reported by Bai et al. [22], who showed a mean of 1.92% with a range from 0 to 12%. Interestingly, Duckers et al. detected a higher mean value of 4.3%, ranging from 0 to 13% [24].

When the different apoptotic indices were correlated, only MA –evaluating exclusively apoptotic cells– was significantly correlated with casp3-AI ($p=0.01$), implying that these two methods may estimate a similar event, while mummified cells possibly represent a different process, as these cells show features of cell death that are incompatible with the classical definition of apoptosis [15].

The variation in the results reported in the literature can be partly attributed to artifacts, resulting in misleading findings. Indeed, there has been reported that pitfalls can occur in the assessment of each of the above methods. Starting with conventional morphology criteria, underestimation of the AI can be produced due to rapid cell shrinkage or phagocytosis of apoptotic bodies before they become apparent in histological sections [4]. Sometimes morphological features of apoptosis can be confused or overlap with those typical of necrosis, as with surface blebbing, a common marker of necrosis as well as of apoptosis [32]. Furthermore, typical features of a cell undergoing apoptosis appear in situations other than apoptosis i.e. superaggregation of chromatin can also occur in necrosis [33]. Being an asynchronous process, the apoptotic process can be detected as a phenomenon with marked structural discrepancies, which are associated with earlier or latest phases [34]. Finally, lack of experience in the evaluation of data [35] and intra-observer subjectivity [5] are additional factors influencing the variability of results.

In our series, we observed cases with no morphological evidence of apoptosis, which showed TUNEL reactivity suggesting that those methods, although evaluating the same event, may lead to different results. Although the TUNEL assay is a method that identifies DNA degradation in apoptotic cells, in some instances it can be positive in necrotic cells as well [4,36]. Moreover, since apoptosis can be induced, although rarely, without DNA degradation, the TUNEL assay could

miss a number of cells undergoing apoptosis [4]. Prefixation and fixation times [7], as well as histological sectioning can produce non-specific TUNEL reactivity [37]. Additionally, during the TUNEL procedure, endogenous endonucleases are released, resulting occasionally in false positive staining [38], while false positivity can also be encountered in processes other than apoptosis, such as autolysis [8,36].

Although immunohistochemical expression of active caspase 3 is widely used in evaluating apoptosis, this process can be executed independently of caspases [14,39]. Furthermore, caspases perform non apoptotic functions [40], thus their partition in cell death procedures should not be considered as single action.

In the present study, when the different apoptotic indices were correlated, MA–evaluating exclusively apoptotic cells – was significantly correlated with casp3-AI ($p=0.01$), implying that these two methods may estimate a similar event, while mummified cells possibly represent a different process, as these cells show features of cell death that are incompatible with the classical definition of apoptosis [15].

In summary, apoptosis can be measured with many and different methods. The variability in the results shows that caution should be exercised, as various detection methods may evaluate different aspects of PCD. This intracellular event should be extensively analysed by using in parallel morphological, biochemical and molecular approaches, as it seems to play an important role in the pathogenesis of cHL.

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