Determination of Marrow Involvement in Non-Hodgkin's Lymphoma by Immunocytochemical Studies

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Abstract : **Bone marrow involvement in non-Hodgkin's lymphoma (NHL) alone can raise the clinical stage from I and II (localized disease) to IV (advanced disease). Multiparameter assessment is thus required to detect early marrow involvement (positive marrow) besides the conventional morphologic evaluation of bone marrow biopsy and marrow aspirates. Determination of lymphocyte subsets in the marrow is considered useful if the phenotype of NHL is known. To prove this possibility, we have performed immunocytochemical studies on marrow aspirate smears to determine CD3+ T-cells and CD19+ B-cells by using the alkaline phosphatase-labeled streptavidin-biotin method in 8 cases of NHL with positive marrow (4 B-cell, 4 T-cell NHL), 11 cases of NHL with negative marrow (8 B-cell, 3 T-cell NHL), and 11 cases of controls including Hodgkin's disease (2), acute leukemia (4), SLE (1), anemia (1), inflammatory bowel disease (1), polycythemia vera (1), and Wegener's granulomatosis (1). CD3+ T-cells and CD19+ B-cells were enumerated by counting 500 nucleated marrow cells. The results showed that the mean CD3/CD19 ratios in B-cell NHL and T-cell NHL with positive marrow were 1.8 and 20.5, while those in B-cell NHL and T-cell NHL with negative marrow and in controls were 3.3, 3.1, and 2.6, respectively. Moreover, cytologic difference between CD3+ T-cells and CD19+ B-cells was observed in 1 case of B-cell NHL and 3 cases of T-cell NHL with positive marrow, while such a difference was absent in NHL with negative marrow and in controls except in 1 case of T-cell NHL with negative marrow. Therefore, immunocytochemical studies are helpful to determine marrow involvement in NHL.**

Keywords: marrow, non-Hodgkin's lymphoma, B-cell, T-cell, immunocytochemistry

เรื่องย่อ : การตัดสินการแพร่กระจายมายังไขกระดูกในน็อน-ฮอดจ์กินส์ลิมโฟมาด้วยวิธีอิมมูโนไซโตเคมี

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การแพร่กระจายมายังไขกระดูกในน็อน-ฮอดจ์กินส์ลิมโฟมาเพียงอย่างเดียวสามารถเปลี่ยนระยะของโรคจากระยะที่ 1 และ 2 (เฉพาะที่) เป็นระยะที่ 4 (แพร่กระจาย) เมื่อตรวจพบ การตรวจหาการแพร่กระจายมายังไขกระดูกในระยะเริ่มแรกด้วยวิธีการต่าง ๆ นอกเหนือไปจากการตรวจดูไขกระดูกด้วยกล้องจุลทรรศน์ธรรมดาจึงมีความสำคัญยิ่ง การตรวจหาชนิดของลิมโฟไซต์ในไขกระดูกเพื่อเทียบกับชนิดของลิมโฟมาเซลล์จัดว่ามีประโยชน์เพื่อการวินิจฉัยดังกล่าว ด้วยวิธีการย้อมอิมมูโนไซโตเคมีเพื่อดูจำนวนของที-เซลล์ (ซีดี3+) และบี-เซลล์ (ซีดี19+) ในไขกระดูกของผู้ป่วยลิมโฟมาที่มีการแพร่กระจาย 8 ราย, ที่ยังไม่มีการแพร่กระจาย 11 ราย, และผู้ป่วยอื่น ๆ ที่ไม่ใช่ลิมโฟมา 11 ราย พบว่าค่าเฉลี่ยของอัตราส่วนระหว่างซีดี 3 และซีดี 19 ในผู้ป่วยลิมโฟมาที่มีการแพร่กระจายมีการเปลี่ยนแปลงที่แตกต่างไปจากผู้ป่วยลิมโฟมาที่ยังไม่มีการแพร่กระจายและผู้ป่วยอื่น ๆ ที่ไม่ใช่ลิมโฟมา ค่าดังกล่าวในผู้ป่วยลิมโฟมาชนิดบี-เซลล์ที่มีการแพร่กระจายเท่ากับ 1.8 ในผู้ป่วยลิมโฟมาชนิดที-เซลล์ที่มีการแพร่กระจายเท่ากับ 20.5 ขณะที่ค่าในผู้ป่วยลิมโฟมาชนิดบี-เซลล์และชนิดที-เซลล์ที่ยังไม่มีการแพร่กระจายและในผู้ป่วยอื่น ๆ ที่ไม่ใช่ลิมโฟมาเท่ากับ 3.3, 3.1 และ 2.6 ตามลำดับ นอกจากนี้ยังพบความแตกต่างในรูปร่างของเซลล์ระหว่างที-เซลล์และบี-เซลล์ในลิมโฟมาชนิดบี-เซลล์ 1 รายและชนิดที-เซลล์ 3 รายที่มีการแพร่กระจายมายังไขกระดูกโดยไม่พบความแตกต่างดังกล่าวในกลุ่มที่ไม่มีการแพร่กระจายและที่ไม่ใช่ลิมโฟมายกเว้นลิมโฟมาชนิดที-เซลล์ 1 รายที่ไม่มีการแพร่กระจายมายังไขกระดูก ดังนั้นวิธีการย้อมอิมมูโนไซโตเคมีมีประโยชน์ในการตัดสินการแพร่กระจายของลิมโฟมามายังไขกระดูก

INTRODUCTION

Marrow involvement in patients with aggressive non-Hodgkin's lymphoma (NHL) is one of the important prognostic factors.[1] The presence of marrow involvement alone can raise the clinical stage from I and II (localized disease) to IV (advanced disease). Multiparameter assessment is required to detect marrow involvement, depending on the number, cytologic features, and distribution of lymphoid cells in the marrow.[2,3] Bone marrow biopsy from bilateral posterior iliac crests with at least 2 cm long core biopsy is recommended in conjunction with examination of marrow aspirate smears and sections in order to detect marrow involvement; the yield is approximately 20% to 30%.[2-4] Using immunophenotyping studies, we can classify most NHL into 2 major groups as B- and T-cell lymphomas.[5-8] Immunophenotyping may detect early or minimal marrow involvement by lymphoma if the predominant subset of lymphoid cells corresponds with the phenotype of lymphoma.[2,3,9] The most reliable method for determining the antigenic profile of lymphoid cells in marrow aspirates requires flow cytometry.[2,3,10,11] However, the alternative is immunocytochemistry performed on marrow aspirate smears using alkaline phosphatase-labeled streptavidin-biotin (AP-LSAB) method.[12]

MATERIALS AND METHODS

Four unstained marrow aspirate smears from patients with suspicion of NHL or known cases of B-cell or T-cell NHL were collected. Controls were patients who did not have NHL but underwent marrow examination for other reasons. One marrow smear from each patient was stained with Wright's stain and lymphoid cells were enumerated from counting 500 nucleated cells in the marrow. B- and T-cell population were determined by lineage specific markers, monoclonal antibodies to CD19 for B-cells and to CD3 for T-cells, applied in the immunocytochemistry using AP-LSAB method.[12] Briefly, the unstained air-dried marrow aspirate smears were fixed in fresh acetone for 90 seconds, rinsed with phosphate buffer saline (PBS) for 5 minutes, incubated in 3% normal horse serum for 5 minutes, applied with

primary mouse anti-human antibody (CD3 or CD19) for 60 minutes, rinsed with PBS for 5 minutes twice, applied with secondary biotinylated rabbit anti-mouse antibody for 30 minutes, rinsed with distilled water, counterstained with Mayer's hematoxylin for 30 seconds, rinsed with tap water, and then mounted with glycerine, a water-based mounting media. Positive cells showed red staining on the cytoplasmic membrane and in the cytoplasm. Then B-cells (CD19+) and T-cells (CD3+) were enumerated by counting 500 nucleated cells in the marrow. The other marrow smear from each case was used as a negative control by using the method described above but omitting the primary antibody. The predominant population of lymphoid cells (B- or T-cell) were compared to the phenotype of lymphoma in each case. A conventional evaluation of marrow aspirates, sections, and bone marrow biopsy was used to determine marrow involvement in each case.[2,3]

RESULTS

Thirty cases comprising 8 cases of NHL with marrow involvement (4 B-cell and 4 T-cell phenotypes), 11 cases of NHL without marrow involvement (8 B-cell and 3 T-cell phenotypes), and 11 control cases including Hodgkin's disease (2 cases), acute leukemia (4 cases), systemic lupus erythematosus (1 case), anemia (1 case), inflammatory bowel disease (1 case), polycythemia vera (1case), and Wegener's granulomatosis (1 case) were collected for analysis. In all cases, immunocytochemistry brought out more lymphoid cells when compared to conventional enumeration of lymphoid cells in Wright's stained marrow smears. The percentage of total lymphoid cells including small lymphocytes and atypical lymphoid cells was higher in NHL with marrow involvement than that in NHL without marrow involvement or in the control group. (Table 1) The mean percentage of total lymphoid cells in the 8 cases of NHL with marrow involvement was 30.2% (range, 11.8-90.0%), while that in NHL without marrow involvement was 8.2% (range, 4.0%-12.2%) and that in the control group 7.1% (range, 1.0%-12.9%).

The mean percentage of CD19+ B-cells or CD3+ T-cells was much higher than usual in NHL with marrow involvement either of corresponding B- or T-cell phenotype when compared to that in NHL without marrow involvement or the control group. (Table 1) In B-cell NHL with marrow involvement, the mean percentage of CD19+ B-cells was 16.0%, while that in B-cell NHL without marrow involvement, T-cell NHL, or the control group was lower than 10.2%. In T-cell NHL with marrow involvement, the mean percentage of CD3+ T-cells was 49.5%, while that in T-cell NHL without marrow involvement, B-cell NHL, or the control group was lower than 17.6%. The mean value of CD3/CD19 ratio in NHL with marrow involvement was much different from that in the normal donors from the study by Clark et al.[13] The mean CD3/CD19 ratios in B-cell and T-cell NHL with marrow involvement were 1.8 and 20.5, respectively; while that in normal donors was 3.7.[13] The mean CD3/CD19 ratio in NHL without marrow involvement or the control group was close to that in normal donors. (Table 1) However, the values of these parameters in an individual case did not follow the above findings even in the results from Clark's series on normal donors.[13] (Table 2)

Concerning the cytologic difference between CD3+ and CD19+ cells, we found this finding helpful to distinguish between neoplastic and reacting lymphoid cells in 4 cases of NHL with marrow involvement (50%). Only one case of NHL without marrow involvement, a case of T-cell NHL, showed such a difference. None was found in the control group. (Table 2)

DISCUSSION

Due to the importance of marrow involvement in NHL,[1] multiparameter assessment is required for its detection.[2,3] In addition to conventional morphologic evaluation of marrow aspirate smears, sections, and bone marrow biopsy, immunophenotyping of formalin-fixed marrow has been proven to be a useful technique for diagnosing marrow involvement in NHL, particularly of B-cell phenotype.[14] Immunophenotyping may detect early or minimal marrow involvement by lymphoma if the predominant subset of lymphoid cells corresponds with the phenotype of lymphoma.[2,3,9] Flow cytometry has been considered to be the most reliable method

Table 1. Determination of lymphoid cells in marrow aspirate smears in 30 patients with or without lymphoma

Categories (number of cases)	Mean percentage of cells in the marrow (range)			
	Total lymphoid cells[1]	CD3	CD19	CD3/CD19
Normal marrow[2] (10)	24.7 (9.0-39.0)	46.4 (19.0-69.0)	11.7 (5.0-27.0)	3.7 (1.7- 9.8)
B-cell NHL with BM+[3] (4)	16.5 (11.8-21.8)	17.6 (16.2-23.0)	16.0 (7.0-37.2)	1.8 (0.4- 3.4)
B-cell NHL with BM-[4] (8)	8.3 (4.0-12.2)	16.4 (4.6-36.0)	10.2 (0.6-34.4)	3.3 (0.7- 7.7)
T-cell NHL with BM+ (4)	44.0 (16.8-90.0)	49.5 (11.8-90.0)	7.7 (0.4-18.2)	20.5 (2.5-45.0)
T-cell NHL with BM- (3)	7.9 (6.2- 9.8)	14.7 (13.0-17.3)	5.0 (3.4- 6.7)	3.1 (2.6- 3.8)
Control group[5] (11)	7.1 (1.0-12.9)	13.0 (3.2-34.6)	5.5 (2.2-12.2)	2.6 (1.0- 7.9)

[1] Counting 500 nucleated marrow cells in Wright's stained marrow aspirate smears
[2] Study in 10 normal donors by using flow cytometry[13]
[3] Marrow involvement by conventional evaluation
[4] Negative marrow (no marrow involvement by conventional evaluation)
[5] Including Hodgkin's disease (2 cases), acute leukemia (4 cases), systemic lupus erythematosus (1 case), anemia (1 case), inflammatory bowel disease (1 case), polycythemia vera (1 case), and Wegener's granulomatosis (1 case).

for determining the antigenic profile of lymphoid cells in marrow aspirates;[2,3,10,11] however, at least one study has shown that flow cytometric immunophenotyping is not a cost-effective replacement for good morphologic evaluation in lymphoma staging.[10] Moreover, small marrow sample is not possible for flow cytometric study; the alternative is immunocytochemistry performed on marrow aspirate smears using AP-LSAB method.[12] This technique also allows cytologic evaluation of the positive cells under light microscopy.

The present study has demonstrated the usefulness of immunocytochemistry performed on marrow aspirate smears using AP-LSAB method to determine the phenotype of lymphoid cells in the marrow as well as cytologic evaluation of the positive cells. The phenotype of NHL is needed to determine the marrow involvement in this situation. The present study has demonstrated that the mean percentage of CD19+ B-cells or CD3+ T-cells was much higher than usual in NHL with marrow involvement either of corresponding B- or T-cell phenotype when compared to that in NHL without marrow involvement or the control group.

Contamination of blood in the marrow is one of the most obvious factors influencing the number of B- and T-cells in the marrow aspirate as we know that T-cells outnumber B-cells in the blood. Clark et al. has demonstrated a mean CD3/CD19 ratio in the blood of 14.8 (range, 3.4-27.7).[13] The increased number of small T-cells in the marrow should be interpreted with awareness of this information.

It is noteworthy that the number of bone marrow lymphoid cells and the CD3/CD19 ratio alone

Table 2. Bone marrow (BM) lymphoid cells and CD3/CD19 ratio in 30 patients with or without lymphoma

Type of NHL or disease	BM lymphoid cells	CD3/CD19
NHL with marrow involvement		
B-cell, follicular, small cleaved cell type	21.8%	0.4*
B-cell, follicular, small cleaved cell type	15.8%	3.4
B-cell, diffuse, mixed small and large cell type	16.4%	1.1
B-cell, diffuse, mixed small and large cell type	11.8%	2.3
T-cell, small lymphocytic type	31.6%	2.5
T-cell, diffuse, mixed small and large cell type	16.8%	5.0*
T-cell, lymphoblastic type	90.0%	45.0*
T-cell, anaplastic large cell type	37.4%	29.5*
NHL without marrow involvement		
B-cell, follicular, large cell type	10.4%	3.5
B-cell, diffuse, mixed small and large cell type	9.0%	1.1
B-cell, diffuse, mixed small and large cell type	4.0%	7.7
B-cell, diffuse, large cell type	7.0%	1.0
B-cell, diffuse, large cell type	10.0%	1.4
B-cell, diffuse, large cell type	12.2%	5.6
B-cell, diffuse, large cell type	8.2%	0.7
B-cell, diffuse, large cell type	5.6%	5.3
T-cell, diffuse, mixed small and large cell type	9.8%	3.8
T-cell, anaplastic large cell type	6.2%	2.9
T-cell, mycosis fungoides	7.7%	2.6*
Control group		
Hodgkin's lymphoma	12.9%	1.0
Hodgkin's lymphoma	4.0%	2.6
Acute lymphoblastic leukemia	7.0%	3.6
Acute non-lymphoid leukemia	1.0%	1.0
Acute non-lymphoid leukemia	11.0%	2.0
Acute non-lymphoid leukemia	12.4%	2.3
Systemic lupus erythematosus	3.0%	2.2
Anemia	4.0%	1.4
Inflammatory bowel disease	8.0%	2.1
Polycythemia vera	7.2%	2.4
Wegener's granulomatosis	7.6%	7.9

*Presence of cytologic difference between CD3+ and CD19+ cells

cannot be used to determine the marrow involvement without the knowledge of the phenotype of lymphoma. For example, if one looks at the case of diffuse large B-cell lymphoma without marrow involvement (Table 2), one will see the high percentage of bone marrow lymphoid cells (12.2%) and the CD3/CD19 ratio of 5.6 leading to the suspicion of marrow involvement by T-cell lymphoma. However, no cytologic difference between CD3+ and CD19+ cells is noted and certainly no large B-cell is detected. Similarly, one may suspect marrow involvement by B-cell lymphoma in another case of B-cell lymphoma without marrow involvement (Table 2) if one just looks at the number of bone marrow lymphoid cells (8.2%) and the CD3/CD19 ratio of 0.7. But this marrow is negative by conventional evaluation because no large B-cell is detected. However, one may argue that this may be a case of morphologic discordance in diffuse large cell lymphoma between the lymph node and bone marrow reported in the literature.[15-19] To determine this possibility, one needs to have evidence of the same clonal immunoglobulin gene rearrangement in the lymph node and bone marrow.[20-24]

At present, there is not only one technique capable of detecting marrow involvement by NHL. Flow cytometry and molecular analysis for clonal immunoglobulin gene or T-cell receptor gene rearrangement may be more sensitive for detecting minimal marrow involvement.[10,11,20-24] Cases of early stage NHL with morphologically negative bone marrow could potentially be restaged as Stage IV on the basis of flow cytometry or molecular analysis results. But the clinical importance of minimal bone marrow involvement by NHL needs further evaluation.[11] False negative results by these sophisticated techniques, however, do occur; thus emphasizing the necessity to use multiparameter assessment of all available results from morphology, immunohistochemistry, molecular biology and clinical data.[2,3,11,23,24]

In summary, this study demonstrated that immunocytochemistry could help to determine marrow involvement in NHL if the CD3/CD19 ratio was altered (≤ 1.8 for B-cell NHL and ≥ 20.5 for T-cell NHL) or there was cytologic difference between CD3+ T-cells and CD19+ B-cells or both. It is also important to emphasize the usefulness of the determination of lymphocyte subsets in the marrow only when the phenotype of NHL is known.

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