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EXPRESSION OF CD 95 (FAS/APO-1) IN NON-HODGKIN'S LYMPHOMA

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Apoptosis mediated by the CD95 (FAS/ APO-1) molecule plays a crucial role in the regulation of B-cell immune response. In this study, the expression of CD95 was examined by flow cytometry & immunohistochemistry in 52 cases of newly diagnosed non Hodgkin's lymphomas (NHLs) and in 12 cases of reactive hyperplastic lymph node (RHL). Also, serum soluble FAS Ligand level (s FAS L) was assessed in these patients as well as in 10 healthy control with matched age and sex .Compared to diffuse large cell lymphoma and Burkitt's lymphoma, low grade lymphoma more frequently expressed CD95. However, CD95 expression did not correlate with ESR , LDH and β2 microglobulin. In this study, sFASL was significantly different in different histopathological subtypes and stages of NHLs and the highest level was in high grade lymphoma and stage IV . It was significantly correlated with ESR , LDH and β2 microglobulin . So, the infrequent expression of CD95 in high grade lymphoma suggests an association between loss of CD95 expression/ function and more aggressive tumour grade. So s FAS L may be putative independent prognostic factor and may be an impediment to the development of FAS-based therapies for NHLs.

Key words: Non Hodgkin's lymphoma, CD95.

INTRODUCTION

A lot of literatures has revealed that CD95 (FAS-APO1) is a type I transmembrane glycoprotein belonging to the tumor necrosis factor (TNF), nerve growth factor receptor family which is broadly expressed on hematopoietic cells including activated B and T cells, NK cells and myeloid cells. Only minority of resting peripheral blood B and T cells express FAS protein. Thus, the CD95 system plays a key role in regulating the immune system especially in T-cell homeostasis[1,2-3].

Apoptosis not only plays an important role in tissue homeostasis, but it is also a mechanism used by the immune system in the elimination of virus-infected cells and tumour cells in the acquisition of self tolerance by depletion of autoreactive T-cells and in the termination of immune responses by removal of reactive T and B cell clones[2]. At least two pathways inducing apoptosis have been identified. It can be mediated by certain serine proteases (granzymes A & B) and perforin which are cytotoxic proteins present in the cytoplasm of cytotoxic T-cells and natural killer (NK) cells and it can be induced by interaction between the cell surface molecule CD95 (FAS, APO-1) on the target cells and its ligand (FASL) on the cytotoxic cells[3].

Activation-induced cell death (AICD) in activated T cells is mediated by CD95 ligand (CD95L) receptor interaction[4]. Stimulation of long-term activated T cells through the T cell receptor (TCR)/CD3 complex induces the production of CD95L, which activates the CD95 pathway by binding to the CD95 surface receptor in an autocrine, paracrine fashion. Signaling through CD95 after ligand-dependent crosslinking of surface receptors requires the recruitment of FADD and caspase 8 into a death-inducing signaling complex (DISC). Caspase 8, an upstream component of the caspase cascade during death receptor-mediated apoptosis, is activated at DISC and

catalyzes the cleavage of downstream caspases[5]. Releasing apoptogenic factors from mitochondria and caspase activation seems to be a key event in apoptosis induced by cytotoxic drugs. In addition, some studies suggested that CD95L receptor system is involved in drug-induced apoptosis in various cell types. by activation of CD95 death signaling pathway and this may contribute to the role of CD95 in the era of anticancer therapy [6].

Several cytokines and the receptors for some cytokines are involved in the pathogenesis and progression of malignant lymphomas. CD95 belongs to the TNF-receptor suprefamily and may mediate apoptosis if triggered by CD95-ligand or specific antibodies. Recently soluble CD95 (a splice variant of the surface-bound molecule) was detected in the serum of patients with lymphoid leukemias and other disorders[7]. CD95 ligand (CD95L) is capable of inducing apoptosis of lymphoid cells by cross-linking with its natural receptor FAS[8].Its soluble (sFASL) can block apoptosis induced by FAS ligand in vitro, and elevated serum concentration of sFAS ligand may be associated with lymphoproliferation and autoimmune disease[9].Soluble FAS ligand has recently been implicated for immune evasion and aggressive behaviour in malignancy[10].

As since, apoptosis mediated by the CD95 (FAS/APO-1) molecule plays a crucial role in the regulation of the B-cell immune response. So, resistance to FAS mediated death may be an important factor in B-cell transformation in vivo. So, a putative defect in FAS/FASL pathway may thus favour the development of malignant B-cell population and disruption of FAS system that may play a role in the pathogenesis of some lymphomas.

So, in the present study, we aimed to investigate CD95 (FAS-APO-1) expression in NHLs to gain insight into putative role of this apoptotic molecule in different histopathological subtypes and clinical stages of lymphoma. Also we examined the levels of soluble FAS ligand in untreated malignant lymphoma patients in order to correlate this molecule with clinical, histological and laboratory parameters.

PATIENTS AND METHODS

This study was carried out on 52 newly diagnosed non-Hodgkin's lymphoma. They included 30 males and 22 female and their ages ranged from 11 to 68 years with a mean ± SD of 37.45 ± 18.83 ys. They were selected from departments of General Surgery and Clinical Oncology, Masnoura University Hospital, during the period from November 1998 to December 2001. Patients were diagnosed by clinical findings, peripheral hemogram, bone marrow biopsy and the diagnosis was confirmed by histopathological examination of the affected lymph node or organ biopsy. 40 patients had nodal affection while 12 patients had extranodal organ affection (Table 1) Laboratory tests including erythrocyte sedimentation rate (ESR), lactate dehydrogenase (LDH) and β2 microglobulin were performed. Biopsies from 12 patients with reactive lymphadenitis were selected to be positive control for FAS staining. In addition ,10 patients with matched age and sex were selected to act as a control group. Patients were grouped histopathologically according to the working classification into:

Low grade lymphoma (LGL) comprised 18 cases {eight of them had extranodal marginal zone lymphoma of mucosa associated lymphoid tissue (MALT) and the other 10 cases had follicular lymphoma (FL)}.

Intermediate grade lymphoma (IGL) comprised 22 cases {eight cases had diffuse large cell (DLL) lymphoma and the other 14 cases had diffuse mixed small and large cell lymphoma (DML)}.

High grade lymphoma (HGL) comprised 12 cases {four cases had Burkitt's lymphoma (BL) while the other 8 cases had lymphoblastic lymphoma (LL)}.

According to Ann-Arbor staging system the patients were classified into 10 cases in stage I, 12 cases in stage II, 16 cases in stage III and 14 cases in stage IV.

Forty patients were followed up after treatment either by chemotherapy, radiotherapy or surgery combined with radiotherapy or chemotherapy. Thirty patients reaching complete remission (CR), 6 reaching partial remission (PR) while the other 4 were in relapse and serum soluble FAS ligand levels were reassessed.

Methods:

Serum soluble FAS ligand levels were assessed at time of diagnosis and at CR, PR and relapse using Sandwich enzyme- linked immunoassay from MBL (Medical and Biological Laboratories CO, LTD, Nogoya, Japan)

CD95 (FAS-APO-1) expression was investigated both by flow cytometry and immunohistochemistry in lymph node or organ biopsy after obtaining patient informed consent .

CD 95 expression by Flow Cytometry:

Saline preserved lymph node biopsy was used .Using mouse anti human CD95 (purified IgG conjugated) fluoroscein isomer thiocyanate (FITC) (serotec Ltd) and trition X100 cell pellets were prepared. After washing and resuspension by phosphate buffer, cell pellets were incubated for 30 minutes up to 24 hours at 4 °C and then CD95 expression was measured by flow cytometry (Epics profile II- coulter).

The isotopic control runned first followed by cell pellets. The percent of CD95 positive cells were determined by setting electronic gates to exclude 95% of positive cells fluorescence in the isotopic control (Fig. 1).

CD 95 expression by immunohistochemistry:

Paraffin section from lymph node biopsy was used. After deparafinzation and rehydration immunostaining protocol was used (Histastain from Zymed Laboratories INC.) using a three step streptavidin-biotin-peroxidase technique. After blocking endogenous peroxidases (using 3% Hydrogen peroxide in absolute methanol), staining was performed using a biotinylated rabbit anti mouse and streptavidin peroxidase conjugate. Streptavidin peroxidase was binding to biotin residues on linking antibody and the presence of peroxidase could be revealed by addition of substrate chromogen solution, which is catalyzed by peroxidase converting it into deposit which can demonstrate the location of antigen (Fig. 2). Staining of germinal center of the reactive lymph node was monitored as positive control for CD95 staining[12].

Statistical analysis:

The tests were run on an IBM Compatible computer using SPSS / PC4 statistical package (SPSS/inc ChicagoIL)

Results were reported as median, range, Kruscal Wallis test and Spearman's rank correlation coefficient test

RESULTS

The results of this study can be summarized in (The Tables 2-7).

(Table 2) represents the expression of CD95 by immunohistochemistry and flow cytometry in different histopathological subtypes in NHLs. The more frequent expression of CD95 was in low grade lymphoma and the highest frequency was in MALT lymphoma (87.5%).

(Table 3) represents the expression of CD95 by flow cytometry and immunohistochemistry in different stages of NHLs. The highest frequency was in stage I (70%).

(Table 4) represents s.FAS ligand levels in different histopathological subtypes and different stages of NHLs compared to the control group. From this table there is significant difference of FAS ligand between different histopathological subgroups and different stages and the highest level was in high grade lymphoma (BL&LL) and in stage IV.

(Table 5) represents correlation coefficient of CD95 expression and sFAS ligand levels and age, ESR, LDH and β2 microglobulin. There is significant positive correlation of s.FAS ligand levels & non significant correlation of CD95 expression and all parameters.

(Table 6) represents comparison of soluble FAS ligand level at time of diagnosis in patients achieving complete remission, partial remission and relapse. There is high significant difference in these groups and the lowest level of s.FAS ligand was at time of diagnosis in those reaching complete remission.

(Table 7) represents comparison of soluble FAS ligand level at complete remission, partial remission and relapse.The lowest level of sFAS L was at CR.

Table (1): ***Different sites of lymphoma affection.***

Sites	*No*	*Procedure*
(A) Nodal		Excision biopsy
1. Cervical	34	
2. Supraclavicular	3	
3. Axillary	1	
4. inguinal	2	
(B) Extranodal		
1. Gastric	2	Radical gastrectomy and oesophagojejunostomy
2. Small intestinal	3	Resection anastomosis
3. Ileocecal	4	Right hemicolectomy
4. Splenic	3	Splenectomy

Table (2): *Expression of CD95 (by immunohistochemistry and flow cytometry) in different histopathological types of NHLs.*

Lymphoma	*Number of cases*	*CD95 by immunohistochemistry*		*CD95 by flow cytometry*	
		No	*%*	*No*	*%*
Low grade lymphoma					
FL	10	7	70	7	70
MALT	8	7	87.5	6	75
Intermediate grade lymphoma					
DLL	8	3	37.5	2	25
DML	14	11	78.5	10	71.4
High grade lymphoma					
BL	4	1	12.5	1	12.5
LL	8	2	25	2	25

Table (3): *Expression of CD95 (by immunohistochemistry and flow cytometry) in different stages of NHLs.*

	CD95 by immunohistochmistry		*CD95 by flow cytometry*	
	No	*%*	*No*	*%*
Stage I no of cases (10)	8	80	7	70
Stage II no of cases (12)	6	50	6	50
Stage III no of cases (16)	4	25	4	25
Stage IV no of cases (14)	3	21.4	3	21.4

Table (4): *Serum CD95 ligand in different histopathological subtypes and different stages of NHLs compared to control group.*

	S. CD95 L (ng/ml)			
	Median		*Range*	
Control	0.08		0.04 0.11	
	Histopathol.subtypes SFASL (ng/ml)		*Stages of NHLs SFASL (ng/ml)*	
	Median	Range	Stage	Median	Range
FL	0.16	0.10-0.9	Stage I	0.17	0.12-1.07
MALT	0.18	0.13-1.13			
DLL	0.21	0.14-1.32	Stage II	0.21	0.16-1.53
DML	0.26	0.16-1.42			
BL	0.28	0.17-1.64	Stage III	0.26	0.18-1.82
LL	0.29	0.18-1.71	Stage IV	0.30	0.19-1.91
Kruscale walis test	X 32 .68			X 30.31	
P value	< 0.001			< 0.001	

Table (5): *Correlation coefficient between CD95 & s. CD95 ligand and age, ESR, LDH, β2 microglobulin*

Parameter	CD95		CD95 ligand	
	R	p	r	P
Age (ys.)	0.19	0.23	0.31	0.04
ESR (1st h/mm)	0.27	0.06	0.35	0.011
LDH (U/L)	0.28	0.06	0.36	0.016
β2 microglobulin (ng/ml)	0.24	0.09	0.59	0.000

Table (6): *Comparison of soluble CD95 ligand level at time of diagnosis in patients achieving complete remission partial remission and relapse.*

	No	s.CD95 ligand (ng /ml)	
		Median	Range
Complete remission	30	0.12	0.10-0.6
Partial remission	6	0.21	0.15-1.46
Relapse	4	0.28	0.18-1.68
Kruscal Walis test		X2 32.97	
P value		< 0.001	

Table (7): *Serum CD95 ligand level in NHLs patients at complete remission, partial remission and relapse.*

	No	S. CD95 ligand(ng/ml)	
		Median	Range
CR	30	0.10	0.06-0.21
PR	6	0.12	0.9-0.56
Relapse	4	0.22	0.16-1.22
Kruscale walis test	X^2 26.22		
P value	< 0.001		

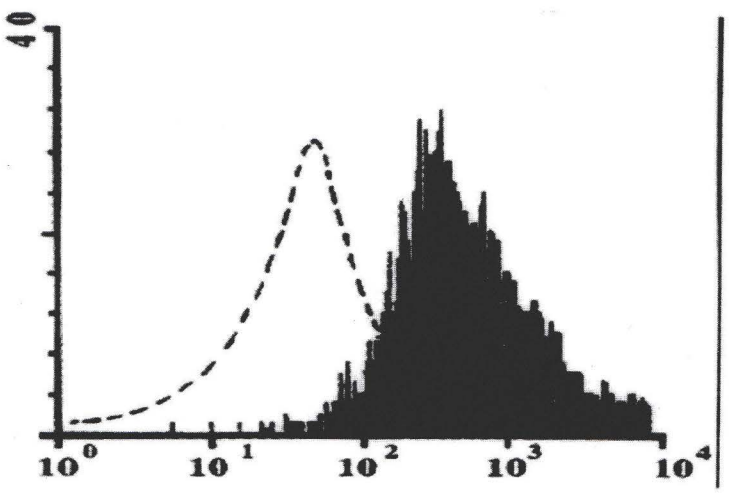
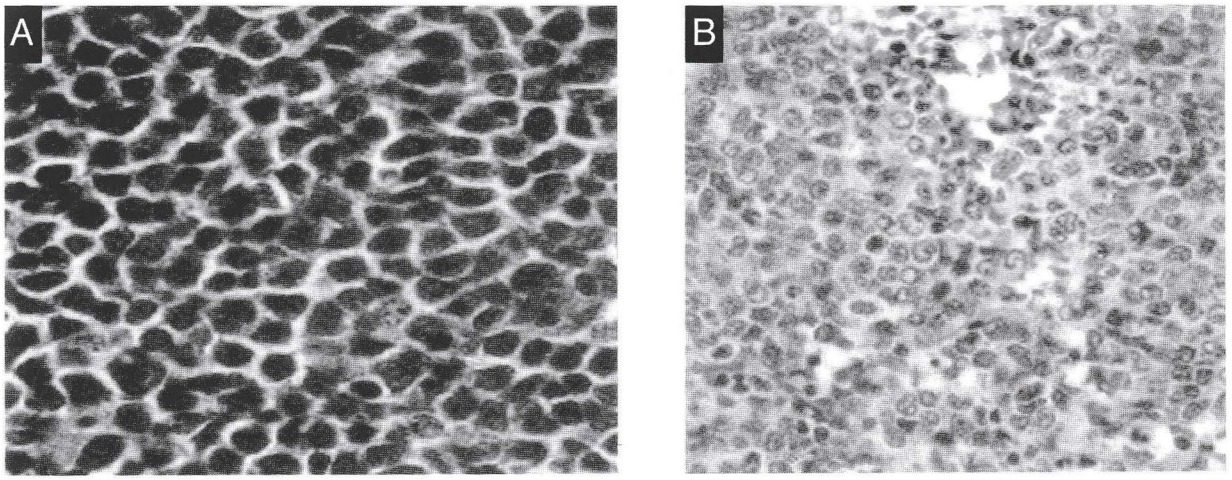


Fig. (1) CD-95 expression by flow cytometry



(a) (b)

Fig. (2) CD-95 expression by immunohistochemistry

DISCUSSION

CD95 (FAS/APO-1) and s.CD95 ligand play a key role in apoptosis of cells of the immune system. They act as effector molecules of cytotoxic T lymphocytes and function in the elimination of activated lymphocytes during down regulation of the immune response[13].

The FAS receptor is capable of inducing apoptosis of lymphoid cells and is expressed in some NHLs. It is assumed that lymphoma cells may possess structural or functional defects of FAS or FAS associated protein resulting in the failure to trigger apoptotic cell death[14].

In order to investigate these apoptotic markers and their role in clinical behaviours of lymphoma, CD95 expression by flow cytometry and immunohistochemistry and serum soluble CD95 ligand level were assessed in 52 newly diagnosed NHLs and in 12 patients with reactive lymphadenitis (to act as positive control for FAS staining) and in 10 healthy individuals with matched age and sex (to act as a control group for s. FAS ligand level).

The results of this study revealed that CD95 expression was high in low grade lymphoma (70% in FL and 87.5% in MALT) than in high grade lymphoma (12.5% in BL and 25% in LL). In intermediate grade lymphoma, CD95 expression was high in DML (87.5%) than in DLL (37.5%) .These results are in agreement with those reported by Nguyen et al,[15] and Seeberger et al , [16].The difference of CD95 expression in different grades and histopathological subtypes can be explained by that FAS is expressed on B cells at a restricted developmental stage and on activated B cells but not on native mature B cells and may be contributed to elimination of infiltrating cells by inducing apoptosis[17] .The differences in the expression of CD95 may be contributed to the difference in clinical behaviour between different grades and histopathological subtypes of NHLs. These results suggest that there is a potential mechanism for tumor immune escape in NHLs and there is resistance of CD95 mediated apoptosis either by decreased expression of CD95 (as in high grade lymphoma) or by loss of functional CD95 expression and consequently self regulatory mechanism.

In this study the highest level of CD95 expression was in stage I lymphoma (70%) while the lowest level was in stage IV(21.4%).We postulate that infrequent expression of CD95 may mediate local or systemic tissue damage and immune evasion and may contribute to the clinical aggressiveness of these tumours.

Serum soluble FAS ligand in this study was significantly different in different histopathological subtypes and in different stages of NHLS. The highest level was in high grade lymphoma (BL&LL) and in stage IV. These observations are in agreement with findings in a study of Yufu et al ,[18] This result supports the hypothesis that in lymphoma patients there is a defect in cell apoptosis of FAS/FAS ligand system Alternatively, this decrease could be the result of the soluble FAS ligand in the sera especially high level in high grade lymphoma and stage IV. These facts seem to support that additive effect of the high soluble FAS ligand on resistance to FAS mediated apoptosis and subsequently more aggressive tumor.

In this study CD95 expression was not correlated with

age,ESR,LDH or B2 microglobulin while these parameters were positively correlated with soluble FAS ligand. So, The data presented in this study strongly suggested that tumor cells may escape immune survillance by the presence of high soluble FAS ligand and the level of soluble FAS ligand may be a biological marker and may be an independent prognostic factor.

Soluble FAS L levels was low - at time of diagnosis-in those reaching CR than those reaching PR or relapsed after therapy. Moreover, after follow up it was low in CR than in PR and relapse these findings were consistent with those of Niitsu et al, [19] who stated that a high serum s. FAS Level is associated with a poor outcome of aggressive non Hodgkins lymphoma

So, the findings of this study should provide new insight into understanding clinical behaviour of the tumor in response to therapy as high FAS L level is associated with more aggressive NHL and the remission rate was significantly decreased in the subgroup having high serum sFASL level at time of diagnosis .So, it may have possible relevance in formulating approaches to cancer therapy

From this study we can conclude that infrequent expression of CD95 in high grade lymphoma suggests an association between loss of CD95 expression/function and more aggressive tumor grade.Serum soluble CD95 ligand may serve as putative marker for active diseases. Thus, intact CD95 system plays a key role in determining sensitivity or resistance towards anticancer therapy. The induction of apoptosis in FAS/FASL system might be a novel and effective approach for lymphoma immunotherapy.

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