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High Expression Apoptotic Proteins; P53, FAS, and BAX Associated with Down Regulation BCL-2 in Tuberculosis Granulomas: An Immunohistochemistry Study

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ABSTRACT

Background: *Apoptosis can be stimulated or inhibited by different signals or cell-cycle arrest proteins, each plays a specific role during Mycobacterial infection. This study analyses the expression and co-relation of apoptotic and anti- apoptotic proteins (P53, FAS, BAX and BCL-2) in human Tuberculosis granulomatous tissue reactions by immunohistochemistry.*

Materials and Methods: *The formalin fixed paraffin embedded blocks of different biopsy specimens from 40 documented TB patients were studied by immunohistochemical staining for expression of P53, FAS, BAX and BCL-2 proteins; 28, 26, 12, 32 paraffin blocks, respectively.*

Results: *In epithelioid macrophages of TB granulomas, high positivity for P53 (100%), BAX (83.3%), and FAS (57.7%) was associated with down regulation of BCL-2 (12.5%). Although, the reaction of surrounding small lymphocytes was vice versa. The multinucleated giant cells also revealed positive reaction for P53 (92.8%) and BAX (83.3%) proteins.*

Conclusion: *In comparison to previous findings, our studies revealed immunolocalization of P53, FAS, and BAX in MTB granulomas by a simple routine pathological method. These findings also re-emphasize the value of immunohistochemical studies in regard to the role of apoptotic proteins in MTB infection pathogenesis. These studies may lead to further effective and better therapeutic strategies in TB patients.****(Tanaffos 2005; 4(13): 11-19)***

Key words: *Apoptosis, Tuberculosis, Granuloma, Immunohistochemistry*

INTRODUCTION

Both virulent and attenuated Mycobacterium Tuberculosis (MTB) induce cell apoptosis in macrophages(1, 2, 3, 4, 5, 6, 7).The process is under genetic control (programmed) and can be activated by different regulatory signals. Each of these signals plays a specific role in the complex cascade of programmed cell death and induced balance between them that represents the survival or death of bacilli inside macrophages. Among them; P53, FAS (APO), BAX and BCL-2 are considered as the important regulatory proteins.

P53 is a 53 Kd DNA-binding phosphoprotein

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which is located on the short arm of chromosome 17. The Wild Type (WT) P53 protein may accumulate in the setting of P53 gene activation, including hypoxia and DNA damage. If DNA damage is too extreme for timely repair, apoptosis ensues and WT P53 protein leads to apoptotic cell death. Endogenous Nitric Oxide (NO) induces DNA damage and also results in WT P53 accumulation (8). It has been already shown that following ingestion of mycobacteria, NO induction occurred in macrophages (9). Thereafter, these macrophages expressed increased number of P53 on their surfaces and in doing so they were more susceptible to apoptosis (10).

FAS (APO) is another regulatory receptor which is present on surface of target cells including alveolar epithelial cells and fibroblasts. FAS ligand promotes apoptosis of macrophages expressing FAS and markedly curtails MTB viability (5). Thereby, the expression of FAS on the surface of macrophages is another notable determinant in susceptibility of AMs to MTB induced apoptosis (11).

BAX, a mammalian 21- Kd proapoptotic protein, shares homology with BCL-2, an anti-apoptotic factor. There is an intimate relationship between BAX and BCL-2 in cell death regulations. If the balance is in favour of BAX, apoptosis commences.

Otherwise, BCL-2 prevents cells from undergoing apoptosis (6). Apoptosis of macrophages during MTB infections has been reported in association with up-regulation of BAX and down-regulation of BCL-2 (1).

Most of the present information were obtained from the investigations performed on macrophages during MTB infection in vitro. Until now, only a few reports have investigated the apoptotic and anti-apoptotic proteins in MTB granulomatous tissue reactions of human models, and most of the studies in regard to tuberculosis and apoptosis were performed on animal models in cytologic specimens. Hence, in present study we were determined to study the localization of these proteins involved in MTB granulomatous tissue reactions in human. For this purpose, parallel sections of formalin fixed paraffin embedded blocks, were analyzed for P53, FAS, BAX, and BCL-2 by immuno-histochemistry staining.

MATERIALS AND METHODS

40 paraffin embedded blocks from 40 patients, which were diagnosed as "Chronic granulomatous inflammation" were selected and retrieved from the archive of pathology department, National Research Institute of Tuberculosis and Lung disease (NRITLD) between 1999 and 2004. The patients' records were reviewed by infectious specialist.

The diagnostic criteria for TB diagnosis were as follows: 1) Granulomatous tissue reaction; with or without necrosis, 2) Microbiologic confirmation (sputum smear/culture), and 3) Response to therapy. All cases were under treatment with anti TB drugs and were followed up in the TB clinics of our center. Seven specimens with the foreign body type granulomas were selected as control group.

Immunohistochemistry (IHC) staining:

Staining was performed on formalin, fixed paraffin embedded tissue sections by streptoavidin biotin. Immunoperoxidase method using DAKO LSAB2 Kit.The primary antibodies, dilution and method of unmasking used in this study were as follows: Anti P53 (Do7); 1/50, Anti FAS (APO-7); 1/50, Anti BAX (Rabbit); 1/350, Anti BCL-2 (clone 124); 1/40. All of the primary antibodies are from DAKO Carpinteria, USA and sections, except for FAS, were subjected to autoclave unmasking at 120 ˚C, for 10 minutes. FAS was unmasked by using proteinase K for 20 minutes at RT. Due to problem of tissue inadequacy in each block and ethic points, all immunostains were not performed on each block; it means that on each block one or more stains were done. The number of paraffin embedded blocks,

which are immunostained for P53, BCL-2, BAX, and FAS are; 28, 32, 12, 26 respectively.

Immunostaining evaluation:

The results of IHC stainings of epithelioid cells and lymphocytes, surrounding the granulomas, for P53, BCL-2, FAS (APO) and BAX were expressed in semi-quantitative fashion according to the estimated percentage of positive cells.

The immunostains for the multinucleated giant cells were expressed only as positive or negative. The immunoreactivities of the components of granulomas for the above mentioned proteins tested were evaluated by observer and were graded on a scale of 0 to 3 in which; 0 = no reaction, 1= mild reaction, 2= moderate reaction, and 3= strong reaction.

The results were reviewed by a supervisor pathologist. There was no significant disagreement between the ratings given by two observers.

RESULTS

Out of 40 studied cases, 14 were female and 26 were male with age ranging from 3 to 77 years. Their clinical and image findings are summarized in table 1 and table 2 and the sites of biopsies are presented in table 3.

Table 1. Clinical findings in tuberculosis patients.

Clinical findings	Number
Abdominal pain	2
Cough(Dry/productive)	20
Dyspnea	15
Fever &Chills	9
Hemoptysis	1
Lymphadenopathy	6
Malaise	1
Pleuretic chest pain	5
Sweating	2
Knee pain	1
Breast pain	1
Skin lesion	1

Table 2. Chest imaging findings of tuberculosis patients.

Chest imaging findings	Number
Cavitary lesion	1
Empyema	2
Fibrosis	4
Pneumothorax	4
Infiltration	8
Lobar collapse	3
Lung collapse	1
Lung nodule	1
Lymphadenopathy	2
Lymph node calcification	3
Pleural effusion	26
Pleural thickening	8
Negative image	3

Table 3. Site of biopsy in tuberculosis patients.

Site of Biopsy	Number
Breast	1
Lung	5
Lymph Node	10
Pleura	22
Skin	1
Synovium	1

The patients had the following diagnostic criteria shown in Table 4. As pathologic diagnosis; 33 cases had necrotizing granulomatous inflammation and 7 cases had non-necrotizing granuloma. As microbiological confirmation; 13 cases were either smear or culture positive and responded to treatment, in the other 27 cases were considered as another diagnostic criteria for TB. In 20 cases, pleural ADA was measured and in 14 cases, ADA was elevated. The synovial ADA was evaluated in the single case of tuberculosis arthritis. All of the 40 patients received anti TB drugs and 38 cases were followed up for 9 months in TB clinics of our center.

Immunohistochemical results:

The results of IHC staining are shown in table 5. Overall, epithelioid macrophages revealed prominent positivity for P53 (28/28, 100%), FAS (15/26, 57.7%), and BAX (10/12, 83.3%) proteins and showed down regulation of BCL-2 (4/32, 12.5%) positive reactions. Co- expression of P53, FAS, BAX and P53, FAS and P53, BAX in epithelioid macrophages were seen in 4, 12, 4 specimens, respectively.

Table 4. Summary of pathologic and diagnostic confirmatory data and sites of biopsies of 40 tuberculosis cases.

Sites of Biopsies	Pathologic Data		Diagnostic Confirmatory Data	
	Necrotizing Granuloma	Non Necrotizing Granuloma	Bacteriologic Confirmation	Response to Treatment
Breast	1	-	1	-
Lymph Node	9	2	6	5
Lung	5	-	2	3
Pleura	16	5	3	18
Skin	1	-	-	1
Synovium	1	-	1	-
Total	33	7	13	27

In contrast to epithelioid cells, the lymphocytes were nearly totally negative for P53, FAS, and BAX.

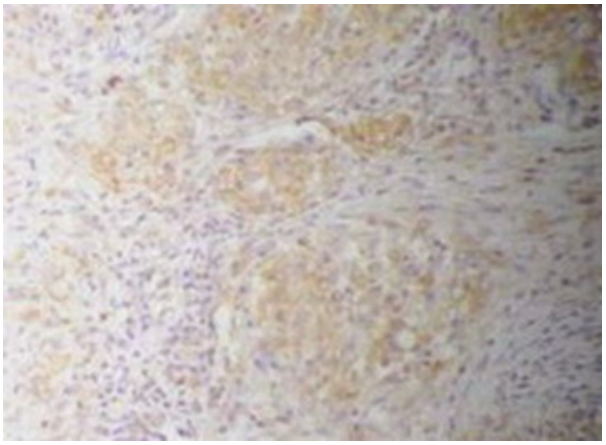
Positive reaction in multinucleated giant cells, P53, FAS and BAX were (26/28, 92.8%), (0/2, 0%), (9/12, 83.3%), respectively.

Nuclear positivity for P53 in multinucleated giant cells was heterogenous in each of them. There was no reactivity for BCL-2 in giant cells but the surrounding small lymphocytes were positive for it. Capillary endothelial cells, some background fibroblasts and some of the germinal center large lymphocytes showed mild reactivity for P53 with some of background fibroblasts being positive for FAS. The intensity of staining for P53 and FAS was prominently stronger in non necrotizing granulomas and in peripheral epithelioid cells than epithelioid cells adjacent to necrotic areas and also in non-necrotizing granulomas more than necrotizing granulomas. In control foreign body type granulomas (the epithelioid cells), P53 was positive in all 7 specimens (100%), and FAS in 1 out of 7 (14.2%) specimens. None of them revealed positivity for BAX and BCL-2.

Table 5. Immunohistochemical staining results of tuberculosis granulomas.

Staining	No.	Result	Epithelioid cells N (%)	Lymphocytes N (%)
P53	28	0	0(0)	27(96.4)
		1	3(10.7)	0(0)
		2	7(25)	1(3.6)
		3	18(64.3)	0(0)
		TP	28(100)	1(3.6)
Bcl-2	32	0	28(87.5)	1(3.1)
		1	3(9.4)	0(0)
		2	1(3.1)	3(9.4)
		3	0(0)	28(87.5)
		TP	4(12.5)	31(96.9)
BAX	12	0	2(16.7)	12(100)
		1	4(33.3)	0(0)
		2	2(16.7)	0(0)
		3	4(33.3)	0(0)
		TP	10(83.3)	0(0)
FAS	26	0	11(42.3)	25(96.2)
		1	4(15.4)	0(0)
		2	3(11.5)	0(0)
		3	8(30.8)	1(3.8)
		TP	15(57.7)	1(3.8)

0=Negative
1= Mildly positive
2= Moderately positive
3= Strongly positive
TP=Total positive



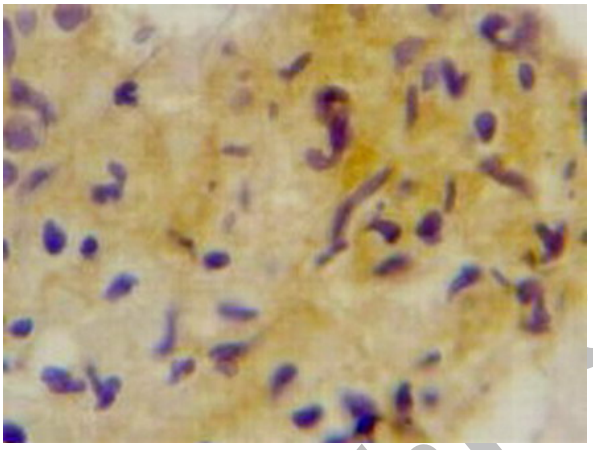
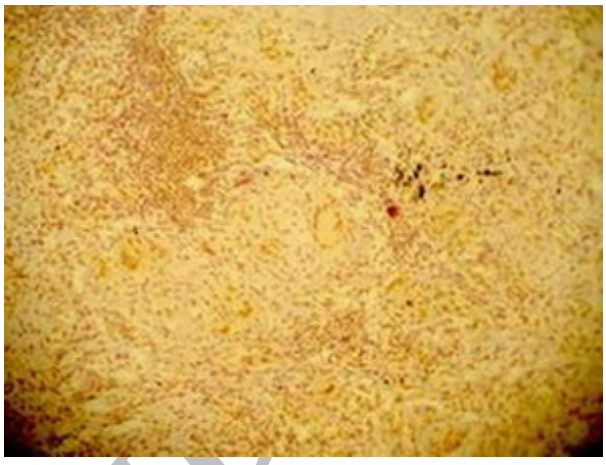


Figure 1,2. BAX cytoplasmic positivity in epithelioid macrophages in TB granuloma.



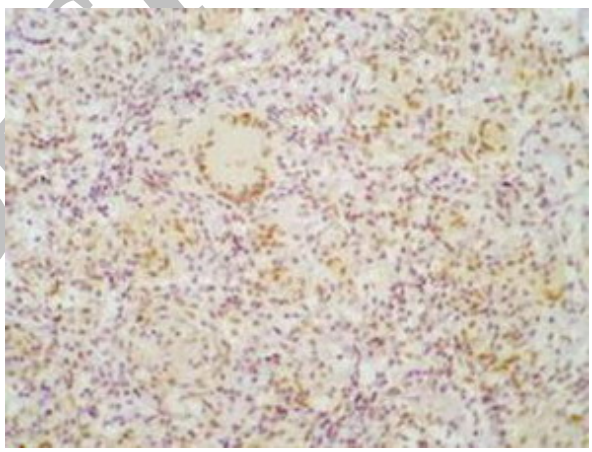


Figure 4, 5. Strong P53 positivity in both epithelioid and multinucleated giant cells in TB granulomas.

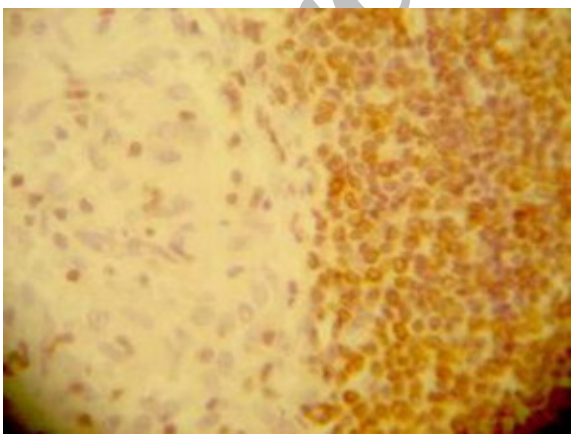


Figure 3. Bcl-2 negative reaction of epithelioid macrophages in TB granuloma.

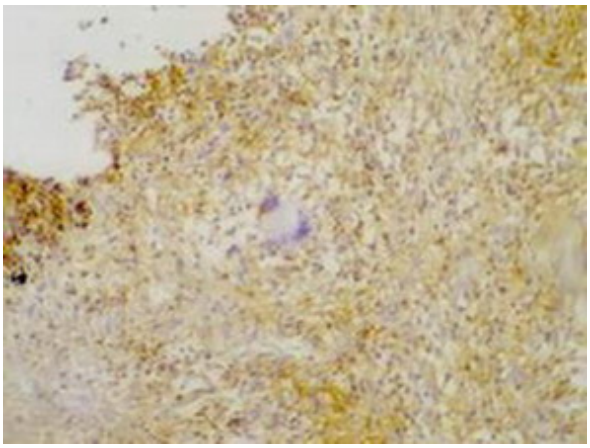


Figure 6. FAS positive reaction in TB granulomas.

DISCUSSION

The present study showed high expression of P53, FAS, and BAX proteins in epithelioid macrophages of tuberculous granulomas on biopsy specimens of human tissue by IHC method. There is prominent immunolocalization of P53 and FAS proteins in the peripheral epithelioid cells, more than epithelioid cells adjacent to the necrotic area and also in non necrotizing granulomas more than necrotizing granulomas. These findings may be indicative of positive reaction for these proteins in vital epithelioid cells.

P53: Wild type P53 accumulation has been also reported in sarcoidosis, foreign body granulomas, and in granuloma annulare (12, 13,14).

Recently, the immunocytochemical determination of P53/P21/ Waf 1/ Cip 1 and on TUNNEL detection of apoptosis in monocytes populations cultured infected with MTB revealed that the P53 and p21, Waf/Cop1 expression and apoptotic index were significantly higher in MTB than in controls (15). The suggested main triggering factors for P53 accumulation in MTB infections were TNF- α and NO (10, 9, 8). Therefore, up regulation of P53, supposed to be compatible with host defense strategies (Safe guard) which are called intracellular sanctuaries in tuberculosis (16).

In our cases expression of FAS on the surface of macrophages and also in epithelioid cells is a notable determinant in susceptibility of these cells to MTB induced apoptosis. FAS–FAS ligand mediated apoptosis of human macrophages infected with MTB has been reported in vivo studies (11, 17). FAS ligand promotes apoptosis of macrophages expressing FAS and markedly curtails MTB viability. Hence the expression of FAS on the surface of macrophages and also in epithelioid cells in our study is a notable determinant in susceptibility of these cells to MTB induced apoptosis. Capping Lipoarabinomannan inhibits macrophage apoptosis, possibly via modulation of FAS and FAS ligand interaction (5).

This study also showed that up regulation of apoptotic proteins, was strongly associated with down regulation of BCL-2 protein in epithelioid macrophages. In microassay analysis of human macrophages and alveolar epithelioid cells, Danelishvilli et al. revealed that high expression of BAX and BAD proteins associated with down regulation of BCL-2 (1). Whereas, Mogga et al showed over expression of BCL-2 in a population of host macrophages containing high MTB antigens, while BAX expression was reduced. He suggested that in doing so the MTB may escape the host's cellular response (19). Rojas et al. also found BCL-2 down regulation of human mononuclear phagocytes after infection with MTB (10). As our results in this study, with similar method, Fayyazi et al., immunohistochemically showed that the majority of vital CD68 positive macrophages surrounding caseous foci are negative for anti- apoptotic BCL-2, but positive for the proapoptotic protein BAX (20). Overall, the process of apoptosis can be sub divided into 3 functionally distinct phases; 1- Initiation phase, 2-Common effector decision phase, and 3-The degredation phase (21). In this view, our data in addition to the present findings in literature, may suggest that some initiation factors such as NO and N Ramp I activate apoptosis signals during MTB infection, probably via up regulation of wild type P53, FAS (APO) and BAX in epithelioid macrophages and simultaneously down regulate BCL-2 protein, undergoing macrophage apoptosis in granulomas. Meanwhile, Mannosylated Lipoarabinomannan (ManLAM) reversed the effect of MTB on P53 and BCL-2 expression and inhibits the apoptosis (18). Thus apoptotic process is an important defensive mechanism to mycobacterial survival and growth. Hence, in tuberculosis, chronic type of the disease and dissemination of the infection

might be related to the paucity of apoptosis in macrophages.

In conclusion, by a simple routine pathologic method; IHC, we showed the strong localization of apoptotic proteins P53, FAS, and BAX was associated with down regulations of BCL-2 in epithelioid cells of tissue sections of human tuberculous granulomas, re-emphasizing the importance of apoptosis in MTB infection tissue immunoresponse via regulating the above proteins. Like Dr. Erokhin et al., we believed that histological and immunohistochemical studies of tuberculous inflammation may specify the mechanism of pathogenesis of TB and serve as the basis for early diagnosis of the disease and timely correction of performed treatment in order to enhance its efficacy (22). Further evaluations regarding definitive apoptotic mechanisms will hopefully lead to manipulate key integral proteins to initiate apoptosis, holding the key to effective therapeutic strategies in treating serious tuberculous infections especially multiple drug resistance (MDR) TB. It is recommended to perform further studies by using more complex molecular pathologic methods e.g. PCR and TUNNEL on paraffin embedded tissue sections that could be more helpful, clarifying more details.

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