In situ localization and mRNA expression analysis of chicken BAX Inhibitor-1

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Abstract: Background: Bax Inhibitor-1 (BI-1) was originally described as testis enhanced gene transcripts involved in development. It is BI-1 is one of the few cell death suppressors conserved in animals and plants. Also BI-1 is involved in development, response to biotic and a biotic stress and probably represents an indispensable cell protectant. It was reported to response to biotic and a biotic stress and probably represents an indispensable cell protectant. BI-1 found to suppress cell death induced by mitochondrial dysfunction. The blot analysis predicted that BI-1 is a single copy gene present in all kinds of vertebrates. It was mapped to rat chromosome 7, mouse chromosome 15, swine chromosome 5 and human chromosome 12q12–q13. It works to inhibit the BAX role. Material and method: In this study Lipopolysaccharides (LPS) Stimulated spleen cells cDNA were used as a tester and non-stimulated ones were used as a driver for suppressive subtractive hybridization (SSH). Then RT-PCR was performed using both forward and reverse primers designed from the SSH obtained fragment. The probed PCR product was then used for in situ hybridization. This study data leads to the identification of chicken BAX Inhibitor-1 fragment. Results: The obtained fragment was about 200 bp covering the area from 525- to the 3′ -end of the human BI-1 homolog. The expression analysis showed a wide variation in tissues and cell lines. In situ studies revealed that mRNA expression is elevated in LPS stimulated tissues. Discussion: In this approach a homolog for a chicken baxI-1 gene was described. The chicken BAXI-1 gene found to be expressed in many tissues and cell lines in different levels. The stimulation time course also was found to have a wide effect on both spleen and IN24 cell lines under study. The BAXI-1 gene was localized by in situ hybridization and the effect of LPS stimulation was investigated in each of bursa, thymus and spleen.
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1. Introduction

Programmed cell death (PCD) is considered to be a vital phenomenon for multicellular organisms because of its involvement in removing unnecessary or harmful cells during normal development or under pathological conditions. It is an evolutionarily conserved mechanism to eliminate unwanted cells commonly occurring during development, as well as in many physiological and pathologic processes (Green, 2000, Reed, 2001 and Ferri, and Kroemer, 2001). Disturbance of the PCD machinery leads to developmental damage, disease and tumour genesis. Thereby, genetic abnormalities as well as pathogenic influence can lead to loss of PCD control. A lot of mechanisms of PCD in animals are well studied. One important mechanism that leads to a morphologically and biochemically defined type of PCD, apoptosis, can be triggered by BAX proteins, that interfere with mitochondrial function. This endogenous death machinery is triggered via two principal signaling pathways. One involves the death receptors; on ligand binding these receptors associate with an adaptor protein to recruit procaspase-8, forming the DISC3. The other pathway is stimulated by various anticancer agents, and irradiation to release cytochrome *c* and Smac/DIABLO from the mitochondrial IM space to the cytosol, resulting in activation of effector caspases and induction of apoptosis (Kroemer, 1999, Du *et al*., 2000, Verhagen *et al.*, 2000, and Halestrap *et al.*, 2000). BAX Inhibitor-1 (BAXI-1) was originally described as testis enhanced gene transcript in mammals. Studies showed that it is a single copy gene present in all kinds of vertebrates (Grzmil *et al.*, 2003). It was predicted that BAXI-1 is a membrane spanning protein with 6 to 7 transmembrane domains and a cytoplasmic C-terminus sticking in the endoplasmatic reticulum and nuclear envelope. Proteins similar to BAXI-1 are present in other eukaryotes, bacteria, and even viruses encode BAXI-1 like proteins. It was also stated that BAXI-1 is involved in development, response to biotic and abiotic stress and probably represents an indispensable cell protectant. BI-1 appears to suppress cell death induced by mitochondrial dysfunction, reactive oxygen species or elevated cytosolic Ca2+ levels (H¨uckelhoven, 2004). It was mapped to rat chromosome 7, mouse chromosome 15, swine chromosome 5 and human chromosome 12q12–q13.11–13 Rat BAXI-1 transcript sizes differ due to the presence of two alternative polyadenylation sites at the 3_ end (H¨uckelhoven, 2004). *In situ* localization of BAXI-1

transcripts or of BAXI-1 promoter activity should provide further evidence for BAXI-1 expression in tissues of enhanced cell death or survival. The fact that BAXI-1 transcripts were preferentially isolated from tissues with high cell death rates indicates a possible function for BAXI-1 in general cell survival (Grzmil *et al*., 2003). BAXI-1 function as a cell death suppressor was first described after screening for human proteins that can inhibit cell death induced by mouse BAX expression in yeast (Xu and Reed, 1998). BAXI-1 does not physically interact with BAX and no clear association of BAXI-1 with mitochondria could be demonstrated thus far, a physical interaction of BI-1 and mitochondria seems not very likely. However, BI-1 can interact with other human cell death regulators, *e.g.* Bcl-2 and Bcl-X*L* , apparently via their BH4 domain that is specific for antiapoptotic Bcl-2 family members.17 BAXI-1 might affect other signalling pathways downstream of mitochondria as well (Xu and Reed, 1998 and Bolduc *et al*., 2003). BAXI-1 was isolated as a suppressor of SW480 cell death induced by tumour necrosis factor-related apoptosis inducing ligand (TRAIL), which has been suggested as potential therapeutic agent against cancer because it kills cells in many tumour lines but obviously does not affect most healthy cells.

2. Material and Methods

A- Preparation of different Tissues and cell lines

Chicken of 12-week-old white leghorn HB15 antigen free were used in this study. Tissues (Bone marrow, brain liver, kidney, spleen, thymus, heart, lung and bursa of Fabricius) were isolated and subjected to molecular study in this work. Cell lines, macrophage cell line HD11 (Beug *et al*., 1979), B lymphoblastoid cell line 1104B (Hihara *et al*., 1974), chicken hepatoma cell line LMH (Kawaguchi *et al.*, 1987), T lymphoblastoid cell line MSB1 (Akiyama and Kato, 1974) and monocytic leukemia cell line IN24 (Inoue and Sato, 1988) had been biocultured in Iscove's modified dulbeccos medium containing from 8-10% fetal bovine serum (FBS). It left to grow up in bio-oven at 5% CO_2 and 38^{0}C then mRNA and coda were prepared for BI-1 expression analysis by RT-PCR.

B- Complementary DNA preparation

I isolated the total RNA from the tissues and cell lines used in this work, the total RNA then purified from both tissues and cell lines by using GeneJET^{TM} RNA purification kit, K0731 and K0732 (Fermentas, Life science). After that, cDNA were prepared using Oligo-dT cDNA extraction kit (Promega). To perform subtractive suppression hybridization (SSH), chicken spleen cDNA was prepared from chicken spleen cells stimulated by lipopolysaccharides LPS 10g/ml (rough strain) from *Salmonella typhimurium* SL1181 (RE mutant) (Sigma, U. S. A.) in Iscove's medium for 3, 6, and 12 hrs. To prepare this cDNA and perform SSH, we used PCR-selectTMcDNA subtraction kit (Clontech, Heidelberg, Germany) according to the manufacturer manual with some modification.

C- Preparation of expression analysis and PCR reaction

The chicken cDNA for BAXI-1 expression analysis was prepared from all studied tissues and cell lines by reverse transcriptase (RT)-PCR by using the primers 5′- ATGTGTGGCTTCGTGCTCTTCG-3′ as a forward primer and 5′-TCACTTCTTCTCCTTCTTCT-3′ as reverse primers. Chicken tissue and cell lines cDNA was amplified using that cDNA as a template and amplitaque gold amplification enzyme kit. The PCR amplilification was done using PTC-100^{TM} by thermal controller (MJ, U.S.A.) according to the following program, The reaction mixture was incubated at 95 ^{0}C for 10 min, denaturized for 30 sec. at 95 ^{0}C, annealed for 30 sec. at the optimal temperature which decided to be 49 ^{0}C and extended at 72 ^{0}C for 2 min. the reaction had been done for 30 cycles and the finally incubated at 72 ^{0}C for 10 min for final extension the expression analysis was explored by using agarose gel electrophoresis technique.

D- *In Situ* Hybridization

I checked the expression of mRNA of chicken BAXI-1 in spleen, thymus and bursa. Frozen sections of chicken organs were sectioned in from 9-11m thickness. For in situ hybridization I used digoxygenin-labeled (Roche Applied Science) probes. Both sense and antisense probes were prepared from the purified PCR product of 180 bp length that obtained from subtractive suppression hybridization (SSH) according to the manufacturer manual with the following few modifications. Both sense and antisense transcripts had been Linearized with T3 and T7/sp6 RNA polymerase. In situ ready frozen Sections were, fixed in (4% paraformaldehyde in 0.1 % DEPC treated PBS) for 30 min., after that incubated in 0.1% active DEPC-PBS 15 min x 2 times for inactivation of RNase. That as followed by immersion of slides in DEPC-treated 5 x SSC

By this way section are ready for hybridization. Before hybridization sections were prehybridized at 58℃ for 2hr in 50% formamide /5 x SSC buffer, 40 µg/ml salmon sperm DNA. Hybridization then had been done for 18 hrs at 58℃, by 400 ng/ml probe of DIG-labeled chicken BAXI-1 fragment, in 50%

formamide /5 x SSC buffer, 40 µg/ml salmon sperm DNA, this step was carried out in 50% formamide /5 x SSC buffer saturated chamber. After hybridization, slides washed by 2 x SSC buffer at RT.1 h in 2 x SSC at 65℃., then, 1 h in 0.1 SSC and 5 min equilibration in buffer 1 (Tris 100 mM, NaCl 150 mM, pH 7.5). After that, slides were incubated O/N with anti-DIG antibody,Pod-coupled, diluted 1:200 in buffer 2 (buffer 1 with 0.5% Boehringer Blocking reagent) at 4℃ .Then hybridized slides had been washed by 2 x 15 min in buffer 1. Slides then equilibrated for 5 minutes in buffer 3 (Tris 100 mM, NaCl 150 mM, $Mgcl_2$ 5o mM, pH 9.5).

Finally, slides were stained with substrate kit for peroxidase (vector® NovaRED, Funakushi, JAPAN) for 30 min. Then washed with tap running water for 15 min and then stained with methelene green for 5 min., dried and mounted and checked for the mRNA of interest.

3. Results

A- Obtaining the subtraction fragment

More than 300 clones of the subtraction clones were subjected to DNA sequence by using an automated applied biosystem model ABI-300 sequencing system. The sequenced clones were analyzed by homology search in the DDBJ/Gen Bank. I got a many differentially expressed clones among it was that show homology with human BAXI-1. The BAXI-1 homolog was around 200 bp covering the position from 525- to the 3′ -end of the human BAXI-1. The subtracted fragment figure (1) showed more than 76% homology with human counterpart human BAXI-1.

B- Gene expression of chicken BI-1

The profile of chicken BAXI-1 expression in different chicken tissues figure and cell lines was reported in figure (2). The effect of time course of stimulation on this gene expression in organs (spleen, thymus and bursa) and cell line (IN24) was presented in figure (2) and figure (3) respectively. Chicken BAXI-1 showed a high expression in spleen, thymus, bursa and testis. Moderate expression was recorded among liver, kidney, bone marrow and lung, very low expression was reported in both muscle and heart. The time course of stimulation showed small change in thymus. The BAXI-1 expression level found to be decreased by the stimulation time in spleen. The cell lines examination showed a high expression in IN24, HD11, MSB1, LMH and 1104B cell lines, somewhat low expression was in CEC-32 cell line. The expression was very low at 2 hr 6 hr it reached its beak by 4 hours. After 12 hours the level increased again figure (3).

C- Chicken BI-1 localization by in situ hybridization

The chicken baxI-1 was localized in both normal and LPS-stimulated spleen, thymus and bursa. Results of spleen stimulation showed that mRNA was increases due to LPS stimulation even the reaction was found also in non-stimulated spleen figure (4). In thymus, the BAXI-1 mRNA was increased in both cortex and medulla figure (5). In bursa the reaction was high in all the bursa lobules and the signal was very week in the non stimulated bursa figure (6).

A.

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Chicken   ATGTGTGGCTTCGTGCTCTTCGACACTCAGCTCATCATTGAGAAGGCAGAGAGCGGAGAT
Human     ATGTGTGGCTTCGTCCTTTTTGATACTCAACTCATTATTGAAAAGGCCGAACATGGAGAT
          ************** ** ** ** ***** ***** ***** ***** **    ******
Chicken   AAGGATTACATCTGGCACTGCGTGGATCTCTCCCTGGATTTTGTCAACATCTTCCGAGAG
Human     CAAGATTATATCTGGCACTGCATTGATCTCTTCTTAGATTTCATTACTGTCTTCAGAAAA
           * ***** ************ * ******* * * *****  * *   ***** ** *
Chicken   CTGCTGATGATCCTGGGGGTGAATGAGAACAAGAAGAAGGAGAAGAAGTGA---
Human     CTCATGATGATCCTGGCCATGAATGAAAAGGATAAGAAGAAAGAGAAGAAATGA
          *  *****  * ****** *  ** *******   ************  **
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B.

Figure 1. Sequence comparison between chicken BAXI-1 fragment and human BAXI-1. The identical residues among the two sequences were underlined by stars in (a) and enclosed in boxes (b).

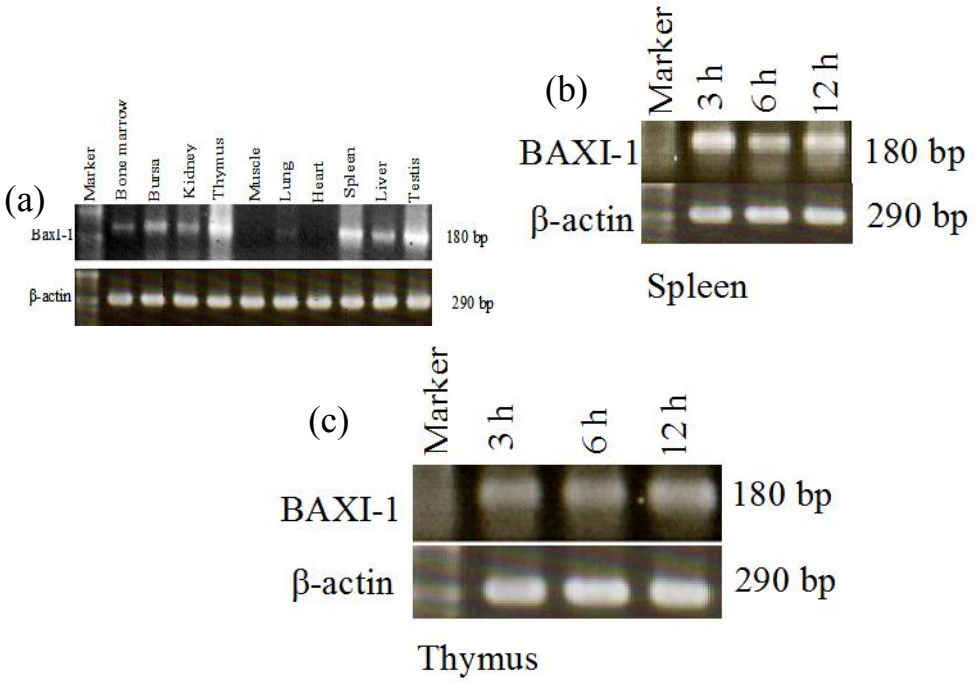

Figure 2. Expression analysis of chicken BAXI-1 in various tissues (a), cell lines (b): Time course stimulation effect on spleen, thymus, bursa (c) and T-cell fraction and B-cell fraction of chicken spleen (d).

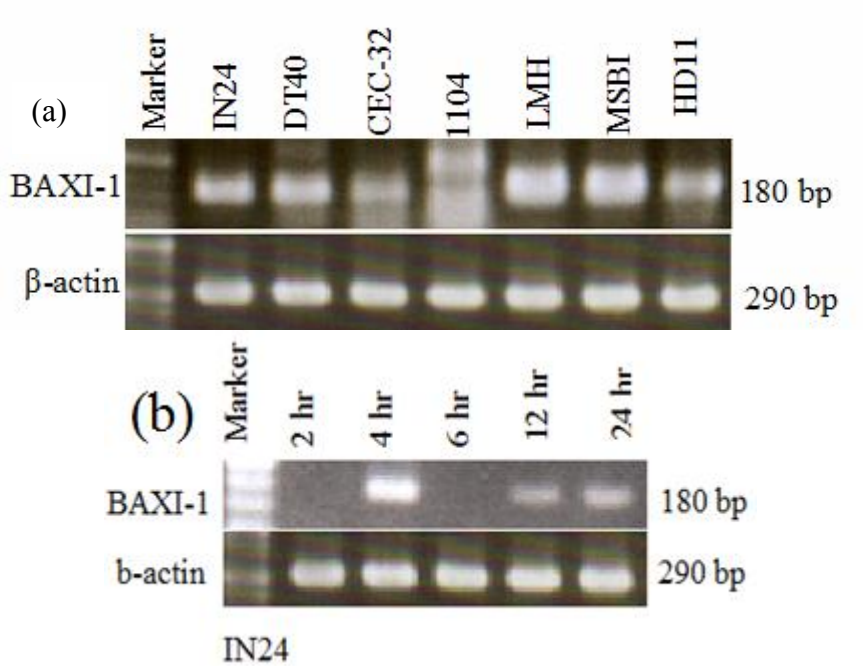

Figure 3. Expression analysis of chicken BAXI-1 in various cell lines (a). time course stimulation effect on In24, HD11, LMH (c).

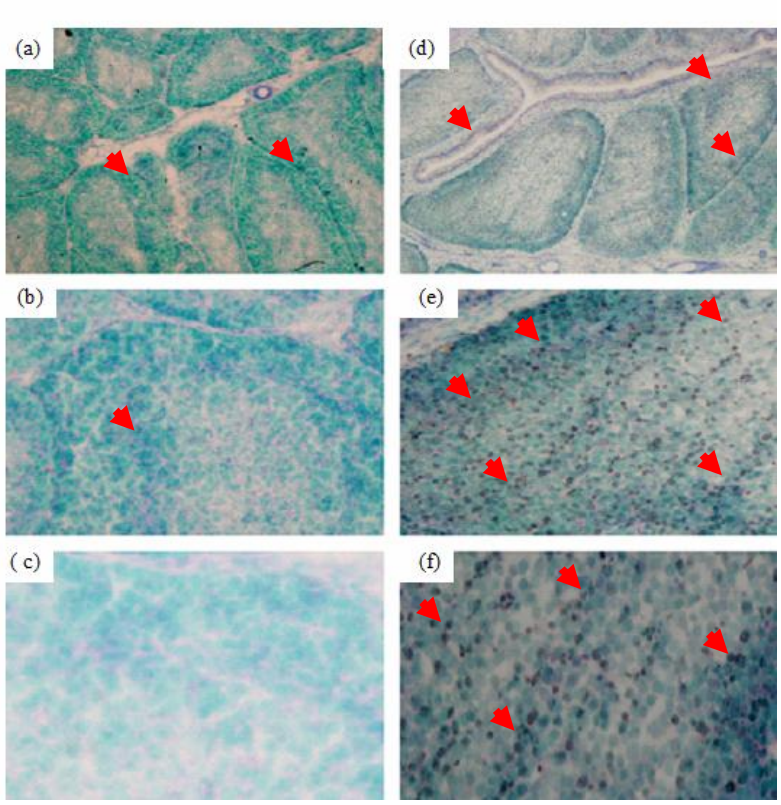

Figure 4. In situ localization of chicken BAXI-1 in LPS stimulated (d, e, f) and non-stimulated bursa (a, b, c). Arrows show some of the positive cells. (a and d X4), (b and e X20), (c and f X40).

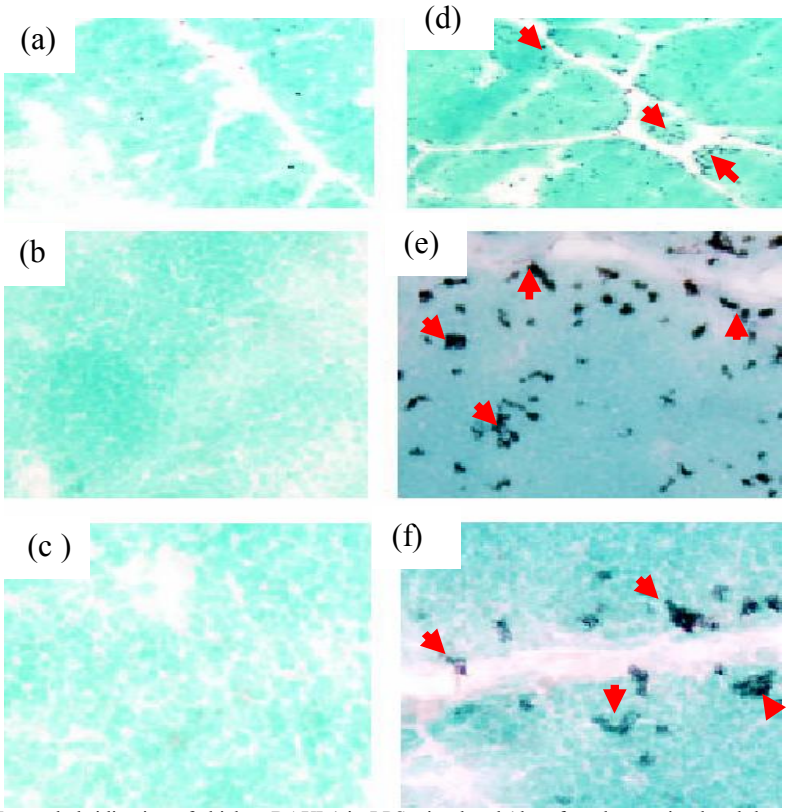

Figure 5. *In situ* hybridization of chicken BAXI-1 in LPS stimulated (d, e, f) and non-stimulated thymus (a, b, c). Arrows show some of the positive cells. (a and d X4), (b and e X20), (c and f X40).

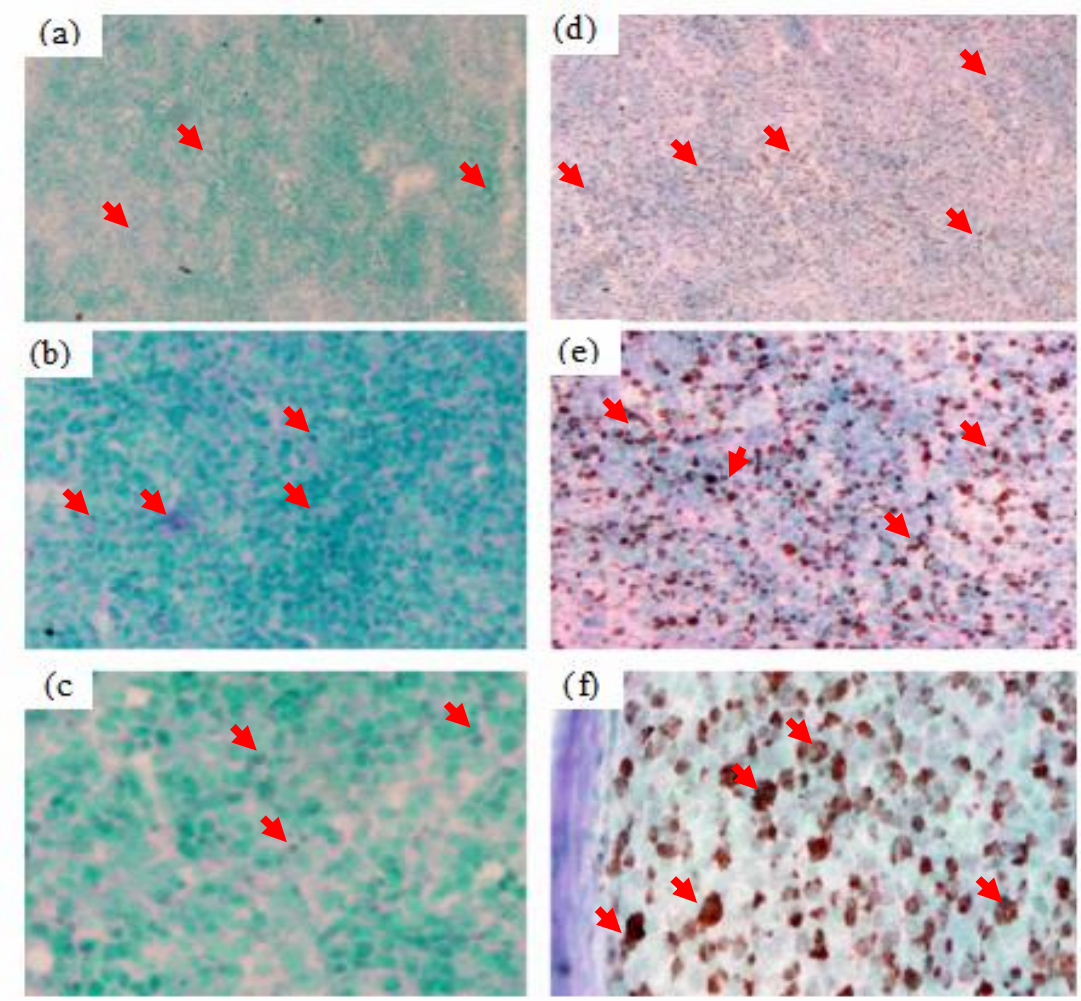

Figure 6. mRNA localization by *in situ* localization of chicken BAXI-1 in LPS stimulated (d, e, f) and non-stimulated spleen (a, b, c). Arrows show some of the positive cells. (a and d X4), (b and e X20), (c and f X40).

4. Discussion

A- Suppressive subtractive hybridization (SSH) chicken baxI-1 fragment

BAX is a proapoptotic Bcl-2 family member that play key roles in the regulation of apoptosis (Deborah *et al.,* 2006). BAX Inhibitor-1 (BAXI-1) was originally described as testis enhanced gene transcript in mammals (H¨uckelhoven, 2004). Among the important approaches to clone the novel gene is the suppressive subtractive hybridization which represents an effective experimental one in identifying and cloning genes displaying differential expression. By Appling subtraction method we can subtract all similar genes between the tester and driver. This strategy had been applied to chicken spleen cells stimulated with LPS as tester and non stimulated one as driver cDNA. Many clones were analyzed by the DNA sequencer and the clone that shows a homology with human baxI-1 was subjected to further studies. We got a 200 bp of chicken BAXI-1 homolog that show more than 76% homology with that of cloned human gene (accession number AY736129).it was reported by many research groups that human BI-1 proteins share a relatively high level of identity with other BI-1 proteins different species (Nathalie *et al*., 2003). The alignment of the SSH fragment with the nucleotide of human BAXI-1 recorded that the obtained fragment runs from the 3′ end upstream till the position 525 (Fig.1). The whole ORF of the human BAXI-1 is about 711 bp so it may will be easy to clone the rest of the gene in the 5′ direction by RACE in the future work.

Expression examination of chicken BAXI-1

BAXI-1 was first cloned from adult rat testis as so-called testis enhanced gene transcript (TEGT) and

subsequently identified in mouse and human (H¨uckelhoven, 2004). Some reports stated that RNA blot analysis indicated that BAXI-1 is widely expressed *in vivo*, including heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (Qunli and John, 1998). In this experiment a surprising diversity of expression of chicken BAXI-1 was observed. Taking these concepts in mind we can explain the diverse expression of chicken BAXI-1 in most of chicken different organs as it shown in figure (2). As it shown in figure chicken BAXI-1 is expressed in very high level in testis coda and also high in each of spleen and thymus. The expression level was moderate in bursa, bone marrow, liver and kidney and also expressed in the rest of studied organs with respect to the difference in expression level in each one this also can be supported by the pervious reports. The expression of BAXI-1 in all of these organs was reported to be due to the nature of BAXI-1 protein where it was reported that this protein is found in many positions (cytoplasm, endoplasmic reticulum). The amino acid sequence of human BAXI-1 found to have from 6-7 Tran membrane domains (TM) (Bolduc *et al.*, 2003) this also explain its expression in different tissues. In spit of reporting a high level of human BAXI-1 expression in lung and heart, in chicken tissue the expression in both organs was very low but was very high in chicken testis as in human. Regarding cell lines expression level, it was found that chicken BI-1 is expressed in all cell lines under study. The expression level showed to be very high in In24, MSB1 and LMH and three cell lines and this support the finding which report that BI-1transcripts were preferentially isolated from tissues with high cell death rates indicating a possible function for BAXI-1 in general cell survival (Villalva *et al.,* 2002). Examination the effect of stimulation time on the level of chicken BAXI-1 expression showed that chicken spleen showed a gradual decline in expression level by the stimulation time where BAXI-1 is an antiapoptotic protein so its expression normally will be affected by the apoptotosis promoting inducers. In thymus the level did not affected so much. This time course stimulation in IN24 cell line showed a peak of expression level after 4 hours of stimulation followed by decreasing the level then the expression level was recovered by the 12 hours and on. The course of chicken BAXI-1 had been found to be very wide and mostly is expressed in all chicken tissues and cell lines examined.

***In Situ* hybridization study of chicken BAXI-1**

The BAX inhibitor-1 is functionally and structurally conserved in eukaryotes. The direct binding of BAXI to BAX usually occurred by selective BH3-only proteins which might provide another level of control within the family (Kim *et al.*, 2009 and Zhang *et al.*, 2009). The molecular interactions contributing to this specificity remain to be fully elucidated (Chen *et al.*, 2005). BAXI-1 transcripts or of BAXI-1 promoter activity should provide further evidence for BAXI-1 expression in tissues of enhanced cell death or survival. The fact that BAXI-1 transcripts were preferentially isolated from tissues with high cell death rates indicates a possible function for BAXI-1 in general cell survival (Grzmil *et al.*, 2003). Frozen sections of chicken spleen, thymus and bursa of Fabricius were prepared and hybridized with a labeled probe prepared from the RT-PCR product of the cloned fragment. In all the three organs the stimulation by LPS showed a remarked expression of chicken BAXI-1 mRNA (Figs.4, 5 and 6). It had been reported that most eukaryotic BAXI-1 proteins comprise RXR and/or KKXX-like amino acid sequences close to the C-terminus representing motifs that typically mediate ER retention of membrane proteins with the C-terminus in the cytoplasm (Shikano, 2003). Interestingly, the charged C-terminus of BAXI-1 that might be also involved in protein-protein interaction is important for cell death regulation. Also the sequence data in chicken subtracted fragment showed the motif KKXKK at the C-terminus confirming the reported investigations. Therefore the signal was so power full in the stimulated section in comparison with that non stimulated ones. The signal in chicken spleen is very strong and this confirm the reports that stated that human BI-1have to role in programmed cell death (PCD) regulation in Eukaryotes where many factors Both endogenous and environmental stress can induce PCD in eukaryotes. Finally, PCD depends on proteolytic activities, which may be directly or indirectly controlled by BAXI-1 function. Also it was stated that BAXI-1 expression might be regulated by stress factors and upstream PCD regulators.

Complete the cloning and investigation chicken BAXI-1 and study its biology will leads to more information about chicken cellular PCD and various signal transduction biology.

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