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Original Research Article

Pro- Apoptotic and anti-apoptotic protein expression in apoptosis

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A B S T R A C T

Apoptosis is a physiological process that entails the programmed death of the cells. Although apoptosis has a functional role in normal development and tissue homeostasis, aberrant triggering of the process by toxicants may lead to abnormal function or disease. Bcl-2 was the first member to be identified of a growing family of genes that regulates cell death in either positive or a negative fashion. Members of the Bcl-2 family of proteins play a major role in governing this mitochondria-dependent apoptotic pathway, with proteins such as bax functioning as inducers, and proteins such as Bcl-2 as suppressors of cell death. The present study was aimed to investigate the mRNA expression of pro-apoptotic proteins like Bid, Bad, Bax and anti apoptotic protein Bcl2 in ventral prostate of infected rats were studied.

Introduction

The most common and well-defined form of programmed cell death (PCD) i.e. apoptosis, is essential for embryonic development, immune-system function and the maintenance of tissue homeostasis in multicellular organisms (McGill and Fisher, 1997). Apoptosis is now studied as a cascade of proteases and endonucleases, where oligonucleosomal DNA laddering and cleavage of a variety of substrates by cysteine proteases have become the modern Gold-Standards (Reed, 2004). To keep the apoptotic programme under control, caspases are initially expressed in cells as inactive procaspase precursors.

When initiator caspases-such as caspase-8 and caspase-9 are activated by oligomerization, they cleave the precursor form of effector caspases such as caspase-3, caspase-6, caspase-7 (Salvesen and Dixit, 1997; Thornberry and Lazebnik, 1998).

There are two pathways by which caspase activation is triggered – the extrinsic and intrinsic apoptotic pathways. The extrinsic pathway is activated by the engagement of death receptors on the cell surface. The intrinsic pathway is triggered by various extracellular and intracellular stresses, such as growth-factor withdrawal, hypoxia, DNA damage and oncogene induction (Budihardjo *et al.,* 1999).

A series of biochemical events is induced the results in the permeabilization of the outer mitochondrial membrane, the release of cytochrome c and other proapoptotic

molecules, the formation of the apoptosome – a large protein complex that contains cytochrome c, apoptotic protease activating factor 1 (APAF1) and caspase-9 and caspase-3 activation. Among these processes, only the permeabilization step is regulated, in that anti-apoptotic members of the Bcl-2 family can stop the march towards apoptotic death (Cory and Adams, 2002). Bcl-2 family proteins share several homology regions (Bcl-2-homology/BH domains) crucial for both their dimerization and apoptosis-modulatory functions (Oltvai and Korsmeyer, 2000). This family includes Bax, Bcl-x, and Bad, Bag, Bag-1, Bak, Bik, Hrk/hara T kiri and others. The pro apoptotic Bax protein, cloned through its ability to co-immunoprecipitate with Bcl-2 (McGill and Fisher, 1997).

The proapoptotic member of the Bcl-2 family Bax can directly cause mitochondria to release cytochrome c. The ability of some Bcl-2 family members, including Bax, to form ion channels has suggested that these proteins open pores in the outer mitochondrial membrance, allowing exit of cytochrome c. Finally cytochrome c release from mitochondria, that triggers caspase activation, appears to be largely mediated by direct or indirect ROS action. The caspase cascade ultimately triggers cell death through the destruction of cellular proteins, which are important for cell viability (Antonsson *et al.,* 1997).

Materials and Methods

Isolation of Total RNA

Total RNA was isolated from the frozen tissues of infected rats using RNA isolation (TRI reagent) following the method of Chomczynski and Sacchi (1987). The total RNA obtained is free from protein and DNA contamination.

Single step guanidium acid phenol method emphasizes on the ability of guanidium isothiocyanate (GTIC) to lyse cells, denature protein and inactivate intracellular ribonuclease rapidly. The presence of β-mercaptoethanol in the mixture enhances the solubilization properties of the GITC extraction buffer. Acid phenol extraction (pH<5.0) selectively retains cellular DNA in the organic phase and aids in the extraction of proteins and lipids. The addition of chloroform further removes lipids and establishes two distinct phases, an organic phase containing the DNA, proteins and lipids and an aqueous containing the RNA (Chomczynski and Sacchi, 1987).

Reagents

TRI reagent: Phenol, guanidium isothiocyanate, urea, detergents, buffering agents and stabilizers,

Chloroform (molecular grade),

Isopropanol (molecular grade),

75% ethanol, **Diethylpyrocarbonate (DEPC) water – 0.2% (or) sterile water**

To 100mg tissue, 1ml of TRIR was added and homogenized well. Following homogenization, the homogenate was stored for 5min at 4ºC to permit complete dissociation of nucleoprotein complexes. To this, 0.2ml of chloroform/ml of RNA reagent was added, shaken vigorously for 15seconds and centrifuged at 12000×g for 15min at 4ºC. After centrifugation, the upper aqueous phase containing RNA were separated. The volume of the aqueous phase was about 40-50% of the total volume of the homogenate.

The aqueous phase was carefully transferred to a fresh eppendorf micro centrifuge tube

without disturbing the interphase. Equal volume of isopropanol was added to the eppendorf tube and then centrifuged at 12000×g for 10min at 4ºC. RNA got precipitated and formed as a white pellet at the bottom of the tube. The supernatant was removed and pellet was washed twice with 75% ethanol (1ml of 75% ethanol/1ml of initial solvent used). Subsequently, the eppendorf tube was vortexed and centrifuged at 7500 × g for 15min at 4ºC. At the end of the procedure, the pellet was dried under vaccum for 5min. Care was taken not to allow the RNA pellet to dry completely, as it will greatly decrease its solubility. The RNA pellet was then dissolved in 30µl of 0.2% sterile water and placed in a water bath at 60ºC for 10 min to ensure complete solubility of RNA. The RNA sample was subsequently vortexed for 10 min and stored at -80ºC.

Quantification of RNA

Diluted RNA sample was quantified spectrophotometrically by measuring the absorbance (A) at 260nm. An absorbance of 1 OD is equivalent to RNA concentration of 40µg/ml. Therefore, the yield can be calculated by multiplying the absorbance at 260nm with dilution factor and 40µg. By determining the absorbance of the sample at 260nm and 280nm, one can assess the purity of the RNA. This is a reflection of the protein contamination in the sample. A ratio of absorbance at 260/280nm is generally considered as good quality RNA (>1.8) (Fourney *et al.,* 1988).

Reverse Transcription-Polymerized Chain Reaction (Rt-Pcr)

RT-PCR is an approach for converting and amplifying a single stranded RNA template to yield abundant double stranded DNA product. It involves two steps as: 1. **First strand reaction:** Complementary DNA (cDNA) is made from an mRNA template using dNTPs & Reverse transcriptase. The components are combined with a DNA primer in a reverse transcriptase buffer for an hour at 37°C. **2. Second strand reaction:** After the reverse transcriptase reaction is complete, cDNA has been generated from the original single strand mRNA, standard PCR (called the “second strand reaction”) is initiated.

Reverse Transcription

RT-PCR is a method used to amplify cDNA copies of RNA. It is the enzymatic conversion of mRNA into a single cDNA template. A specific oligodeoxy nucleotide primer hybridized to the mRNA and is then extended by a RNA dependent DNA polymerase to create a cDNA copy.

Reagents

First Strand buffer(5x): (250mM Tris-HCl (pH8.3), 75mM KCl, 15mM $MgCl_2$

dNTP (2.5µM each dNTP)

OligodT (10µM): Stock (2µg/µl): 147.1µg of oligodT dissolved in 73.55µl RNase free water Working: 3µl of stock diluted with 37µl of sterile water

Superscript III Reverse Transcriptase (200U/µl): Isolated from an E.Coli strain carrying the Reverse Transcriptase gene from M-MuLV (Moloney Murine Leukemia Virus). The RT Kit was purchased from INVITROGEN

Template (RNA-1.5µg for 20µl reaction)

RNase free water

For each step mixed gently and spinned briefly and incubated in the thermal cycler at 25ºC for 5 minutes, 50^0C for 45 minutes,

70°C for 15 minutes and finally maintained at 4°C for 5 min. After the reaction, samples were stored at -20ºC or proceed to the PCR.

Polymerized Chain Reaction

Reagents

Master Mix components (2X)

KAPATaq DNA Polymerase (Isolated from the thermophilic bacterium Thermus Aquaticus purified from recombinant Escherichia coli), Buffer (Mg^{2+} and 0.4mM each dNTP, (USA), Forward primer (6µM), Reverse primer (6µM), cDNA- Template, RNase and DNase free Water.

The purpose of a PCR (Polymerase Chain Reaction) is to make a huge number of copies of a gene. There are three major steps in a PCR, which are as follows:

Total reaction of 20µl

Amplified product was analyzed by Agarosez gel electrophoresis with ethidium bromide staining. Agarose gel electrophoresis is an effective method for the identification of purified DNA molecules (Sambrook *et al.,* 1989).

Results and Discussion

Fig 1, shows the Bid, Bad & Bax mRNA expression in ventral prostate.The mRNA expression of Bid, Bad & Bax was significantly increase in ventral prostate of infected rats while compared with control rats. ROS initiates apoptosis in ventral prostate by upregulating the gene expression of pro-apoptotic.

Fig 2, illustrates the Bcl2 mRNA expression in ventral prostate of adult rats. The mRNA expression of Bcl2 was significantly decreased in ventral prostate of infected rats while compared with control rats. ROS initiates apoptosis in ventral prostate by down regulating the gene expression of anti-apoptotic Bcl-2 family members.

Reaction	**Temperature (^{o}C)**	**Time**
Initial Denaturation	94	2 min
Denaturation	94	30 sec
Annealing	Varies	30 sec
Extension	72	30 sec
Final extension	72	4 min

Components		**Volume (µl)**
Master mix (2µM)		10
Gene of interest	Forward primer	2
	Reverse primer	2
Internal control	Forward primer	2
	Reverse primer	2
RT sample		1
Sterile Water		5
Total Reaction		**20**

Fig.1 Bid, Bad and Bax Mrna Expression in Ventral Prostate of Infected Adult Rats

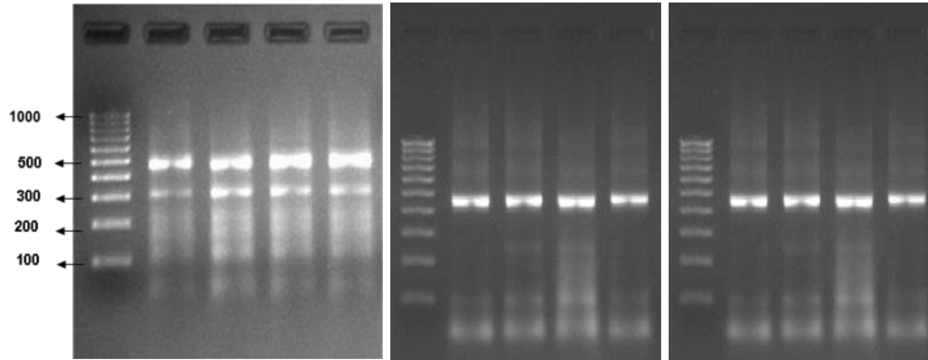
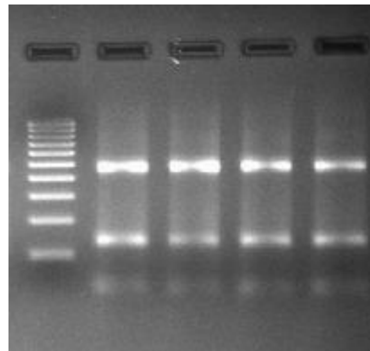
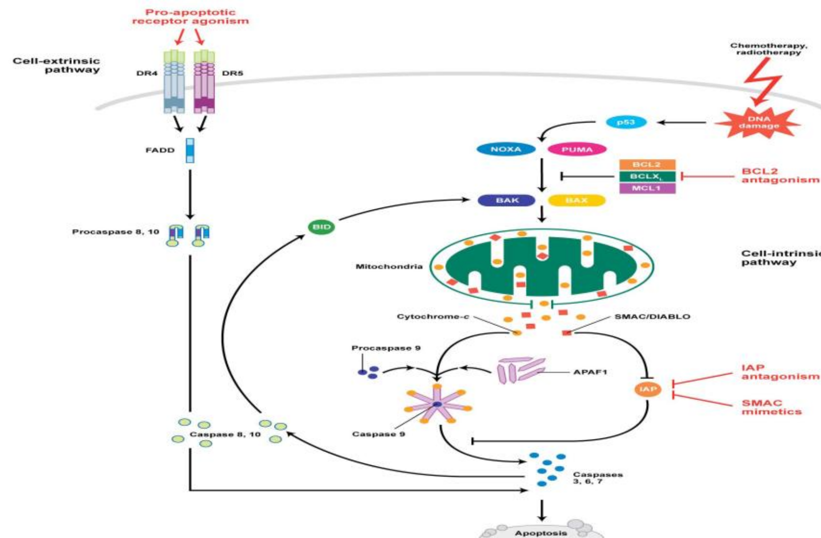


Fig.2 Bcl2 Mrna Expression in Ventral Prostate of Infected Adult Rats



Intrinsic and Extrinsic Pathway Signaling for Apoptosis (Ashkenazi, 2002)



In conclusion, apoptosis is an active process, characterized by mitochondrial swelling, chromatin condensation and formation of apoptotic bodies and eventually activation of caspases (Thornberry *et al.*, 1998; Shi, 2004). In the present study, increased

mRNA expression of pro-apoptotic proteins such as Bid, Bad, Bax enlighten ROS mediated DNA damage in the ventral prostate of Infected rats. Which leads to the increased production of apoptotic proteins whereas simultaneously down regulates the expression of Bcl2 through scavenging ROS. The mRNA expression of apoptotic proteins Bid, Bad, Bax were increased in the ventral prostate of infected rats. ROS initiates apoptosis in ventral prostate by upregulating the gene expression of pro-apoptotic and down regulating the gene expression of anti-apoptotic Bcl-2 family members.

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