Chapter 5

Mechanism of anticancer activities
INDUCTION OF APOPTOSIS IN HUMAN BREAST CANCER CELLS BY ACETONE FRACTION OF _H. ADAKODIEN_ ROOT EXTRACT

INTRODUCTION

Apoptosis plays an active role in the physiological cell turn over during adult life, embryonic development and normal functioning of immune system. On the other hand dysregulation of apoptosis plays an important role in the pathophysiology of different disorders, such as neurodegenerative diseases, auto immunity and cancer. (Thompson CB 1995). It is an accepted statement that tumor cells possess many different mechanisms to become intensive to external insults or internal damages and, therefore thwart the execution of the apoptotic machinery.

Now a days intensive research is underway to understand the factors that can lead to dysregulation of apoptosis and to develop new drugs targeting apoptosis as a therapeutic approach. It is clear that therapeutic windows in cancer therapy are relatively narrow, and tumor cells become resistant to many chemotherapeutic drugs after repeated use.

It is well known that most of the currently used anticancer agents induce apoptosis in tumor cells. The induction of the endogenous death machinery can be initiated through the activation of two principal pathways (Sun X M et. al., 1999). One involves the binding of ligands to death receptors, such as CD95 and tumor necrosis factor - receptor as subsequent recruitment of caspase – 8 into the death signaling complex. The other pathway is triggered by a number of apoptotic stimuli, such as anticancer agents or irradiation, and is essentially controlled at the mitochondrion. (Ashkenazi A et. al.,1998). An initial event in the
mitochondrial pathway is the release of cytochrome C from the mitochondria into the cytosol, which together with dATP and Apaf – 1 autocatalyses the activation of caspase – 9. (Ashkenazi A et al., 1998).

It is found that Holostemma has effective cytotoxic effect, on mouse cells. But there are no studies on the mechanism of cell death induced by Holostemma. Recent studies on mouse embryonic fibroblast cells suggest that apoptosis may be involved in the cytotoxicity of certain drugs. (Jaiswal A S et al., 2002).

The aim of the study is to investigate whether the cell death induced by Holostemma occurs through apoptosis. Flow cytometry provides a method for sorting a heterogeneous mixture of biological cells into two or more containers, one cell at a time, based up on the specific light scattering and fluorescent characteristic of each cell. The flow cytometric analysis indicates that the cell death induced by the treatment of Holostemma adakodien involves the activation of apoptosis cascade.

Materials and methods are described in Chapter II.7.
RESULTS

_H-adakodien_ inhibits growth of MDA MB231 and MCF – 7 cells (human breast cancer cells) in a concentration dependent manner.

The acetone extract caused a clear concentration dependent inhibition of growth of the breast cancer cells MDA MB231 and MCF. 7 Cell viability was assayed by reduction of MTT at 48h (Fig.5.3) after the addition of various concentrations (37.50 to 300 µg/ml.) of _H.adakodien_ acetone extract.

_H.adakodien_ acetone extract induces Morphological Alterations, Nuclear Condensation and translocation of phosphatidyl serine.

The phenotypic characteristics of _H.adakodien_ treated cells were evaluated by microscopic inspection of over all morphology. Treatment of cells with 300 µg/ml _H.adakodien_ acetone extract for 24hr resulted in the formation of nuclear condensation, which was clearly evident in the inverted fluorescent microscopy (Fig.5.4).

To confirm whether the cytotoxic effects induced by _H.adakodien_ root acetone fraction in these cells involve apoptotic changes, cells were examined for characteristic apoptotic patterns.

Up on treatment of cells with different concentration of _H.adakodien_ root acetone fraction, nuclear condensation (examined by staining the cells with ethidium bromide and acridine orange) was visible in MDA MB231 and MCF.7 cells at 24h (Fig.5.4)
*H. adakodien* induces apoptosis, not necrosis.

To assess whether the cell death induced by *H. adakodien* involves typical changes encountered during apoptosis, we utilized double labelling techniques using annexin V Flous/PI to distinguish between apoptotic and necrotic cells. We first looked for changes in phosphatidyl serine on the cell membrane. Under defined salt and calcium concentrations, annexin V is predisposed to bind PS that is translocated on to the cell surface in the very early stages of apoptosis. Hence apoptotic cells were detected with annexin V, labeled with fluorescein isothiocyanate and photographed with a camera attached fluorescent microscope. Addition of PI cannot enter the cells in the early stages of apoptosis when the membrane integrity is intact. MDA MB 231 and MCF.7 clearly showed the apoptotic changes on treatment with *H. adakodien* root acetone fraction. The corresponding phase contrast microscopy of control and treated cells are shown in Figure.5.1 & 5.2.

Effect of *H. adakodien* root acetone fraction on nuclear fragmentation.

The nuclei of MDA MB231 and MCF.7 cells were stained Hoechst 3342 and assessed by microscopy. Figure.5.4 indicates that control cells had intact cell nuclei, while *H. adakodien* treated cells showed significant nuclear fragmentation, characteristic of apoptosis where as the controlled cells shows a decrease in nuclear fragmentation.

The process of apoptosis is fundamental in the developmental and homeostatic maintenance of complex biological systems. Dysregulation or failure of normal apoptotic mechanism will contributed to transformation of cell and provide a growth advantage to cancer cells. It is characterized by cell shrinkage, chromatin condensation, DNA, fragmentation and the activation of specific cystein proteases. (Fischer U et.al., 2005).
Translocation of phosphatidyl serine (PS) from inner to outer cytoplasmic membrane is a characteristic feature of apoptosis. This externalization exposes PS membrane for annexin binding (conjugated with fluorescein) and can be visualized through a fluorescence microscope or can be sorted using a flow cytometric instrument.

P2 represents annexin negative population and P3 represents annexin positive population. There was a significant increase in the apoptotic population, annexin positive population (from 17 to 70%), after treatment with HA for 24 hours in MCF-7 cells.
Fig. 5.3 Cytotoxic Evaluation of HA using MDA MB 231 & MCF-7 cells at 48 hours - MTT assay

A dose dependant growth inhibition is observed in both the cell lines upon treatment with a HA (37.50 to 300μg/ml)

Fig. 5.4 INDUCTION OF APOPTOSIS IN HUMAN BREAST CANCER CELLS BY H. ADAKODIEN (AF) (MCF-7, MDA MB 231)
Staining of apoptotic nuclei using AO/EtBr and Hoechst dye

i) Cells after treatment with HA were stained with different dyes to evaluate apoptosis. Ethidium bromide is selectively taken up by the apoptotic cells and stained the condensed nuclei (stained yellow) whereas the control cells have taken only acridine orange (stained green).

ii) Hoechst staining could demonstrate condensed apoptotic nuclei (after treatment with HA 300μg/ml) as observed as condensed white dots.
DISCUSSION

The process of apoptosis is fundamental in the developmental and homeostatic maintenance of complex biological systems. Dysregulation or failure of normal apoptotic mechanism will contribute to transformation of cell and provide a growth advantage to cancer cells. It is characterized by cell shrinkage, chromatin condensation, DNA, fragmentation and the activation of specific cystein proteases. (Fischer U et.al., 2005).

Different studies from our laboratory indicates that *H. adakodien* root acetone fraction shown to have antitumor activities in mice. It also found to increase the life span of EAC bearing animals. Cytotoxic effect was also observed *in vitro* EAC cells. However limited studies have been carried out to assess the detailed mechanism of anti tumor effect of the acetone fraction of *H. adakodien* root.

The present study was designed to determine whether the cytotoxicity and antitumor effect is due to the induction of apoptosis. The present set of investigations was therefore initiated to study the apoptotic potential of acetone fraction of *H. adakodien*. The studies shown that acetone fraction markedly reduced the cell viability in a concentration dependent manner, both in MDA MB 231 and MCF.7 cells at 48 hrs. (Fig.5.3.)

The suppression of cell proliferation may be due to induction of cell death. In the context of cell death it is important to emphasize the difference between apoptotic and non apoptotic types of cell death. In contrast to apoptosis, necrosis is a passive process occurring as a result of acute damage that disables the cell to maintain the basic energetic functions. In contrast apoptosis is always a finely regulated process initiated in response to physiological stimuli or to an environmental damage. (Nicotera P et.al., 1998).

In the early stages of apoptosis while the cell membrane is still intact, phosphatidyl serine to which Annexin V binds specifically is
translocated to the extracellular leaflet of the membrane. Our flow
cytometric data (Fig.5.1 & 5.2) revealed that the mode of cell death is
apoptosis but not necrosis. This result indicated that the acetone fraction
of *H. adakodien* could induce apoptosis in human breast cancer cells.
(MDA MB231 and MCF .7 cells).

Another significant change during apoptosis is the condensation of
nuclei and fragmentation of the nucleus. The results with AO/ethidium
bromide and also with Hoechst 3342 dye confirmed the induction of
apoptosis in human breast cancer cells (MDA MB 231 and MCF.7)
(fig.5.4.).

It can be concluded from the above results that acetone fraction of
*H. adakodien* root extract inhibits the growth of MDA MB231 and MCF.7
cells in concentration dependent manners. The cytotoxic and anti tumor
effect of acetone fraction of *H. adakodien* is through the induction of
apoptosis as demonstrated by the present data.