Development of Novel Anti-cancer Drugs Based on Studies of Molecular Signaling in Apoptosis Using Biophysical Methods

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Abstract
Apoptosis (programmed cell death) is a very important cellular process that plays an important role in cancer. In this review, we summarize our research in three related areas: (1) Using single living cell analysis techniques to obtain important insight about the signalling mechanisms of apoptosis in mammalian cells. (2) Development of novel molecular bio-sensors for detecting caspase activation within a single living cell based on the fluorescence resonance energy transfer (FRET) method. These sensors not only enable us to study the regulating mechanisms of enzyme activation in an *in vivo* condition, they also provide a powerful tool for high-throughput screening of new drugs. (3) Using such biosensors, we identified several purified compounds in TCM (traditional Chinese medicine) that could be developed into novel anti-cancer drugs.
Keywords: Apoptosis, GFP, FRET, Anti-cancer drug, biosensor

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1. Introduction

Programmed cell death (also called "apoptosis") is a controlled cell suicidal process that allows our body to destroy damaged or unwanted cells in an orderly way (Kerr et al., 1972). The study of apoptosis has gained widespread attention in recent years due to its newly discovered roles in a variety of pathological processes. For example, when DNA is damaged in a cell and cannot be repaired, the cell will enter apoptosis to avoid the formation of abnormalities in the tissue. Thus, failure of programmed cell death can cause cancer. Besides tumor genesis, apoptosis is also found to be associated with numerous pathological disorders, including strokes, Alzheimer's disease, auto-immune disease, AIDS, etc.

In the last few years, a large number of studies have been conducted aiming to understand the process of apoptosis on a cell and molecular basis. In recent years, we have been using a combination of innovative imaging and molecular techniques to study signaling mechanism of apoptosis in a single living cell. These techniques include labeling specific proteins with color mutants of GFP (Green Fluorescent Protein) (Heim et al., 1995) to study their sub-cellular localization and dynamic degradation, and measurements of protein-protein interaction using the FRET (Fluorescence Resonance

Energy Transfer) method (Pollok et al., 1999). Our approach has certain advantages. First, since these optical methods are non-invasive, we can preserve the organization and structure of the cell during the biological study. Second, by conducting the study in a single cell, we can avoid the problem of cell synchrony, which would be difficult to achieve in a cell population study. Third, in the single cell study, we can correlate the temporal- and spatial-dependent changes of a specific signaling molecule with a particular cellular event. Finally, using the FRET technique, we can measure the dynamics of a specific protein-protein interaction or the activation of a given enzyme within a single cell. Such measurements can complement very well with studies using conventional biochemical methods.

Since many important diseases, including cancer and neurodegenerative diseases, are related to defective or excessive programmed cell death, drugs that can either facilitate or block programmed cell death can both be potentially useful in treating diseases. Hence, our research not only leads to a better understanding of the mechanisms regulating apoptosis, it can also help us to discover new drugs against major diseases.

2. Study the Signaling Mechanism of Apoptosis

2.1 Apoptosis and its Signalling Pathways

The signaling pathways that direct the apoptotic process is very complicated. There are many external signals that can trigger the initiation of apoptosis, including UV-irradiation, activation of the "death domain" via the TNF (Tumor Necrosis Factor) receptor, treatment of hormone (e.g. glucocorticoid) and chemotherapy drugs (e.g. camptothecin) (Martin et al., 1991; Nagata, 1997) (see Fig. 1). As for internal signals, apoptosis involves a programmed cascade of intracellular events, centering on the activation of a class of cysteine proteases called "caspases" (Cohen, 1997). Some of these caspases (such as caspase-8 and caspase-9) are "initiators" of the apoptotic process, while others (such as caspase-3) are "executioners". Besides caspases, a number of gene products are also known to be key players in processing apoptosis (Adams et al., 1998; Gross et al., 1999). Among them, the Bcl-2/Bax families play a particularly important role. When these genes are over-expressed (or mutated), some of them can sensitize cells to undergo apoptosis, while others may prevent cells from entering programmed cell death. At present, it is known that there are three major apoptotic pathways, including: (1) The death domain pathway, (2) The mitochondria pathway, and (3) the ER pathway. In many cells, the mitochondria pathway is the most important one. A large variety of apoptotic stimuli can trigger the Bax family proteins to aggregate at the mitochondrial outer membrane and cause mitochondria to release a number of apoptotic factors, including cytochrome c (Cyt-c) and Smac/DIABLO (Desagher et al., 2000; Du et al., 2000; Wang, 2001). The released Cyt-c then induces the "apoptosis protease-activating factor" (Apaf-1) protein to form a multimeric complex to recruit and activate procaspase-9, which in turn activates procaspase-3. The released Smac, on the other hand, can neutralize a set of caspase inhibitors, known as

IAP (Verhagen et al., 2000). The combination of activation of caspases and removing of their inhibitors finally causes cell death. A summary of the major signaling pathway of apoptosis mediated by mitochondria is shown in Fig.1.

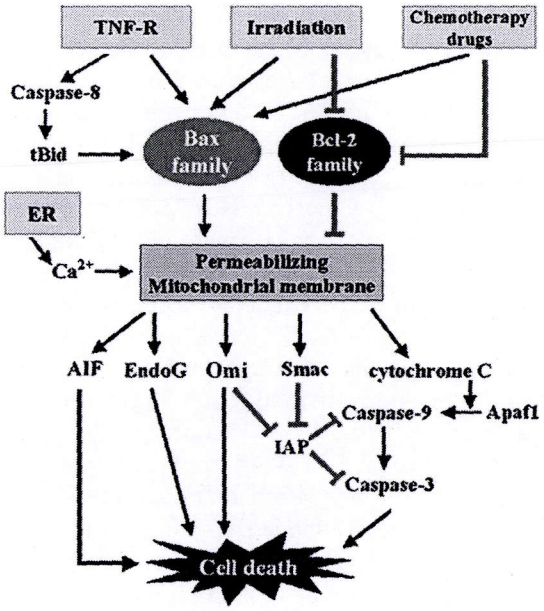

Figure 1: An overview of the mitochondria-dependent signalling pathways of apoptosis.

2.2 Apoptosis and Cancer

The prevention and treatment of cancer is one of the most important medical challenges in the modern time. It became known recently that defects in apoptosis are the major cause for cancer formation. Both Bcl-2 and p53 genes have been shown to play important roles in apoptosis and cancer. For example, the Bcl-2 gene was found at the chromosomal breakpoint of t (14; 18) in a human leukaemia line and a follicular lymphoma (Tsujimoto et al., 1985). And Bcl-2 may promote tumour cell survival by blocking programmed cell death (Hockenbery et al., 1990; McDonnell et al., 1989). P53 was the first tumour suppressor gene linked to apoptosis. It is now known that over half of human cancers have mutations in p53, and loss of p53 function can both disable apoptosis and accelerate tumour development in transgenic mice (Attardi et al., 1999; Ryan et al., 2001). Moreover, functional mutation or altered expression of p53 downstream effectors (Bax, Bak, and Apaf-1) or upstream regulators (ATM, Mdm2, and $p19^{ARF}$) occur in human tumours (Di Cristofano et al., 2000; Kondo et al., 2000; Soengas et al., 2001). And overexpression of IAPs, particularly survivin, which can

inhibit caspase activation, is commonly observed in many types of human tumours (Deveraux et al., 1999).

2.3 Apoptosis and Chemotherapy

Besides tumourigenesis, defect in apoptosis is also a major cause of drug resistance in cancer cells during chemotherapy treatment. Most of the drugs act by inhibiting DNA synthesis through binding to DNA or interfering with production of nucleic acids. Some can affect the synthesis of microtubules or other mitotic proteins. It became evident only very recently that most chemotherapeutic drugs kill cancer cells through the mechanism of apoptosis (Lowe et al., 2000). For example, cisplatin, a chemotherapeutic drug that links DNA strands, kills cancer cells by activating the apoptotic machinery (Mellor, 2006; Moggs et al., 1996). Taxol, a microtubule damaging reagent, can cause Bcl-2 phosphorylation, thereby inactivating Bcl-2 and suppressing its anti-apoptotic function. This allows cell death to be executed inside the cancer cell (Haldar et al., 1998).

Currently, the biochemical mechanisms by which tumour cells develop resistance to anticancer drugs are still not well understood. There is growing evidence that disruption of apoptotic pathways contributes to drug resistance. For example, loss of p53 function correlates with multi-drug resistance in many tumour types (Wallace-Brodeur et al., 1999). And mutations or altered expression of Bcl-2-related proteins can drastically alter drug sensitivity in experimental models (Schmitt et al., 2000; Wei et al., 2001; Zhang et al., 2000). Furthermore, altered expression of Apaf-1 and IAP has been correlated with drug resistance in various tumours (Deveraux and Reed, 1999; Soengas et al., 2001).

If cancer is caused by the failure of the apoptotic machinery, how can we treat cancer by chemotherapy, which is supposed to induce apoptosis in cancer cells? The answer is that the apoptotic process can be activated and regulated through multiple pathways. Some cancer cells are defected only in some, but not all, of the apoptotic pathways. So, with detailed knowledge of the relationship between cancer and apoptosis, we can design drugs to target the non-defective apoptotic pathways of a given cancer.

2.4 Using Biophysical Techniques to Study the Signaling Mechanism of Apoptosis

In the last decade, we have been combining molecular and biophotonic methods to study the apoptotic mechanisms in a single living cell. These single cell analysis techniques were developed relatively recently due to technological advancement in two areas: (1) advanced living cell imaging techniques, (e.g., confocal microscope), and (2) fluorescent labelling of target proteins by gene-fusion with GFP or other fluorescent proteins. (For details, see recent reviews (Lippincott-Schwartz et al., 2001; Sekar et al., 2003; Zimmermann et al., 2003)). In the last five years, we have conducted a series of studies on the apoptotic signalling pathways using single cell analysis. It is well known that cyt-c release was associated with TNF-induced apoptosis (Weiss et al., 1988). But, what is the mechanism of Cyt-c release? One major hypothesis is that, a mega-channel

traversing across the outer and inner mitochondrial membranes called "PT pore" is induced to open during apoptosis (Desagher and Martinou, 2000; Green et al., 1998). Solutes and water in the cytosol then enter mitochondrial matrix through this channel, causing the mitochondrion to swell and rupture its outer membrane. As a result, Cyt-c and other proteins in the inter-membrane space are released. This hypothesis has been widely cited in many studies, but so far, there is still a lack of conclusive evidence to prove or disprove it. In order to provide a critical test of this swelling theory, we used fluorescence imaging techniques to measure the dynamic re-distribution of GFP-labelled Cyt-c in living HeLa cells, and used a red color fluorescent dye, Mitotracker, to image the morphological change of mitochondria at the same time (Fig.2). We found that mitochondria did swell when HeLa cells were induced to enter apoptosis by UV treatment. The swelling, however, occurred only after Cyt-c was released. Our result strongly suggests that Cyt-c release in apoptosis is not caused by mitochondrial swelling (Ungermannova et al., 2005).

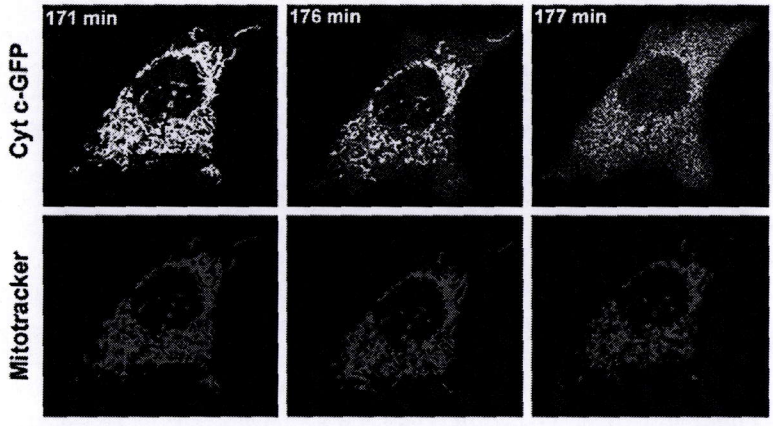

Figure 2: Using GFP-gene fusion to study apoptotic signaling in a single living cell. Time-dependent measurements of Cyt c-GFP distribution and Mitotracker in a living HeLa cell during UV-induced apoptosis. (See Gao et al, 2001).

After this study, we were interested in investigating the alternative releasing mechanisms. It has been suggested recently that formation of "channels" in the outer mitochondrial membrane by Bax-like proteins may be responsible for the release of mitochondrial proteins, such as Cyt-c and Smac (Desagher and Martinou, 2000). Several different models have been proposed: (a) Bax may form channels by itself; (b) Bax may form chimeric channels with the voltage-dependent anion channel (VDAC) protein; (c) Bax may form a "protein-lipid complex" to destabilize the mitochondrial membrane (Desagher and Martinou, 2000). We decided to test these models by examining the dynamic re-distribution of GFP-labeled Bax during UV-induced apoptosis using living-cell imaging techniques. We studied the dynamic re-distribution of GFP-labeled Bax during UV-induced apoptosis in single living HeLa cells. Bax was

initially found to be localized in the cytosol in a diffused pattern. Then, during the progression of apoptosis, Bax was observed to translocate from the cytosol to mitochondria and began to form small punctate structures there. Soon after that, more Bax proteins were found to aggregate in mitochondria to form large clusters. About half an hour later, the cell shrank and died. The Bax aggregates were composed of hundreds of molecules, and thus were far larger than "channels". Hence, we think that the lipid-protein complex formed by Bax in the outer mitochondrial membrane is probably the most viable model for explaining the release of mitochondrial proteins during apoptosis (Zhou et al., 2004).

3. Using FRET-based Biosensors to Discover new Anti-cancer Drugs by Screening Active Components in TCM (Traditional Chinese Medicine)

TCM has been used to treat many chronic diseases in China and some of the Asian countries for several thousand years. The pharmacological mechanisms of most TCM, however, are not well known. Recently, there is evidence suggesting that some of the components of TCM may act through regulating apoptosis. The challenge now is to have a high throughput method to screen active compounds in TCM that can induce apoptosis in cancer cells.

The activation of caspase-3 is the most important event that occurs during the execution stage of apoptosis, since cell death is inevitable once this protease is activated. Thus, caspase-3 activation is considered an early and reliable marker for assaying apoptosis. Several years ago, we developed a special technique that uses the FRET (fluorescence resonance energy transfer) method to detect the activation of caspase in a single living cell (Chang et al., 2003). The basic principle of this method is to fuse a CFP (cyan fluorescent protein) and a YFP (yellow fluorescent protein) with a peptide linker containing a caspase-specific cleavage site, DEVD (Fig.3A). Before caspase is activated, energy can be transferred directly from the donor (CFP) to the acceptor (YFP), so that when the donor is excited, fluorescence emission from the acceptor can be detected. When the target caspase is activated, it cleaves the linker and causes the donor and acceptor to separate. Thus, the FRET effect is effectively eliminated (Fig.3B). Using these sensors, we were able to measure the dynamics of caspase-3 activation in a signal living cell during UV-induced apoptosis (Figs.3B & C) (Chang et al., 2003). Later, using the same FRET method, we have also generated probes that can detect the activation of caspase-8 during TNFα-induced apoptosis in a single living cell (Chang et al., 2003).

Using this FRET biosensor (called "sensor C3"), we can detect the activation of caspase-3 in a single living cell (Chang et al., 2003). We have generated a stable HeLa cell line that automatically produces the CFP-DEVD-YFP fusion protein (i.e., sensor C3). Previously, we have used these sensor C3 cells to monitor the caspase-3 activation during UV or TNF induced apoptosis (Chang et al., 2003). Results from those *in vivo* apoptotic studies indicated that the reduction of the emission ratio (YFP/CFP) of sensor C3 in response to caspase-3 activation reached 4 fold. The strong FRET effect and high

sensitivity of this probe allow us to use cells grown in the 96-well culture plate to quickly screen a large number of active components of TCM. Fluorescent intensity can be measured using a fluorescent plate reader equipped with a special set of excitation and emission filter for detecting FRET effects. The successful candidates will be further analysed for their anti-cancer activities by standard biochemical approaches.

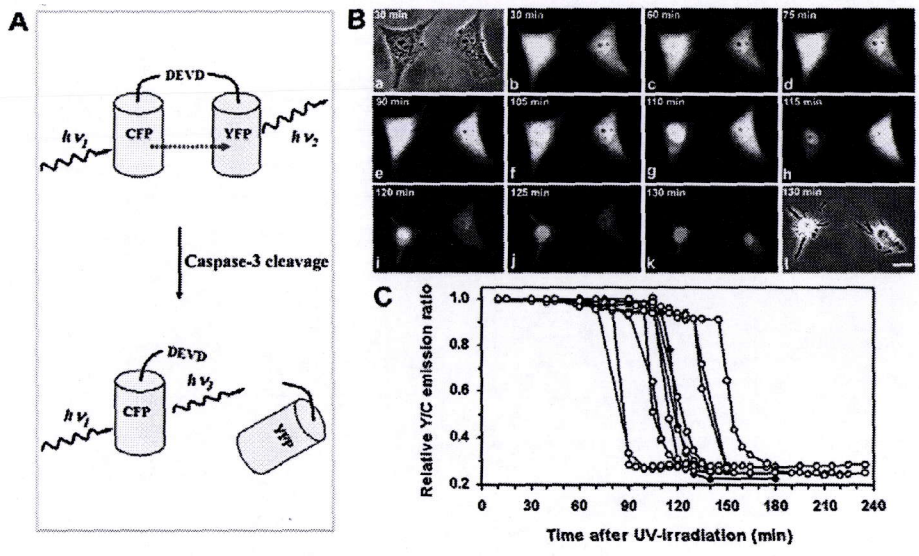

Figure 3: Using sensor C3 to detect caspase-3 activation within a single cell. (A) The design of the FRET biosensor C3. (B) Dynamic FRET changes in response to caspase-3 activation in two living HeLa cells. The emission of sensor C3 shifted from YFP to CFP (panels **b-k**) after UV treatment. (C) The relative emission ratio of YFP/CFP from 11 different UV-induced apoptotic cells was plotted as a function of time. (See Luo et al, 2001).

In fact, we have demonstrated that our sensor C3 cells can be used as a high throughput screening platform for identifying anti-cancer drugs by testing this system with a variety of known anti-cancer compounds, including paclitaxel, vincristine, etoposide, hydroxyurea and camptothecin (Tian et al., 2007). Vincristine and paclitaxel are well known anti-cancer drugs. They can arrest cells in mitosis by preventing mitotic spindle assembly (Mollinedo et al., 2003; Yang et al., 1997). These mitotic arrested cells subsequently will enter apoptosis. Figure 4a shows the changes in the Y/C emission ratio from Sensor C3 cells during the course of paclitaxel treatment at three different concentrations. About 50% of the total reduction in the Y/C emission ratio was seen from cells treated with 10 nM paclitaxel for ~36 h, and a more significant decrease of nearly 75% in the Y/C ratio was seen from cells treated with 50 nM paclitaxel for

over 30 h. No fluorescent change was seen at a lower concentration of paclitaxel (1 nM) and the control cells.

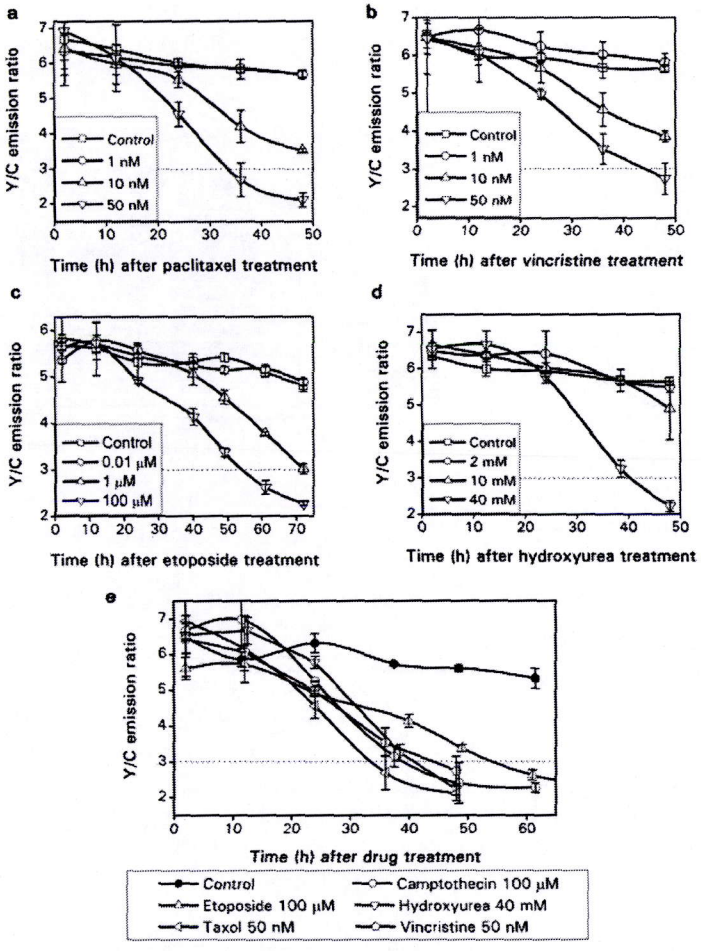

Figure 4: High throughput drug screening using sensor C3. A significant reduction in the Y/C emission ratio can be seen from Sensor C3 cells treated with different anti-cancer drugs: paclitaxel (a), vincristine (b), etoposide (c), and hydroxyurea (d). A summary of FRET effects in Sensor C3 cells in response to multiple anti-cancer drugs at their optimal concentrations (e). The error bars represent the standard deviation from three independent experiments. (See (Tian et al., 2007).

Similarly, we demonstrated that our system can easily detect the anti-cancer effect of vincristine. Here, three concentrations of vincristine (1 nM, 10 nM and 50 nM) were added to Sensor C3 cells. Screening results showed that 10 nM of vincristine reduced

the Y/C emission ratio to about 4, and a higher concentration of vincristine at 50 nM further reduced the Y/C ratio to less than 3 (as indicated by the dotted line) within 48 h (Figure 4b).

The third drug we tested was etoposide, an inhibitor of DNA topoisomerase II. Etoposide can block DNA synthesis during S phase of the cell cycle, and then induce cells into apoptotic cell death (Fearnhead et al., 1994). From the screening results in Figure 4c, we can see that the effective concentration of etoposide for activating apoptosis is between 1-100 μM. Also, significant reductions in the Y/C emission ratio were seen 60 h after addition of the drug, suggesting that etoposide may need more time to activate caspase-3 and induce apoptotic cell death in these Sensor C3 cells than paclitaxel and vincristine.

We are now using this technique to screen active components of TCM that have anti-cancer potential. For example, *Salvia Miltiorrhiza Bunge* is an herbal plant that has been widely used in TCM for the treatment of chronic hepatitis, liver fibrosis, hematological abnormalities and heart diseases. Danshen is the dried root of *Salvia Miltiorrhiza Bunge* and many of its ingredients have been purified and can be classified into two groups (Tang et al., 1992). The first group is phenolics; the second group is diterpene quinone pigments such as tanshinones. Over 40 tanshinones have been isolated, among them tanshinone IIA, cryptotanshinone, tanshinone I and dihydrotanshinone are the major constituents (Wu et al., 1991). The tanshinones have a common structural feature of planar phenanthrene quinone, which shares certain similarities to anti-tumour drugs such as anthracyclines and anthracenedinoes. Recently, it was reported that tanshinone IIA had growth inhibitory effects in several human cancer cell lines (Liu et al., 2000; Park et al., 1999). It was suggested that some of the Danshen ingredients inhibited cell growth by inducing them to undergo apoptosis (Liu et al., 2000; Sung et al., 1999; Yoon et al., 1999). At present, we are using the sensor C3 technique to identify the most potent components of Danshen in their anti-tumor effect.

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