Measurement of Cytochrome C Release by Zenyth 340 from Mitochondria is a Tool to detect early Steps for Initiating Apoptosis

1. Introduction

Apoptotic cell death is a fundamental feature of virtually all cells (5). It is an indispensable process during normal development, tissue homeostasis, development of the nervous system and the regulation of the immune system. Insufficient or excessive cell death can contribute to human disease, including cancer or degenerative disorders (14). The highly coordinated and stereotyped manner of this induced cell death suggests that the cells activate a common death program, towards which diverse signal – transducing pathways converge (2,17,18).

The mitochondria turned out to participate in the central control or executioner phase of the cell death cascade (1). Cytochrome C was identified as a component required for the crucial steps in apoptosis, caspase-3 activation and DNA fragmentation (8). Cytochrome C was shown to redistribute from mitochondria to cytosol during apoptosis in intact cells (6a,b).

Mitochrondrial cytochrome C is a water – soluble protein of 15 kDa with a net positive charge, residing loosely attached in the mitochrondrial intermembrane space. Cytochrome C functions in the respiratory chain by interaction with redox partners. It is highly conserved during evolution. Like most mitrochondrial proteins cytochrome C is encoded by a nuclear gene and synthesized as a cytoplasmic precursor molecule, apocytochrome C, which becomes selectively imported into the mitochondrial intermembrane space. The molecular mechanisms responsible for the translocation of cytochrome C from mitochondria to cytosol during apoptosis are unknown.

A reduction in mitochondrial transmembrane potential has been reported to accompany early apoptosis (7). The release of cytochrome C into the cytosol leads to an activation of an apoptotic program via activation of a caspase dependent pathway (12,15,13,4). Cytochrome C achieves this goal by interaction with other cytosolic factors forming a complex (apoptosome) composed of cytochrome C, Apaf-1, dATP and Apaf-3/caspase 9 (10,11,3). Bcl-2 on the other hand was shown to be able to prevent apoptosis by blocking the release of cytochrome C from mitochondria (18).
Measurement of cytochrome C release from the mitochondria is a tool to detect the first early steps for initiating apoptosis in cells. Cytochrome C release in the cytosol occurs prior to the activation of caspases and DNA fragmentation which is considered the hallmark of apoptosis.
Detection of cytochrome C released from the mitochondria to the cytoplasm can be achieved by a selective lysis of the cell membrane.

Very recently it has been shown that this mitochondria dwelling molecule can be detected in the medium already 1h after apoptosis. Moreover, elevated cytochrome C levels were observed in serum from patients with hematological malignancies. In the course of cancer chemotherapy, the serum-cytochrome C level grew rapidly and it decreased gradually as the patient was cleared from malignant cells. Thus, serum-cytochrome C monitoring might serve as a clinical marker indicating the onset of apoptosis and cell turn-over in vivo (9).

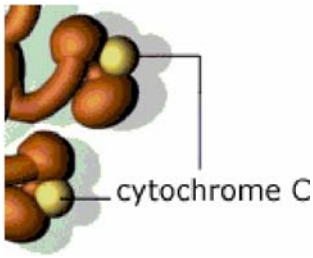

Fig.1: Cytochrome C is a key modulator in early steps of apoptosis (formation of apoptosome)

2. Materials

- Human Cytochrome C ELISA (Bender Medsystems)
- Anthos Zenyth 340 Microplate Reader with Evaluation Software
- Adjustable single- and multichannel micropipettes
- Beakers, flasks, cylinders necessary for preparation of reagents

3. Specimen Collection

Cell culture lysates, human whole blood and human serum will be suitable for use in the assay. Remove serum from the clot or red cells, respectively, as soon as possible after clotting and separation.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Clinical samples should be kept at 2° to 8°C and separated rapidly before storing at -20°C to avoid loss of bioactive Cytochrome C. If samples are to be run within 24 hours, they may be stored at 2° to 8°C. Avoid repeated freeze-thaw cycles.

4. Test Protocol

Natural human serum samples were applied to **Bender MedSystems Instant ELISA™** microplates. For incubation times and wash cycles refer to the corresponding instruction manual. Samples were measured with a **Zenyth 340** reader at 450nm, reference measurement was taken at 620nm. Alternatively extracts from cell lysates can be applied to microplates. See guidelines for preparation of cell extracts below:

4.1 Cell Lysis procedure (cell culture samples)

a. Spin down cells for 15 minutes at 1200 rpm.
b. Wash cell pellet once in cold PBS.
c. Re-suspend cells in Lysis Buffer to a concentration of 1.5 x 10^6 cells/ml.
d. Incubate for 1 hour at room temperature with gentle shaking.
e. Centrifuge cells at 1000 x g for 15 minutes.
f. Dilute the supernatant in Assay Buffer at least 50-fold (5µl supernatant + 245µl Assay Buffer) for the assay. Aliquot and store supernatant not used immediately at –70°C.

4.2 Cell Lysis procedure (whole blood samples)

a. Spin down 1 ml of whole blood for 15 minutes at 1200 rpm.
b. Remove plasma (supernatant) carefully.
c. Re-suspend cell pellet in 3ml Lysis Buffer.
d. Incubate for 1 hour at room temperature with gently shaking.
e. Spin down for 15 minutes at 1000 x g.
f. Dilute the supernatant at least 10-fold in Assay Buffer and assay immediately. Aliquot supernatant not needed and store at –70°C.

4.3 Serum samples

Dilute serum samples before assaying 1:2 in assay buffer (e.g. 150µl serum sample + 150µl assay buffer).

a. Mix all reagents thoroughly without foaming before use.
b. Determine the number of Microwell Strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards.

Each sample standard, blank, and optional control sample should be assayed in duplicate. Remove extra Microwell Strips coated with Monoclonal Antibody (murine) to human Cytochrome C from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.

c. Wash the microwell strips twice with approximately 300 µl Wash Buffer per well with thorough aspiration of microwell contents between washes. Take care not to scratch the surface of the microwells.

After the last wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing or place upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.

5. Calculation of Results

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean.

- Standard curve is automatically calculated by the Zenyth 340 microplate reader. In addition the instrument features 4 different modes of curve fitting:

- point to point
- linear regression
- cubic spline
- 4 parameter fit

- To determine the concentration of circulating Cytochrome C for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding Cytochrome C concentration.

- For samples which have been diluted according to the instructions given in this manual , the concentration read from the standard curve must be multiplied by the dilution factor.

-Note: Calculation of samples with an O.D. exceeding 2.0 may result in incorrect, low Cytochrome C levels. Such samples require further dilution with Assay Buffer in order to precisely quantify the actual Cytochrome C level.

- It is suggested that each testing facility establishes a control sample of known Cytochrome C concentration and runs this additional control with each assay. If the values obtained are not within the expected range of this control, the assay results may be invalid.

Standard	Cytochrom c concentration (ng/ml)	O.D. (450nm)	O.D. Mean	C.V. (%)
1	5.00	2.105	2.196	5.8
	5.00	2.286		
2	2.5	1.205	1.187	2.2
	2.5	1.168		
3	1.25	0.613	0.594	4.6
	1.25	0.574		
4	0.63	0.264	0.254	5.5
	0.63	0.244		
5	0.31	0.122	0.112	12.6
	0.31	0.102		
6	0.16	0.063	0.066	5.3
	0.16	0.068		
7	0.08	0.047	0.048	1.5
	0.08	0.048		
Blank	0.00	0.029		
	0.00	0.026		

Fig. 2: Shows representative data of a standard curve. Mean values and CV -values were calculated by Zenyth 340 software.

6. Results/Summary

The ideal platform for absorbance measurement for the **Bender MedSystems Cytochrome C ELISA** turned out to be the **Anthos Zenyth 340 microplate reader.** This absorbance detector allows through its very flexible software a rapid and convenient read out and data processing. **Bender MedSystems' Instant ELISA** technology and **Anthos reader Zenyth 340** provide a complete solution for busy customers in Biotech and High Throughput laboratories. Both products ensure significant boost of production.

7. Literature

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