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Design and development of a whole-cell luminescent biosensor for detection of early-stage of apoptosis

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ABSTRACT

We present here a novel whole-cell biosensor to detect early-stages of apoptosis based on Apaf-1 oligomerization and apoptosome formation using the split luciferase strategy. The amino-fragment (1–416 amino acids) and carboxy-fragment (395–550 amino acids) of firefly luciferase were fused to amino-terminal of Apaf-1. The cotransfected HEK cells were then treated with doxorubicin for induction of apoptosis. The performance of our biosensor for monitoring of programmed cell death over 24 h was investigated by measuring bioluminescence activities. We observed a significant increase (~15 fold) in luminescence signal compared to control cells 4 h after apoptosis induction. It reached a maximum activity over 10 h (~155 fold). Moreover, juxtapositioning of Apaf-1 monomer and apoptosome formation occur about 5 h earlier than the appearance of significant caspase3/7 activity upon induction of apoptosis by doxorubicin. The time–response curve of split luciferase shows a sigmoidal pattern which indicates cooperativity in oligomerization of Apaf-1 upon binding of cytochrome *c*. This biosensor can be used as a new platform, based on the protein fragment complementation strategy for assessing potential chemotherapeutic drugs as well as a sensitive and dynamic system in the time- and dose-dependent studies of apoptosis.

1. Introduction

Apoptosis, programmed cell death, is an active regulatory response in both physiological and pathological conditions (Taylor et al., 2007). Apoptosis is a highly organized, evolutionarily conserved and genetically regulated pathway for maintaining homeostasis (Taylor et al., 2007; Riedl and Salvesen, 2007; Ow et al., 2008; Okada and Mak, 2004; Fuchs and Steller, 2011) and a powerful way for demolition of unwanted cells during development of multicellular organisms (Taylor et al., 2007). Apoptosis is characterized by morphological exchanges including cell contraction, extensive plasma membrane budding, chromatin condensation, fragmented nucleus and apoptotic bodies formation (Riedl and Salvesen, 2007; Ow et al., 2008; Okada and Mak, 2004; Fuchs and Steller, 2011).

The three main pathways of cell death activation are referred to as the mitochondria-associated apoptosome formation (intrinsic), the binding of extracellular death ligand (extrinsic) and the cytotoxic lymphocyte-initiated granzyme B (Cullen and Martin, 2009; Shi, 2002). These pathways lead to the activation of a family of zymogen cysteine proteases known as caspases (Shi, 2002; Pan et al., 1998). The activated forms of these enzymes participate in a cascade that ultimately cleaves a set of proteins, resulting in dismantling of the cell (Cullen and Martin, 2009). Defect of apoptotic pathways brings about a wide range of pathologic conditions such as cancer, autoimmune diseases and spreading of viral infections (Fuchs and Steller, 2011).

A lot of evidence shows that mitochondria acts as an important regulator for promoting or inhibiting programmed cell death via intrinsic pathway (Ow et al., 2008). In this pathway, various cellular stresses such as DNA-damaging reagents, heat shock, many types of oxidative stress and many other forms of cellular damages (Riedl and Salvesen, 2007) result in apical caspase (caspase-9) activation through the formation of caspase-activating complex which is termed as apoptosome (Ried et al., 2005; Reubold et al., 2011).

The apoptosome formation is triggered by release of cytochrome *c* from mitochondria and oligomerization of Apaf-1 molecules which in turn promotes the activation of caspase-9 (Pan et al., 1998; Ried et al., 2005; Reubold et al., 2011; Yuan et al., 2010). Apaf-1 comprises of an amino-terminal caspase recruitment domain (CARD), a central nucleotide-binding oligomerization domain (NOD), and carboxy-terminal multiple WD40 repeats (Reubold et al., 2011; Yuan et al., 2010; Hu et al., 1998; Adrain et al., 1999; Zou et al., 1997). The WD-40 domain is thought to be in charge of binding to cytochrome *c* and has a

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regulatory role in Apaf-1 function (Hu et al., 1998; Adrain et al., 1999). Apaf-1 exists in a globular latent monomer in cytoplasm, so its CARD domain and part of its NOD domain are held between the WD-40 repeats in a locked autoinhibited form (Hu et al., 1998; Adrain et al., 1999; Zou et al., 1997).

It has been suggested that cytochrome *c* upon release from mitochondrial inter-membrane space binds to the WD-40 repeats (Hu et al., 1998) and causes conformational changes in Apaf-1 which exposes the nucleotide binding sites to dATP/ATP. The binding of (d)ATP to NOD domain of Apaf-1 brings about a further conformational change in Apaf-1 and generates a semi-open Apaf-1 conformation (Riedl and Salvesen, 2007). Concurrently with the hydrolysis of the bound (d)ATP and (d)ADP exchange leads to Apaf-1 oligomerization and a wheel-shaped signaling platform, the apoptosome, is formed (Riedl and Salvesen, 2007; Reubold et al., 2011). The apoptosome formation promotes the activation of caspase-9 as the initiator caspase which propagates a cascade of further caspase activation (effector caspases) events (Hu et al., 1998; Adrain et al., 1999; Zou et al., 1997; Liu et al., 1996).

So far, several models of the apoptosome structure are extracted from modeling studies (Yuan et al., 2010) and all of them are speculative and determination of the detailed structural organization of the apoptosome remains uncertain and somewhat controversial and waits for a structure at an atomic resolution (Riedl and Salvesen, 2007). However, the crystal structure of an Apaf-1 monomer has been determined recently (Reubold et al., 2011). Nonetheless, the different scenarios confirm that in the apoptosome structure the CARD domains of Apaf-1 take in a ring-like arrangement required for the recruitment of procaspase-9 (Riedl and Salvesen, 2007).

A great majority of modern research in cancer therapy and drug discovery focusses on development of compounds that blocks the cell cycle, inhibiting the DNA polymerase enzyme and finally inducing apoptosis (Taylor et al., 2007; Fuchs and Steller, 2011). In concert with these progressions several techniques have been developed for apoptosis detection. These methods mainly rely on one of the programmed cell death features (Fuchs and Steller, 2011).

We report here, for the first time, the construction of a novel apoptosis reporter system based on Apaf-1 oligomerization and apoptosome formation model upon release of cytochrome *c* from mitochondria at the preliminary phase of apoptosis (Cullen and Martin, 2009).

Firefly luciferase with high quantum yield, the ability of different color emissions (Hosseinkhani, 2011) and suitable structural properties is widely used for design of biosensors (Nazari and Hosseinkhani, 2011). To develop this apoptosis reporter, we have employed the split firefly reporter strategy (Ozawa et al., 2001; Villalobos et al., 2007; Paulmurugan and Gambhir, 2003; Ozawa, 2006), wherein a firefly luciferase is genetically split into two non-functional fragments so that luciferase activity is effectively abolished and then these fragments are fused to potentially interacting protein partners and upon induction if the target proteins interact, the fragments of the reporter protein are brought within proximity leading to signal generation that can be measured (Ozawa, 2006; Hashimoto et al., 2011; Fan-Minogue et al., 2010; Misawa et al., 2010).

In our design, amino-terminal and carboxy-terminal portions of firefly luciferase were fused to amino-terminal of Apaf-1. The human embryonic kidney cells (HEK293T) were transfected with generated constructs and induction of apoptosis was carried out in optimal concentration of doxorubicin. Subsequent to the release of cytochrome *c* from mitochondria and apoptosome formation, luciferase fragments are partially reconstituted and in the presence of luciferase substrates light was emitted similar to other reconstituted luciferases (Villalobos et al., 2007; Paulmurugan and Gambhir, 2003; Ozawa, 2006). We have also shown that this reporter can be used as a platform for assessing potential of chemotherapeutic drugs as well as a sensitive and dynamic system in the time- and dose-dependent studies.

2. Materials and methods

Details on extraction of RNA, reverse transcription, polymerase chain reaction and cloning of Apaf-1 gene are provided in Supplemental information.

2.1. Plasmid construction

The PCR-amplified amino-terminal fragment, 1–1248 bases, of the luciferase sequence was derived from pGL3-Control Vector (Promega) by the use of forward: 5′-CTA GCTAGC ATG GAA GAC GCC AAA AAC-3′ and reverse: 5′-CTA TGT CGA CTC CGC CTC CTC CAG ATC CGC CTC CAC CTG ACC CGC CAC CTC CAC TAT CCT TGT CAA TCA AGG CGT TGG TC-3′ primers. The carboxyl-terminal fragment, 1194–1650 bases, of the luciferase sequence was derived by the use of forward: 5′-GTA GCTAGC ATG GCT CCT ATG ATT ATG TCC GGT TAT GT-3′ and reverse: 5′-ACT ATG TCG ACT CCG CCT CCA CCT GAT CCG CCT CCT CCA GAT CCG CCT CCA CCT GAC CCG CCA CCT CCA CTC ACG GCG ATC TTT CCG CCC TTC-3′ primers.

According to this strategy (Ozawa, 2006), the split luciferase fragments of 1–416 amino acids (N-Luc) and 395–550 amino acids (C-Luc) were produced and used for making split reporter (Fig. 1).

Based on previous reports (Misawa et al., 2010; Takeuchi et al., 2010), a flexible Gly–Ser linker was added to the reverse primers of N-luc and C-luc and a Kozak consensus sequence was optimized with initiating ATG codon for proper initiation of translation in the forward primers of N-luc and C-luc fragments. The purified fragments were digested by *BamHI*/*Nhe*I and then cloned into the *BamHI*/*Nhe*I digested/dephosphorylated pcDNA3.1 (+) vector, harboring Apaf-1 gene and transformed into XL10 Gold *Escherichia coli* competent cells (Sambrook, 2001). Positive clones were selected by colony PCR and screened for the correct sequence by double digestion and sequencing.

2.2. Cell culture and transfection

Human embryonic kidney cells (HEK293T) were cultured in Dulbecco's modified eagle's medium (DMEM, high glucose; Invitrogen) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Invitrogen) solution at 37 °C in an

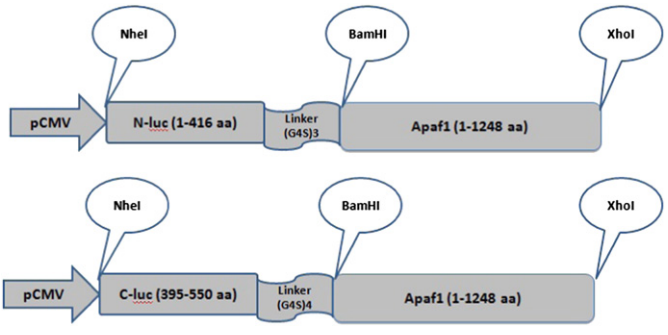

Fig. 1. General scheme of different plasmid constructs. N-luc, the N-terminal fragment of firefly luciferase (1–416 amino acids) connected to N terminus of Apaf-1 through $(GGGGS)_3$ flexible linker and C-luc, the C-terminal fragment of firefly luciferase (395–550 amino acids) connected to N-terminus of the other Apaf1 through $(GGGGS)_4$ flexible linker under the control of the CMV promoter.

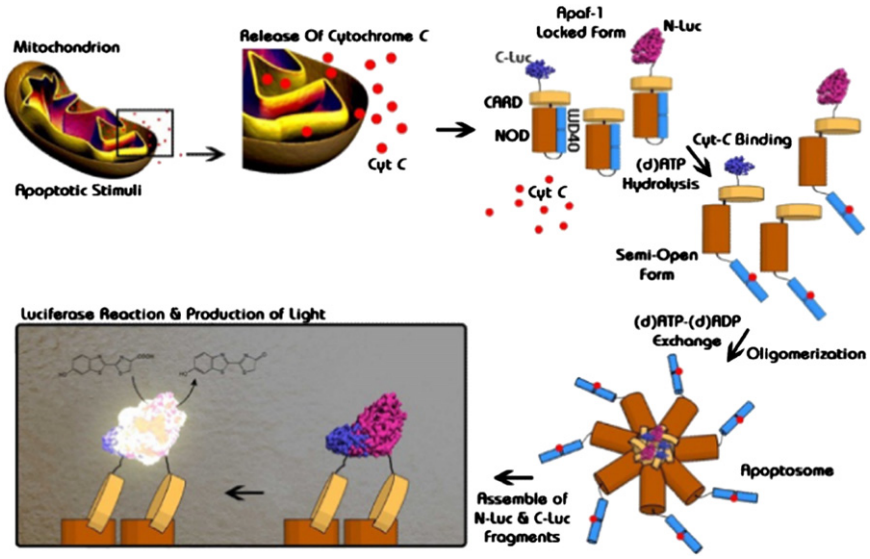

Fig. 2. General scheme of the apoptosis sensor. Schematic representation explains the protein-fragment complementation strategy of split luciferase for monitoring apoptosis induction based on Apaf-1 oligomerization upon cytochrome *c* release from mitochondrion.

incubator with 5% CO_2. Transfection with plasmid mixture was performed in 80% confluent 24-h-old cultures of 293T cells in 24-well plates using the calcium phosphate transfection method. The transfection mixture for a 24-well plate was prepared by gently mixing 25 μg of plasmids containing the N-luc–Apaf-1 construct, 25 μg of plasmids containing C-luc–Apaf-1 construct, and 62.5 μl of sterile $CaCl_2$ and total volume of above solution was adjusted to 500 μl with sterile water, then 500 μl of sterile 2X HBS (50 mM HEPES, pH 7.05, 10 mM KCl, 12 mM dextrose, 280 mM NaCl, 1.5 mM Na_2HPO_4) was added drop-wise under gentle agitation. The solution was mixed for 2 min by vortex and incubated 15 min at room temperature. Two hours before transfection, the growth medium was replaced with 400 μl of fresh medium preheated at 37 °C and 40 μl of transfection mixture was applied drop-wise to each well and incubated at 5% CO_2, 37 °C overnight (14–16 h). Following the incubation period, the medium containing the transfection mixture was replaced with 400 μl of fresh complete growth medium.

2.3. Caspase assay

The transfected cells were treated in triplicate with increasing concentrations of doxorubicin (0, 0.1, 0.2, 0.5, 1, 2, 5, and 10 μM) and assayed for caspase3/7 activity at indicated times using a carboxyfluorescein FLICA apoptosis detection kit (Immunochemistry Technologies, LLC, part# 94) by a fluorescent microplate reader (Biotek, Germany). Cells were also observed under a fluorescence microscope (Nikon, Japan) using a band pass filter (excitation 490 nm, emission 520 nm) to view the green fluorescence of activated-caspase cells.

2.4. Bioluminescence assay of reconstituted reporter

The transfected cells were exposed in triplicate to 0.5 μM of doxorubicin (Ebendoxo, EBEWE Pharma Ges). Cells were washed twice with cold PBS, trypsinized, pelleted by centrifugation at various time points and then resuspended in 200 μL of cold buffer A (10 mM HEPES–KOH pH 7.5, 1.5 mm $MgCl_2$, 10 mM KCl, 1 mM Na-EDTA, 68 mM sucrose, and 1 mM PMSF). The cells were then allowed to swell on ice for 10 min, and then mixed for 30 s by vortex. The hypotonic buffer A caused the cells to swell and burst (Liu et al., 1996). The cell debris was pelleted by centrifugation at 4 °C, 12,000 rpm for 30 s. The supernatant cell lysate represented was removed for luciferase assay. The activity was immediately monitored as a luminescence signal produced upon addition of 20 μL of luciferase assay complex (25 mM Tris, 10 mM $MgSO_4$, 1 mM luciferin, and 2 mM ATP) to 20 μL of cell lysate using a luminometer (Berthold Detection System, Germany) as reported earlier (Nazari and Hosseinkhani, 2011). For measuring luciferase activity in intact cells, the cells harvested at indicated times were resuspended in 50 μl D-luciferin (5 mM, dissolved in PBS). Immediately, cells were placed for 10 min in a 37 °C, 5% CO_2 incubator, and then analyzed by a luminometer.

3. Results

3.1. Design of pre-early apoptosis detection sensor

Although the first crystal structure of recombinant Apaf1 has been solved very recently (Reubold et al., 2011), the detailed structural organization of the apoptosome complex still is uncertain (Riedl and Salvesen, 2007). Based on electron microscopic image of apoptosome (Yuan et al., 2010), several speculative models of the apoptosome structure are proposed (Riedl and Salvesen, 2007; Yuan et al., 2010). In order to direct confirmation of this model (Riedl and Salvesen, 2007) and also to monitor apoptosis in preliminary phase, a novel split luciferase complementation assay based on apoptosome model is developed (Fig. 2). In this strategy (Villalobos et al., 2007; Ozawa, 2006), the luciferase reporter has to be rationally dissected into two fragments, according to previous reports (Takeuchi et al., 2010; Ishimoto et al., 2011). The best complement pair was from 1 to 416 amino acids as the N-terminal fragment and from 395 to 550 as the C- terminal part. Based on this information, the amino- (1–416 amino acids) and carboxy- (395–550 amino acids) terminal fragments of firefly luciferase were separately fused to the N-terminus of Apaf-1. For enhancing the folding and the flexibility of luciferase fragments (Ozawa, 2006), a flexible linker composed of $(Gly–Gly–Gly–Gly–Ser)_n$ amino acids is inserted at the junction between the luciferase fragments and Apaf-1 [$(G_4S)_3$ linker for N-luc and $(G_4S)_4$ linker for C-luc fragment] (Fig. 1). We presumed that, if N-luc–Apaf1 and C-luc–Apaf1 participate in apoptosome formation, upon apoptosis induction, the N-and

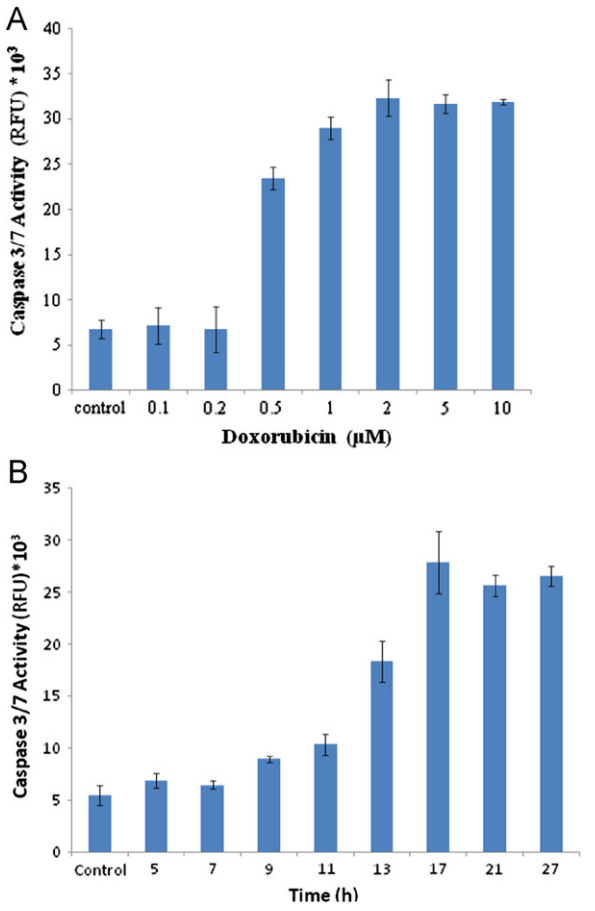

Fig. 3. (A) Dose–response curve of doxorubicin-induced apoptosis activation of caspase3/7 in HEK293T cells. The cells were transiently transfected with 2 μg of established plasmids (C-luc–Apaf1 plasmid and N-luc–Apaf1 plasmid). (B) Doxorubicin-induced, time-dependent apoptosis and caspase-3/7 activation in HEK293T cells. Values are mean ± SD of three separate experiments.

C-terminal fragments of firefly luciferase would reconstitute as a functional reporter and exhibit luminescence (Fig. 2).

3.2. Induction of apoptosis by doxorubicin in transfected HEK293T cells

Doxorubicin (Adriamycin) is a well-known chemotherapeutic agent which is used in treatment of a wide variety of tumors (Rebbaa et al., 2001). Its interaction with cellular DNA along with apoptosis activation via intrinsic pathway is believed to be a principal mechanism of its biological activity (Rebbaa et al., 2001). Caspase 3/7 assays were performed to assure that doxorubicin has induced apoptosis in transfected HEK293T cells.

To determine the optimal concentration of doxorubicin to induce apoptosis in HEK293T cells, the cotransfected cells (with exogenous Apaf-1) were treated with various concentrations of doxorubicin (0, 0.1, 0.2, 0.5, 1, 2, 5, and 10 μM) for 24 h. Activities of caspase 3/7 were assayed with the apoptosis detection kit. As shown in Fig. 3A doxorubicin induced a dose dependent activation of caspase3/7 in transfected HEK293 cells and maximum activity (~4.7 fold) was observed at 2 μM. No further increase in caspase 3/7 activity was observed upon treatment to higher concentrations of doxorubicin.

5 μM of doxorubicin induced a significant increase in caspase 3/7 activity (~3.5 fold) as compared to control sample without doxorubicin treatment that is consistent with substantial

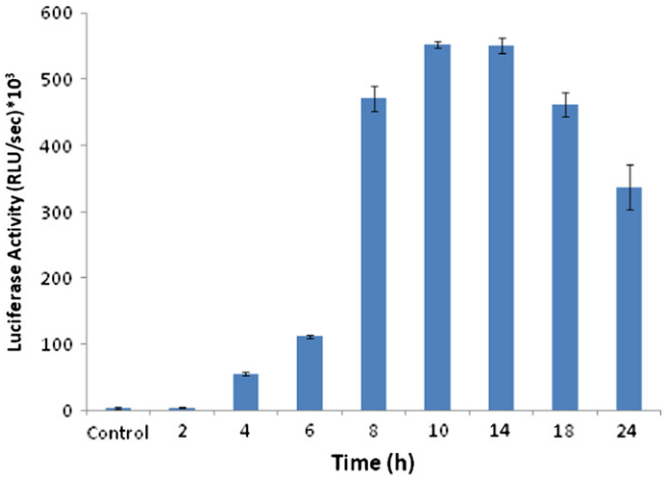

Fig. 4. Doxorubicin-induced, time-dependent apoptosis activation upon apoptosome formation in HEK293T cells. The transfected HEK293T cells with both the established plasmids were incubated with 0.5 μM doxorubicin. Luciferase activities were measured to determine the time course of the apoptosis activation. Values are mean ± SD of three separate experiments.

induction of apoptosis. Therefore, for following experiments 0.5 μM concentration of doxorubicin was chosen.

3.3. Time-dependent response of caspase 3/7 activation

To investigate the time course of activation of caspase 3/7 and also determination of the optimal time of exposure, the cotransfected HEK293T cells were treated with 0.5 μM of doxorubicin for 5, 7, 9, 11, 13, 17, 21 and 27 h. Caspase 3/7 activity was measured according to a procedure as explained under the experimental procedure. As indicated in Fig. 3B, 0.5 μM doxorubicin induced a remarkable increase in caspase 3/7 activity (~2.6 fold) within 9 h and reached to its maximum response (~8 fold) within 17 h. For further confirmation of these results, we studied the time course of apoptosis activation using the fluorescence microscopy technique. The cotransfected cells were treated with 0.5 μM of doxorubicin, and at indicated times the cells were washed twice with cold PBS and treated with FLICA probe, according to manufacturer's instructions, (Immunochemistry Technologies, LLC) fluorescence was monitored using a Nikon fluorescence microscope equipped with a suitable filter. Consistent with the previous results (Fig. 3B) we found that 0.5 μM doxorubicin caused a significant increase in apoptosis activation in the transfected HEK293T cells after 9 h (Fig. 1S, Supplementary information).

3.4. Reconstitution of split luciferase upon apoptosome formation

To prove the feasibility of apoptosis detection at preliminary stage of apoptosome formation, HEK293T cells were cultured in a 24-well plate, cotransfected with 2 μg of both N-luc–Apaf1 and C-luc–Apaf1 plasmids and then treated with 0.5 μM doxorubicin. Total cell number was identical in all experiments. The performance of our sensor for monitoring of programmed cell death over 24 h was investigated by measuring bioluminescence intensities via a procedure as explained under experimental section. As indicated in Fig. 4, we observed a time dependent increase in luminescence intensity following induction of apoptosis over 14 h and then it was decreased. We also observed a significant increase (~15 fold) in luminescence signal compared to control cells 4 h after apoptosis induction. It reached a maximum activity over 10 h (~155 fold), reflecting increasing apoptosis levels. We also assessed the applicability of split luciferase reporter for monitoring the doxorubicin induced apoptosis in intact cells. Fig. 5A and B

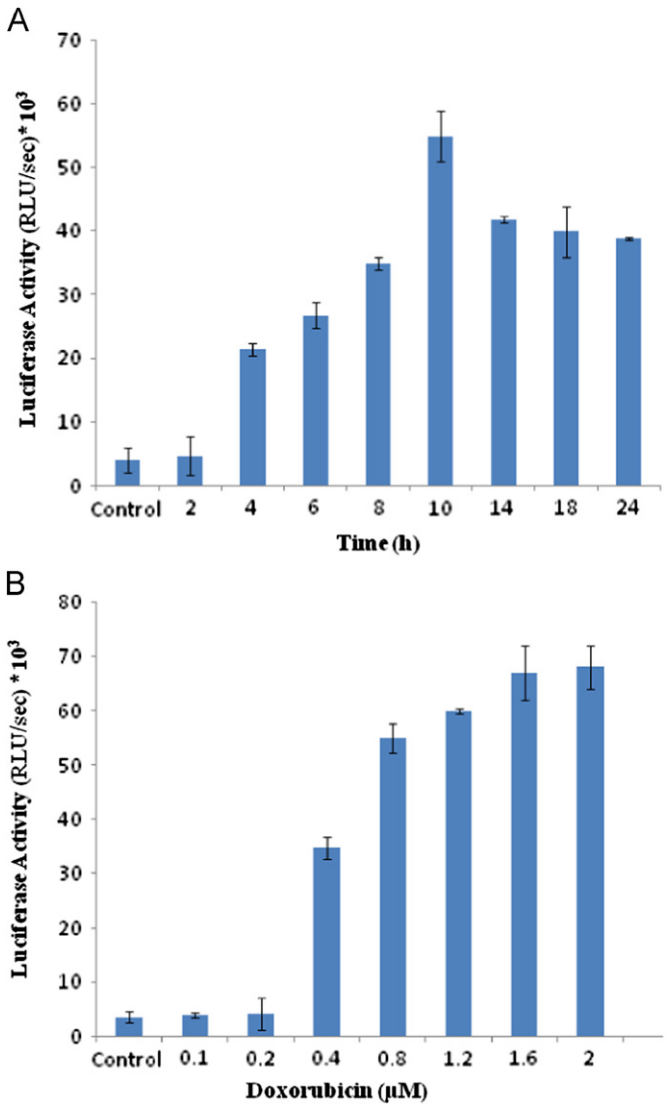

Fig. 5. (A) Time-dependent apoptosis activation in intact cells and (B) dose-dependent apoptosis activation can be detected in intact cells. The cells were transiently transfected with 2 μg of established plasmids (C-luc–Apaf1 plasmid and N-luc–Apaf1 plasmid) and incubated with different concentrations of doxorubicin for 10 h. Values are mean ± S.E. of three separate experiments.

shows a significant time- and dose-dependent increase in bioluminescent activity, respectively. These results are in agreement with cell lysate analysis data and demonstrate that oligomerization of Apaf-1 produces a suitable platform for reconstitution of the luciferase fragments and proves that this sensor can potentially detect apoptosis upon cytochrome *c* release.

3.5. Enzymatic Activity of the N- and C-terminal half of luciferase

To investigate whether the constructed N- and C-fragments of luciferase lost their enzymatic activity, each fragment of luciferase was separately expressed in HEK293T cells and bioluminescence intensities were measured subsequent to apoptosis induction in presence of 0.5 μM doxorubicin after 10 h. According to Fig. 2S, neither of N-terminal half nor C-terminal half of luciferase could independently produce significant signal (B and C) as compared to mock cells (A) while in the cotransfected cells with both plasmids a remarkable signal (~30 folds increase) was produced. According to this result and bioinformatic analysis, both composition and length of the used flexible linker are suitable for reconstitution of luciferase fragments.

4. Discussion

The intrinsic pathway of apoptosis is developing through formation of an apoptosome complex (Okada and Mak, 2004). Several speculative models of the apoptosome structure have been proposed from modeling studies (Riedl and Salvesen, 2007) based on indirect evidences and its low resolution electron micrograph (Reubold et al., 2011). Our findings here represent a direct evidence for oligomerization of Apaf-1 molecules and apoptosome formation under apoptosis condition at cellular level (Fig. 2). That is to say, if had it not been for the "right" juxtaposition of the Apaf-1 molecules in the apoptosome complex upon induction of apoptosis and release of cytochrome *c*, luminescence activity of the split firefly luciferase would have not been observed.

Another interesting result is the behavior of oligomerization upon apoptosis induction by doxorubicin. As indicated in Fig. 6, in cells harboring split-luciferase conjugated to Apaf-1, reconstituted luciferase activity brought about with a sigmoidal curve which may indicate cooperative oligomerization of Apaf-1 monomers upon binding of cytochrome *c*. In fact, it may be suggested that increase of incubation time with doxorubicin will increase the ligand (cytochrome *c*) concentration within the cell, thereby causing cooperative association of Apaf-1 monomers. Thus, the cooperative oligomerization of Apaf-1 enables them to bring faster as much split luciferase together as it would if the monomer association were independent. This type of cooperativity has been observed in different classical models of multi-subunit proteins such as hemoglobin.

The apoptosis assays involve use of many different modalities including cytomorphological alterations, DNA fragmentation, detection of caspases, membrane alterations and mitochondrial assays (Fuchs and Steller, 2011). However, release of cytochrome *c* is superior to the mentioned methods and thereby allows the earlier detection of apoptosis (Cullen and Martin, 2009).

In this study, we initially sought to establish a new platform, based on protein fragment complementation strategy for detection of apoptosis at preliminary phase. This approach started with split-ubiquitin (Johnssonand Varshavsky, 1994) which has been developed for several reporter proteins such as β-galactosidase

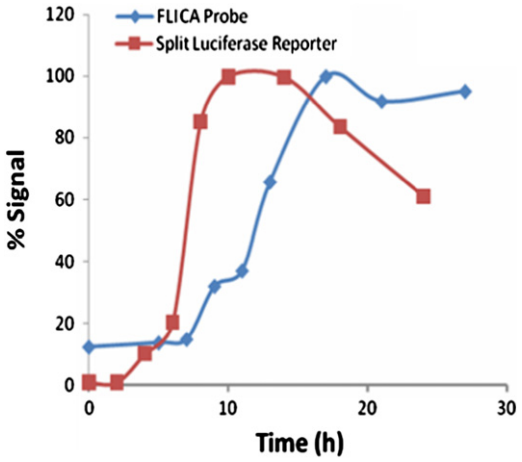

Fig. 6. Comparison of time–response curves. Monitoring of apoptosis progress is compared by activity of caspase3/7 and split-luciferase complementation assay. The signal at each time and experiments are relatively compared with the highest obtained signal in each set of measurements.

(Mohler and Blau, 1996), lactamase (Galarneau et al., 2002), dihydrofolate reductase (Michnick et al., 2000), GFP (Park and Raines, 2000), and luciferase (Ozawa et al., 2001).

According to the numerous interesting properties of firefly luciferase especially production of detectable signal and high quantum yield (Hosseinkhani, 2011), we turned our attention to designing a new sensor based on the split luciferase strategy (Ozawa, 2006). The amino- (1–416 amino acids) and carboxy- (395–550 amino acids) terminal fragments of firefly luciferase were separately fused to the N-terminus of Apaf-1 and a flexible linker composed of (G_4S) was inserted between interacting proteins and the fragments of firefly luciferase to enhance proper folding, as illustrated in Fig. 1. The negligible signal observed upon separate transfection of each half of luciferase (N-luc–Apaf1 and C-luc–Apaf1) suggested that background luciferase was minimal as compared with control cells and indicated that luciferase activities of both N- and C-terminal halves of luciferase were completely lost (Fig. 2S). The ability of reconstitution of split luciferase fragments in transiently cotransfected HEK293T cells with both N-luc–Apaf1 and C-luc–Apaf1 vectors in presence and absence of doxorubicin was also tested. We observed a significant recovered luciferase activity (30-fold after 10 h) in cotransfected HEK293T cells treated with 0.5 μM doxorubicin as compared to non-induced cells (Fig. 2S). However, in contrast to previous reports on other split firefly luciferase systems (Paulmurugan and Gambhir, 2003), higher signal to background ratio was achieved (Fig. 6) presumably through lower interaction between latent form of N-Luc–Apaf1 and C-Luc–Apaf1 monomers.

The ability of doxorubicin in apoptosis induction (Rebbaa et al., 2001) was initially confirmed by measurements of caspase 3/7 activity. As it is obvious in Fig. 1S, upon treatment of the cells with FLICA probe (Bedner et al., 2000), high-affinity fluorescent ligand to activated caspase, a green fluorescence was observed. Owing to the high degree of autofluorescence of cellular components and nonspecifically accumulation of FLICA probes within the cells (Hickson et al., 2010), the lower signal to background ratio was observed (Bedner et al., 2000) as compared with our bioluminescent split reporter (Fig. 6). Time-dependent studies of split luciferase reporter revealed a 6-fold increase in intact cell and a 150-fold increase in cell lysate bioluminescence activity (Figs. 4 and 5A) 4 h after apoptosis induction and further showed a maximum bioluminescence activity over 10 h, after which a gradual decline was observed.

In comparison with the results extracted from fluorescent tests, FLICA probe (Fig. 3B and 1S), we showed that the use of the split luciferase method produces a strong and stable signal solely over 4 h under apoptosis condition and significantly decrease the time required for determining drug efficacy compared with caspase 3/7 assay. However, according to the best of our knowledge, caspase assay is the earliest detection methods of apoptosis induction which have ever been reported (Bedner et al., 2000).

Recently, several attempts have been made using luminescent reporter systems for apoptosis monitoring. Intervention of a DEVD caspase 3/7 cleavage site in circular permuted luciferase (Hickson et al., 2010; Coppola et al., 2008; Laxman et al., 2002) and application of a modified firefly luciferase substrate (Hickson et al., 2010), Z-DEVD-aminoluciferin, provide similar condition for apoptosis assay. However, a limitation of these strategies is the low intensity of luminescent signal due to insufficient digestion of the DEVD sequence during apoptosis which in turn offers the obligation for long time incubation for complete digestion of caspase site and obtaining the detectable luminescent signal (Bedner et al., 2000; Hickson et al., 2010). As our split luciferase reporter doesnot face this challenge and the luciferase measurements can immediately be conducted at the time of interest.

Although the approach outlined in this study cannot be directly used in clinical investigations, but it can serve as a versatile tool for in vivo imaging and preclinical drug discovery. In addition, this bioluminescent reporter can be optimized through production of different stable cell lines harboring the split luciferase apoptosis reporter for use in a wide range of therapeutic applications in cancer drug discovery field because of its ease and speed of performance, short acquisition time, and concurrent measurement of multiple cell lines.

5. Conclusion

In summary, according to the results presented in this manuscript, we have shown for the first time the construction of an Apaf-1 split luciferase biosensor for apoptosis monitoring upon release of cytochrome *c*. It has been shown that the designed biosensor can be used for earlier detection of apoptosis and is a new platform for assessing potential chemotherapeutic drugs as well as a sensitive and dynamic system in the time- and dose-dependent studies of apoptosis. Moreover, the current reporter system revealed the molecular architecture of apoptosome and cooperativity in oligomerization of Apaf-1 subunits.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2012.06.034.

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