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ARTICLE *in* IN VITRO CELLULAR & DEVELOPMENTAL BIOLOGY - ANIMAL · SEPTEMBER 2012

Impact Factor: 1.15 · DOI: 10.1007/s11626-012-9542-8 · Source: PubMed

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DOI 10.1007/s11626-012-9542-8

REPORT

Dynamics of caspase-3 activation and inhibition in embryonic micromasses evaluated by a photon-counting chemiluminescence approach

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Received: 29 May 2012 / Accepted: 31 July 2012 / Editor: Tetsuji Okamoto, Michele Schultz

Abstract Caspases are key enzymatic components of the intracellular apoptotic machinery, and their role in mammalian systems is often studied using fluoromethylketone (FMK) inhibitors. Despite many advantages of such approach, efficiency of the inhibitor and membrane permeability speed are often questioned. This work therefore focuses on an exact evaluation of caspase-3 FMK inhibition dynamics in camptothecin-induced mesenchymal micromasses. Two parameters of caspase-3 FMK inhibitor were investigated: first, the stability of the inhibitory potential in the time course of cultivation and, simultaneously, the dynamics of caspase-3 FMK inhibition after camptothecin-induced apoptosis peak. A photon-counting chemiluminescence approach was applied for quantification of active caspase-3. The sensitivity of the photon-counting method allowed for evaluation of active caspase-3 concentration in femtogram amounts per cell. The inhibitor penetrated the cells within the first minute after its application, and the peak of caspase-3 started to decline to the blank level after 30 min. The inhibitory effect of the FMK inhibitor was unchanged during the entire 48 h of cultivation.

Keywords In vitro primary cultures · Caspases · Camptothecin · FMK inhibitor

Interest in apoptosis keeps rapidly increasing in the last decades as this phenomenon plays fundamental roles in development and homeostasis and has been applied in many therapeutical approaches (Strasser et al. 2011). Apoptosis can proceed by different pathways; however, cleavage of caspases is one of the molecular hallmarks of most apoptotic cell deaths. Caspase-3 along with caspase-6 and caspase-7 belongs to a death trio of executioner pro-apoptotic caspases and is considered as the central caspase representing the point of no return (Kroemer et al. 2009). These cystein proteases are activated posttranslationally in the cleavage cascade starting from initiation caspase down to executioner ones. The functional changes at protein level limit advantages of expression analysis using nucleic acids and available amplification methods. Moreover, knock out of caspase is often lethal (Hakem et al. 1998; Kuida et al. 1996, 1998; Varfolomeev et al. 1998). Therefore, pharmacological inhibition of caspases is an important tool to study the roles of these enzymes in mammalian systems, and fluoromethylketones (FMK) are widely used. These molecules can rapidly manipulate the activity of caspase; however, their effectiveness related to their stability and membrane penetration potential used to be questioned (Abraham and Shaham 2004).

Here, we established primary mesenchymal cell cultures (micromasses) and applied a camptothecin treatment to induce caspase-3 cleavage in order to investigate the dynamics of following FMK inhibition. Modulation of caspase machinery using FMK inhibitors was performed several times in the explant cultures such as the limb providing a striking example

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Published online: 14 September 2012

of apoptosis in organ shaping (Chautan et al. 1999; De Valck and Luyten 2001), also with special focus on caspase-3 (Kudelova et al. 2012). As the explants take advantage of intact organs, the embryonic micromasses, cultured clusters of enzymatically separated cells, allow for direct evaluation of individual cells after treatment and were shown very useful also for apoptosis research (Roy et al. 2010).

To create cultures of micromasses, front limbs at embryonic day (E) 12.0 were obtained from wild-type pregnant mice (strain CD1) according to an agreed experimental protocol (Laboratory Animal Science Committee of the Institute of Animal Physiology and Genetics, CAS, v.v.i., Czech Republic). Limbs were mechanically disintegrated in a medium (PSA with FCS, 9:1) followed by enzymatic disintegration with 300 μl of Dispase II (Invitrogen, Carlsbad, CA) in a final volume of 2.7 ml medium, then incubated in a shaker (1,000 rpm/1–1.5 h/37°C) and vortexed, each for 15 min. Three millilitres of micromass medium (MM) (26.4 ml F12, 17.6 ml Dulbecco's modified Eagle medium (DMEM), 5 ml FCS, 0.5 ml Pen/Strep, 0.5 ml L-glutamine) were added to the cell suspension and then centrifuged at 1,000×*g*/5 min. The resuspension of sediment in 3 ml of MM by centrifugation through the cell strainer with washing of 3 ml MM and then centrifugation at 1,000×*g*/5 min were performed. According to the formula $V[\mathrm{ml}] = X \times 250,000\,\mathrm{x6[ml]}/2 \times 10^7$(cell counting (X) in Burker's chamber), the calculated volume was added to the sediment and was used for 10-μl aliquots to make spots in cultivation plates (Fig. 1).

After 1 h/37°C incubation, 2 ml of MM was added to each cultivation plate and then cultured according to the experimental design in the incubator (37°C/5% CO_2). The MM was changed the next day with simultaneous addition of camptothecin. Camptothecin (Sigma-Aldrich, Saint Louis, MO) was dissolved directly in MM at the concentration of 5 μM (Hsiang et al. 1985; Saeki et al. 2011). Caspase-3 inhibitor (FMK004, R&D Systems, Minneapolis, MN) was dissolved in the dimethylsulfoxide (DMSO, Sigma-Aldrich) and applied in the final concentration of 100 μM.

The experimental design involved several steps. First, quantification of active caspase-3 was followed in the control group (without any treatment) and experimental group (camptothecin treatment) to detect the caspase-3 activation peak. Samples were collected and measured before initialisation of the cultures, prior to addition of camptothecin and then in 1-h intervals. Subsequently, a FMK caspase-3 inhibitor was added to the cultures at the peak time, and dynamics of inhibition was followed in four parallel-running groups: (1) control of cultivation (DMEM only), (2) vehicle control (DMEM+DMSO), (3) camptothecin-induced group and (4) inhibited group (medium+FMK). Sample aliquots were taken every 5 min. The cell populations were collected in 2-h intervals by sucking the micromass medium out and adding 1 ml of 0.25% trypsin to each plate, washing in 1 ml of sterile PBS and then centrifuging at 3,000 rpm/10 min. The supernatant was discarded and the sediment resuspended in 1 ml of micromass medium. Cells were counted in Burker's chamber, in 50 large squares two times to find the average and then determine the number of cells per volume. A portion of 15 μl of cell suspension with evaluated number of cells was well mixed with 15 μl of CASPASE-GLO™ 3/7 (Promega, Madison, WI) reagent. A part of 20 μl of this mixture was injected into a specially designed detection chamber of a volume of 30 μl made of stainless steel. The highly polished inner surface and conical shape of the chamber are designed to reflect all the luminescence to the photomultiplier tube (PMT) detection window. The chemiluminescence (CL) emission was monitored immediately after mixing for a period of 1 h, sufficient to catch the steady-state plateau of the signal. The maximum signal was taken as a representative value proportional to the amount of caspase-3. After each experiment, the chamber was consecutively washed by acetonitrile and water.

Caspase-3 activation peak was detected in the experimental group treated with camptothecin as compared with the control group without any treatment (Fig. 2*A*). The difference between control and experimental groups started to be clearly apparent 6 h after camptothecin treatment, and the peak was achieved and maintained between 8- and 10-h intervals.

Dynamics of caspase-3 FMK inhibition was evaluated after camptothecin peak, and the cells were exposed to the inhibitor 1–60 min. To guarantee a quick elimination of the inhibitor from the medium, not only all cells (free cells require centrifugation time to deplete the medium) but also adherent cells only (rapid wash off) were used for evaluation. As shown in Fig. 2*B*, the inhibitor must have penetrated the cells during the

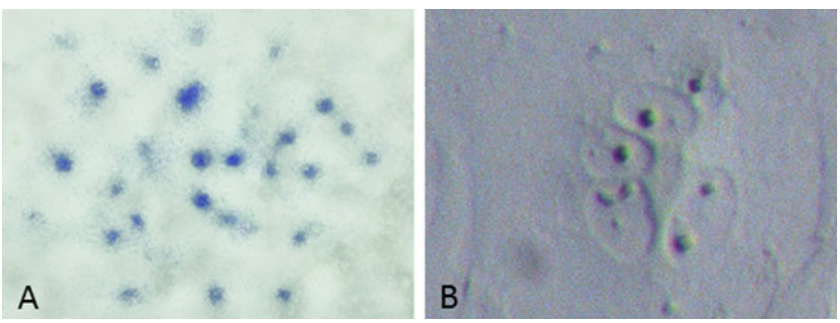

Figure 1. (*A*) Microscopic image of front limb micromass cultures after Alcian Blue staining (magnification, ×40) with (*B*) a detailed view at individual cells (magnification, ×250).

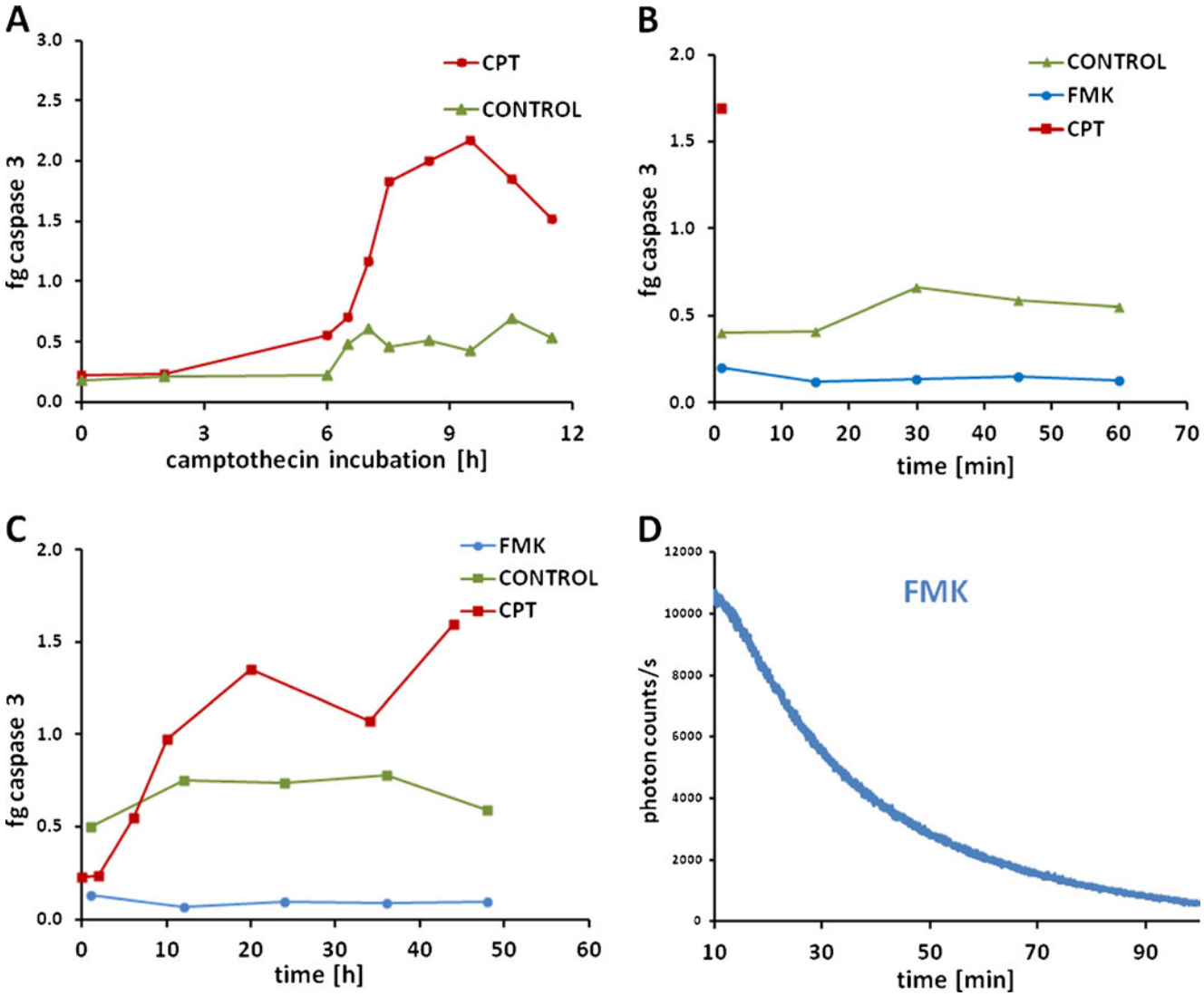

Figure 2. (*A*) Amount of active caspase-3 per cell. Camptothecin-induced caspase-3 activation peak (*red line*) in the experimental group in comparison with the control group (*green line*) cultures in the time course after treatment. (*B*) Penetration of FMK inhibitor during cultivation evaluated after camptothecin peak. Inhibition effect of FMK inhibitor in the experimental group during 60 min (*blue line*) in comparison with the control group (*green line*). Camptothecin-induced apoptosis peak shown by the *red squares*. (*C*) Stability of FMK inhibitor during the whole time of cultivation. The experimental group showed constant inhibition effect during 48 h of cultivation (*blue line*) and maintenance at lower level in comparison with the control group (*green line*). (*D*) Dynamics of caspase-3 inhibition in the experimental group with FMK inhibitor and CPT in the measurement course based on the CL emission signal (*blue line*).

first minute. Nevertheless, the complete inhibition effect in such a short time could be explained by binding to the active caspase-3 later during the sample processing (takes 30 min). In order to analyse this possibility in detail, the CL level was evaluated during the whole measurement course (90 min). Indeed, the effect of the FMK inhibitor was obvious after the CL emission caught the maximum signal, and then the signal rapidly declined indicating the inhibitory effect as soon as after 30 min (Fig. 2*D*). In camptothecin-treated and control groups, the signal achieved the steady-state plateau and did not change as in the blank measurement (data not shown). The value of the signal was proportional to the amount of cell populations.

Simultaneously, stability of the inhibitor was checked every 12 h during the experiment time course to follow any sign of degradation due to culture conditions. To exclude any effect of DMSO on cell survival and increase of cleaved caspase-3, medium only and DMSO-treated cells were compared. The inhibitory effect of the FMK inhibitor during the whole time of cultivation was constant (Fig. 2*C*). The amount of active caspase-3 in the experimental group (camptothecin treatment with FMK inhibitor) was maintained even at a lower level in comparison with the control group (without treatment) as there is always some number of apoptotic cells in the culture giving a slight caspase-3 positive signal (as seen also in Fig. 2*B* and *C*).

There is an array of methods available for apoptosis detection (Martinez et al. 2010), and caspases are often evaluated as hallmarks of apoptosis as their cleavage is not random but very selective. Active caspase detection *in situ* is limited to immunohistochemistry with possible transfer of specific tissue bound cell populations using laser capture microdissection and individual cell labelling for flow cytometry (Matalova et al. 2010). Flow cytometry in general is a method of choice for active caspase evaluation, and several modifications allowing for higher specificity have been developed based particularly on new fluorescent substrate and tagging of the cleaved caspase molecules (Hug et al. 1999; Kohl et al. 2005, Cai et al. 2011). As the flow cytometry results inform about the proportion of caspase-3-positive cells to the total number of cells, the photon-counting method may adjust the result up to femtogram content per one caspase-3-positive cell. Moreover, the described CL procedure is a one-step mixing system without any washing steps and expensive immobilized agents. In other approaches, fluorescent correlation spectroscopy, microfluidic techniques and fluorescent resonance energy transfer were used to evaluate caspases in cell lysates (Martinez et al. 2010). With respect to dynamics, most of the methods provide data at the nanomolar concentrations. Using the CL approach, measurement at femtogram level was achieved.

Extremely high sensitivity of CL stems from several reasons. An interference of scattered excitation light, background fluorescence of other species present in the sample, as well as any photobleaching can be excluded. Moreover, a slow but long-lasting generation of photons is amenable to the application of the photon-counting detection regime, the most sensitive method for the quantitative measurement of light. Conventional PMT with nanosecond time resolution furnished with fast electronics enabling a gigahertz acquisition of the signal can be used as detection elements. Another advantage of this instrumentation is an easy processing of digital signal which can easily be discriminated from the dark signal and other interfering phenomena.

In summary, quantification of caspase-3 using the photon-counting CL approach in embryonic micromasses clearly demonstrated activation and inhibition dynamics in an *in vivo*-like system which indicate that penetration of the FMK inhibitor and following caspase inhibition, at least in individual cells, occurs within minutes. This method can be easily applied as an alternative to biotinylated inhibitors also in other systems as it demonstrates not only penetration but also the inhibitory effect of FMK inhibitors.

Acknowledgments This study was supported by the Grant Agency of the Czech Republic, project P502/12/1285 (UVPS Brno) and P206/11/2377 (IACH v.v.i.). IAPG runs under IRP IPAG no. AVOZ 5045015, IACH under OP EC CZ. 1.07/2. 3 00/20. 0182; MMCI is supported by the European Regional Development Fund and the State Budget of the Czech Republic (RECAMO, CZ.1.05/2.1.00/03.0101). Thanks to Assoc. Prof. M. Buchtova for her great help with the setup of the micromass culture system.

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