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LIVE IMAGING BY TIME-LAPSE MICROSCOPY CAN MORE CLEARLY EVALUATE THE ANTI-APOPTOTIC STATE OF PRIMARY HEPATOCYTES ISOLATED FROM THE *DRS* KNOCKOUT MOUSE

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Abstract
To investigate the anti-apoptotic state of primary hepatocytes isolated from the *drs* knockout (KO) mice, Fas-mediated apoptosis was observed by flow cytometry and time-lapse microscopy. Treatment with anti-Fas antibody plus actinomycin D or cycloheximide induced over 50% apoptosis for primary hepatocytes based on annexin V staining. At 24 hr later under these apoptotic stimuli, the sub G1 fraction of hepatocytes isolated from the *drs* KO mice was significantly decreased in comparison to wild C57BL/6 mice. In contrast, sequential time-lapse microscopic analysis revealed clearer and larger differences in the apoptotic responses of primary hepatocytes from the *drs* KO and wild-type mice from 4 hr to 24 hr after apoptotic induction. The time-lapse microscopic analysis clearly indicated the hepatocytes isolated from the *drs* KO mouse to be more resistant to the apoptotic stimuli than other methods. The time-lapse microscopy is very useful for the sequential observation of viable primary hepatocytes.

Key words: time-lapse imaging, apoptosis, *drs* knockout mouse

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Introduction

A dysfunction of apoptosis is associated with cancer[1] and genetic alterations of apoptosis-related genes, such as TP53 and RB1 are found in human hepatocellular carcinoma[2-4]. Genetically modified mice with a tendency to develop liver cancer such as

TP53 and E2F have a malfunction of apoptosis[5-8]. The analysis of apoptosis in cultured cell lines is important and common-place. However, most cultured cell lines have acquired different characteristics than normal tissues or tumors in culture. Analyzing primary cells from various tissues in a genetically modified mouse that is prone to develop cancer provides more significant results, since primary cells more closely resemble cells in a living organism.

Many techniques have been reported for evaluating apoptosis. Apoptosis was originally defined based on morphological hallmarks, such as nuclear condensation and fragmentation, membrane blebbing and cell shrinkage, which can be observed by microscopy[9,10]. The annexin V assay for cell surface phosphatidylserine exposure and the sub G1 fraction assay for fragmented DNA are other methods for detection of apoptosis[11]. The time-lapse microscope is a novel device for viable cell observation[12-14]. In this method, a marked single hepatocyte can be observed sequentially and intensively in a culture chamber.

Primary hepatocytes isolated from the *drs* KO mice were examined. *Drs* was originally isolated as a suppressor of *v-src* transformation and reported to be involved mainly in ER stress-induced apoptosis[15]. It was reported that *drs* KO mice have a high tendency to develop malignant tumors, including lymphomas, lung adenocarcinomas and hepatomas over six months after birth[16]. In this study, the behavior of primary hepatocytes was observed upon apoptotic induction. The results indicated that the time-lapse microscopic analysis is more useful to show that the primary hepatocytes isolated from the *drs* KO mice were resistant to apoptosis than other methods.

Materials and Methods

Animals

All experiments were approved by the animal care committee of the Hamamatsu University School of Medicine, and all procedures regarding animals were conducted according to institutionally approved protocols and guidelines. About 7 to 9 week-old male CB57BL/6CR mice (SLC, Japan) and *drs* KO mice[16] were used for these experiments.

Isolation of primary hepatocytes

Primary hepatocytes were isolated from liver tissue specimens by the two-perfusion method. First, pre-perfusion buffer (20 mM HEPES/0.5 mM EGTA/900 mg/L glucose in Ca^{2+} free Hanks solution, pH 7.2) was used to wash out the blood and then the tissue was perfused with collagenase buffer (5 mM $CaCl_2$/0.005% collagenase/0.005% trypsin inhibitor/Hanks solution) for 10 min. Finally, the liver tissue was digested in the same buffer for 10 min at 37°C. The liver cells were suspended in DMEM containing 10% fetal bovine serum (FBS). Cell viability was 90-95% based on the trypan blue exclusion method. The cells were cultured at 2×10^6 cells/ml in culture medium (DMEM containing 10% FBS, 40 U/L insulin and 16 mg/L dexamethasone) on 35 mm glass based dish (Iwaki, Japan) in a humidified 5% CO_2/95% air at 37°C.

Apoptosis induction

After overnight culture, the cells were washed twice with phosphate-buffered saline (PBS) to eliminate dead cells. Dishes containing hepatocytes with high viability (more than 90%) were separated into four groups: group 1. culture medium only as a control, group 2. medium containing 0.75 mg/ml anti-Fas antibody (Jo2, BD Biosciences), group 3. medium containing 0.75 mg/ml anti-Fas antibody plus 0.05 mg/ml actinomycin D (Sigma), group 4. medium containing 0.75 mg/ml anti-Fas antibody plus 0.05 mg/ml cycloheximide (Sigma).

Annexin V-PE staining and apoptosis index

Annexin V-PE and propidium iodide (PI) double staining was performed with non-permeabilized cells. Then the cells were observed using a fluorescence microscope. Apoptotic cells were stained

using an Annexin V-PE Apoptosis Detection Kit (BD Biosciences). These experiments use 5 mg/ml PI instead of the 7-amino-actinomycin D in the kit. A monolayer culture ($1\text{-}2\times10^5$ cells/dish) was used for fluorescence microscopy. The hepatocytes on the dishes were observed and recorded using a fluorescence microscope imaging system (Nikon, Biostation) at dual excitation wavelengths of 440 nm (for Annexin V) and 530 nm (for PI) and dual emission wavelengths of 520 nm (for Annexin V) and 620 nm (for PI). More than three 35 mm-dishes were evaluated under the same conditions in at least 3 fields for one 35 mm dish. The apoptosis index (%) = (Annexin V-positive cell number/total observed cell number) $\times$100.

Flow cytometric analysis of cell cycle and apoptosis

The treated cells were evaluated by the sub G1 fraction on flow cytometry every 4 hr to 24 hr after stimulation. At the scheduled time, the cells were treated with EDTA and trypsin to remove the hepatocytes from the dish. Detached cells were fixed with 70% ethanol and the cell nuclei were stained with PI. The DNA content of 20,000 cells was monitored in one analysis with an Epics XL flow cytometer (Beckman Coulter). The mature hepatocytes were gated and analyzed using the Epics XL SYSTEMII software program (Beckman Coulter) during the analysis of the cell cycle parameters or the Multicycle software (Beckman Coulter) for peak detection of sub G1 hypoploid apoptotic cells.

Time lapse imaging of cultured hepatocytes

The isolated hepatocytes were observed under the Fas-mediated apoptosis induction using time-lapse microscopy. The apoptosis index in the time lapse imaging is the percentage of the apoptotic cell number to the total cell number every 4 hr after stimulation. More than three 35 mm dishes were evaluated under the same conditions and at least 3 fields for one 35 mm dish. Cells were kept in a 5% CO_2 incubation chamber at 37°C for 24 hr after stimulation. Time lapse imaging was performed using a computer-assisted fluorescence microscope (Olympus, LCV 100) equipped with an objective lens (Olympus, UAPO 40x/340 N.A.=0.90), a halogen lamp, a red LED (620 nm), a CCD camera (Olympus, DP30), differential interference contrast (DIC) optical components and interference filters.

Western blot analysis of caspase-3

The cells were lysed in an NP40 lysis buffer (150 mM NaCl, 1% Nonidet P-40 and 50 mM Tris-HCl, pH 7.4) and the immunoblotting of cell extracts using anti-caspase-3 antibody (# 9662, Cell Signaling Technology) was performed as described previously[17].

Statistical Analysis

The data were analyzed using Student's t-test and the Mann-Whitney U test. The level of significance was $p<0.05$.

Results and Discussions

There are several methods to detect apoptotic cells[9-11]. Annexin V staining is a familiar method to determine if a cell is in an apoptotic state[11]. Annexin V-PE staining was used to detect apoptotic hepatocytes (Fig. 1a-b). The adequate concentration of anti-Fas antibody was determined based on the kinetics (Fig. 1c). Because high concentrations of anti-Fas antibody induced both apoptosis and necrosis, it was not possible to obtain an adequate apoptosis index using anti-Fas antibody alone. The cells were treated with actinomycin D or cycloheximide in addition to anti-Fas antibody according to a previous study[18]. Finally, primary hepatocytes were cultured in the medium containing 0.75 mg/ml anti-Fas antibody plus 0.05 mg/ml actinomycin D or 0.05 mg/ml cycloheximide. The treatment with actinomycin D or cycloheximide alone, could not obtain the sufficient apoptotic stimulations (data not shown).

The sub G1 subpopulation represents apoptotic cells with fragmented DNA content. This method

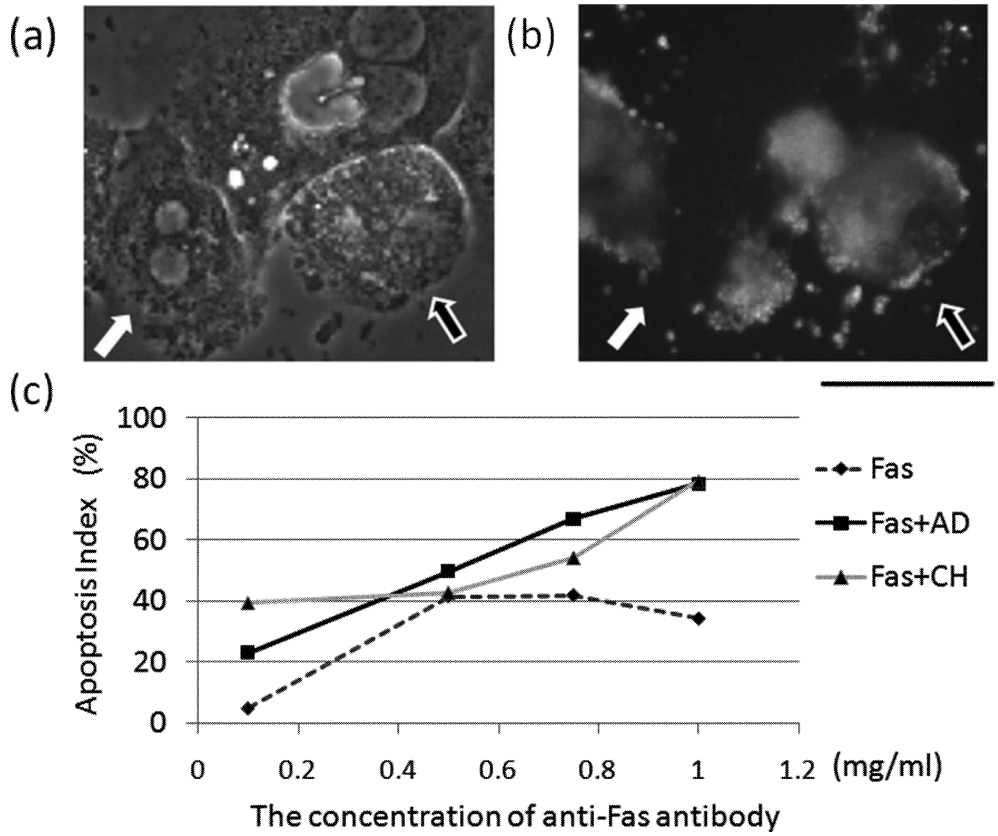

Fig. 1. Microscopic view of hepatocytes stained with annexin V and the kinetics of Fas-triggered apoptosis of mouse primary hepatocytes
The primary mouse hepatocytes are shown on the microscopic images (a) and the fluorescence images of annexin V-PE staining (b). The viable hepatocyte (white arrows) was not stained with annexin V and the hepatocyte with nuclear fragmentation (black arrows) was stained with annexin V at the cell membrane. The scale bar represents 50 μm. (c) Mouse primary hepatocytes were incubated with various concentrations of anti-Fas antibody at 37°C with/without 0.05 mg/ml actinomycin D or 0.05 mg/ml cycloheximide. The apoptosis index was determined as the percentage of annexin V-positive apoptotic cells observed by fluorescent microcopy. The experiments were repeated three times. ◆: anti-Fas antibody only, ■: anti-Fas antibody plus 0.05 mg/ml actinomycin D (AD), ▲: anti-Fas antibody plus 0.05 mg/ml cycloheximide (CH).

is one of the standard methods to measure the degree of apoptosis[11]. We measured the sub G1 fraction of both hepatocytes before and after apoptotic stimulation. At 24 hr, the sub G1 fraction was significantly smaller in the hepatocytes from the *drs* KO mouse than in those from wild-type mouse (*drs* KO vs. Wild-type 63.2±5.91% vs. 87.1±2.77%, $p<0.05$, Fig. 2e, f, g). However, the differences from 4 hr to 20 hr were not statistically significant (Fig. 2a-d, g).

Next, the apoptosis of primary hepatocytes was evaluated using time-lapse microscopy. The behavior of isolated mouse hepatocytes could be observed individually. When the record of 24 hr observation was pressed into a short movie, the figures were captured as TIFF files at 10 min intervals and edited 6 figures (information about one hour) into one second. Therefore, the total length

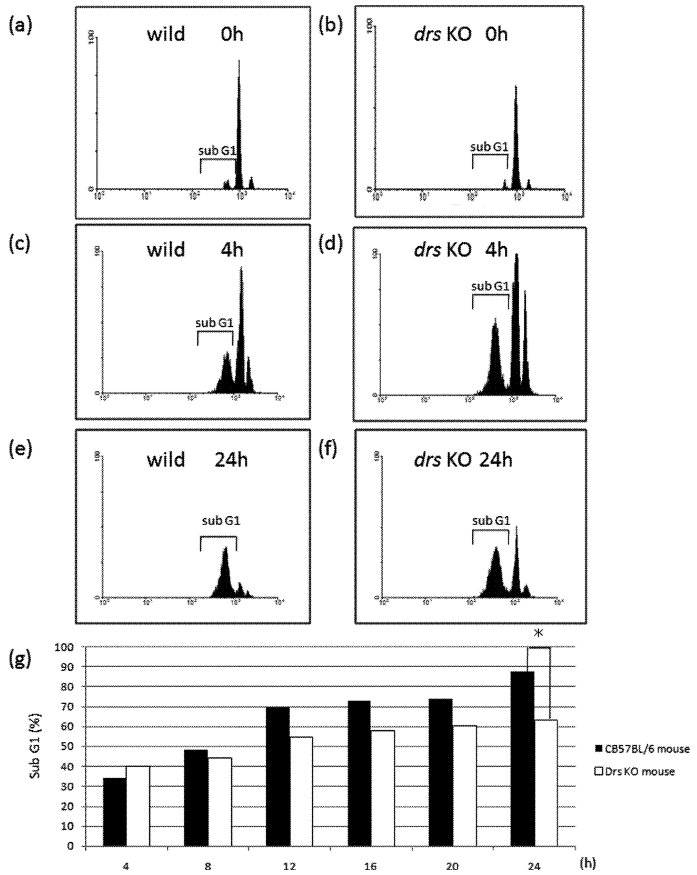

Fig. 2. FACS analyses of hepatocytes isolated from *drs* knockout mice.
DNA content analyses of the primary hepatocytes isolated from wild-type mice (a, c, and e) and *drs* KO mice (b, d and f) at 0 hr (a and b), 4 hr (c and d) and 24 hr (e and f) after apoptotic stimulation. The sub G1 fraction are marked. (g) Time course of sub G1 fraction of wild-type and *drs* KO primary hepatocytes. The sub G1 fraction of primary hepatocytes from *drs* KO mice (light bars) are significantly decreased compared to those from the wild-type mice (dark bars) only 24 hr after apoptotic stimulation. $*=p<0.05$

of a short movie was edited into 24 seconds per 24 hr. In this way, it was possible to precisely observe the state of apoptotic cells. The apoptosis index of *drs* KO mouse was significantly decreased from 4 hr to 24 hr after apoptotic induction. The primary hepatocytes isolated from the wild-type mouse began to be apoptotic from 4 hr and more than 50% of hepatocytes were apoptotic at 24 hr in cells treated with of anti-Fas antibody plus actinomycin D. In contrast, the primary hepatocytes from the *drs* KO mouse were resistant to apoptosis (Fig. 3).

Because of the discrepancy of the results obtained by FACS and time-lapse microscopy, the apoptosis reaction was confirmed using the biochemical approach. The best biochemical hallmark of apoptosis is the activation of caspase. There are three major apoptotic pathways. The first one is death receptor pathway in which FADD activates caspase-8 upon binding of FasL and TNF to Fas and TNF receptor, respectively. Anti-Fas antibody mimics FasL. The second is mitochondrial pathway in which apoptotic and antiapoptotic Bcl

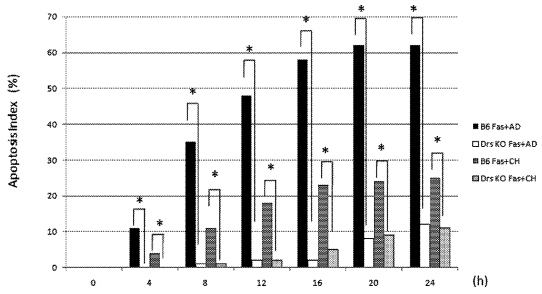

Fig. 3. Apoptosis index of primary hepatocytes on time-lapse live imaging.
The apoptosis index was calculated as the percentage of viable cell number in the total cell number by time lapse imaging analyses. The values of the primary hepatocytes isolated from *drs* KO mice were clearly low in comparison to those from wild-type mice at 4 hr to 24 hr after apoptotic stimulation. $*=p<0.05$. Fas, anti-Fas antibody; AD, actinomycin D; CH, cycloheximide.

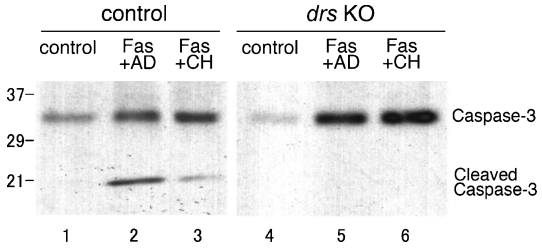

Fig. 4. Caspase-3 was not activated in hepatocytes isolated from *drs* KO mice upon anti-Fas antibody plus actionomycin D or cycloheximide stimulation. Primary hepatocytes isolated from wild-type (lanes 1-3) and *drs* KO mice (lanes 4-6) were untreated (control; lanes 1 and 4) and treated with anti-Fas antibody plus actinomycin D (Fas+AD; lanes 2 and 5) or anti-Fas antibody plus cycloheximide (Fas+CH; lanes 3 and 6) for 8 hr were lysed and cell extracts (40 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis, transferred to a nylon membrane and then the membrane was incubated with anti-caspase-3 antibody. Inactive caspase-3 (32 kDa) and active cleaved caspase-3 (21 kDa) are shown.

family proteins play important roles. When the cells are treated with actinomycin D or cycloheximide, new protein synthesis of Bcl family proteins is blocked and the amounts of antiapoptotic proteins are decreased more than apoptotic proteins due to shorter half-life of antiapoptotic proteins. When the balance tilts to apoptosis, cytochrome c is released and activates caspase-9. The third one is endoplasmic reticulum (ER) pathway in which ER stress induces activation of caspase-7 and caspase-12. All these activated caspases activate finally caspase-3. So, detection of active caspase-3 in cells and tissues is important for confirming apoptosis induced by a wide variety of apoptotic signals[19]. The activation of caspase-3 was measured in both-type hepatocytes 8 hr after induction. The active or cleaved caspase-3 was detected in wild-type mouse hepatocytes. In contrast, the cleaved caspase-3 was not detected in *drs* KO mouse hepatocytes (Fig. 4). This result confirmed that the results of time lapse microscopy were correct.

It is difficult to handle primary hepatocytes with a low proliferation rate unlike established cell lines and cancer cells. Dead or broken cells are likely to attach the dish wall. The time-lapse live-image analysis could select the suitable cells for judging apoptosis. During detaching cells with trypsin and EDTA for flow cytometric analysis, some hepatocytes were broken and numbers of broken cells might increase when cells were treated with apoptotic stimuli. However, FACS analysis could not exclude the broken cells. Previous reports using microscopy indicated that the primary hepatocyte starts apoptosis about 4 hr after the induction[18], which is consistent with the current results. In summary, a time-lapse image analysis is thus considered to be very useful in the sequential observation of viable and apoptotic primary hepatocytes.

References

1) Hanahan, D. and Weiberg, R.A. (2000) The hallmarks of cancer. *Cell*, **100**, 57-70.
2) Thorgeiersson, S.S. and Grisham, J.W. (2002) Molecular pathogenesis of human hepatocellular carcinoma. *Nat. Genet.*, **31**, 339-346.
3) Edamoto, Y., Hara, A., Biernat, W., Terracciano, L., Cathomas, G., Riehle, H.M., Matsuda,

M., Fujii, H., Scoazec, J.Y. and Ohgaki, H. (2003) Alterations of RB1, p53 and Wnt pathways in hepatocellular carcinomas associated with hepatitis C, hepatitis B and alcoholic liver cirrhosis. *Int. J. Cancer*, **106**, 334–341.

4) Hussain, S.P., Schwank, J., Staib, F., Wang, X.W. and Harris, C.C. (2007) TP53 mutations and hepatocellular carcinoma : insights into the etiology and pathogenesis of liver cancer. *Oncogene*, **26**, 2166–2176.

5) Storer, R.D., French, J.E., Haseman, J., Hajian, G., LeGrand, E.K., Long, G.G., Mixson, L.A., Ochoa, R., Sagartz, J.E. and Soper, K.A. (2001) P53+/- hemizygous knockout mouse : overview of available data. *Toxicol. Pathol.*, **29** suppl., 30–50.

6) Calvisi, D.C. and Thrgeirsson, S.S. (2005) Molecular mechanism of hepatocarcinogenesis in transgenic mouse models of liver cancer. *Toxicol. Pathol.*, **33**, 181–184.

7) Halazonetis, T.D., Gorgoulis, V.G. and Bartek, J. (2008) An oncogene-induced DNA damage model for cancer development. *Science*, **319**, 1352–1355.

8) Conner, E.A., Lemmer, E.R., Omori, M., Wirth, P.J., Factor, V.M. and Thorgeirsson, S.S. (2000) Dual functions of E2F-1 in a transgenic mouse model of liver carcinogenesis. *Oncogene*, **19**, 5054–5062.

9) Wyllie, A.H., Kerr, J.F. and Currie, A.R. (1980) Cell death : the significance of apoptosis. *Int. Rev. Cytol.*, **68**, 251–306.

10) Kerr, J.F., Wyllie, A.H. and Currie, A.R. (1972) Apoptosis : a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer*, **26**, 239–257.

11) Kajstura, M., Halicka, H.D., Pryjma, J. and Darzynkiewicz, Z. (2007) Discontinous fragmentation of nuclear DNA during apoptosis revealed by discrete "sub G1" peaks on DNA content histogram. *Cytometry*, **71**, 125–31.

12) Sakaue-Sawano, A., Kurokawa, H., Morimura, T. *et al.* (2008) Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. *Cell*, **132**, 487–498.

13) Yamanaka, Y., Tamplin, O.J., Beckers, A., Gossler, A. and Rossant, J. (2007) Live imaging and genetic analysis of mouse notochord formation reveals regional morphogenetic mechanisms. *Dev. Cell*, **13**, 884–896.

14) Debeir, O., Megalizzi, V., Warzee, N., Kiss, R. and Decaestecker, C. (2008) Videomicroscopic extraction of specific information on cell proliferation and migration in vitro. *Exp. Cell Res.*, **314**, 2985–2998.

15) Tambe, Y., Isono, T., Haraguchi, S., Yoshioka-Yamashita, A., Yutsudo, M. and Inoue, H. (2004) A novel apoptotic pathway induced by the *drs* tumor suppressor gene. *Oncogene*, **23**, 2977–2987.

16) Tambe, Y., Yoshioka-Yamashita, A., Mukaisho, K. *et al.* (2007) Tumor prone phenotype of mice deficient in a novel apoptosis-inducing gene, *drs*. *Carcinogenesis*, **28**, 777–784.

17) Chandra, A., Itakura, T., Yang, Z., Tamakoshi, T., Wang, B., Ueki, T., Sato, K., Uezato, T. and Miura, N. (2006) Neurogenesin-1 differentially inhibits the osteoblastic differentiation by bone morphogenetic proteins in C2C12 cells. *Biochem. Biophys. Res. Commun.*, **344**, 786–791.

18) Ni, R., Tomita, Y., Matsuda, K., Ichihara, A., Ishimaru, K., Ogasawara, J. and Nagata, S. (1994) Fas-mediated apoptosis in primary cultured mouse hepatocytes. *Exp. Cell Res.*, **215**, 332–337.

19) Mazumder, S., Plesca, D. and Almasan, A. (2008) Caspase-3 activation is a critical determinant of genotixic stress-induced apoptosis. *Methods Mol. Biol.*, **414**, 13–21.