FEBS Letters 453 (1999) 356–360 FEBS 22209

Imaging of caspase-3 activation in HeLa cells stimulated with etoposide using a novel fluorescent probe

Shin Mizukami[a], Kazuya Kikuchi[a], Tsunehiko Higuchi[a], Yasuteru Urano[a], Tetsuo Mashima[b], Takashi Tsuruo[b,c], Tetsuo Nagano[a,*]

[a] *Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*
[b] *Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, 1-37-1 Kami-Ikebukuro, Toshima-ku, Tokyo 170-8455, Japan*
[c] *Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan*

Received 29 March 1999; received in revised form 17 May 1999

Abstract Microscopic visualization of intracellular enzyme activity can provide information about the physiological role of the enzyme. Caspases are cysteine proteases that have critical roles in the execution of apoptosis. General fluorometric substrates of caspase-3, such as DEVD-MCA, are unsuitable for imaging because they are excited at short wavelength, so we designed and synthesized novel fluorescent probes that are excited at suitable wavelengths for detecting caspase-3 activity in living cells. Using one of these probes, we succeeded in microscopic visualization of caspase-3-like activity within HeLa cells treated with etoposide. The caspase-3-like activity was increased in the cytosol at first, then expanded to the whole cell.

Key words: Apoptosis; Caspase-3; Fluorescence; Imaging

1. Introduction

Apoptosis is a form of cell death that is accompanied by characteristic changes in the cell, such as chromatin condensation, formation of apoptotic bodies, DNA degradation, etc. Cysteine proteases called caspases play important roles in apoptosis. Nicholson et al. classified caspases into three groups according to their substrate specificity [1]. Among these groups, the group II caspases (caspase-2, -3 and -7), whose preferred tetrapeptide sequence for cleavage is DEXD (X is an unspecified amino acid), are reported to cleave several apoptosis-related proteins, for instance poly-ADP-ribose polymerase (PARP) [2], which is involved in DNA repair, genome surveillance and integrity [3,4]. Caspase-3 is also required for DNase activation [5].

Microscopic visualization of caspase-3-like activities in cells would provide valuable information about the apoptotic mechanism. However, DEVD-MCA and its derivatives [6], which are generally used to detect caspase-3-like activities, can only be used in cell-free systems, because DEVD-MCA is excited at 380 nm and autofluorescence of cells excited at around 380 nm interferes with measurements. In this report, we describe the design and synthesis of novel fluorescent probes to allow direct visualization of caspase-3-like activity in cells. These probes are excited at long wavelength, so cellular autofluorescence is suppressed. We used one of the probes to visualize the activities of caspase-3-like proteases in HeLa-S3 cells during the induction of apoptosis by treatment with etoposide.

*Corresponding author. Fax: (81) (3) 5841-4855.
E-mail: tlong@mol.f.u-tokyo.ac.jp

Abbreviations: Ac-DEVD-MCA, acetyl-Asp-Glu-Val-Asp-MCA; MCA, 4-methylcoumarinyl-7-amide; Z-Asp-CH_2-DCB, benzyloxycarbonyl-Asp-$CH_2OC(O)$-2,6-dichlorobenzene; DMSO, dimethyl sulfoxide; GDEVDGVK, Gly-Asp-Glu-Val-Asp-Gly-Val-Lys; LY, lucifer yellow; CDCF, 6-carboxydichlorofluorescein; CTMR, 5-carboxytetramethylrhodamine; CXR, 5-carboxy-X-rhodamine; HPLC, high-performance liquid chromatography; ODS, octadecylsilica; PBS, phosphate-buffered saline

2. Materials and methods

2.1. Materials

Ac-DEVD-MCA and Z-Asp-CH_2-DCB were purchased from Peptide Institute (Osaka, Japan). Recombinant human caspase-1 was kindly donated by Dr. D.W. Nicholson. Recombinant human caspase-3 was purchased from Funakoshi (Tokyo, Japan). Recombinant human caspase-6 and caspase-7 were purchased from Pharmingen (San Diego, CA, USA). Etoposide was purchased from Sigma (St. Louis, MO, USA). All double-labeled peptides and DEVD-MCA were stored at −20°C as 200 μM stock DMSO solutions. Protease assay (PA) buffer contains 20 mM HEPES (pH 7.5), 10 mM dithiothreitol, 10% glycerol, 0.1% CHAPS, and 100 mM NaCl.

2.2. Synthesis of double-labeled peptides

We applied a method developed by Geoghegan et al. [7,8], using periodate oxidation of N-terminal serine. SGDEVDGVK (Peptide Institute; 1.1 mg, 1.0 μmol) was dissolved in 25 mM sodium phosphate buffer (pH 7.0). This solution was treated with 30 μl of 40 mM $NaIO_4$ in 25 mM sodium phosphate buffer (pH 7.0) for 30 min. Then, 120 μl of 30 mM lucifer yellow CH dipotassium salt (LY; Sigma) in 100 mM sodium acetate (pH 4.5) was added to the solution, the reaction mixture was wrapped with foil to exclude light, and the mixture was stirred gently for 18 h at room temperature. The product, LY-GDEVDGVK, was purified by preparative HPLC (Gilson, Middleton, WI, USA) with an octadecylsilica (ODS) column (GL Sciences, Tokyo, Japan). Lyophilized LY-GDEVDGVK was dissolved in 300 μl of 100 mM sodium acetate, and 2.1 mg (4.0 μmol) of 5-carboxytetramethylrhodamine succinimidyl ester (CTMR-SE, synthesized in two steps from *N*,*N*-dimethylaminophenol and trimellitic anhydride) in 50 μl of CH_3CN was added. After 30 min, LY-GDEVDGVK-CTMR was purified by preparative HPLC. MALDI-TOF MS *m/z* 1715 (theoretical $MH^+ = 1715$).

CDCF-GDEVDGVK-CTMR and CDCF-GDEVDGVK-CXR were synthesized by the same method as LY-GDEVDGVK-CTMR, except for the usage of 6-carboxy-2′,7′-dichlorofluorescein hydrazide (CDCF, synthesized based on the literature [9]) and 5-carboxy-X-rhodamine succinimidyl ester (CXR-SE, synthesized in two steps from 8-hydroxyjulolidine and trimellitic anhydride) instead of LY and CTMR-SE. MALDI-TOF MS *m/z* 1730 (theoretical MH^+ of CDCF-GDEVDGVK-CTMR = 1729), 1832 (theoretical MH^+ of CDCF-GDEVDGVK-CXR = 1833), respectively.

2.3. Protease assay

Fluorometric substrates (4 μM) were incubated with 10 ng of recombinant caspases for 1 h at 37°C in 50 μl of PA buffer. Then, 450 μl of cold water was added to the reaction mixtures and the fluorescence intensity was measured. Excitation and emission wavelengths of LY-

PII: S0014-5793(99)00755-3

GDEVDGVK-CTMR, CDCF-GDEVDGVK-CTMR (and CDCF-GDEVDGVK-CXR) and DEVD-MCA are 430 nm/530 nm, 505 nm/525 nm and 380 nm/460 nm, respectively.

2.4. Cell culture and induction of apoptosis

HeLa-S3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 μg/ml kanamycin and 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2/95% air.

The cells were treated with etoposide (20 μg/ml) to induce apoptosis. After 24 h, the cells were harvested, washed twice with PBS, and lysed in lysis buffer (10 mM HEPES (pH 7.4), 5 mM dithiothreitol, 2 mM EDTA, 0.1% CHAPS). This lysate (50 μg protein) was incubated at 37°C with 4 μM of each synthetic fluorometric probe or DEVD-MCA in PA buffer. The total volume of the reaction mixture was 50 μl. After incubation for 2 h, 450 μl of cold water was added to the reaction mixture and the fluorescence intensity was measured.

2.5. Microinjection

HeLa-S3 cells were grown on a poly-D-lysine-coated glass-bottomed dish (MatTek, Ashland, MA, USA). CDCF-GDEVDGVK-CTMR (200 μM) was dissolved in microinjection buffer (10 mM Tris, pH 7.4, 25 mM KCl), and injected into the cells with an Eppendorf injection system attached to an Olympus IX70 microscope. Each cell was injected for 0.1 s at a pressure of 100 hPa. After the injection, the cells were incubated for 7 h in a CO_2 incubator for stabilization.

2.6. Microscopic visualization of caspase activation

The injected cells were observed with a fluorescence microscopic imaging system, Argus 50 (Hamamatsu Photonics, Shizuoka, Japan), with MBP490, DM505 and BA515IF filter units. After post-incubation, the injected cells were washed twice with PBS and suspended in Hanks' balanced salt solution (pH 7.4) containing 1% fetal bovine serum warmed at 37°C. The dish was transported to the microscope and the cells were treated with 10 μg/ml etoposide. The fluorescence images were captured at intervals of 10 min for 14 h.

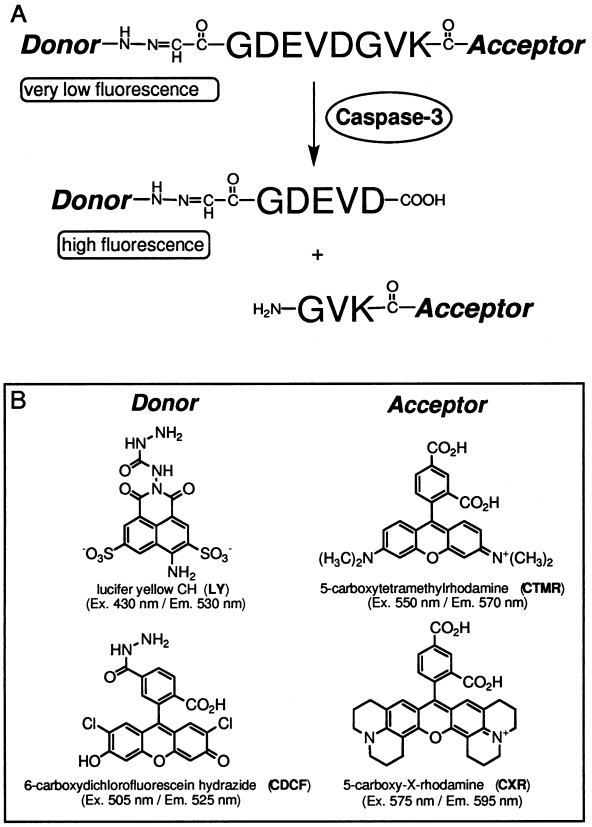

Fig. 1. A: Structure of the synthetic fluorometric probes and mechanism of detection of caspase-3. B: Structures of the fluorophores used as donors and the acceptors. GDEVDGVK is Gly-Asp-Glu-Val-Asp-Gly-Val-Lys.

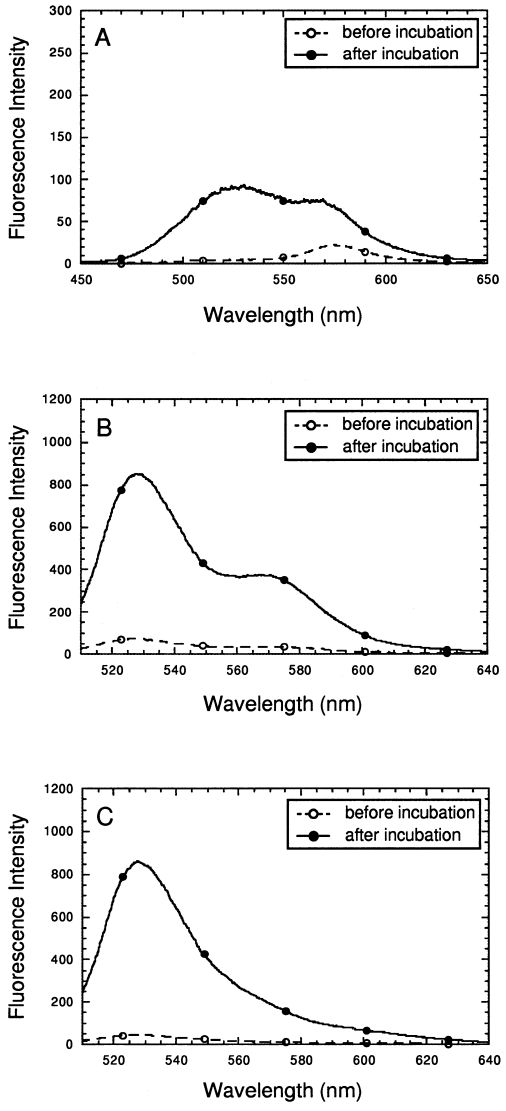

Fig. 2. Emission spectra of the synthetic fluorometric probes before (open circles) and after (closed circles) incubation with recombinant human caspase-3. A: LY-GDEVDGVK-CTMR. B: CDCF-GDEVDGVK-CTMR. C: CDCF-GDEVDGVK-CXR. All synthetic probes (2 μM) were incubated with recombinant human caspase-3 (0.2 μg/ml) for 4 h at 37°C.

3. Results

3.1. Design and synthesis of fluorescent probes for detecting caspase activity

It is known that PARP is cleaved by caspase-3-like proteases (group II caspases) during apoptosis and the cleavage site sequence is -GDEVDGV- [2]. Using an oligopeptide containing -GDEVDGV-, we designed novel fluorescent probes to detect the activities of caspase-3 and its related caspases (Fig. 1A). Two different fluorophores were conjugated to the two terminals of the oligopeptide: one fluorophore (called the donor) was conjugated to the N-terminus of the oligopeptide, and the other (called the acceptor) to the C-terminus. LY and CDCF were chosen as donors, and CTMR and CXR as acceptors, based on their spectral characteristics (Fig. 1B). When we measured the fluorescence spectra of these compounds, the emissions of the donors were highly quenched. Presumably the acceptor absorbs the donor's energy, because

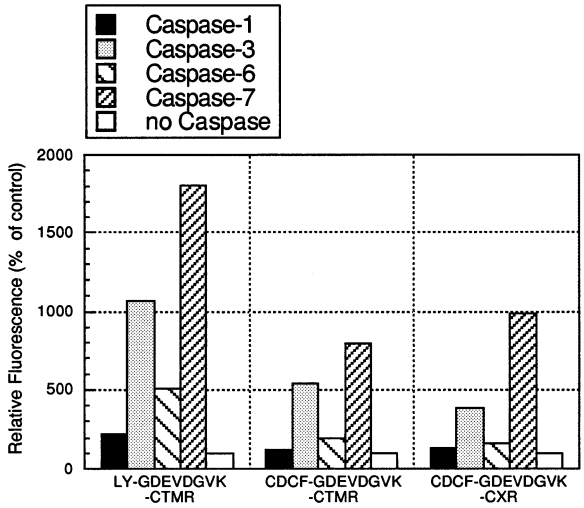

Fig. 3. Fluorescence intensity of the synthetic fluorometric probes after incubation with several caspases. All synthetic probes were incubated with 0.2 μg/ml of recombinant human caspases (-1, -3, -6 and -7) at 37°C. After 1 h, the reaction mixtures were diluted 10-fold with cold water, and the fluorescence spectra were measured. Relative fluorescence value is expressed as a percentage of the control fluorescence value in the absence of enzyme.

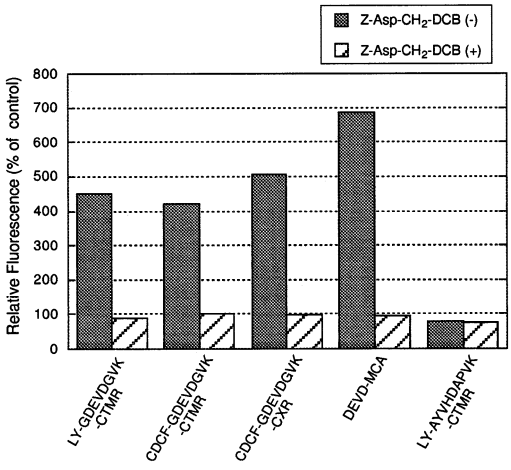

Fig. 4. Detection of caspase-3-like activity from HeLa-S3 cells treated with etoposide by using the synthetic fluorescent probes. All synthetic probes and DEVD-MCA were incubated for 2 h at 37°C with cell lysates of HeLa-S3 cells untreated (control) or treated with 20 μg/ml etoposide for 24 h. Z-Asp-CH_2-DCB was added at 100 μM. Relative fluorescence is expressed as a percentage of control values. GDEVDGVK and AYVHDAPVK are Gly-Asp-Glu-Val-Asp-Gly-Val-Lys and Ala-Tyr-Val-His-Asp-Ala-Pro-Val-Lys, respectively.

the conformation of the peptide in aqueous solution would allow the donor and acceptor to be in close proximity. Strong fluorescence was expected to be generated after cleavage of the peptide, since the donor and acceptor would no longer be adjacent.

When the three double-labeled peptides were incubated with recombinant human caspase-3, the fluorescence intensity of all the donors increased greatly (Fig. 2). These increases were completely inhibited by addition of Z-Asp-CH_2-DCB [10], which is an irreversible caspase inhibitor (data not shown). Then we examined whether these compounds were cleaved by other recombinant human caspases (caspase-1, -6, and -7). Caspase-7, which belongs to the same family as caspase-3 [1], cleaved them as effectively as caspase-3, while caspase-1 and caspase-6 showed little activity (Fig. 3). When DEVD-MCA was incubated with caspase-6 under the same conditions, the fluorescence intensity reached about 60% of that obtained with caspase-3 (data not shown).

3.2. Detection of caspase-3 activity from apoptotic cells

Etoposide is a topoisomerase II inhibitor that induces apoptosis in various cell lines [11]. We stimulated HeLa-S3 cells with etoposide to induce apoptosis and prepared the cell lysate. To test whether it is possible to detect caspase-3-like activity using the synthetic fluorometric probes, the probes were incubated with the cell lysate. All the fluorometric probes were cleaved when they were incubated with cell lysate prepared from apoptotic cells, and most of the proteolytic activity was suppressed by adding Z-Asp-CH_2-DCB to the reaction mixture (Fig. 4). Moreover the background fluorescence intensity, due to cleavage by other intracellular proteases, was much smaller than the background of DEVD-MCA. The results suggested that these probes can be used to detect caspase-3-like activity in living cells under a microscope. The fluorescence of another synthetic probe, LY-AYVHDAPVK-CTMR, which was designed as a fluorometric probe to detect caspase-1 activity, was not increased by incubation with etoposide-treated cell lysate as compared to the control. We could also detect caspase-3-like activity from apoptotic THP-1 cells with the synthetic probes (data not shown).

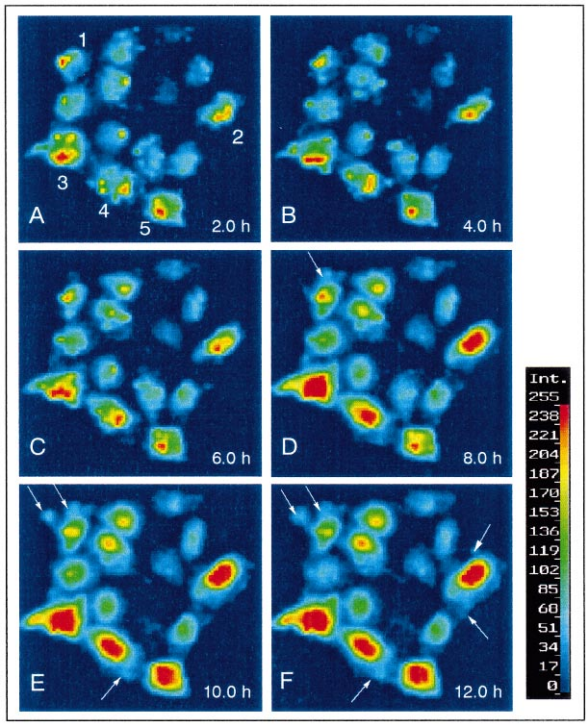

Fig. 5. Fluorescence images of CDCF-GDEVDGVK-CTMR-injected HeLa-S3 cells A: 2 h, B: 4 h, C: 6 h, D: 8 h, E: 10 h and F: 12 h after treatment with 10 μg/ml etoposide. Arrows show the cell membrane blebs. The cells were incubated at 37°C under humidified air. Five cells are numbered 1–5 as shown in A.

3.3. Microscopic visualization of caspase-3-like activity in living HeLa-S3 cells

To examine the feasibility of microscopic visualization of caspase-3-like activity, CDCF-GDEVDGVK-CTMR was microinjected into HeLa-S3 cells. The injected cells were treated with etoposide after post-incubation, and the fluorescence images of the donor were captured at intervals of 10 min for 14 h. Green fluorescence of the donor gradually increased from about 5 h after the treatment with etoposide (Fig. 5). The increase of the fluorescence was initially observed in the cytosol, then the caspase-3-like activity was observed through the whole cell including the nucleus.

In some of the cells, the fluorescence intensity decreased rapidly after the increase. When untreated cells were observed with the fluorescent probe, the fluorescence of the donor hardly increased as compared with the treated cells.

4. Discussion

Caspases have very important roles in the apoptotic process, and caspase-3 and its related caspases such as caspase-2 or -7 cleave many proteins related to apoptosis [2,5,12–15]. Therefore, observation of temporal and spatial changes of caspase-3 activity in the cell with fine resolution is important to clarify the mechanism of apoptosis. We considered that imaging of caspase-3 would be one of the best approaches. DEVD-MCA and its derivatives are widely used in experiments to measure caspase-3-like activity, but they cannot be applied to imaging of living cells, because their excitation wavelength is short in the UV region. In this paper, we synthesized novel synthetic fluorometric probes, double fluorescence-dye-labeled peptides, which were superior to DEVD-MCA with regard to specificity and sensitivity. All these synthetic fluorometric probes were excited at long wavelength in the visible region, and should be suitable for imaging experiments with low background fluorescence.

We chose dichlorofluorescein as a fluorophore (donor) for the synthetic probes, because it has a high fluorescence quantum yield, it is excited at long wavelength and it is very stable. Dichlorofluorescein has a lower pK_a value than fluorescein, and its fluorescence intensity is independent of the solvent pH at around neutral pH. Tetramethylrhodamine and X-rhodamine were chosen as acceptors because their absorbance spectra overlap with the emission spectrum of dichlorofluorescein. Lucifer yellow was chosen as another donor because its emission maximum (530 nm) is similar to that of dichlorofluorescein (525 nm).

We initially designed these fluorescent probes based on the principle of fluorescence resonance energy transfer (FRET). FRET is a phenomenon that can be observed when the donor is near ($<$ about 100 Å) the acceptor [16]. When FRET occurs, the fluorescence of the donor is absorbed by the acceptor and the fluorescence of the acceptor is observed. However, when the synthetic probes were excited in aqueous solution, the acceptor's fluorescence was not observed, even though the donor's fluorescence was absorbed, except in the case of LY-GDEVDGVK-CTMR. Since FRET was observed when the probes were dissolved in methanol, we assume that the donor and the acceptor did not interact with each other in methanol because methanol is less polar than water, and in an aqueous solution the fluorescence of the acceptor was not observed because the donor and the acceptor interacted intramolecularly in a hydrophobic manner.

When the synthetic probes were incubated with recombinant human caspase-3, the fluorescence intensities of the donors increased, as expected (Fig. 2). To preclude the possibility that the synthetic probes were cleaved by other caspases such as caspase-6, the probes were mixed with other caspases. These double-labeled probes were hardly cleaved by caspase-6, although DEVD-MCA was cleaved by caspase-6. Accordingly, these synthetic probes are superior to DEVD-MCA for detecting caspase-3-like activity in living cells. However, the synthetic probes were also cleaved by caspase-7, which belongs to the same group as caspase-3.

Since we could detect caspase-3-like activity in the lysate of HeLa-S3 cells treated with etoposide (Fig. 4), we tried to image caspase-3-like protease activity in apoptotic HeLa-S3 cells. Because none of the synthetic probes was membrane-permeable, we used a microinjection technique. We chose CDCF-GDEVDGVK-CTMR for this experiment because it is excited at a longer wavelength (ex. 505 nm) and has a higher fluorescence intensity than LY-labeled compounds (ex. 430 nm).

When the fluorescence images of CDCF-GDEVDGVK-CTMR in etoposide-treated HeLa-S3 cells were captured (Fig. 5), an increase of the fluorescence appeared from about 5 h after the treatment with etoposide. After 8 h, blebbing of the cell membrane was observed and the number of the blebs was increased at 10 h and 12 h (cells **1**, **2** and **4** in Fig. 5), then the cells died. This result provides direct evidence that blebbing of the cell membrane follows activation of caspase-3-like proteases. The fluorescence intensity was initially augmented outside the cell nucleus, and in the second step the intensity was expanded to the whole cell including the nucleus (cells **2**–**5**). When the activity was observed through whole cells, the fluorescence intensity in the nucleus was apparently higher than in the cytosol, compared with the nuclear pattern of the Hoechst dye staining images. In order to confirm that the cleaved product (CDCF-GDEVD) does not localize to the nucleus, we injected it into the cells. The distribution of the cleaved product did not change after 6 h. However, it is necessary to confirm under confocal microscopy for the determination of nuclear activity, because the thickness of the cell around the nucleus does affect the fluorescence intensity under this condition.

Moreover, the fluorescence of some cells decreased rapidly after the gradual increase. The reason for this decrease is presumed to be that when the cells died of apoptosis, the cell membrane was fractured and the probes leaked out into the medium. The fluorescence of cell **1** decreased after the capture of Fig. 5F. In another experiment, the degradation of the nucleus was observed when the cells start to bleb.

In conclusion, we could image the intracellular activity of caspase-3-like proteases under a fluorescence microscope by using a new fluorescent probe. It should be possible to extend this method to measure protease activity in various biological samples, for instance, neuronal tissues, nematodes, etc.

Acknowledgements: We thank Dr. D.W. Nicholson (Merck) for providing purified recombinant human caspase-1.

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