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Original Article

Granulysin induces cell death with nuclear accumulation

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Exogenously added granulysin was reported to kill mammalian target cells. The sites of actions and molecular mechanisms of granulysin in target cell killing, however, are presently unclear. We here examine the effects of granulysin with the target HeLa cells transiently expressed with GFP-fused 9 kDa granulysin. Endogenously expressed GFP-fused granulysin was preferentially localized in the nucleus and induced apoptotic cell death accompanying with phosphatidylserine translocation and nuclear condensation in a caspase-independent manner. These results suggest that granulysin enters the nucleus of target cells and induces apoptosis.

Key words: GFP fusion proteins, caspase-independent cell death, nuclear translocation

Introduction

Ca^{2+}-dependent granule exocytosis is one of the mechanisms by which cytotoxic lymphocytes, such as NK cells and CTLs, kill target cells to eliminate tumor cells and cells infected with intracellular pathogens from the body[1,2]. Upon contact with target cells these cytotoxic lymphocytes release cytotoxic granule proteins, such as perforin and granzymes, into the intercellular space. Perforin has been elucidated to make target cells permeable in their cell membrane, which allows granzymes to enter the target cells leading to apoptotic cascade reactions.

Cytotoxic lymphocytes produce another cytotoxic granule protein, granulysin[3], which has been shown to co-localize with perforin and granzymes in the cytotoxic granules[4,5]. Granulysin is synthesized as a 15 kDa precursor form and processed to a 9 kDa mature active form in the cytotoxic granules[6]. Granulysin resembles saposin-like proteins (SAPLIP) in that, apart from structural similarity, it kills a broad spectrum of bacteria, fungi and parasites by directly affecting their membrane structures[13]. Importantly, granulysin also shows cytotoxic and apoptosis-inducing effects on various mammalian tumor cells, suggesting its critical role in the lymphocyte-mediated cytotoxicity[7,8,9]. Consequently, much attention is given to the mode of action of granulysin to aid in the understanding of the self-defense mechanisms exhibited by lymphocytes. Multiple mechanisms have been proposed for the granulysin-induced apoptosis, including ceramide generation via sphingomyelinase activation, activation of some caspases and induction of translocation of apoptosis inducing factor (AIF) from the mitochondria to the nuclei[7,11,12,15]. These findings were obtained from experiments in which granulysin was exogenously added to intact target cells. It has been supposed that action of granulysin in such experiments occurs primarily in the cell surface plasma membrane[7]. In addition to such a cell surface-mediated pathway, granulysin is also shown to directly enter the target cell cytoplasm in a perforin-dependent manner[13]. The latter event is considered to occur principally when NK cells and CTLs kill their targets in various physiological and pathophysiological settings because perforin and

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granulysin are generally co-expressed in these cytotoxic lymphocytes. Nevertheless, little is known about the actions of granulysin that is introduced directly into the cytoplasm. Furthermore, information on intracellular localization of granulysin within the target cell, which would be helpful for elucidating its sites of actions, is presently scant. Recently, Sekiya and colleagues showed that adenovirus vector-mediated transfer of granulysin induced apoptotic cell death in HuD antigen-expressing Colon 26 cells[10], although in their experimental conditions the numbers of apoptotic cells were very few and the precise correlation between granulysin expression and apoptosis at the cellular level remained unclear. In this study, to address these questions, we took advantage of the GFP fusion system[18] and examined at the single cell level the effects and localization of endogenously expressed granulysin using HeLa tumor cells as targets.

Materials and Methods

Plasmid construction

To construct GFP-fused proteins, cDNAs for 9 kDa granulysin {corresponding to amino acid sequence G^{63} through R^{136} of the full-length granulysin}[13], and granzyme B (I^{21} through C-terminus) were amplified by RT-PCR with Pfu polymerase (Stratagene, CA) using total RNA from normal peripheral blood mononuclear cells as a template. Each PCR product was cloned into the HindIII/BamHI (for granulysin) or the BspI/KspI (for granzyme B) restriction sites, respectively, of GFP C-terminal fusion vector (Clontec, CA).

Cell culture and transfection

HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin and streptomycin. For transient expression of GFP-fused proteins, $5x10^3$ of HeLa cells were inoculated into the ϕ12-mm glass base dish (Asahi Techno Glass, Tokyo, Japan) a day before transfection. The cells were then, transfected with 25 ng of each plasmid DNA using FuGENE 6 reagent (Roche, Basel, Switzerland) and cultured for times indicated.

Western blot analysis

Transfected HeLa cells were directly lysed with SDS-sample buffer (62.5 mM Tris-HCl pH6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.01% BPB). Proteins in the cell lysates were resolved on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel/Relliehausen, Germany). The membranes were probed with an anti-GFP polyclonal antibody (1:1000, Clontec, CA) followed by incubation with a horseradish peroxidase-conjugated anti-rabbit IgG. The antigens were visualized using ECL-western blotting detection reagents (Life Science Products, CA).

Detection of apoptotic cells

Cells were first stained with Hoechst 33342 (H33342) (SIGMA-ALDRICH, MO) for 10 min, washed with PBS and incubated with Alexa Fluor^{TM}594-conjugated annexin V (Molecular Probes, OR) in the annexin V binding buffer (MBL, Nagoya, Japan) for 15 min at room temperature. After washing with annexin V binding buffer, cells were fixed with 4% paraformaldehyde and observed under a fluorescence microscope BX60 (OLYMPUS, Tokyo, Japan) or a confocal laser microscope Radiance 2000 (Bio-Rad, CA).

Intracellular staining of granulysin

Transfected HeLa cells were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature and permeabilized with 0.5% Tween 20-containing PBS. Then, the cells were stained with anti-granulysin monoclonal antibody (RC8)[19] followed by Alexa FluorTM586-cnojugated anti-mouse IgG (Molecular Probes, OR).

Caspase inhibitor treatment

To block caspases, z-VAD-fmk (MBL, Nagoya, Japan) or DMSO (vehicle control) was added to a final concentration of 50 μM or 0.1%, respectively, to the transfected HeLa cell cultures at 6 h and 24 h after transfection.

Results

Expression of GFP-fused proteins in transfected HeLa cells.

In this study, we used granzyme B, a typical apoptosis-inducing molecule, as a positive control to validate our GFP fusion system. The 9 kDa granulysin and granzyme B linked to GFP at their amino termini were expressed in HeLa cells by the transient transfection method. Western blot analysis illustrated that the GFP (31 kDa), GFP-fused 9 kDa granulysin (GFP-granulysin, 40 kDa) and GFP-fused granzyme B (GFP-

granzyme B, 60 kDa) were expressed in respective transfected HeLa cell populations (Fig. 1A). The subcellular localization was then examined by confocal laser microscopy. At the early time points after transfection (10 h), GFP-granulysin was detected throughout the cell, including nuclear, membrane and cytoplasmic regions, with predominance of nuclear localization (Fig. 1B). At 24 h after transfection, the nuclear accumulation of GFP-granulysin became maximum (Fig. 1C). Examination with anti-granulysin mAb[19] showed the similar nuclear accumulation of GFP-granulysin (Fig. 1D). In contrast, GFP alone was distributed evenly throughout the cell, while GFP-granzyme B was mainly localized at small cytoplasmic granules (Fig. 1C). Thus the nuclear translocation seen with the GFP-granulysin chimera protein was considered to be owing to a property of 9 kDa granulysin by itself.

GFP-granulysin induced posphatidylserine (PS) translocation and nuclear condensation

To examine the effects of granulysin on the morphological features of the target cells, we observed GFP-granulysin-transfected HeLa cells at various time points after the transfection by confocal laser microscopy. Significant morphological changes were not evident until a day after transfection. On day 2, we found that parts of the cells expressing GFP-granulysin became rounded and condensed, which are thought to be initial signs of apoptosis. On day 3, the signs of apoptosis were more frequently observed among GFP-granulysin-expressing cells (data not shown). To characterize the granulysin-induced cell death at the molecular level, the transfected cells were doubly stained with H33324 and annexin V, an indicator for PS translocation (an initial apoptotic marker). As shown in Fig. 2A, a substantial part of GFP-granulysin-expressing HeLa cells was stained with annexin V and showed condensed nuclei at 48 h after transfection. At

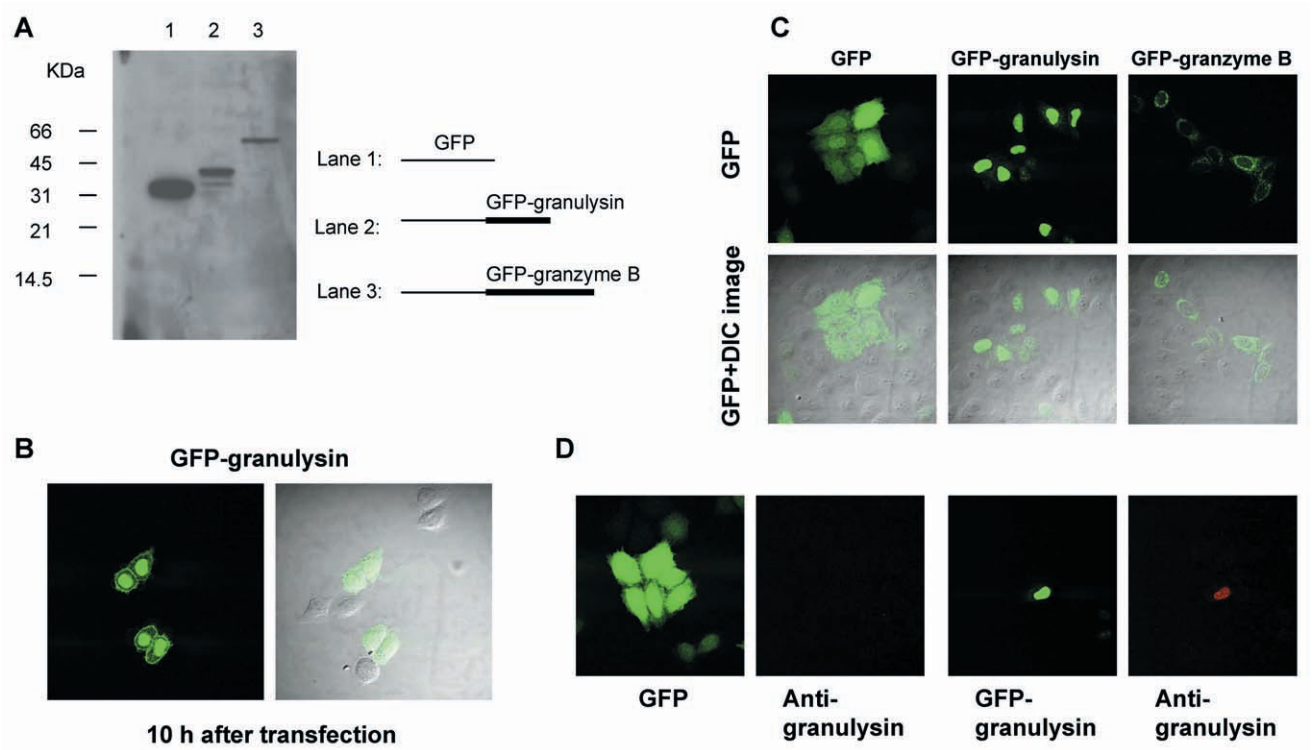

Fig. 1. Subcellular localization of GFP-fused granulysin and granzyme B. **A**. HeLa cells transfected with GFP (lane 1), GFP-granulysin (lane 2) or GFP-granzyme B (lane 3) vector were cultured for 24 h and directly subjected to Western blot analysis with anti-GFP antibody. Schematic structures of GFP and GFP fused proteins are illustrated in the right panel. **B**. HeLa cells transfected with the GFP-granulysin vector were observed under a confocal laser microscope (left panel:GFP, right panel:GFP+DIC image) 10 h after transfection. **C**. HeLa cells transfected with GFP (left panels), GFP-granulysin (central panels) or GFP-granzyme B (right panels) vector were observed under a confocal laser microscope (upper panels:GFP, lower panels:GFP+DIC image) 24 h after transfection. **D**. HeLa cells transfected with GFP (left two panels) or GFP-granulysin (right two panels) vector were stained with anti-granulysin monoclonal antibody RC8 24 h after transfection, and then examined for expression of GFP (green) and granulysin protein (red) under a confocal laser microscope.

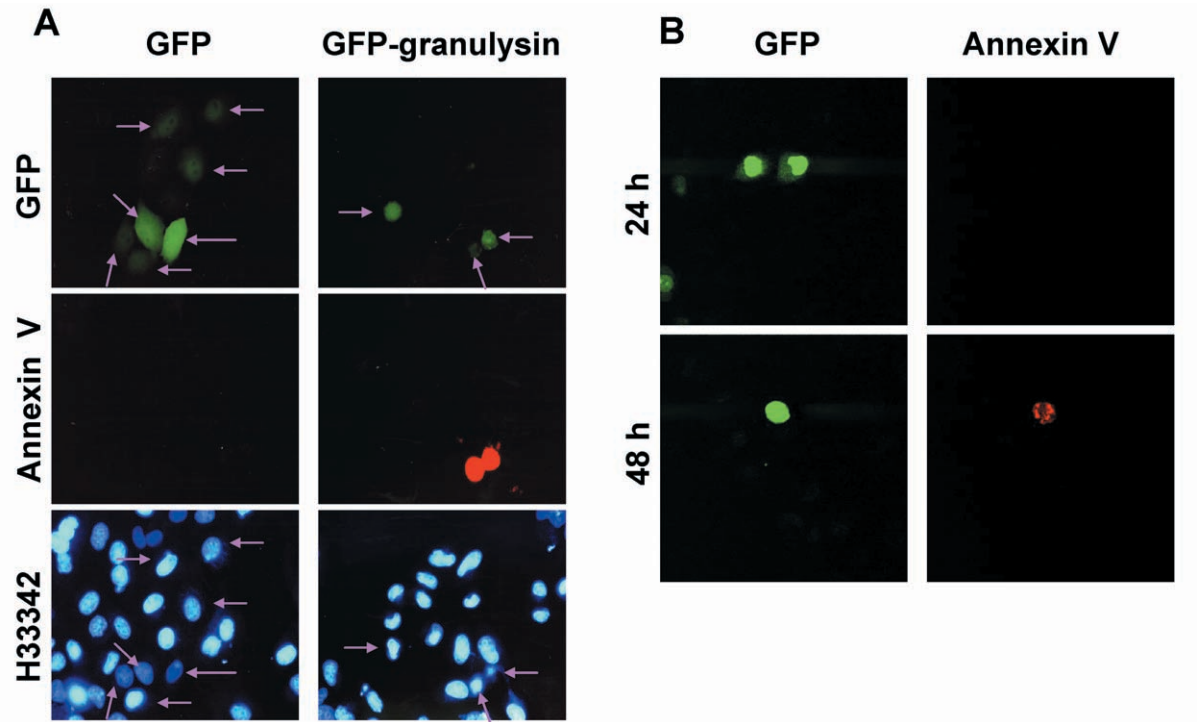

Fig. 2. PS translocation and nuclear condensation in GFP-granulysin-transfected HeLa cells.
A. HeLa cells transfected with GFP (left panels) or GFP-granulysin (right panels) vector were examined for their annexin V binding and nuclear condensation (H33342) by fluorescence microscopy at 48 h after transfection. **B**. HeLa cells transfected with GFP-granulysin vector were examined for GFP expression (left panels) and annexin V binding (right panels) at 24 h and 48 h after transfection by confocal laser microscopy.

24 h after transfection, however, such apoptotic events (PS translocation and nuclear condensation) were little observed even though nuclear translocation of GFP-granulysin was nearly completed at this time point (Fig. 2B).

GFP-granulysin induced cell death with slower kinetics than GFP-granzyme B

To further characterize the GPF-granulysin-induced cell death, we next performed a kinetics study. As shown in Fig. 3A, GFP-expressing HeLa cells showed no significant staining with annexin V throughout the culture periods, indicating that GFP expression by itself had little, if any, cytotoxic effect. In contrast, among the GFP-granulysin-expressing cell population annexin V-positive cells was gradually increased until 72 h after the transfection (Fig. 3A). Compared to the GFP-granulysin transfectants, GFP-granzyme B-expressing cells became positive for annexin V staining with faster kinetics (Fig. 3A), suggesting some differences in the underlying mechanisms.

A caspase inhibitor did not affect granulysin-induced cell death

We finally examined whether or not the GFP-granulysin-induced cell death is caspase-dependent. For this end, we applied a versatile caspase inhibitor, z-VAD-fmk[7] to our experimental system. As shown in Fig. 3 B-a and C, treatment with z-VAD-fmk on its own had no cytotoxic effect on the control GFP transfectants. As expected from the previous studies[20], the caspase inhibitor significantly inhibited the GFP-granzyme B-induced PS translocation (Fig. 3 B-c and C). In contrast, the same treatment had no significant effects on the GFP-granulysin-induced PS translocation and nuclear translocation of the fusion protein (Fig. 3 B-b and C). These results demonstrated that endogenously expressed GFP-granulysin induced apoptotic cell death in HeLa cells in a caspase-independent manner.

Discussion

In this study we for the first time provide evidence showing that, when introduced into the cytoplasm, 9 kDa granulysin is preferentially translocated to the

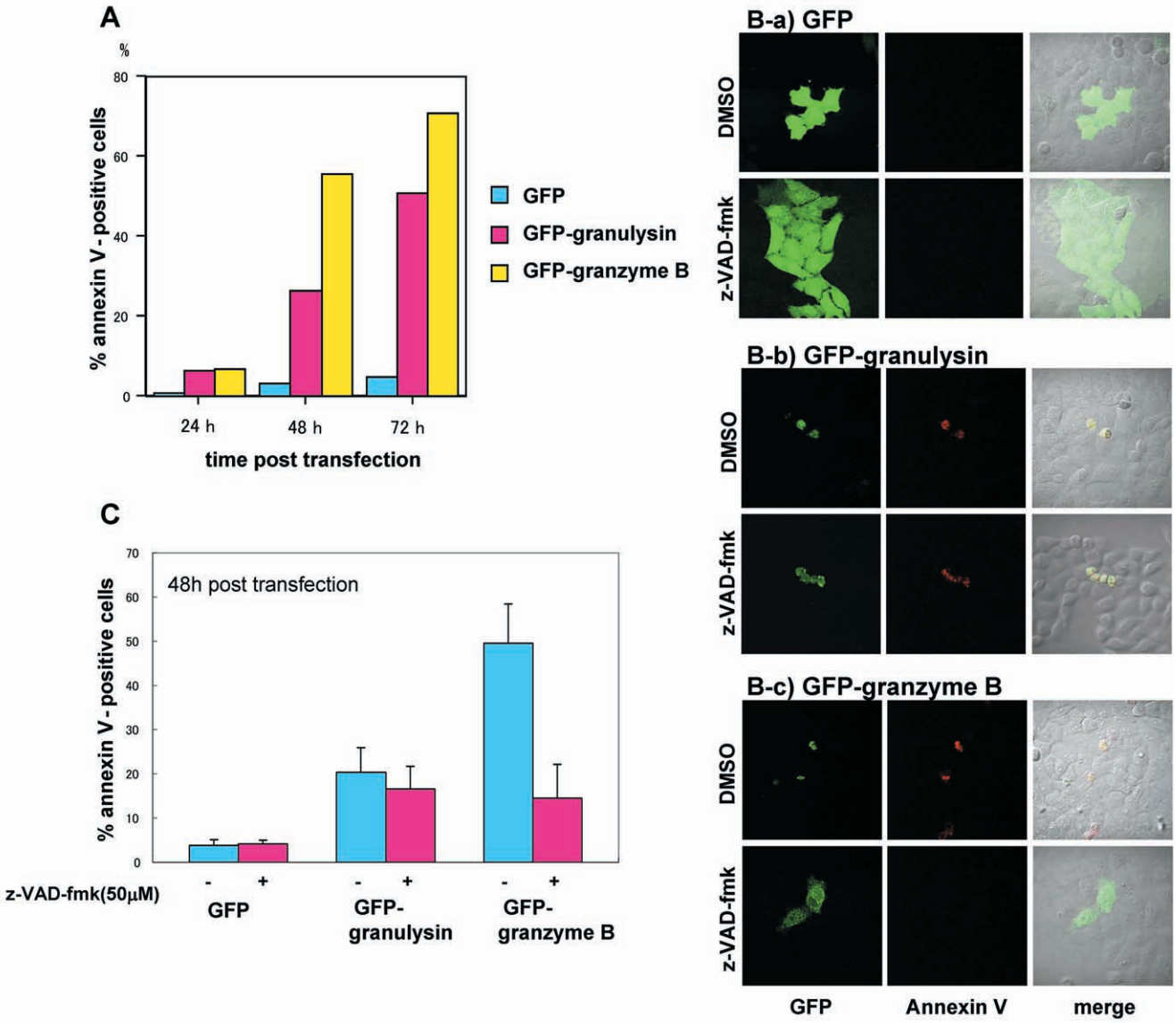

Fig. 3. Effects of z-VAD-fmk on GFP-granulysin- induced apoptosis.
A. HeLa cells were transfected with GFP (blue column), GFP-granulysin (red column) or GFP-granzyme B (yellow column) vector, and frequencies of annexin V-positive cells among each GFP-positive cell population were examined at indicated time points by confocal microscopy. **B**. HeLa cells were transfected with GFP (a), GFP-granulysin (b) or GFP-granzyme B (c) vector cultured for 48 h in the presence of 0.1% DMSO (vehicle control) or 50 μM z-VAD-fmk, and examined for their annexin V binding by confocal microscopy. **C**. Frequencies of annexin V-positive cells among GFP-positive cell populations in HeLa cell transfectants treated as described in B are shown.

nucleus and induces apoptotic cell death with PS translocation and nuclear condensation in a caspase-independent manner. Importantly, exogenously added granulysin was also reported to cause PS translocation and nuclear condensation in a caspase-independent manner[7]. Such a cell surface-mediated pathway, namely by an autocrine/paracrine mechanism, of granulysin was unlikely to occur in our experimental system because GFP-granulysin was not released from the transfected cells and apoptotic cells were exclusively observed among the GFP-granulysin-positive cells. Most recently, it was shown that exogenously applied granulysin was rapidly taken up into the cytoplasm of Jurkat cells[21]. These findings together imply that the primary target molecule(s) of granulysin may exist in the cytoplasm or nucleus rather than the cell surface membrane. This concept might be of primary importance to understand the apoptosis-inducing

mechanism of granulysin.

Previous studies demonstrated that exogenously applied granulysin caused mitochondrial damage leading to release of apopsosis-inducing mitochondrial proteins such as AIF[12,21]. AIF has been shown to translocate to the nucleus and cause nuclear condensation in a caspase-independent manner[12]. Although we did not provide information on effects of GFP-granulysin on mitochondria in the target cells, our observation that the caspase-independent nuclear condensation was also seen in our experimental system (data not shown), suggests possible involvement of AIF in the GFP-granulysin-induced apoptosis. Since no significant accumulation was seen in mitochondria, direct action of GFP-granulysin on mitochondria may be unlikely in our system. It is therefore conceivable that the initial event that leads to nuclear translocation of AIF can be triggered by granulysin in the cytoplasm or nucleus and the translocated AIF then plays critical role in the granulysin-induced apoptosis.

In this study GFP-granulysin was shown to have a tendency to accumulate to the nucleus. We did not show that 9 kDa granulysin by itself (without GFP moiety) has a property to accumulate to the nucleus, however, 9 kDa granulysin has overall charge of +11 by basic amino acids rich segments[3] and computer analysis with PSORT II (prediction of protein localization sites in animal cells) predicts its nuclear localization. Moreover, it was shown that, when applied exogenously to Jurkat cells, E. coli-derived recombinant 9 kDa granulysin entered the cells and partly localized to the nucleus within 20 min after application[21]. The nuclear accumulation of GFP-granulysin is thus considered to reflect the nature of native 9 kDa granulysin.

The nuclear accumulation of GFP-fused granulysin was also observed in other cell lines, Jurkat, PC12, K562 and COS-7. Among these cell lines, Jurkat and PC12 cells showed GFP-granulysin-dependent apoptosis with PS translocation and nuclear condensation similar to HeLa cells, while no clear apoptotic signs were observed in COS-7 cells up to 48 h after transfection (our unpublished data). These results suggested that susceptibility to granulysin-induced apoptosis differs with cell types. Several apoptosis-related proteins such as CAD/ICAD, granzymes, AIF and caspases show nuclear translocation prior to apoptotic events[14-17]. The precise correlation between the nuclear translocation of granulysin and various apoptotic events, including AIF translocation, PS translocation and nuclear apoptosis, remains to be examined.

The GFP-fused granulysin we used in this study appeared to retain full properties of granulysin. Therefore, such a strategy may be useful to identify yet unknown cellular component(s) that interact with granulysin since the tag moiety can be used for the affinity purification without disrupting the interaction.

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