Bioluminescent reporters to monitor killer cell-mediated delivery of granzymes inside target cells

Intracellular profiling of granzyme M activity

Stefanie A.H. de Poot1, Elisabeth A. van Erp1, Jan Meeldijk1-2, Roel Broekhuizen1, Roel Goldschmeding1, Marjolein C. Olthof1, Elisabeth M.P. Steeghs1, and Niels Bovenschen1-2

1Department of Pathology, University Medical Center Utrecht, Utrecht, The Netherlands
2Laboratory of Translational Immunology, University Medical Center Utrecht, Utrecht, The Netherlands

Corresponding author:
Dr. Niels Bovenschen, Department of Pathology, University Medical Center Utrecht, Heidelberglaan 100, Utrecht 3584 CX, The Netherlands, tel: +31 88 7556565; fax: +31 88 7569593; e-mail: n.bovenschen@umcutrecht.nl

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To the editor:

With great interest, we have read the recent paper by Vrazo et al. in Blood, who report on the use of luciferase-based biosensors to detect proteolytic activity of granzymes (Gr)A, B and K. They show that these sensors can be used to profile granzyme delivery by natural killer (NK) cells inside tumor cells in real time. However, humans express five granzymes, and a specific sensor for GrM is lacking. GrM is expressed by lymphocytes of both the innate and adaptive immune system, and plays a role in the control of cancer, viruses, and inflammation. Here, we report the development and comparison of two distinct variants of GrM gain-of-function biosensors: the GrM GloSensor, which is similar to the sensors described by Vrazo et al., and the GrM iGLuc sensor (Figure 1A).

We have previously characterized the extended substrate specificity of human GrM using complementary positional proteomics, identifying AKMPL↓AAEEE as an optimal cleavage recognition motif. Based on this sequence, we developed two GrM sensors and corresponding mock sensors (P1 Leu replaced by Ala). The GrM GloSensor is based on a circularly permuted firefly luciferase (FLuc), locked in an inactive state by a short peptide-linker. Upon GrM-mediated cleavage of the linker, the FLuc becomes active. The GrM iGLuc sensor is a fusion of gaussia luciferase (GLuc) and murine pro-interleukin 1β (IL1β). When GrM cleaves off the IL1β pro-domain, the GLuc monomerizes and becomes active.

We synthesized the GrM and mock sensors in vitro using cell-free transcription/translation, and subsequently treated them with purified recombinant GrM or inactive GrM-SA mutant (catalytic Ser replaced by Ala) (Figure 1B). Both GrM sensors were activated by GrM in a concentration-dependent manner, but not by GrM-SA. The corresponding mock sensors were not activated, indicating that activation depends on GrM-mediated cleavage after the Leu
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residue in the peptide recognition sequences. Consistent with these data, visualization of the
fluorescently-labeled GrM sensors by SDS-PAGE showed that the full-length Glo (~61 kDa)
and iGluc (~60 kDa) sensors were cleaved by increasing concentrations of GrM, but not by
GrM-SA, resulting in the formation of the expected subunits (~36/~25 and ~40 kDa,
respectively) (Figure 1C). To determine the specificity of the GrM sensors, they were incubated
with all purified human granzymes (Figure 1D). Of these, only GrM led to robust activation of
the GrM sensors, and none of the granzymes could activate the mock sensors.

We expressed the sensors in OPM-2 multiple myeloma cells and confirmed expression by
immunoblot and flow cytometry (data not shown). Recombinant GrM activated the GrM
sensors in freeze-thaw lysates of OPM2 cells in a concentration-dependent manner, whereas
GrM-SA did not (Figure 1E). Intracellular delivery of recombinant GrM via pore-formation
with streptolysin O in tumor cells also led to GrM sensor activation (data not shown).

To determine whether GrM activity can be profiled inside tumor cells that are attacked by
cytotoxic lymphocytes, we co-cultured GrM sensor-transduced OPM-2 cells with NK cells
(KHYG-1), which express high levels of GrM. Increasing effector:target ratios resulted in
increased activation of both GrM sensors (Figure 1F). Activation of the iGLuc sensor was
much stronger, suggesting that the iGLuc sensor may outperform the GloSensor in a more
physiological setting. The corresponding mock sensors were not activated. Activation of the
sensors occurred within 2 hours, which is compatible with the proposed kinetics of granzyme
delivery. Similar results were obtained in HeLa cervix carcinoma cells (Suppl. Figure S1).

Primary NK and lymphokine activated killer (LAK) cells also activated the GrM sensors
(Figure 1G and Figure 1H, respectively). In addition, sensor activation by LAK cells correlated
with cell death induction in target cells (Figure 1I). To confirm that GrM sensor activation was
due to GrM, iGLuc sensor-transduced OPM-2 cells and KHYG-1 NK cells were (pre-
incubated with the cell-permeable GrM-specific inhibitor Ac-KVPL-cmk\(^8\). The tetrapeptide sequence KVPL is specific for GrM and is not recognized by the closely related neutrophil elastase and cathepsin G\(^9\). Treatment with Ac-KVPL-cmk completely inhibited NK cell-mediated activation of the GrM iGLuc sensor (Figure 1J).

Our data nicely complement the recent data of Vrazo and coworkers\(^1\), who developed GrA/K/B biosensors to profile tumor cell death kinetics induced by killer cells. We now report two new protease-cleavable biosensors that specifically track GrM proteolytic activity in the course of cytotoxic lymphocyte-induced cell death. This allows further studies to monitor entry and functional activity of all granzymes in target cells during cancer development, inflammation, and virus infections.
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Author contribution:
Contribution: SAHdP participated in the design of the study, performed experiments, analysed and interpreted the data, and wrote the paper. EAvE, JM, RB, MCO, and EMPS performed experiments. RG participated in the design of the study and interpreted the data. NB participated in the design of the study, analysed and interpreted the data, and wrote the paper.

Conflict-of-interest disclosure:
The authors declare no conflict of interest.

References


Figure legend

Figure 1. Intracellular profiling of GrM activity by bioluminescent gain-of-function biosensors. (A) Schematic overview of the GrM GloSensor (left) (based on Promega backbone) and the GrM iGLuc sensor (right). The GloSensor circularly permuted FLuc molecule is locked in an inactive conformation by a short peptide-linker containing a GrM-specific recognition sequence (AKMPL↓AAEEE). Upon cleavage of the linker by GrM, the FLuc subunits undergo a conformational change, resulting in the formation of active FLuc. The GrM iGLuc sensor is a fusion protein of pro-IL-1β and GLuc. Upon cleavage of the AKMPL↓AAEEE recognition sequence by GrM, the prodomain is separated from the IL-1β and GLuc domains, leading to GLuc monomerization, resulting in sensor activation and secretion. (B) GrM GloSensors and iGLuc sensors were produced in vitro using a cell-free transcription/translation system (Protease-Glo™ Assay and TNT Sp6 High-Yield Wheat Germ Master Mix, Promega), and were subsequently incubated at 37°C for 30 minutes with increasing concentrations of purified GrM or GrM-SA (an inactive GrM mutant in which the catalytic Ser residue has been mutated to Ala) in 50 mM Tris, 150 mM NaCl, pH 7.4. Resulting relative luminescence units (RLU) were detected with a Veritas Microplate Luminometer (Promega). (C) The GrM and mock GloSensors and iGLuc sensors were labeled with fluorescent green lysines (FluoroTect Green Lys, Promega) during in vitro transcription/translation and incubated at 37°C for 30 minutes with indicated concentrations of GrM, or the maximal dose of GrM-SA. Samples were separated on SDS-PAGE and scanned on a Typhoon 9410 scanner (GE Healthcare). Full-length GloSensor (~61 kDa) and its cleavage fragments (~36 and 25 kDa), and full-length iGLuc sensor (~60 kDa) and its largest cleavage fragment (~40 kDa) are visible. (D) GrM GloSensors and iGLuc sensors were produced as in (B), and incubated at 37°C for 30 minutes with recombinant GrA, GrB, GrH, GrK, or GrM (50
Granzymes were produced in *Pichia pastoris* and granzyme activity was verified as described previously.\(^\text{10}\) (E) GrM sensors were cloned into a lentiviral pLV plasmid for transduction of OPM-2 multiple myeloma cells. Sensor expression was verified with immunoblot and flow cytometry (using anti-firefly luciferase and anti-FLAG antibodies). Freeze-thaw lysates (10 µg) of GloSensor or iGLuc-transduced OPM-2 cells were treated with indicated concentrations of GrM or GrM-SA for 1 hour at 37°C, after which Bright-Glo or Stop&Glo reagent (Promega) was added and luminescence was measured. (F) GloSensor-transduced OPM-2 cells were co-cultured with KHYG-1 cells for two hours in the presence of 300 µg/ml D-luciferin in increasing effector:target (E:T) ratios. iGLuc sensor-transduced OPM-2 cells were co-cultured with KHYG-1 cells in increasing effector:target (E:T) ratios. After 2 hours, 4.4 µM coelenterazine (CTZ) was added and luminescence was measured. (G) Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using density gradient centrifugation. FACS sorting was then used to isolate CD3-CD56+ primary NK cells. NK cells were stimulated with IL-2 for 20 hours and subsequently co-cultured with sensor-expressing HeLa target cells for 3 hours. Sensor activation was detected with LARII (Dual Luciferase kit, Promega) or CTZ for the GloSensors and iGLuc sensors, respectively. (H) PBMCs were stimulated with IL-2 for 4 days to generate lymphokine activated killer (LAK) cells. These were then co-cultured with sensor-expressing HeLa cells as described in 1G. (I) Target cell death induced by co-culture with LAK cells was measured after 16 hours using AnnexinV/propidium iodide (PI) FACS. AnnexinV/PI double negative cells were considered viable (with viability set at 100% for untreated cells). (J) iGLuc-transduced OPM-2 cells and KHYG-1 cells were pre-treated with 100 µM GrM-inhibitor Ac-KVPL-cmk or DMSO (vehicle control) for 30 minutes, after which they were co-cultured in the presence of Ac-KVPL-cmk or
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175 DMSO for 2 hours as in (F). All data in this figure are depicted as mean ± SD and represent at least three independent experiments.
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