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ORIGINAL PAPER

Detection of Cancer Cell Death Mediated by a Synthetic Granzyme B-like Peptide Fluorescent Conjugate and the same Peptide Binding in Bacteria

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Abstract Granzyme-mediated apoptosis, supported by pore-forming perforin, plays an important role in CD8+ T lymphocytes (CTL)-dependent cellular immunity protection against both cancer and viral infection. Quantitative and qualitative problems with CTL are potential contributing factors to disease progression. The feasibility of developing CTL-independent cellular immunity is desired but must first overcome the barrier of CTL-independent target cell recognition. Granzyme B with its strong pro-apoptotic activity in many different target cells is investigated for use in the CTL-independent cellular immunity approach, and granzyme B or its bioactive peptides without the enzymatic activity are more desirable for use. Native granzyme B with enzymatic activity is usually investigated in cancer cells for its mediation of apoptosis by detection of DNA fragmentation. Detection of cell death mediated by such peptides in cancer cells is needed to demonstrate the potential therapeutic purposes. We show with never-before-seen microscopic images using fluorescence microscopy that a synthetic granzyme B-like peptide fluorescent conjugate (GP1R) can: 1) mediate cell death of different cancer cells via membrane extrusion, 2) bind to constitutively expressed binding targets in different cancer cells and bacteria, and 3) promote bacterial phagocytosis. The putative binding targets may serve as a universal pathologic biomarker detectable by GP1R. Our data taken together demonstrate the potential applications of GP1R for use in CTL-independent target cell recognition and target cell death induction. It may lead to development of rapid targeted detection and new treatment of cancer, viral and bacterial infections. The new treatment may show mutual benefits for two or more diseases.

Keywords Fluorescence microscopy · Cancer · Granzymes · Cell death · Bacteria · Virus

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Introduction

CD8+ T lymphocytes (CTL) play an essential role in innate cellular immunity and immunotherapy to induce apoptosis in many different target cells via either the granule-exocytosis model or the Fas ligand-receptor model [1–10]. Cancer cells and virally infected cells in general, but not bacteria, have long been regarded as target cells of CTL. In vivo, cellular immunity is CTL-dependent not only for the release of cytolytic granules but also for the target cell recognition. Many different antigens may be needed to activate different target-cell-specific CTL. Quantitative or qualitative problems with CTL are potential contributing factors to the progression of tumor growth and viral infection. Getting the benefit of defensive protection like cellular immunity against many different types of pathologic cells, individually or combined, without the dependence of CTL would be desirable. However, overcoming the barrier of the target cell recognition without CTL is difficult.

The granule-exocytosis model requires pore-forming perforin and granzyme-mediated apoptosis to effectively kill neoplastic cells like cancer cells and virally infected cells like HIV infected T-cells [11, 12]. Among five different granzymes known in humans, granzyme B is found to be strongly pro-apoptotic [13]. Motyka et al. demonstrated that granule-purified native granzyme B, without the presence of perforin, gained cell entry and induced cell death [14]. Studies on the substrates and inhibitors of granzyme [15] did not indicate if

granzyme B or granzyme B-like peptides without the enzymatic activity are able to induce similar cell death without perforin. Developing such bioactive peptide and detecting its interaction with different target cells are needed to move forward the CTL-independent cellular immunity approach. Native granzyme B with enzymatic activity is usually investigated in cancer cells for its mediation of apoptosis by detection of DNA fragmentation [16]. Our objective is to determine if a synthetic granzyme B-like peptide-fluorescent conjugate such as GP1R (BioJENC, USA) has binding affinity and ability to induce cell death in different cancer cells that can be viewed by fluorescence microscopic methods. Another objective is to determine if bacteria may also be the potential target cells of CTL.

Materials and Methods

Cancer Cell Culture

The cancer cell lines used were MDA-MB-231 of breast cancer, PANC-1 of pancreatic cancer, and HeLa of cervical cancer (with HPV DNA integration). All cell lines were purchased from ATCC (Manassas, USA) and maintained according to ATCC's instructions. A non-cancer cell line, HUVEC, was used as negative control. All cell lines were propagated in RMPI medium (Invitrogen, USA) supplemented with 10 % heat inactivated fetal calf serum (FCS) and penicillin and streptomycin. The cells were maintained at 37 °C in a humidified air atmosphere with 5 % CO_2, which is the same incubation condition used for the cell death induction experiments. Fresh cell cultures and methanol prefixed cell cultures were used in the cell death induction and/or cell binding experiments that were done twice in duplicate.

Cell Binding and Cell Death Induction Experiments

GP1R, a synthetic granzyme B-like peptide conjugated with rhodamine fluorescent dye (BioJENC, USA), was used in incubation with cancer and non-cancer cells pretreated with and without methanol fixation. In the cell binding experiments, cancer and non-cancer cells prefixed with methanol on a slide were incubated with GP1R (300–500 μL of 1:1000 dilution in phosphate buffered saline or PBS) for 5 min at room temperature. After rinsing the slides gently twice with distilled water or PBS, the cells on the slides were covered with PBS and a cover slip for visualization by light microscopy and fluorescence microscopy. Microscopic images were captured by AxioCamMR3.

In the cell death induction experiments, fresh cancer cells in suspension (about 10^4 cells in 100 μL of RPMI medium with 10 % heat inactivated FCS) without methanol fixation were incubated without and with GP1R (10 μg in 10 μL) for 4 h and 24 h at 37 °C. After 4 h or 24 h incubation, an aliquot (10 μL) of cell suspension was added to the slide with PBS (90 μL) and covered with a cover slip for visualization by light microscopy and fluorescence microscopy. Trypan blue stain was added to the cells after incubation for use as an indicator of cell viability.

The methanol-fixed cells and fresh cells in the cell binding and cell death induction experiments were examined for the presence of fluorescent deposits, an indication of the binding of GP1R. In the cell death induction experiments, the presence of trypan blue stain inclusion in cells was an indication of cell non-viability.

Bacterial Binding and Phagocytosis Experiments

For bacterial binding experiments: Gram-positive *Staphylococcus aureus* and Gram-negative *E. coli* in mixed culture and pure culture were prefixed with methanol and incubated with GP1R (300–500 μL of 1:1000 dilution in phosphate buffered saline or PBS) for 5 min at room temperature. After rinsing the slides gently twice with distilled water or PBS, the cells on the slides were covered with PBS and a cover slip for visualization by light microscopy and fluorescence microscopy. The bacterial cell binding experiments were done twice in duplicates.

For bacterial phagocytosis experiments: The bovine whole blood samples from infected cows were donated samples by Dr. Donald G. Luther. The EDTA-whole blood sample (100 μL) was incubated with GP1R (10 μL) for 24 h at 37 °C. After 24 h incubation, an aliquot (10 μL) of cell suspension was added to the slide with PBS (90 μL) and covered with a cover slip for visualization by light microscopy and fluorescence microscopy. The bacterial phagocytosis experiments were done twice in duplicates.

Results

Cell Binding Experiments

Using fluorescence microscopy, fluorescent deposits were detected in cancer cells of all three cancer cell lines with and without methanol fixation, indicating cell binding of GP1R even in binucleated cells (Fig. 1). A similar GP1R-binding pattern inside a methanol-fixed binucleated breast cancer cell (Fig. 1a) and a binucleated pancreatic cancer cell without methanol-fixation (Fig. 1b and c) is shown. Methanol-fixed non-cancer cells of HUVEC did not show similar pattern of intense fluorescent deposits (Fig. 1d).

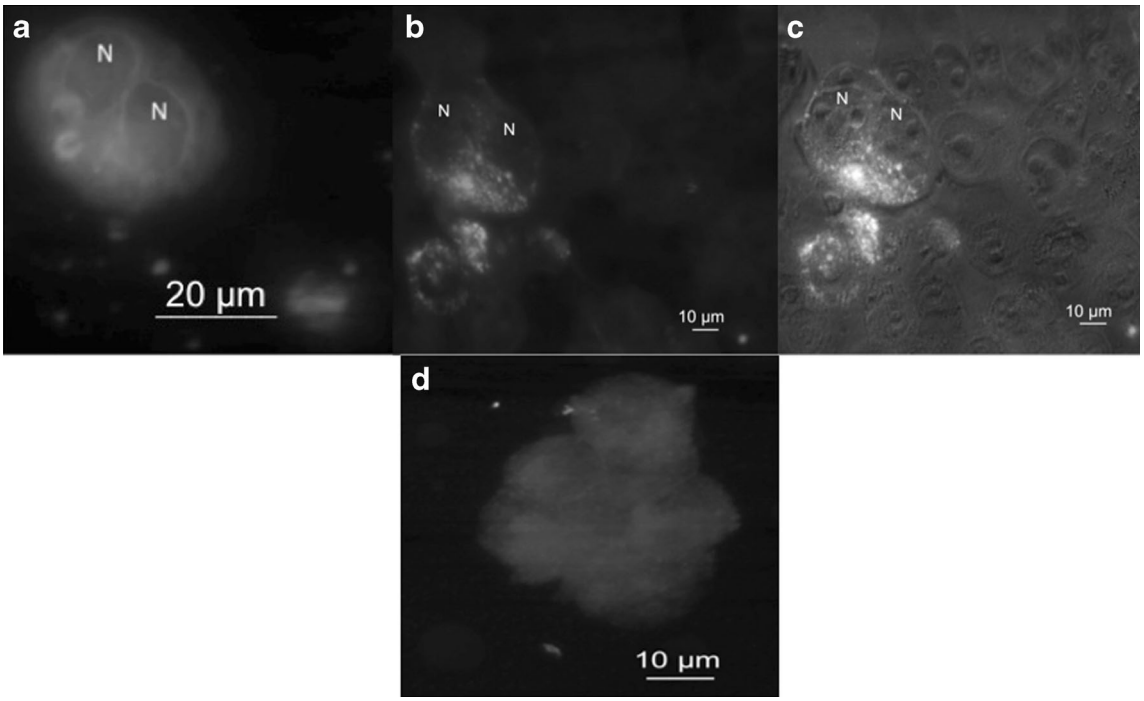

Fig. 1 Fluorescence microscopy of binucleated cancer cells with GP1R-binding. Fluoroscopic images of a binucleated breast cancer cell with methanol pre-fixation (**a**) and a binucleated pancreatic cancer cell without methanol pre-fixation (**b** and **c**) with GP1R-binding are shown in comparison to methanol-fixed non-cancer cells of HUVEC (**d**). The presence of fluorescent deposits in cells indicates binding of GP1R to corresponding cellular binding targets. In **a**, **b**, and **d** the microscopic images were recorded using fluorescence microscopy; in Fig. 1c the microscopic image was recorded using light microscopy with Nomarski optics and fluorescence microscopy combined. Prior to image recording, both methanol-fixed MDA-MB-231 breast cancer cells and HUVEC non-cancer cells had a 5-minute incubation at room temperature with GP1R and fresh PANC-1 pancreatic cancer cells without methanol-fixation had a 4-hour incubation at 37 °C with GP1R. A similar GP1R-binding pattern with the majority of intense fluorescence deposits discernible in the cytosol was shown in cancer cells of both cell lines but not in non-cancer cells of HUVEC. N as shown denotes nucleus. Scale bars for each panel are as shown. A representative of about 5 binucleated cells and non-binucleated cells in each field examined ($n=5$) in duplicates is shown

Cell Death Induction Experiments

Cell death mediated by GP1R as shown by the presence of trypan blue stain inclusion was detected in HeLa cervical cancer cells with GP1R incubation (Fig. 2b) when compared with cells incubated without GP1R (Fig. 2a). A phenomenon of membrane extrusion was detected in GP1R-positive PANC-1 pancreatic cancer cells (Fig 2c) and HeLa cervical cancer cells (Fig. 2e) after 24 h incubation with GP1R. The phenomenon of membrane extrusion is also discernible in microscopic images of the same GP1R-incubated HeLa cells with trypan blue stain as recorded by light microscopy (Fig. 2d). In Fig. 3, both the early (Fig. 3a and b) and late stage (Fig. 3c) of membrane extrusion detected in breast cancer cells are shown.

Bacterial Binding and Phagocytosis Experiments

The presence of fluorescent deposits was detected in both methanol-fixed Gram-negative *E. coli* (arrows) and Gram-positive *Staphylococcus aureus* (Fig. 4a). A relatively uniform distribution of fluorescent deposits discernible on the cell surface of *E. coli* in a pure culture is shown in Fig. 4b. GP1R-positive bacteria detected within the phagosome (arrow) of a phagocyte in the bovine whole blood specimen after 24 h incubation with GP1R is shown (Fig. 4c).

Discussion

A recent news report has shown the unexpected HIV-clearance benefit of cancer treatment in two cancer patients also infected with HIV [17]. These cancer patients who had received bone marrow transplant were found to be HIV free for months. The HIV-clearance is suspected to be the work of the transplanted immune system cells. It raises the hope of an emerging treatment mutually beneficial for these two life-threatening diseases. However, bone marrow transplant is not without serious risk, as the patient's own bone marrow has to be destroyed before receiving the transplant. The results of our study may give the first indication that similar mutual benefit may be attained using a pharmacologic composition comprising a synthetic granzyme B-like peptide such as GP1R, without the need of bone marrow transplant. We show

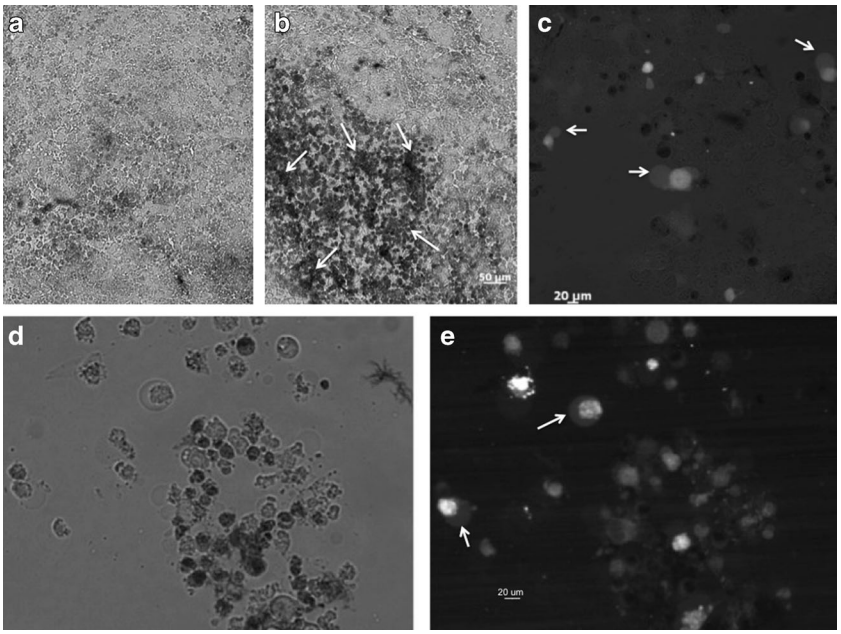

Fig. 2 Trypan blue viability stain and GP1R-binding stain in cancer cells. Trypan blue inclusion in cells is used as an indication of non-viable cells or cell death. HeLa cervical cancer cells incubated without GP1R for 24 h at 37 °C did not show discernible trypan blue inclusion (**a**), while many HeLa cells incubated under the same condition in the presence of GP1R (**b**) showed very intense trypan blue inclusion (*arrows*). Phenomenon of membrane extrusion (*arrows*) in PANC-1 pancreatic cancer cells (**c**) and HeLa cervical cancer cells (**e**) detected after 24 h incubation at 37 °C with GP1R are shown. Microscopic images of GP1R-incubated HeLa cervical cancer cells with trypan blue stain as recorded using light microscopy (**d**) are compared with the fluoroscopic images of the same cells with the membrane extrusion phenomenon (*arrows*) as recorded using fluorescence microscopy (**e**). Scale bars for each panel are as shown (**a** has the same scale bar as **b**, and d as **e**). Shown is a representative of about 50 cells in each field examined ($n=5$) in duplicates

never-before-seen fluoroscopic images of cell binding and cell death induction mediated by GP1R in different cancer cells including cancer cells with a suspected viral association. Using fluorescence microscopy, we demonstrate that GP1R are interactive with the expected target cells of CTL.

For decades, activated CTL have been known to target and kill pathologic cells like cancer cells and virally infected cells via granzyme-mediated apoptosis. It is unclear how a single species of granzyme B can induce cell death of so many different types of cells. A suggestive explanation is that granzyme B may serve as a master key of cell death induction in the CTL target cells that have a matching lock comprising a preexisting universal pathologic biomarker. The results of our study appear to lend support to this explanation.

In our study, the presence of GP1R fluorescent deposits detected in methanol-fixed cells of all three cancer cell lines including binucleated cancer cells but not non-cancer cells (HUVEC) is significant. It indicates that there are binding targets constitutively expressed in cancer cells (including cancer cells active in growth with mitotic defect as shown in binucleated cells). It also indicates the relatively high sensitivity of GP1R target cell binding in cancer cells. The lack of discernible surface GP1R binding in cancer cells with and without methanol fixation further indicates that internal GP1R

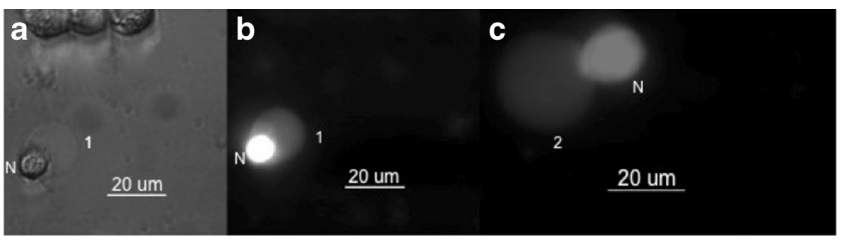

Fig. 3 Different stages of membrane extrusion in breast cancer cells with GP1R incubation. Microscopic images of an MDA-MB-231 breast cancer cell (#1) after incubation with GP1R for 24 h at 37 °C as recorded using light microscopy (**a**) and fluorescence microscopy (**b**) are shown, depicting an early stage of the phenomenon of membrane extrusion. A microscopic image of another breast cancer cell (#2) depicting a near completion of the nucleated (stronger fluorescence) component separated from the plasma membraneous component (weaker fluorescence) as recorded using fluorescence microscopy is shown (**c**). N as shown denotes nucleus. Scale bars for each panel are as shown. A representative of about 50 cells in each field examined ($n=5$) in duplicates is shown

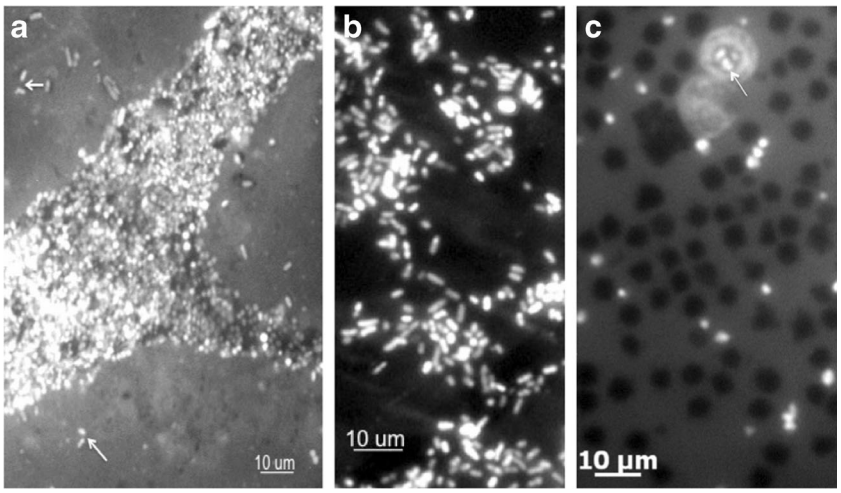

Fig. 4 GP1R-binding in bacteria and phagocytosis of the same. Microscopic images of GP1R-positive *Staphylococcus aureus* and *E. coli* (*arrows*) in a mixed culture pre-fixed with methanol as recorded using fluorescence microscopy are shown (**a**). Fluoroscopic images of *E. coli* bacteria in a pure culture prefixed with methanol depict a relatively uniform distribution of GP1R fluorescent deposits on the cell surface (**b**). The methanol-fixed bacteria were incubated with GP1R for 5 min at room temperature before examination by fluorescence microscopy. GP1R-positive bacteria were discernible in the bovine whole blood sample from an infected animal after 24 h incubation with GP1R and a few GP1R-positive organisms were shown within the phagosome (*arrow*) of a phagocyte (**c**). The non-fluorescent (*black*) microscopic images are red blood cells. Scale bars for each panel are as shown. Representatives of about 100 bacteria in each field examined ($n=5$) in duplicates are shown

binding to these preexisting binding targets is required prior to induction of cell death. This suggests that the preexisting binding targets are likely pathologic cells specific and common in different types of cancer cells including cancer cells with a suspected viral connection like HeLa cells known to have HPV DNA integration. The preexisting GP1R binding targets implicated here in different cancer cells appear to have the attributes of a universal pathologic biomarker and GP1R may serve as the master key of detection. Thus, we now can use GP1R to detect pre-existing binding targets for GP1R or granzyme B-like in cells that have not been previously considered as CTL target cells. Determination of fluorescent GP1R binding quantitatively and qualitatively may result in different binding patterns specific for different types of cancer cells and virally infected cells at different stages of disease progression.

Detection of GP1R binding in both methanol-fixed Gram-positive and Gram-negative bacteria is supporting data to further validate the foregoing suggestive explanation. Cancer cells and virally infected cells are usually regarded as the only direct target cells of CTL-dependent cellular immunity. CTL have only been shown in ADCC (antibody dependent cell cytotoxicity) to facilitate killing of bacteria. Thus, detection of GP1R binding directly to these bacteria is unexpected. The relatively uniform distribution of GP1R fluorescent deposits discernible on the cell surface of *E. coli* (Fig. 4b) is significant. This staining pattern is a sharp contrast to the intense intracellular GP1R fluorescent deposits in cancer cells as shown in Fig. 1. It suggests that the identities of the molecules comprising the GP1R-binding targets of bacteria and cancer cells may be different, but the GP1R binding targets in both are likely the same. Mannose-6-phosphate/Insulin-like growth factor II receptor on the cell surface has been identified as the granzyme B receptor [14]. The GP1R-binding targets in cancer cells as shown here appear to be expressed in the cytosol. Given the unique polysaccharide and peptide composition of the bacterial cell wall, it is possible that the intracellular GP1R-binding targets in cancer cells are likely associated with special glycosylation that are also found on the cell surface of both Gram-positive and Gram-negative bacteria. Using GP1R in colocalization experiments with other cancer/bacteria-related biochemicals might help elucidate the identities of the molecules containing the GP1R-binding targets. This may lead to better understanding of the pathogenesis of cancer and bacterial infection, providing more useful information to help develop effective diagnostics, therapeutics, and preventive medicine.

Detection of the presence of GP1R-binding targets on the bacterial cell surface, but not inside the bacteria, was further supported when GP1R-positive bacteria were also detected within the phagosomes of phagocytes in the bacteria-laden whole blood specimen after GP1R incubation. This mechanism of bacterial elimination is similar to ADCC that uses antibody as opsonin to cover the bacterial cell surface to promote phagocytosis. The bacteria will subsequently be destroyed by the phagocytes. It appears that different locations of the GP1R-binding targets in bacteria and cancer cells not only have resulted in different GP1R-mediated binding patterns but also killing mechanisms. The detection of trypan blue stain inclusion in cancer cells implies that membranes of these cells have been compromised. However, the detection of GP1R-induced membrane extrusion suggests that the

mechanism of destroying the cancer cells beyond repair is losing the membrane. Cells without the membrane or losing the nucleus as shown after GP1R incubation are not expected to survive and grow with or without the showing of trypan blue stain inclusion. This membrane extrusion feature can be used as another cell death indication. The discernible whole membrane separation as shown (with very weak fluorescence) in Fig. 3c attests that the GP1R binding with the putative universal pathologic biomarker inside the cancer cells can induce cell death in situ, independent of CTL. It is obvious that cell entry of GP1R is accomplished without the need of perforin or CTL. Endocytosis may be one possible mechanism for GP1R's entry into the cell given the absence of distinct cell surface staining in methanol-fixed GP1R-positive cancer cells.

Using fluorescence microscopic methods, we show the unexpected GP1R-binding in bacteria, suggesting that bacteria may be a "new" target cell of CTL besides cancer cells and virally infected cells. Our innate CTL-dependent cellular immunity might have always intended bacteria as one of the CTL target cells. In addition to bacteria, other unexpected "new" target cells of CTL may also be found by using GP1R in investigations of other diseases impacted by CTL like systemic lupus erythematosus [18], asthma [19], multiple sclerosis [20], and Alzheimer's disease [21]. It is still unclear what role CTL plays in these autoimmune disorders. GP1R may be used in amyloid beta plaques or proteins from patients with Alzheimer's disease (AD) at different disease stages to determine if there are preexisting GP1R binding targets. This may shed some light on the CTL-associated pathogenesis of AD and possible new direction for better AD diagnosis and treatment.

Granzyme-mediated apoptosis has been shown to be effective in HIV-1 infected cells [12]. Although our study did not use GP1R in virally infected cells like HIV-positive T lymphocytes per se, the GP1R-binding and GP1R-mediated cell death in HeLa cervical cancer cells shown here may suggest that similar GP1R activities are likely to result in these and other virally infected cells. The results of the bacterial phagocytosis experiments further indicate the high binding specificity of GP1R in a biological sample as complex as whole blood specimen. There was no indication of non-specific binding to red blood cells or plasma components. The anticipated presence of numerous active proteases in whole blood did not appear to have significant inhibitory effects on the binding efficacy of GP1R to the targeted microorganisms. There was also no indication of collateral damage to the cells surrounding the intensely stained bacteria despite a relatively high concentration of GP1R used. This is likely attributed to the absence of the preexisting binding targets (or putative universal pathologic biomarker) in those cells. The observed high target-binding specificity of GP1R is important if it is used in body fluids like whole blood to aid the detection and identification of HIV-infected CD4+ T cells or circulating tumor cells or bacteremia. It is of equal importance if GP1R is used in a therapeutic composition with the intent to destroy these disease-causing agents.

In conclusion, we show with never-before-seen fluoroscopic images, that a synthetic granzyme B-like peptide fluorescent conjugate (GP1R) can: 1) mediate cell death of different cancer cells via membrane extrusion, 2) bind to constitutively expressed binding targets in different cancer cells and bacteria, and 3) promote bacterial phagocytosis. The putative binding targets may serve as a universal pathologic biomarker detectable by GP1R. Our data taken together demonstrate the potential applications of GP1R for use in CTL-independent target cell recognition and target cell death induction. It may lead to development of rapid targeted detection and new treatment of cancer, viral and bacterial infections. The new treatment may show mutual benefits for two or more diseases.

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