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研究課題名（和文）Enhancing apoptosis of cancers using systems biology approach.

研究課題名（英文）Enhancing apoptosis of cancers using systems biology approach

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研究成果の概要（和文）：TRAIL promises in controlling cancer but several cancers remain non-sensitive to it. We developed a computational model and predicted that together with PKC inhibition, TRAIL induces 95% cell death. We confirmed this utilizing PKC inhibitor, bisindolylmaleimide I, and PKC-δ siRNA in fibrosarcoma.

研究成果の概要（英文）：Over the last decade, we have developed computational models to interpret instructive cell signaling and high-throughput transcriptome-wide behaviors of immune and cancer cells. Here, we focused to overcome cancer resistance. TRAIL (tumor necrosis factor related apoptosis-inducing ligand), a proinflammatory cytokine, has shown promising success in controlling cancer threat due to its ability to induce apoptosis in cancers specifically, while having limited effect on normal cells. Nevertheless, several malignant cancers, such as fibrosarcoma (HT1080), remain non-sensitive to TRAIL. To sensitize HT1080 to TRAIL treatment, we developed a dynamic computational model based on perturbation-response approach, to predict a crucial co-target to enhance cell death. The model simulations suggested that PKC inhibition together with TRAIL induce 95% cell death. Subsequently, we confirmed this result experimentally utilizing the PKC inhibitor, bisindolylmaleimide (BIM) I, and PKC siRNAs in HT1080.

研究分野：総合生物

キーワード：TRAIL　Cancer　Computational Biology　Modeling

１．研究開始当初の背景

Over the last decade, we have developed computational models and statistical techniques to interpret instructive cell signaling and high-throughput transcriptome-wide behaviors of immune, cancer, and embryonic development cells. Here, our focus was to overcome cancer resistance.

TRAIL (tumor necrosis factor related apoptosis-inducing ligand), a proinflammatory cytokine, has shown promising success in controlling cancer threat due to its ability to induce apoptosis in cancers specifically, while having limited effect on normal cells. Nevertheless, several malignant cancer types, such as fibrosarcoma (HT1080) or colorectal adenocarcinoma (HT29), remain non-sensitive to TRAIL. To sensitize HT1080 to TRAIL treatment, we developed a dynamic computational model based on perturbation-response approach, to predict a crucial co-target to enhance cell death. The model simulations suggested that PKC inhibition together with TRAIL induce 95% cell death. Subsequently, we confirmed this result experimentally utilizing the PKC inhibitor, bisindolylmaleimide (BIM) I, and PKC siRNAs in HT1080.

２．研究の目的

We focused on the resistance mechanisms in TRAIL-stimulated human fibrosarcoma (HT1080) cells. It was recently established that HT1080 resistance mainly occurs through the activation of MAPKs and NF-κB. However, the sensitization of H1080 to TRAIL still remains elusive. Firstly, we developed a dynamic computational model to analyse the time-course activation profiles of several cell survival (IκB, JNK, p38) and apoptotic (caspase-8, -3) signaling molecules. The model, based on perturbation-response approach, does not require the full knowledge of all signaling species and their reaction kinetics. Instead, it uses linear response rules, derived from the fundamental law of information (signaling flux) conservation, to elucidate novel features of population-average cell signaling pathways and has been successfully used for Toll-like receptors (TLRs)-3, -4 and TNF signaling studies.

Our initial model was based on the well-known TRAIL signaling topology. TRAIL-R1 and TRAIL-R2, on binding with TRAIL, form receptor clusters through O-glycosylation and/or palmitoylation. This facilitates the recruitment of FADD, caspase-8 and cFLIP to the intracellular death domain of TRAIL-R1 and -R2, collectively forming primary death-inducing signaling complex (DISC). Still attached to the membrane, the DISC becomes enriched in lipid rafts, subsequently allowing caspase-8 to interact with CUL3/RbxI-based E3 ligase complex. Polyubiquitylation of caspase-8 occurs and the ubiquitin-binding protein p62/sequestosome-1 binds with caspase-8 to detach it from the DISC. Consequently, caspase-8 interacts with RIP1, TRAF2 and IKK-γ to form secondary DISC, which activates downstream NF-κB, MAPKs, and caspase-3, a member of the cysteinyl-aspartate-specific proteases, through the extrinsic pathways.

Using the model, we predicted protein kinase C (PKC) as the most effective target, with over 95% capacity to kill human fibrosarcoma (HT1080) in TRAIL stimulation (1). Here, to validate the model prediction, which has significant implications for cancer treatment, we conducted experiments on two TRAIL-resistant cancer cell lines (HT1080 and HT29).

Here, we tested the previous model prediction by experimentally verifying whether targeting PKC will enhance apoptosis in TRAIL-resistant cancer cell lines. Experiments were performed on TRAIL-induced human fibrosarcoma (HT1080) and human colon adenocarcinoma (HT29) cells, and the cell viability was compared with control normal fibroblasts (TIG-1 and MRC-5). Moreover, to investigate the intracellular mechanisms for resultant cell viability, we measured time-course activation levels of caspase-3, PARP, p38, and JNK. Subsequently, we analyzed the expressions of each PKC isoform member in HT1080 cells. To identify a crucial target member for enhanced cancer apoptosis, we prepared relevant siRNA KD experiments. In summary, our study investigates (i) whether the model prediction of PKC suppression will enhance cancer cell death is true, and (ii) whether computational modeling using perturbation-response approach is valuable for biological research focusing on cancer treatment.

３．研究の方法

Reagents and Cell Culture

Recombinant human TRAIL was purchased from Peprotech. Bisindolylmaleimide I (BIM-I) was purchased from Merck Millipore. Human fibrosarcoma cell lines (HT1080), human embryo fibroblasts (TIG-1), and human colorectal adenocarcinoma cells (HT29) were obtained from Japanese Collection of Research Bioresources (JCRB) cell bank. Human fetal lung fibroblasts (MRC-5) were obtained from American Type Culture Collection (ATCC). HT1080, TIG-1, HT29, and MRC-5 were grown in DMEM (Nissui Seiyaku Co.) containing 10% calf serum, 100 U/mL of penicillin, Streptomycin 100 μg/ml and Amphotericin B 0.25 μg/ml at 37°C in a 5% CO2 humidified atmosphere.

Cell Viability Assay

The cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and trypan blue exclusion. MTT assay: cells (10 × 104) were inoculated in each well and incubated for 24 h. Thereafter, 50 μL of MTT (2 mg/mL in PBS) was added to each well and the plates were incubated for a further 2 h. The resultant formazan was dissolved with 100 μL of dimethyl sulfoxide (DMSO) after aspiration of culture medium. Plates were placed on a plate shaker for 1 min and then read immediately at 570 nm using TECAN microplate reader with Magellan software (Männedorf, Switzerland). Trypan blue exclusion: cells were detached with 1 mL of trypsin and suspended in DMEM. After staining with trypan blue, viable cells were counted using microscopy (n = 3). The percentage of trypan blue exclusive viable cells was determined as a percentage of the total number of cells.

Western Blot Analysis

Anti-PARP, anti-phospho-p38, and anti-β-actin antibodies were purchased from Cell Signaling Technology. Proteins were extracted from the cell lines using radioimmunoprecipitation assay (RIPA) buffer according to the manufacturer's instructions. Next, their concentrations were measured by Bradford protein assay. Equal amounts of protein were loaded in each well and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which was subsequently transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked for 1 h with 5% BSA in TBST on the shaker at room temperature. The membrane was placed on PARP and p–p38 antibody diluted at a 1:1000

proportion in diluent buffer [5% (w/v) BSA and 0.1% Tween 20 in TBS] and incubated overnight at 4°C on the shaker. The membrane was washed three times in TBS as above and incubated with secondary antibody diluted at a 1:10000 proportion for 1 h on the shaker at room temperature. The membrane was again washed three times for 5 min each time as above and finally the results were generated by using an enhanced chemiluminescence (ECL) Western blotting kit.

Enzyme Linked Immunosorbent Assays of Cleaved Caspase-3 and Phosphorylated JNK

Cleaved caspase-3 and phosphorylated JNK concentrations were measured by ELISA Duo Sets IC Kit (R&D Systems) following the instructions of the manufacturer.

Transfection

siRNA duplexes were purchased from Sigma. The transfection of classic PKCs (PKCα, PKCβ, PKCγ), the novel PKCs (PKCδ, PKCε, PKCη, PKCμ, PKCθ), and the atypical PKCs (PKCζ, PKCι) and scrambled siRNA were carried out using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen).

Quantitative Real-Time PCR Analysis

Total cellular RNA was extracted from cells using the TRIzol reagent according to the manufacturer's instructions (Invitrogen). One microgram of RNA was reverse-transcribed using a first-strand cDNA synthesis kit (ReverTra Aceα; Toyobo). Quantitative real-time PCR (qRT-PCR) was performed using SYBR premix Ex Taq (Takara) on the Applied Biosystems StepOnePlus™ according to the technical brochure of the company. qRT-PCR primers used in this study are listed in Table 1. Quantitative measurements were determined using the ΔΔCt method and expressions of GAPDH gene for PKC genes and RPL27 gene for rela, mtor, bcl2, bax, cytoc, and jun were used as the internal control. Melt curve analyses of all qRT-PCR products were performed and shown to produce the sole DNA duplex.

４．研究成果

Previously, we investigated the TRAIL-resistant mechanism in HT1080 cells using a computational model. We predicted that the suppression of a novel pro-survival molecule would result in significant enhancement of apoptosis through signaling flux redistribution. PPI database search indicated that the pro-survival molecule is a member of PKC. To experimentally validate this result, in this project, we investigated the effects of two TRAIL-resistant cancer cells to PKC inhibition.

First, using different doses of PKC inhibitor BIM-I together with various levels of TRAIL stimulation, we observed approximately 99 and 95% cell death occurred for HT1080 and HT29 cells, respectively. Notably, the effect on control TIG-1 and MRC-5 cells were less significant, at approximately 40 and 20% cell death, respectively.

Second, to confirm the mechanism for cell death is through apoptosis, we measured the activations of PARP and caspase-3 over 3 h in TRAIL-stimulated HT1080 cells untreated and treated with BIM-I, and compared with activations of p38 and JNK. We found that PARP, caspase-3 cleavages and p38 phosphorylation were significantly enhanced in BIM-I treated cells, while JNK activity was very low. These results are in consistency with the previous prediction of our computational model. We also investigated the expressions of major pro- and anti-apoptotic genes, and found them to be

mostly repressed at their transcription levels, especially after 1 h for BIM-I treated cells.
Third, to identify the crucial PKC family member for single specific target, we investigated the mRNA expressions of all 10 major isoforms in HT1080 cells. We selected the top four significantly expressed isoforms for developing siRNA KDs, and subsequent experiments demonstrated that PKCδ is a key target for enhancing cell death in TRAIL-resistant HT1080 cells.
It is worthy to mention other previous works that have studied PKC in different cancer types. Although these works have demonstrated the importance of PKC, the investigations were performed in different cell lines or stimulations. In this work, however, we focused mainly on HT1080 and limitedly on HT29 cells. In addition, we bring to the attention the power of using multidisciplinary research to systemically identify a key target that can be experimentally tested. Therefore, to our knowledge, this is the first time the usefulness of a computational model is shown to identify a consistent and key target for regulating TRAIL-resistance. In summary, our work provides further evidence for the utility of systemic approaches in providing effective treatment strategies to tackle complex diseases.

５．主な発表論文等
（研究代表者、研究分担者及び連携研究者には下線）

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Springer, NewYork
Immuno Systems Biology
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○出願状況（計 0 件）

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〔その他〕
ホームページ等

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