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添付資料：研究報告書

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2．研究テーマ

ヒト細胞におけるシャペロン HSP27 と相互作用する分子の検索

3．成果の概要（100字程度）

HSP27 を GST 融合タンパク質として大腸菌で大量に発現する系を構築して、大量に精製した。次に、HSP27 を結合したアフィニティーカラムを作製した。このカラムにヒト細胞の細胞溶解液を流して、カラムに結合する分子だけを回収した、回収された HSP27 結合分子を SDS－ポリアクリルアミドゲル電気泳動で分離し、クーマシーブルー染色により 4 種類存在することが確認された。今後、この HSP27 結合分子について、LC/MS/MS を用いて蛋白質を同定する予定である。

4．研 究 業 績

(1) 学会における発表　(無)　・　有（学会名・演題）

(2) 発表した論文　(無)　・　有（雑誌名・題名）

—日中医学協会助成事業—

ヒト細胞におけるシャペロンHSP27と相互作用する分子の検索

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Abstract

HSP27, a member of the small heat shock protein family, is implicated in diverse biological function. Cytoprotective roles of Hsp27, as a molecular chaperon, include inhibition of caspase activation, prevention from stress-induced disruption of cytoskeleton, and modulation of intracellular redox potential. We recently found that HSP27 is involved in the protective role against UVC-induced cell death in human UVAP-2 cells, possibly via function in DNA repair. In the present study to elucidate the molecular mechanisms of the UVC resistance, we searched for HSP27-interacted proteins in the human cells, by affinity column chromatography using HSP27 protein-binding Sepharose. The HSP27 proteins of wild type and mutated type, in which the residues Ser15, 78 and 82 were substituted for aspartic acids to mimic the phosphorylated HSP27, were expressed as fusion protein with GST in *E. coli*. GST, the wild type (GST-HSP27) and the mutated type (GST-HSP27/3D) of HSP27 fused with GST were purified from *E. coli* using GSH-Sepharose column and then conjugated with NHS-activated Sepharose. Cytosolic and nuclear fractions from cell lysates were prepared from human UVAP-2 cells mock-irradiated and irradiated with UVC (10 J/m^2). Up to now, the cytosolic fraction from the mock-irradiated cells was applied to the affinity columns and eluted with high salt solution. The eluted fraction was subjected to SDS-PAGE analysis, and four protein bands bound to the GST-HSP27 and GST-HSP27/3D columns but not to the GST column were detected in the comparative SDS-PAGE analysis. Identification of the four proteins appeared to specifically bind to HSP27 is now in progress by mass spectrometry.

Keywords　HSP27, UVC, HSP27-inieracted protein, molecular chaperone, human cells

Introduction

Stress events induce the expression of a set of highly conserved proteins called heat shock proteins (HSPs) in prokaryotes and eukaryotes. The expression of these proteins is induced not only by elevated temperature but also other types of stress like heavy metals, H_2O_2, arsenite or alcohols, and increased expression is involved in the protection against stress-induced cellular injury [1-5]. Thus, the more general term 'stress proteins' has been introduced for HSPs, which belong to molecular chaperones. Ultraviolet light (UV) induces significant cellular damage, primarily producing DNA lesions such as thymine dimers and (6-4)photoproducts [6, 7]. Human cells have defense mechanisms to survive UV-induced injury. HSPs may play roles in defense mechanisms since cells preconditioned by heat shock were reported to be resistant to UVB (principally 290- 320 nm in wavelength) irradiation [8-11]. However, the kind of HSP involved in the resistance and the mechanisms underlying the HSP-induced resistance are still elusive. HSP27, a member of the small heat shock protein family, is implicated in diverse biological

function. Cytoprotective roles of Hsp27, as a molecular chaperon, include inhibition of caspase activation, prevention from stress-induced disruption of cytoskeleton, and modulation of intracellular redox potential. [12]. We have established human cell lines differing in their sensitivity to UVC, RSa and UVAP-2 [13-14]. From the comparative studies of these cell lines, we recently found that HSP27 is involved in the protective role against UVC-induced cell death in human UVAP-2 cells, possibly via function in nucleotide excision repair [15] . In the present study to elucidate the molecular mechanisms of the UVC resistance, we searched for HSP27-interacted proteins in the human cells. We first constructed plasmids to express wild type and mutated type of HSP27, in which the residues Ser15, 78 and 82 were substituted for aspartic acids to mimic the phosphorylated HSP27, as fused proteins with gluathione-S-transferase (GST). These fusion proteins were expressed in *E. coli.* and purified and then conjugated with Sepharose. Next, we searched for HSP27-interacted proteins in UVAP-2 cells by affinity column chromatography using Sepharose binding those fusion proteins, and detected several proteins appeared to specifically bind to HSP27.

Materials and methods

Plasmids

pBluescript-HSP27, which contain cDNA sequence of *hsp27* gene, was gift from H. hosoya. (Hiro shima University , Japan)

pBluescript..KS(+)-HSP27/3D mutant , in which 15-Ser, 78-Ser and 82-Ser of *hsp27* were substituted for aspartic acids, was gift from Dr. Weber and Dr. Hicky (University of NEVADA , USA). The sequence of the cDNAs of *hsp27* and *hsp27/3D* were inserted to GST fusion protein expression vector using proper sites for restriction enzymes.

Purification of protein

Pellets of E. coli. expressing GST, the wild type (GST-HSP27) and the mutated type (GST-HSP27/3D) of HSP27 fused with GST were lysed with a buffer (50mM Tris /HCL, pH 7.5, 10mM 2-ME, 1mM EDTA, 50 mM NaCl, 10% Glycerol) containing 0.1% NP-40 , 1 mM PMSF , 0.01mM pepstain A, 0.08 mg/ml lysozyme, and the lysates were incubated for 1 hour at 37° C and then centrifuged at 10,000 x g for 1 hour at 4° C. The supernatants were applied to Glutathione Sepharose 4 Fast Flow (Amersham biosciences AB SE-751 84 Uppsala Sweden) followed by a wash in the lysis buffer , then eluted with 40mM glutathione . The purified proteins were confirmed by SDS-PAGE analysis.

Coupling of proteins with NHS-activated sepharose

GST, GST-HSP27 and GST-HSP27/3D proteins were dialyzed with a buffer (0.2M $NaHCO_3$, pH 8.3, 0.2M NaCl), concentrated by Amicon Ultra-4 (Milipore Corporation Bedford, MA01730 U.S.A) according to the manufacturer' s recommendations. The concentrated proteins were coupled with NHS-activated Sepharose (Amersham Pharmacia Biotech AB SE-751 84 Uppsala Sweden) for 2 hours at room temperature and blocked with 0.5 Tris-HCl, pH 8.3, containing 0.5 M NaCl and then washed with 0.1 M CH_3COONa, pH 4.0, containing 0.5 M NaCl. The blocking and washing were repeated 6 times and finally washed with 50 mM Tris-HCl, pH 7.5, containing 50 mM NaCl.

Cells and culture condition

UVAP-2 cells were established from human RSa cells [13], as a UVC-resistant cell line, by mutagenization with ethyl methanesulfonate followed by UVC irradiation [14]. UVAP-2 cells were cultured in Eagle's MEM (EMEM) (Nissui, Tokyo, Japan) medium supplemented with 10% (v/v) calf serum (CS) (Biosciences PTY Ltd, A.C.N., Australia) at 37° C in a humidified atmosphere containing 5% CO_2.

UVC irradiation

UVC was generated from a 6-W National germicidal lamp (Matsushita Electronic Industrial Co., Osaka, Japan). The intensity of UVC was 1 $J/m^2/s$, which was measured by a UV radiometer, UVR-254 (Tokyo Kogaku Kikai Co., Tokyo, Japan). Cells were seeded in a 100-mm dish and grown to a confluent condition. The cells were irradiated with UVC at an indicated dosage immediately after the medium was removed and then reincubated for an appropriate time as previously described [20]. Mock-irradiated cells were treated in the same manner but without irradiation

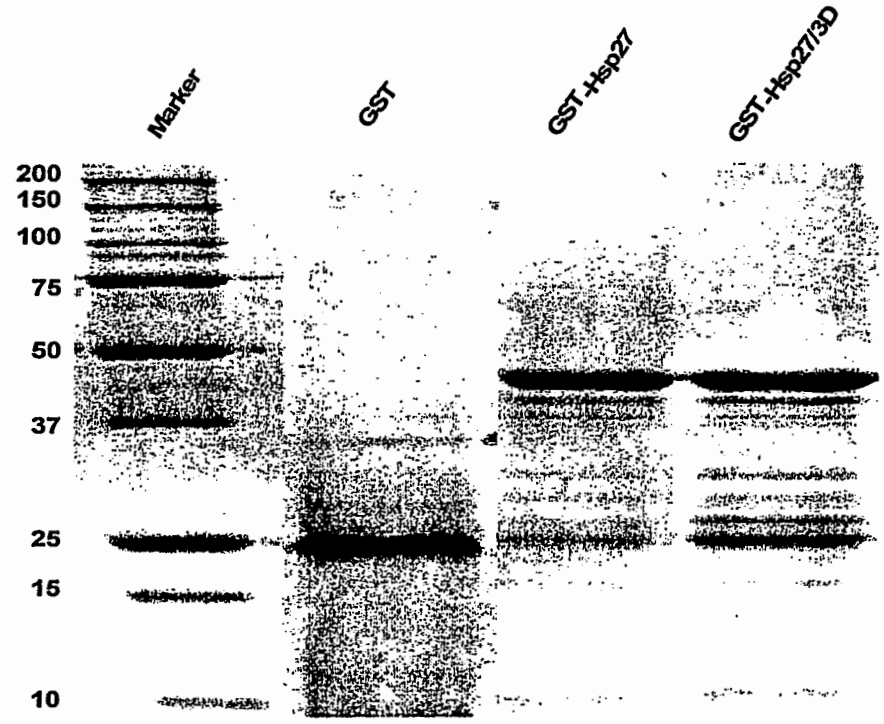
Preparation of cytosolic and nuclear fraction from human UVAP-2 cells

Affinity chromatography

The affinity column was pre-washed with the cell lysis buffer. Cytosolic and nuclear fractions of cell lysates from human UVAP-2 cells mock-irradiated were applied successively to the three affinity column, GST, GST-HSP27 and GST-HSP27/3D binding Sepharose, in their order, washed with the lysis buffer, and then eluted sequentially with a buffer (10mM Tris-HCl, pH 7.5 , 10 mM NaCl , 2.5 mM $MgCl_2$, 2 mM NaF , 2 mM Na_2VO_4) containing 1 M NaCl, then the buffer contaninig 1 M $MgCl_2$, and finally 0.1 M glycine - HCl , pH 3.0. Eluted protein were concentrated using a TCA precipitation method, separated by SDS-PAGE, and then detected by CBB or silver staining.

Results

We purified about 7.2mg GST, 6.1mg GST-Hsp27 and 6.44mg GST-Hsp27/3D from 16L, 16Land 14L L-broth culture E. coli, respectively(figure1). Cytosolic fractions of cell lysates from human UVAP-2 cells mock-irradiated were applied successively to the two affinity column, GST, GST-HSP27 binding Sepharose. There are four proteins appeared to specifically bind to HSP27(figure2).



Figurel The purified proteins were confirmed by SDS-PAGE analysis and stained by CBB

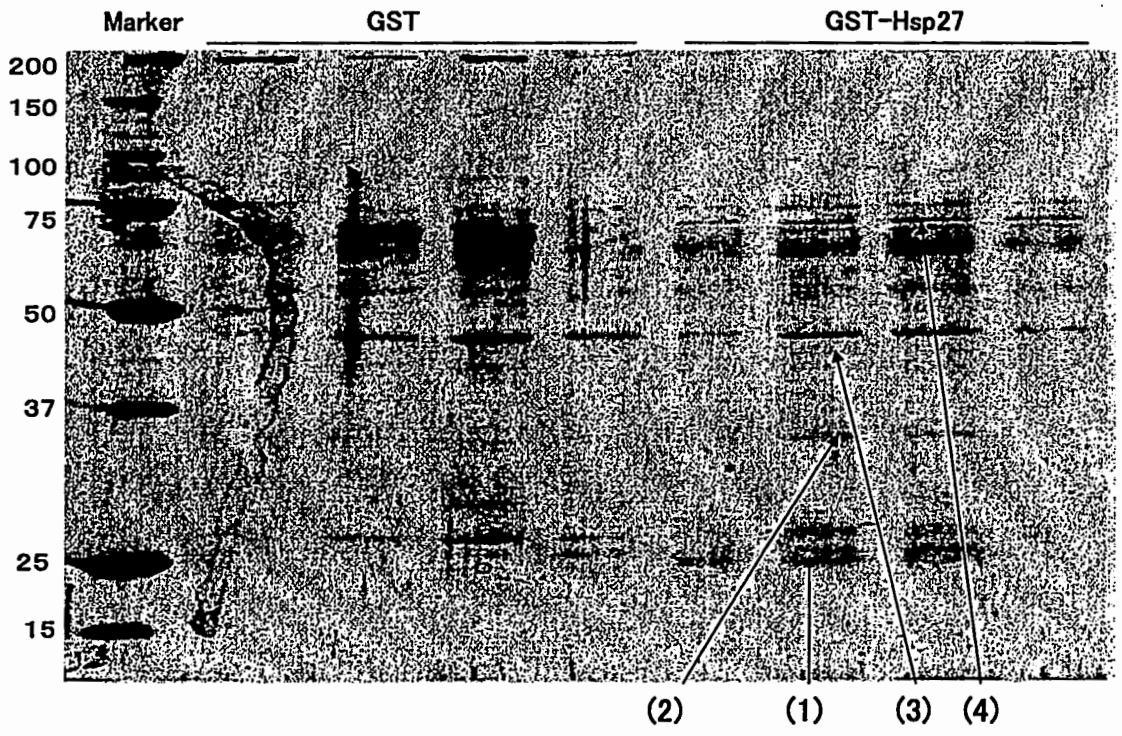


Figure2 Proteins interact with HSP27

Discussion

It was reported that human cells tolerate damage caused by UVB irradiation if they have been preconditioned for heat treatment [7-9], and it was suggested that inducible HSPs play roles in cellular responses after UV irradiation [10]. However, Trautinger et al. [20-21] reported that overexpression of HSP27 did not confer resistance to UVA (principally 320-380 nm in wavelength)- and UVB-induced cell death in a human squamous cell carcinoma cell line. On the other hand, we recently found that HSP27 has the protective role against UVC-induced cell death in UVC-resistant human UVAP-2 cells, possibly via function in nucleotide excision repair [16]. UVC is a useful tool as a DNA damaging agent for investigating DNA repair mechanisms and stress responses in human cells [22-23]. We used UVC as a DNA damaging stressor and found previously the involvement of some other molecules, such as NPM [15] and syndecan 1 [25], in resistance to UVC in human cells.

HSP27, a member of the small heat shock protein family (sHSP), is expressed constitutively at low levels in many cells and tissues. HSP27 expression increases following heat shock or other type of stress and enhances the cellular resistance to different types of stress, including heat shock, oxidative stress [16-17], and cancer chemotherapy agents [19]. It has been previously reported that HSP27 play roles in the cytoprotective role as a molecular chaperon via inhibition of caspase activation , prevention of stress-induced disruption of the cytoskeleton, and the modulation of the intra-cellular redox potential [13].

The molecular mechanisms of the protective roles of HSP27 against UVC via function in the DNA repair is unknown. If HSP27 also functions in the roles as a molecular chaperone, it is expected to interact with other protein. Zhu *et al.* [26] and Kindas-mugge et al. [27] used immuno-precipitation method to search for HSP27-interacted protein; Zhu *et al.* reported that a small amount of actin is co-precipitated by anti-HSP27 antibody in resting platelets, and Kindas-mugge *et al.* found that HSP27 interacts with actin, p53, HSP70 and HSP90, respectively, in a human epidermal carcinoma cell line transfected with *hsp27* cDNA. In this study, we used an affinity column chromatography method using HSP27-binding Sepharose to search for HSP27- and phosphorylated HSP27- interacted proteins in human UVAP-cells mock-irradiated and irradiated with UVC. For the purpose, mutated type of HSP27, in which 15-Ser, 78-Ser and 82-Ser of hsp27 were substituted for aspartic acids, as well as wild type of HSP27 was expressed in E. coli. as fused proteins with GST, and purified. The mutated type was reported to be mimic phophorylated HSP27. Up to now, we identified four proteins appeared to specifically bind to HSP27 in cytosolic fraction in the mock-irradiated cells. The identification of the proteins is now in progress by mass spectrometry.

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