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学位論文要旨

SUMMARY

Testicular Sertoli cells phagocytose apoptotic spermatogenic cells in a manner depending on the membrane phospholipid phosphatidylserine (PS) expressed at the surface of the latter cell type. Although class B scavenger receptor type I (SR-BI) has been presumed to be a phagocytosis receptor of Sertoli cells, whether or not SR-BI binds directly to PS remains unclear. In this study, I examined this issue using a solid-phase protein-phospholipid binding assay. The extracellular domain of rat SR-BI fused with human Fc (SRBIecd-Fc) bound to PS with a dissociation equilibrium constant of 2.4×10^{-7} M, while other phospholipids including phosphatidylethanolamine, phosphatidylinositol, and phosphatidylcholine were poor binding targets. The binding activity was enhanced when $CaCl_2$ was included in the assay or when SRBIecd-Fc was pretreated with *N*-glycanase. A portion of the extracellular domain spanning amino acid positions 33 to 191 (numbered with respect to the amino terminus) fused with Fc (SRBI33-191-Fc) showed activity and phospholipid specificity equivalent to those of SRBIecd-Fc. Finally, SRBI33-191-Fc bound to the surface of PS-exposed apoptotic cells.

Taken together, I conclude that PS-binding activity of SR-BI is responsible for recognition of apoptotic spermatogenic cells by Sertoli cells.

INTRODUCTION

Cells undergoing apoptosis are selectively and rapidly eliminated from the organism by phagocytosis. This process contributes not only to the clearance of unnecessary or spent cells from the body but also to maintaining tissue homeostasis. It is thus of great importance to elucidate the mechanism underlying phagocytosis of apoptotic cells, which presumably consists of various distinct events such as the migration of phagocytes toward the site of apoptosis, the recognition and englufment of apoptotic cells by phagocytes, processing of

engulfed apoptotic cells in phagocytes, and the alteration of gene expression in engulfing phagocytes. Although investigation of these phenomena, especially the recognition step, has recently become intensive, most mechanism still remains unclear.

Phagocytes such as macrophages bind to apoptotic cells, probably by recognizing 'phagosytosis marker molecules' that are expressed on the surface of target cells; the membrane phospholipid phosphatidylserine (PS) is the best-characterized phagocytosis marker. PS, which is normally restricted to the inner leaflet of the membrane bilayer, translocates to the outer leaflet and is exposed to the cell surface during apoptosis by a caspase-mediated mechanism of which the details remain unknown. Externalized PS serves as a phagosytosis marker; that is, phagocytes recognize apoptotic cells by using PS receptors and engulf those cells.

During spermtogenic differentiation, more than half of the differentiating spermatogenic cells die, probably by apoptosis, before they mature into spermatozoa, although the mechanism and functional significance of this phenomenon are unknown. The occurrence of spermatogenic cell apoptosis at various stages of differentiation has been reported. Only limited number of apoptotic spermtogenic cells, however, are detectable when testis sections are histochemically examined. This is probably due to the rapid elimination of apoptotic cells by phagocytosis. In fact, electron microscopic studies with rodent testis sections have revealed that degenerating spermatogenic cells are engulfed by Sertoli cells. Rat Sertoli cells in primary cultures phagocytose apoptotic spermatogenic cells in a manner dependent on PS exposed on the surface of the apoptotic cells and SR-BI present in Sertoli cells. However, whether or not SR-BI directly bind to PS remains to be elucidated. In this study, I verified the possibility that SR-BI directly binds to PS, a phogocytosis marker, using a cell-free solid-phase assay.

RESULTS

1) Binding of extracellular domain of SR-BI to PS

1-1 Preparation of extracellular domain of SR-BI as a Fc-fused protein

To examine the PS-binding activity of SR-BI, the extracellular domain (ecd) of rat SR-BI was expressed as a fusion protein with Fc region of human IgG (SRBIecd-Fc) in a culture cell line and purified by protein A-Sepharose chromatography. The ecd of the Gas6 receptor Axl was similarly obtained as a Fc-fused protein (Axlecd-Fc). The proteins bound to the Sepharose resin were eluted with a buffer (pH 3.0) and analyzed by SDS-polyacrylamide gel electrophoresis. Both recombinant proteins appeared to be nearly homogeneous were detected with an anti-human IgG Fc antibody in Western blotting while only SRBIecd-Fc gave a signal with an anti-SR-BI antiserum.

1-2 Binding of SRBIecd-Fc to PS in a cell-free solid-phase assay

Binding of Fc-fused proteins to plastic plates coated with PS, phosphatidylcholine (PC) was examined by using anti-human IgG Fc antibody. SRBIecd-Fc effectively bound to PS but not to PC in a dose-dependent manner, while Axlecd-Fc did not significantly bind to either phospholipid. To examine phospholipid specificity of SRBIecd-Fc binding, the same assay

was conducted with phosphatidylethanolaine and phosphatidylinositol. The results clearly showed that SRBIecd-Fc did not bind to either phospholipid. Since another aminophospholipid (phosphatidylethanolamine) and anionic phospholipid (phosphatidylinositol) were poor binding targets, I concluded that SRBIecd-Fc specifically binds to PS.

I next examined the effect of Ca^{2+} on PS-binding activity of SRBIecd-Fc. The binding assay was carried out in the presence of varied concentrations of $CaCl_2$. The addition of $CaCl_2$ stimulated the binding activity of SRBIecd-Fc to PS.

1-3 Kinetic analysis for binding of SRBIecd-Fc to PS

Kinetic parameters for binding of SRBIecd-Fc to PS were determined using IAsys biosensor. SRBIecd-Fc bound to a PS-coated cuvette but not to a PC-coated cuvette, while Axlecd-Fc did not show affinity to either cuvette. The dissociation equilibrium constant (K_D) was obtained, and SRBIecd-Fc was found to bind to PS with K_D of 2.4×10^{-7} M.

2) Structure-function analysis of SR-BI

A structure-function analysis of SR-BI was done to narrow the region responsible for PS-binding activity.

2-1 Expression of Fc-fused proteins containing various portions of SR-BI

293T cells were transfected with DNAs that coded for various portions of SRBIecd, and expression of the expected Fc-fused proteins were analyzed by Western blotting with an anti-human IgG Fc antibody. Only the protein containing a region corresponding to amino acid residues 33-191 ($SRBI_{33\text{-}191}$-Fc) was detectable in the culture medium. Other proteins existed within cells and did not seem to undergo secretion. $SRBI_{33\text{-}191}$-Fc was purified from the culture medium by protein A-Sepharose and analyzed for purity by SDS-polyacrylamide gel electrophoresis followed by staining with CBB and Western blotting with either an anti-rat SRBI antiserum or an anti-human IgG Fc antibody. $SRBI_{33\text{-}191}$-Fc was as obtained with purity similar to that of SRBIecd-Fc.

2-2 Binding of $SRBI_{33\text{-}191}$-Fc to PS

I then examined the PS-binding activity of $SRBI_{33\text{-}191}$-Fc. $SRBI_{33\text{-}191}$-Fc bound to plates coated with PS but not to PC-coated plates in a dose-dependent manner. Both the efficiency and specificity of the binding activity were indistinguishable between $SRBI_{33\text{-}191}$-Fc and SRBIecd-Fc. The kinetic analysis using IAsys showed that the dissociation equilibrium constant of $SRBI_{33\text{-}191}$-Fc for binding to PS, 3.6×10^{-7} M, was close to that of SRBIecd-Fc. PS-binding activity of $SRBI_{33\text{-}191}$-Fc was stimulated in the presence of $CaCl_2$. From these results, I concluded that the region between amino acid residues 33-191 is responsible for the PS-binding activity of rat SR-BI.

3) *N*-glycosylation of SR-BI and its effect on PS-binding activity

3-1 *N*-glycosylation of SR-BI in rat Sertoli cells

SR-BI has some putative *N*-glycosylation sites. To test whether *N*-glycosylation of

SR-BI occurred in Sertoli cells, lysates prepared from Sertoli cells of 20-day-old rats were denatured, incubated with *N*-glycanase, and analyzed by Western blotting with an anti-rat SR-BI antiserum. Treatment with the enzyme made the SR-BI signal more discrete and caused a significant reduction of the molecular mass, indicating that SR-BI is *N*-glycosylated in Sertoli cells. The mobility of purified SRBIecd-Fc in a gel similarly increased after treatment with *N*-glycanase, indicating that SRBIecd-Fc is also *N*-glycosylated in 293T cells.

3-2 Effect of *N*-glycosylation on PS-binding activity of SR-BI

To prepare unglycosylated SRBIecd-Fc, 293T cells were transfected with the expression DNA and maintained in the presence of tunicamycin, an *N*-glycosylation inhibitor. When the culture media and whole-cell lysates were examined for the presence of SRBIecd-Fc, the signal that was clearly detectable in a control medium was absent in the medium of a culture with tunicamycin. Instead, multiple discrete signals that possessed mobility faster than that SRBIecd-Fc were obtained with lysates of the cells cultured in the presence of the inhibitor. This indicates that treatment with tunucamycin abrogated maturation and secretion of SRBIecd-Fc and suggests that *N*-glycosylation is needed for the expression of SR-BI. I thus decided to obtain unglycosylated SRBIecd-Fc by treating purified protein with *N*-glycanase. Although complete removal of carbohydrate moieties required the enzyme reaction under the denaturing condition, I found that denaturation step itself inactivated SRBIecd-Fc. Treatment with *N*-glycanase was therefore done without denaturation, and SRBIecd-Fc that underwent partial deglycosylation was prepared. The preparation showed enhanced activity of binding to PS. The result suggests that the glycosyl modification is inhibitory for PS-binding activity of SR-BI.

4) Binding of SRBI33-191-Fc to apoptotic cells with externalized PS

I next examined whether SR-BI binds to PS present at the cell surface. Jurkat cells treated with doxorubicin or left untreated were mixed with SRBI33-191-Fc or Axlecd-Fc and analyzed for the binding of Fc-fused protein by fluorescence microscopy after the addition of an FITC-labeled antibody recognizing Fc. Jurkat cells undergoing doxorubicin-induced apoptosis expressed PS at the surface and retained the integrity of the plasma membrane, which guaranteed that the Fc-fused proteins did not enter the cells. Apoptotic Jurakt cells incubated with SRBI33-191-Fc showed punctate cell surface staining (in green~yellow) in addition to dispersed faint reddish signals at the nucleus that were due to intrinsic fluorescence of DNA-bound doxorubicin. In contrast, Axlecd-Fc-treated cells gave only the nuclear staining, and normal Jurkat cells were not fluorescence even after incubation with SRBI33-191-Fc. These results indicate that SRBI33-191-Fc binds to PS present at the surface of apoptotic Jurkat cells.

DISCUSSION

In this study, I showed that SR-BI directly binds to PS, a marker for the recognition of apoptotic spermatogenic cells by Sertoli cells. This is the first indication of the PS-binding

activity of any phagocytosis-inducing PS receptors that have so far been identified. The obtained dissociation equilibrium constant (2.4×10^{-7} M) was small enough to support the specific interaction between SR-BI and PS. The region spanning amino acid positions 33 to 191 was shown to be responsible for the PS-binding activity of rat SR-BI. Some other proteins are also known to possess the PS-binding activity: examples are protein kinase C (PKC), PKC substrate MARCKS, and annexin V. All these proteins, however, are seemingly unrelated to phagocytosis. The primary structure of the region responsible for the PS-binding activity dose not show similarity with the PS-binding domains of SR-BI and other proteins, such as the C2 domain of PKC, the γ–carboxyglutamic acid domain of Gas6, and the motif FXFXLKXXXKXR identified using an anti-idiotypic monoclonal antibody that recognizes a PS-recognizing monoclonal antibody. It is thus of probable that there are multiple motifs defining the PS-binding activity. Determination of the three-dimensional structures of PS-binding proteins, as has been done with the C2 domain and annexin V, is necessary to determine the common structural basis for the PS-protein interaction, if such a basis exists.

The events lying downstream of the binding of PS to SR-BI are not clear at all. Further experiments are needed to clarify how the binding of PS to SR-BI evokes engulfment of apoptotic spermatogenic cells by Sertoli cells.

学位論文審査結果の要旨

河崎優希氏から提出された学位論文について，上記5名の審査委員による査読の後に平成14年8月5日に口頭発表会が行われた。同日に最終の審査委員会が開かれ，審議の結果，以下のとおり判定された。

本論文に記載された研究は，アポトーシス細胞貪食反応の際の食細胞側受容体として機能するタンパク質の働きを生化学的に解析したものである。精子への分化途中でアポトーシスを起こした精子形成細胞は，セルトリ細胞によって速やかに排除される。この現象は効率のよい精子生産に必要であることが示されている。セルトリ細胞は，アポトーシス精子形成細胞の表層に出現した膜リン脂質ホスファチジルセリン（PS）を認識して貪食を行うことがわかっている。この際にスカベンジャー受容体のひとつであるSR-BIがセルトリ細胞の貪食受容体として働くことが示されているが，SR-BIが直接にPSに結合するかどうかは不明であった。河崎氏は，リン脂質を塗布したプレスチックプレートへの精製タンパク質の結合を調べることにより，SR-BIが240nMの解離定数でPSに特異的に結合することを示した。さらに，この結合活性は509アミノ酸残基からなるラットSR-BIの，細胞外領域の一部に相当するアミノ酸番号(N末が1)33～191の領域に担われていた。また，SR-BIの発現が*N*結合糖鎖の付加により，PS結合活性がカルシウムイオンにより，それぞれ調節されることが示唆された。これらの知見は，これまで報告されている貪食受容体候補分子のいずれについても得られておらず，SR-BIの解析で初めてもたらされたものである。

本研究は，貪食受容体の生化学的解析を行うことにより食細胞によるアポトーシス細胞認識機構に言及したものであり，博士（薬学）の学位に値すると判定された。