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学位授与の要件	課程博士(学位規則第4条第1項)
学位授与の題目	Structural change of ribosomes during apoptosis : degradation and translocation of ribosomal proteins in doxorubicin-treated Jurkat cells (アポトーシス過程におけるリボゾームの構造変化：ドキソルビシンで処理したJurkat細胞でのリボソームタンパク質の分解と局在変化)
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学位論文要旨

SUMMARY

Changes in the amount and localization of human ribosomal proteins during apoptosis were determined. When total lysates of Jurkat cells which had been induced to undergo apoptosis by doxorubicin were analyzed by Western blotting, degradation of three ribosomal proteins, S18, L5 and L14, was detected at 48 h after the induction of apoptosis. Decreases in the amounts of these three ribosomal proteins were also observed when ribosome-enriched fractions were examined. These changes were partly abolished by the addition of the pan-caspase inhibitor z-VAD-fmk. Moreover, formation of the 80S ribosome complex appeared to be inhibited at 48 h after apoptosis induction. On the other hand, the extent of protein synthesis, assessed by measuring the incorporation of [^{35}S]Met into bulk proteins, decreased as early as 12 h after the addition of doxorubicin. These results indicate that changes in the amount of ribosomal proteins and the overall structure of ribosome, occur in apoptosing cells after protein synthesis declines. Finally, analyses by flow cytometry, immunofluorescence, and Western blotting showed that six ribosomal proteins S15, P0, L5, L6, L7, L36a and L41 were relocalized and expressed at the cell surface during apoptosis. The above results collectively indicate that ribosomes are structurally altered in apoptotic cells following inactivation of protein synthesis.

INTRODUCTION

Degradation of chromosomal DNA should result in the complete cessation of gene expression in cells fated to die. However, the rate of protein synthesis decreases even before chromosomal DNA is degraded, suggesting the presence of an active

mechanism of inhibiting gene expression at the level of protein synthesis in apoptotic cells. A variety of proteins and nucleic acids are involved in protein synthesis, and the ribosome stands at the center of the translational machinery. It is reasonable to expect that the inhibition of protein synthesis in apoptotic cells is the consequence of some structural modification(s) of these molecules. In fact, the 28S ribosomal RNA and translational factors have been shown to undergo degradation in apoptotic cells. It is, however, not clear whether these changes lead to structural changes of ribosomes or the inhibition of protein synthesis.

In contrast, changes in ribosomal proteins appear to be more complex. There are some reported changes of ribosomal proteins in apoptotic cells; P0 degrades or is structurally modified, S1 is translocated to the membrane bleb, and S19 is covalently dimerized and secreted. Moreover, the modified S19 serves as a chemoattractant specific for macrophages. These findings indicate that ribosomal proteins are structurally modified in apoptotic cells and may acquire the extra-ribosomal function. However, the previous studies dealt with only a limited number of ribosomal proteins, and changes in the overall structure of the ribosome in apoptotic cells have not been intensively examined. Here I analyzed the amount, structure and localization of ribosomal proteins in apoptotic human leukemic cells using polyclonal antibodies raised against peptides and subunits corresponding to partial amino acid sequences of 79 human ribosomal proteins.

RESULTS

1) *Decrease in the amount of ribosomal proteins during apoptosis*

1-1) Detection of ribosomal proteins by Western blotting

I first determined changes in the amount of ribosomal proteins during apoptosis. Seventy-nine kinds of rabbit antisera were tested for their ability to bind to native ribosomal proteins by Western blotting. Twenty-eight antisera gave signals at the positions corresponding to the expected molecular masses of 11 small subunit proteins: Sa, S3a, S4X, S8, S11, S12, S14, S15, S18, S24 and S30, and 17 large subunit proteins: L5, L7, L7a, L8, L9, L13, L13a, L14, L15, L17, L18, L28, L31, L34, L35a, L36a and L39. I then examined changes in the amount of these ribosomal proteins in apoptotic cells.

1-2) Decrease of S18, L5, and L14 in apoptotic Jurkat cells

Whole-cell lysates were prepared from Jurkat cells that had been exposed to doxorubicin for various lengths of time, and subjected to Western blotting with the 28 kinds of anti-ribosomal protein antisera. Although most ribosomal proteins did not change upon apoptosis induction, three antisera detected decrease and degradation. The signal of S18 became less intense in doxorubicin-treated cells. Anti-L5 and anti-L14 antisera detected signals with faster mobility that disappeared in the presence of the corresponding antigen peptides, indicating that these signals were derived from degradation products of each ribosomal protein. These changes with three ribosomal proteins all became apparent at 48 h or later after the induction of apoptosis. I concluded that three ribosomal proteins, S18, L5 and L14, undergo degradation at late stages of apoptosis.

1-3) Ribosomal protein degradation during caspase-modiated apoptosis

I next asked if the ribosomal protein degradation occurred during caspase-dependent apoptosis. Jurkat cells were treated with doxorubicin in the presence of the pan-caspase inhibitor z-VAD-fmk, and whole-cell lysates were analyzed for ribosomal proteins by Western blotting. Appearance of the presumed degradation products of L5 and L14 was largely, though not completely, abolished. The decrease in the intensity of intact S18 was only marginally inhibited by z-VAD-fmk. These results indicated that degradation of the three ribosomal proteins occurs in a manner partly dependent on the action of caspases. .

1-4) Absence of the three ribosomal proteins in the ribosome

I next examined whether the above changes are observed in the ribosome complex. To do this, ribosome-enrich fractions were prepared from doxorubicin-treated Jurkat cells and analyzed by Western blotting. Decrease of the three proteins was also observed in the ribosome fraction. However, the presumed degradation products of L5 and L14 were not detectable. These results indicated that the three ribosomal proteins are degraded and dissociated from the ribosome complex.

1-5) Degradation of ribosomal proteins in Jurkat cells undergoing Fas-mediated apoptosis

I next examined if the above findings are restricted to cells that are undergoing doxorubicin-induced apoptosis. Jurkat cells, which express the apopotosis receptor Fas, were incubated with CH11, an agonistic anti-human Fas monoclonal antibody, for various lengths of time, and whole-cell lysates were analyzed for the ribosomal proteins S18, L5, and L14. I found that all the three ribosomal proteins degraded in CH11-treated Jurkat cells in a manner similar to that observed with doxorubicin-treated cells. These results indicated that degradation of ribosomal proteins does not depend on the way of apoptosis induction, but is a phenomenon commonly observed during apoptosis.

2) *Failure in the formation of the 80S ribosome complex during apoptosis*

Absence of the three ribosomal proteins might influence the structural integrity of ribosomes. I thus examined the formation of the ribosome complex in doxorubicin-treated Jurkat cells. When ribosome-enriched fractions prepared from doxorubicin-treated Jurkat cells were separated in an agarose/polyacrylamide composite gel and analyzed by Western blotting. The signal from the 80S complex decreased as the culture period was prolonged whereas that from the 40S or 60S subunit was almost unchanged. These changes became apparent at 48 h after induction of apoptosis, and this is when the ribosomal proteins, S18, L5 and L14, started to degrade. These results indicated that formation of the 80S complex was inhibited in apoptotic Jurkat cells simultaneously with degradation of the three ribosomal proteins.

3) *Decrease in the rate of protein synthesis during apoptosis*

In order to correlate the above findings with changes in the rate of protein synthesis, Jurkat cells that had been treated with doxorubicin for various lengths of time were pulse-labeled with a radiolabeled amino acid, and the amount of labeled proteins was determined by SDS-polyacrylamide gel electrophoresis and autoradiography.

Signals derived from labeled proteins became undetectable 24 h after the induction of apoptosis. These results indicated that the inhibition of protein synthesis precedes the structural changes of ribosomes, that is, degradation of the threeribosomal proteins and disappearance of the 80S ribosome complex.

4) *Translocation of ribosomal proteins to the surface of apoptotic cells*

Some ribosomal proteins have been shown to change their localization during apoptosis: S1 is transferred to the membrane bleb, and S19 is secreted from cells after covalent modification. I therefore conducted three different experiments to examine whether any ribosomal proteins are present on the surface of apoptotic Jurkat cells.

4-1) Flow cytometry

I first conducted a flow cytometry analysis. The seventy-nine antisera were tested if they bound to plasma membrane-permeabilized Jurkat cells more intensely than to intact cells in a manner inhibitable by the presence of corresponding antigen peptides. Twenty-eight antisera were selected including those recognizing 10 small subunit proteins: Sa, S2, S4X, S11, S14, S15, S18, S24, S29 and S30, and 18 large subunit proteins: P0, P2, L3, L4, L5, L6, L7, L10, L14, L23a, L29, L35a, L36, L36a, L37, L38, L39 and L41. Jurkat cells that had been treated with doxorubicin were subjected to flow cytometry with those 28 antisera to determine the surface expression of ribosomal proteins. Six antisera, that recognize S15, P0, L5, L6, L36a and L41, bound to apoptotic cells more efficiently than to normal cells. This suggests that those ribosome proteins are present at the surface of apoptotic Jurkat cells.

4-2) Immunofluorescence

To further confirm the surface expression of ribosomal proteins in apoptotic cells, immunofluorescence analyses were adopted. Of the six antibodies positive in flow cytometry, four that recognize P0, L5, L6 and L36a showed more intense binding to the surface of apoptotic cells than to normal cells.

4-3) Western blotting

Finally, proteins present on the surface of Jurkat cells were isolated and analyzed by Western blotting with three anti-ribosomal protein antisera that were usable for this purpose. Jurkat cells were surface labeled with biotin, and the labeled proteins were affinity·purified using avidin-conjugated magnetic beads. When the isolated surface proteins were analyzed with anti-S15, -L5, and -L36a antisera, signals corresponding to all the three ribosomal proteins were detectable only in the surface proteins prepared from apoptotic cells. These results indicated that the three ribosomal proteins exist at the surface of apoptotic Jurkat cells.

The results from the above three experiments accorded with each other and indicated that five large subunit proteins, P0, L5, L6, L36a and L41, and one small subunit protein, S15 are relocated and expressed at the surface of Jurkat cells undergoing doxorubicin-treated apoptosis.

DISCUSSION

I here analyzed changes in the amount and localization of eukaryotic ribosomal

proteins during apoptosis. The analysis by Western blotting of 28 ribosomal proteins revealed that three of them, S18, L5 and L14, underwent degradation and were dissociated from ribosomes during apoptosis induced by the anticancer drug doxo*rubicin or* an agonistic anti-Fas monoclonal antibody. These results suggested that ribosomes lacking particular ribosomal proteins exist in apoptotic cells. Moreover, I found that the amount of the 80S ribosome complex decreased in apoptotic cells. These changes should make ribosomes unfunctional in protein synthesis. However, they do not seem to be the major cause of the decreased rate of protein synthesis in apoptotic cells, because protein synthesis declined well before the changes in the ribosomal proteins became detectable. I speculate that the degradation of ribosomal proteins occurs to completely inactivate gene expression at the final step in the process of apoptosis.

Another possible role of the structural changes of ribosomes is regulation of the extra-ribosomal function of ribosomal proteins. I found that six ribosomal proteins moved to the surface of apoptotic cells. I hypothesize that these proteins can be marker(s) for recognition of apoptotic cells by phagocytic cells. Apoptotic cells are rapidly and selectively eliminated from the organism by phagocytosis, and this is defined by specific recognition of target cells by phagosytes. Phagocytic cells could recognize the externalized ribosomal proteins and engulf the apoptotic cells. However, this is only a hypothesis and needs to be verified by further studies.

学位論文審査結果の要旨

西田純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2月4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，以下のとおり判定した。

本論文は，アポトーシス過程におけるリボソームの変化について解析したものである。タンパク質合成で中心的な役割を果たすリボソームタンパク質は，アポトーシスに伴って分解や局在性の変化を受ける例が報告されている。しかし，これまでの研究ではごく一部のリボソームタンパク質でしか調べられておらず，また細胞の種類に依存するケースも知られている。本研究では79種類のヒトリボソームタンパク質すべてについて網羅的に解析することを試みた。作製された79種類のウサギ抗血清のうち，実際の解析に使用できたのは28種類であったが，このうち3種類でアポトーシスに伴う抗原リボソームタンパク質の分解を認めた。また，これらの分解はリボソームからの遊離を引き起こし，80S リボソームの形成阻害に導くことが強く示唆された。一方，フローサイトメトリー，および蛍光抗体法を用いて，アポトーシス細胞表層におけるリボソームタンパク質の存在を調べ，6 種類のリボソームタンパク質がアポトーシスの初期に細胞表面へ移行することを見出した。

本研究は，アポトーシスの過程で複数のリボソームタンパク質が構造と局在性の変化を受けることを明らかにしたものであり，リボソームタンパク質の新しいリボソーム外機能を示唆したものと高く評価され，博士（薬学）の学位に値すると判定した。