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SELECTIVE INHIBITION OF DEXAMETHASONE-INDUCED APOPTOSIS IN RAT THYMOCYTES BY HERBIMYCIN A

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Summary: DNA fragmentation and cell death in rat thymocytes induced by dexamethasone were inhibited by herbimycin A but not by the other inhibitors of tyrosine kinase including genistein and tyrphostin. Herbimycin A also prevented the inter-nucleosomal DNA fragmentation induced by dexamethasone. On the contrary, apoptosis induced by DNA topoisomerase inhibitors such as camptothecin and etoposide were not affected by herbimycin A. These results demonstrate that dexamethasone-induced apoptosis is specifically inhibited by herbimycin A.

Apoptosis is a process of active cell death and plays an important role in tissue development, cell differentiation and the maintenance of the cell numbers (1,2). It is well known that the morphological and biochemical characteristics of apoptosis are different from those of necrosis (3). Many compounds such as glucocorticoid (4), inhibitors of DNA topoisomerase (5), Ca^{2+} ionophre (6), and ionizing radiation (7) induce apoptosis in several cells. Recently much attention has been focused on the molecular mechanism of apoptosis. The information about signal transduction pathway of apoptosis is required to understand the molecular mechanism of apoptosis. In spite of many efforts, however, the signal transduction of apoptosis is still unclear.

The involvement of protein phosphorylations in the regulation of various cellular events is well established (8). Some protein kinases are implicated in the regulation of apoptosis.

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Phorbol esters inhibit apoptosis in immature rat thymocytes (9) and interleukin-2-dependent T lymphocytes (10), suggesting that activation of protein kinase c results in the inhibition of this type of cell death. Recently, it has been reported that activation of tyrosine kinase was associated with suppression of apoptosis in hemopoietic cells (11). On the other hand, ionizing radiation that induces apoptosis, stimulates tyrosine kinase activity (12). These results are complicated in the involvement of protein tyrosine phosphorylation in apoptosis. In many systems, inhibitors of tyrosine kinase such as genistein, herbimycin A and tyrphostin are used to evaluate the role of this enzyme in various cellular events. During the study to assess the involvement of protein tyrosine phosphorylation in apoptosis using these inhibitors, we have found that apoptosis induced by dexamethasone is selectively inhibited by herbimycin A but not by genistein and tyrphostin. This paper describes an inhibitory effect of herbimycin A on apoptosis induced by dexamethasone but not by the other inducers such as DNA topoisomerase inhibitors.

MATERIALS AND METHODS

Cell culture of thymocytes. Thymocytes were prepared from immature rats (4 weeks). Isolated thymocytes were suspended with RPMI 1640 medium supplemented 10% fetal calf serum (FCS), and centrifuged at 120 x g for 10 min. Resulting cells were washed twice. Finally thymocytes were suspended with RPMI 1640 medium supplemented with 10% FCS at a density of 10 x 10^6 cells/ml. Genistein and herbimycin A were obtained from Wako Pure Chemicals Co. (Osaka, Japan). Camptothecin and etoposide were from Sigma Chemical Co. (St. Louis, MO). Tyrphostin was from Life Technologies, Inc. (Tokyo, Japan). These chemicals were dissolved with dimethylsulfoxide (DMSO) and added to the medium at the final concentration of DMSO of less than 0.2%. No significant effects of the solvent on DNA fragmentation and cell viability were observed at this concentration. All other reagents were the analytical grade available.

Assays. DNA fragmentation was assayed using diphenylamine (13), and was expressed as the percentage of fragmented DNA to the total DNA (fragmented plus intact DNA). Agarose gel electrophoresis was performed as described previously (4). The cell viability was assessed by the release of lactate dehydrogenase (LDH) into the medium. The activity of LDH was measured spectrophotometrically (14) and was expressed as the percentage of total cellular enzyme activity released into the medium. Data were expressed as mean $\pm$ S.E. from 4 to 6 separate experiments.

RESULTS AND DISCUSSION

The effects of various inhibitors of tyrosine kinase on DNA fragmentation induced by dexamethasone are shown in Fig. 1. The treatment of thymocytes with dexamethasone resulted in an increase in DNA fragmentation as described previously (4). Although herbimycin A at the concentration of 0.3 μM did not affect DNA fragmentation in control cells, DNA fragmentation induced by dexamethasone was completely inhibited by this compound. No effect of the other inhibitors on dexamethasone-induced DNA fragmentation was observed. In the absence of dexamethasone, genistein caused a significant increase in DNA fragmentation. This effect of genistein may be due to inhibit DNA topoisomerase II activity rather than tyrosine kinase (15). The concentrations of herbimycin A, genistein (16,17) and tyrphostin (18) tested in this experiment were enough to inhibit the activity of tyrosine kinase. These compounds are thought to inhibit tyrosine kinase activity based on the different mechanism. Sulfhydryl group is involved in the inactivation of the enzyme by herbimycin A (19). On the other hand, genistein (17) and tyrphostin (18) competes ATP as

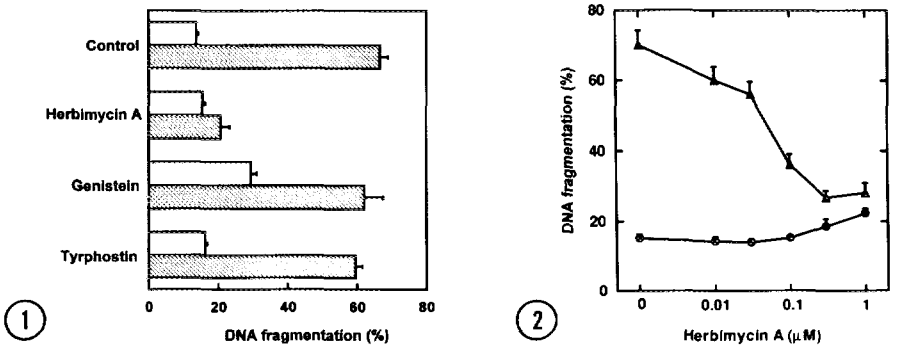

Fig. 1. Effects of various tyrosine kinase inhibitors on DNA fragmentation induced by dexamethasone in rat thymocytes. Thymocytes were incubated for 4 hours in the presence (▨) and absence (□) of 1 μM dexamethasone. The concentrations of herbimycin A, genistein and tyrphostin were 0.3 μM, 100 μM and 20 μM, respectively.

Fig. 2. Effect of herbimycin A on DNA fragmention induced by dexamethasone. Thymocytes were incubated with various concentrations of herbimycin A in the presence (▲) and absence (●) of 1 μM dexamethasone for 4 hours.

the substrate and tyrosine residue of the enzyme, respectively. Therefore, the specificity of herbimycin A for inhibition of dexamethasone-induced DNA fragmentation may be due to the different characteristics for the enzyme inactivation.

The inhibitory effect of dexamethasone-induced DNA fragmentation by herbimycin A was dependent on its concentration (Fig. 2). A significant effect was seen at 0.03 μM herbimycin A. DNA fragmentation induced by dexamethasone disappeared in the presence of more than 0.3 μM of herbimycin. Although herbimycin A had little effect on DNA fragmentation of control cells in this experiment, this compound at the concentration of 1 μM tended to increase in DNA fragmentation. In respect of this point, Azuma *et al.* (15) reported that an incubation of mouse thymocytes with herbimycin A induced apoptosis. Their observation was based on the longer incubation time and higher concentration of herbimycin A than our condition. As shown in Fig. 3, agarose-gel electrophoresis revealed a ladder-like

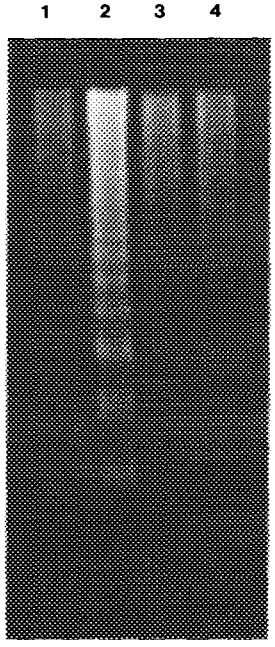

Fig. 3. Effect of herbimycin A on nucleosomal DNA fragmentation induced by dexamethasone analyzed by agarose-gel electrophoresis. Lane 1, control thymocytes incubated for 8 hours; lane 2, thymocytes treated with 1 μM dexamethasone for 8 hours; lane 3, thymocytes treated with 1 μM herbimycin A for 8 hours; lane 4, thymocytes treated with 1 μM dexamethasone and 1 μM herbimycin A for 8 hours.

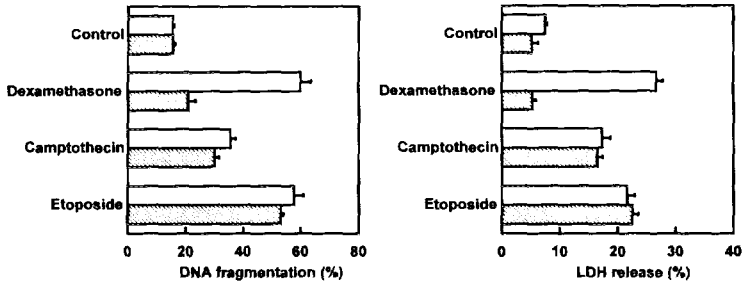

Fig. 4. Effect of herbimycin A on DNA fragmentation and LDH release induced by dexamethasone and DNA topoisomerase inhibitors. Thymocytes were incubated with 1 μM dexamethasone, 10 μM camptothesine and 10 μM etoposide in the presence (▨) and absence (□) of 0.3 μM herbimycin A for 4 hours.

pattern of fragmented DNA extracted from the cells treated with dexamethasone. This ladder pattern of DNA cleavage, the typical biochemical characteristic of apoptosis (4), indicates that dexamethasone causes the inter-nucleosomal DNA fragmentation in thymocytes. Herbimycin A prevented the inter-nucleosomal DNA fragmentation induced by dexamethasone (Fig. 3). In addition to DNA fragmentation, the incubation of thymocytes with dexamethasone caused a significant release of LDH into the medium (Fig. 4). Herbimycin A also prevented the release of LDH induced by dexamethasone. Thus it has been demonstrated that herbimycin A inhibits both DNA fragmentation and cell death release induced by dexamethasone. Fig. 4 also shows camptothecin and etoposide increase both DNA fragmentation and LDH release in rat thymocytes. No effect of herbimycin A on DNA fragmentation and LDH release was observed in the cells treated with these inhibitors of DNA topoisomerase, indicating that herbimycin A is seems to be a specific inhibitor of dexamethasone-induced apoptosis.

It should be noted that herbimycin A specifically inhibits apoptosis induced by dexamethasone but not by DNA topoisomerase inhibitors. Although glucocorticoids induce apoptosis in thymocytes, the molecular mechanism has been still unknown. Some of possible mechanism of the signal transduction of apoptosis by glucocorticoids are postulated; 1) glucocorticoid-induced apoptosis is accompanied by elevation of intracellular concentration of Ca^{2+} in thymocytes, suggesting that sustained elevation of intracellular Ca^{2+} concentration triggers apoptosis (13). 2) bcl-2 blocks glucocorticoid-induced apoptosis when expression of

c-myc is repressed, suggesting that another step in the signal transduction pathway exists between cytostatic phase and the cytolytic phase of apoptosis (20). Generally characteristics of apoptosis induced by glucocorticoids and inhibitors of DNA topoisomerases are quite similar. Thus, it is impossible to discriminate between apoptosis induced by these compounds based on the assay of DNA fragmentation and analysis of DNA cleavage by agarose gel electrophoresis. Recently apoptosis were divided into two process, namely p53 dependent and independent processes (21). Apoptosis induced by ionizing radiation and DNA topoisomerase inhibitors is dependent on the expression of p53. On the other hand, glucocorticoid-induced apoptosis is not involved in the expression of p53 (21). These results indicate that the signal transduction pathway of apoptosis induced by glucocorticoids is differing from that induced by ionizing radiation and DNA topoisomerase inhibitors. The data obtained in this study indicate that herbimycin A is likely to inhibit selectively p-53 independent process of apoptosis. Therefore, herbimycin A is an useful tool for making clear the signal transduction of apoptosis induced by glucocorticoids. The study on the mechanism of inhibition of apoptosis by herbimycin A is now in progress in our laboratory.

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