REVIEW

Modulatory effect of monosodium glutamate on rat thymocyte proliferation and apoptosis

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Abstract

Background: **Monosodium glutamate (MSG) is the sodium salt of glutamic acid, widely spread in modern nutrition. Numerous recent studies have shown the existence of glutamic receptors on different non-neuronal cells, which among others also include lymphocytes and thymocytes.**
Objectives: **The current study was designed to evaluate the prolonged effect of MSG on rat thymocyte proliferation, apoptosis and expression of two apoptosis related proteins, Bcl-2 and Bax.**
Material and methods: **Wistar rats (male) were exposed to monosodium glutamate (MSG) (4 mg/g body wt, i.p.) for 6 consecutive days and sacrificed on 30th and 45th day after last MSG dose. Thymocyte proliferation was evaluated by measuring the expression of proliferating cell nuclear antigen by flow cytometry. Apoptosis was detected using the Annexin V-FITC/PI apoptosis detection kit and cells were analyzed using a flow cytometer. Expression of Bcl-2 and Bax proteins were determined with flow cytometry using respective monoclonal antibodies.**
Results: **The current study results demonstrate that MSG significantly decreased thymocyte proliferation ($p<0.001$) induced by ConA and increased apoptosis rate ($p<0.001$) of the cells during examination period. MSG treatment induced down regulation of Bcl-2 protein while Bax protein levels were not significantly changed.**
Conclusion: **These results indicate that MSG significantly modulates thymocyte proliferation by modulating the apoptosis rate of the cells. The temporal profile of Bcl-2 and Bax expression, after MSG treatment, suggest that down regulation of Bcl-2 protein and resulting change of Bcl-2/Bax protein ratio may be an important event in thymocyte apoptosis, triggered by MSG *(Tab. 1, Fig. 3, Ref. 36)*.**
Key words: monosodium glutamate, thymocytes, apoptosis, Bcl-2, Bax.

One of the commonest food additives in the developed world is monosodium glutamate (MSG), a flavor enhancer. MSG can be found in various concentrations in numerous food products (1). According to its chemical structure, MSG is the sodium salt of glutamic acid (GA). The wide MSG distribution in modern nutrition enables a continuous intake of this substance into organism, which results in accumulation and rise of the GA concentration in blood (2). GA is accepted as an excitatory amino acid neurotransmitter in the mammalian central nervous system. This amino acid acts at multiple receptor type, divided into two main groups: ionotropic glutamate receptors (iGluR) and metabotropic glutamate receptors (mGluR) (3). iGluR (NMDA, AMPA and kainate receptors) form ion channels and mediate fast excitatory glutamate responses, while mGluR are coupled with G proteins and they regulate the activity of a variety of membrane enzymes and ion channels (4). According to their sequence of homology and signal transduction machinery, mGluR are subdivided into Group I (mGlu1 and mGlu5 receptors), Group II (mGlu2 and mGlu3 receptors) and Group III (mGlu4, mGlu6, mGlu7 and mGlu8 receptors) (3). Numerous studies showed that excitotoxicity, induced by excessive activation of glutamate receptors, was associated with some neurodegenerative disorders such as Huntington's and Alzheimer's disease (5, 6). The exact

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mechanism of neuronal cell death, induced by excitotoxins, still remains unknown. However, recent data suggest that necrotic and apoptotic pathways can be activated after over-stimulation of various glutamate receptor subtypes (7, 8).

On the other hand, during the last decade, different studies showed the existence of iGluR and/or mGluR on different non-neuronal cells (9, 4). The initial researches, in the field of human lymphocytes, showed that peripheral lymphocytes bind glutamate with high affinity (10). Later studies indicated that mGluR exist in mice (11), rat thymocytes (12) and human lymphocytes (13). Further researches showed that glutamate increased intracellular concentration of calcium ions in human (14) and rodent lymphocytes (15). However, precise functions of the glutamate receptors on lymphocytes are largely unknown. Therefore, the current study was designed to examine the prolonged effects of MSG on rat thymocyte proliferation and apoptosis and answer the question whether these processes involve changes in Bcl-2 and Bax protein level.

Materials and methods

Animals

Experiments were performed on adult male Wistar rats (120-140g), 8±10 weeks old, bred at the Vivarium of the Institute of Biomedical Research, Medical faculty, Nis, under conventional laboratory conditions.

Materials

Culture medium (CM) was prepared using RPMI 1640 (Sigma, St Louis, Mo., USA), according to the manufacturer instructions. CM containing 25 mM HEPES, 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml) and 10 % fetal calf serum (FCS).

Concanavalin A (ConA) was purchased from Sigma, Munich, Germany. ConA was dissolved in CM at concentration of 5 µg/ml.

Monosodium glutamate (MSG) was obtained from Fluka Chemika AG, Buchs, Switzerland.

The following anti-rat monoclonal antibodies were purchased from Immunotech (Marseille, France): Anti-PCNA (anti-proliferating cell nuclear antigen), anti-Bcl-2 and phycoerythrin (PE)-conjugated anti-rat IgG (H+L). Anti-Bax monoclonal antibody was obtained from Sigma, St Louis, Mo., USA.

Monosodium glutamate animal treatment

Experimental animals were injected intraperitoneally (i.p.), with 1 ml physiologic saline containing MSG (4 mg/g of body weight) and their respective controls (control animals) with 1ml physiologic saline, for 6 consecutive days. Animals (experimental and control) were sacrificed (using ether anesthesia) on 30th and 45th day after last MSG dose.

Preparation of thymocytes and cell culture

The thymus (from experimental and control animals) was extirpated using sterile technique and placed in CM/10 % FCS. Thymocytes were released by teasing thymus through a steel-mesh. Cell suspensions were filtered through sterile nylon-filter to remove stroma and then the cells were washed twice with CM/10 % FCS. Thymocytes were counted and adjusted to a density of $1x10^7$ cells/ml. Cells were cultured in 96-well flat-bottom plates (Sarstedt, Newton, USA), containing a 100 µl of cell suspension ($1x10^6$ cells) in each well. For further evaluation of the proliferative activity, thymocytes were treated with optimal (5 µg/ml) concentration of ConA. All cultures were done in triplicates. The thymocytes were cultured for 72 hours in an incubator (Assab, Sweden) at 37 °C in an atmosphere of 95 % air and 5 % carbon dioxide.

Proliferation assay

We used flow cytometric analysis to measure lymphocyte proliferation by measuring the expression of proliferating cell nuclear antigen (PCNA), an auxiliary cyclin protein necessary for DNA polymerase, maximally expressed in mid S-phase (16). Proliferative activity of thymocytes was evaluated after 72 hours incubation, by using anti-PCNA monoclonal antibody, according to the manufacturer instructions, with minor modifications. Briefly, at the end of the culture period, cells were collected and washed twice in PBS containing 5 % FCS. After that, the cells were fixed in 70 % methanol, for 30 min, at -20 °C. Cells were washed twice with PBS containing 5 % FCS to remove the methanol and incubated in the dark for 1 h at room temperature, with anti-PCNA monoclonal antibody (final concentration 5 µg/ml). After incubation, cells were washed twice and incubated 45 minutes at room temperature with appropriate dilutions of PE-conjugated anti-rat IgG monoclonal antibody. Non-specific binding was detected by the control cells which were incubated with the secondary antibody (PE-conjugated anti-rat IgG) alone. Labeled cells were fixed in 4 % formalin and analyzed (5000 analyzed cells/per sample) on a flow cytometer (Coulter XL-MCL, Krefeld, Germany).

Detection of apoptosis (flow cytometry)

Detection of apoptosis by flow cytometry was performed using the Annexin V-FITC/Propidium iodide (PI) apoptosis detection kit (Immunotech, Marseille, France). Freshly isolated thymocytes (from control and experimental group) were washed with cold PBS containing 5 % FCS. Cells were counted and adjusted to a density of $1x10^6$ cells/ml. 100 µl of cell suspension ($1x10^5$ cells/tube) were resuspended in 100 µl of cold 1x annexin V binding buffer. Annexin V-FITC (5 µl) and PI (2.5 µl) were added to each sample and incubated on ice for 10 minutes in the dark. Cell suspensions were raised to a final volume of 500 µl with cold 1x annexin V binding buffer, placed on ice and kept in the dark until flow cytometric analysis (cells were analyzed by flow cytometry within 30 min). To set up compensation and quadrants, following controls were used: unstained cells, cells stained only with Annexin V-FITC (no PI) and cells stained only with PI (no Annexin V-FITC). Annexin V is a Ca^{2+} dependent phospholipids-binding protein that has a high affinity for phosphodylserine (PS), translocated from the inner leaflet of the plasma membrane to the outer leaflet in apoptotic cells (17). Annexin V-FITC is a sensitive probe for identifying cells that are undergoing apoptosis, because PS exposure occurs early in the apoptotic

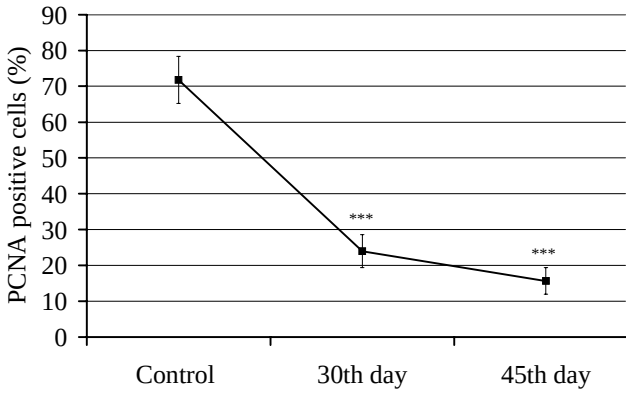

Fig. 1. Effect of MSG on proliferation of rat thymocytes triggered by ConA. Thymocytes from control, T30 and T45 animals were cultivated for 72 hours with ConA (5 μg/ml) and than stained with anti-PCNA monoclonal antibody as previously described. The intensity of thymocyte proliferation was evaluated by measuring the percentage of proliferating cell nuclear antigen (PCNA) positive cells. Values are given as mean percentage±SD of one representative experiment (out of four experiments with similar results).
*Abbreviations: T30 – Animals sacrificed at 30th day after glutamate administration, T45 – Animals sacrificed at 45th day after glutamate administration, ConA-Concavalin A. *** p<0.001 compared to control (non-treated) animals.*

process (18). Cells that are stained positive for Annexin V-FITC and negative for PI were treated as apoptotic cells in the early stage of apoptosis. Thymocytes that stained positive for both Annexin V-FITC and PI represet cells in the late stage of apoptosis. Cells that are stained negative for both Annexin V-FITC and PI were alive whereas only PI positive cells were necrotic cells.

Flow cytometric evaluation of Bcl-2 and Bax levels

The levels Bcl-2 and Bax were measured by flow cytometry as described previously (19), with minor modifications. Briefly, after isolation, thymocytes (from control and experimental group) were collected and washed with PBS containing 5 % FCS. Permeabilization of thymocytes ($1x10^6$ cells/tube) was done using saponin-based permeabilization reagent IntraPrep™ (Immunotech, Marseille, France), according to the manufacturer instructions. Cells were incubated in the dark for 45 minutes at room temperature with anti-Bcl-2 monoclonal antibody (final concentration 2 μg/ml) and anti-Bax monoclonal antibody (final concentration 10 μg/ml). After incubation, cells were washed twice in PBS containing 5 % FCS and incubated 30 minutes in the dark, at room temperature, with PE-conjugated anti-rat IgG monoclonal antibody (diluted 1:100). Labeled cells were fixed in 4 % formalin and analyzed (5000 analyzed cells/per sample) on a flow cytometer. Non-specific binding was detected by the control cells, which were incubated with the secondary antibody alone.

Statistical analysis

Results are presented as the mean±SD of four independent experiments or triplicate samples. Significant differences between the groups were analyzed with Student's t-test.

Results

Modulatory effect of MSG on proliferation of thymocytes stimulated with Con A

The prolonged effects of repeated MSG doses, on the proliferation of rat thymocytes, triggered by optimal (5 μg/ml) concentration of ConA, were tested at 30th and 45th day after MSG treatment. The obtained results, presented in Figure 1, show that MSG administration to animals, significantly decrease thymocyte proliferation, at the 30th day after last MSG dose ($p<0.001$), as compared to proliferation of thymocytes from control (non-treated) animals. As shown in Figure 1, decrease of thymocyte proliferation continued, with maximal inhibition observed at the 45th day after MSG administration.

Effect of MSG on apoptosis rate of rat thymocytes

To investigate whether MSG induced inhibition of proliferation, mediated by increased thymocyte apoptosis, in next experiments we evaluated the effect of MSG administration on rat thymocyte apoptosis. The influence of MSG treatment on thymocyte apoptosis was tested at 30th and 45th day after last MSG dose. We showed that MSG, at concentrations of 4 mg/g of body weight, was able to trigger apoptosis of rat thymocytes, as determined by staining with Annexin-V-FITC/PI (Table 1, Figure 2). Results given in Table 1, show that the percentages of apoptotic cells were statistically higher in animals treated with MSG, than in

Tab. 1. Effect of MSG treatment on apoptosis of rat thymocytes.

Group	Apoptosis (Flow cytometry)			Necrosis %
	Total (%)	Early (%)	Late (%)	
Control	38.2±4.97	36.22±4.86	1.97±0.54	0.87±0.82
T30	80.3±2.18***	20.47±3.7	59.82±5.31	2.02±0.78
T45	84.85±3.29***	13.75±2.52	71.1±5.74	51±1.11

Thymocytes were isolated at 30th and 45th day after MSG administration and then stained with Annexin V-FITC/PI apoptosis detection kit, as previously described. Total percentages of Annexin-FITC positive cells are considered as total percentage of apoptotic cells. Annexin-FITC+/PI- are cells in the early stage of apoptosis whereas Annexin-FITC+/PI+ are cells in the late stage of apoptosis. Values are mean±S.D. of four experiments with 3 rats in each group.
Abbreviations: T30 – Animals sacrificed at 30th day after glutamate administration, T45 – Animals sacrificed at 45th day after glutamate administration. *** p<0.001 compared to control (non-treated) animals.

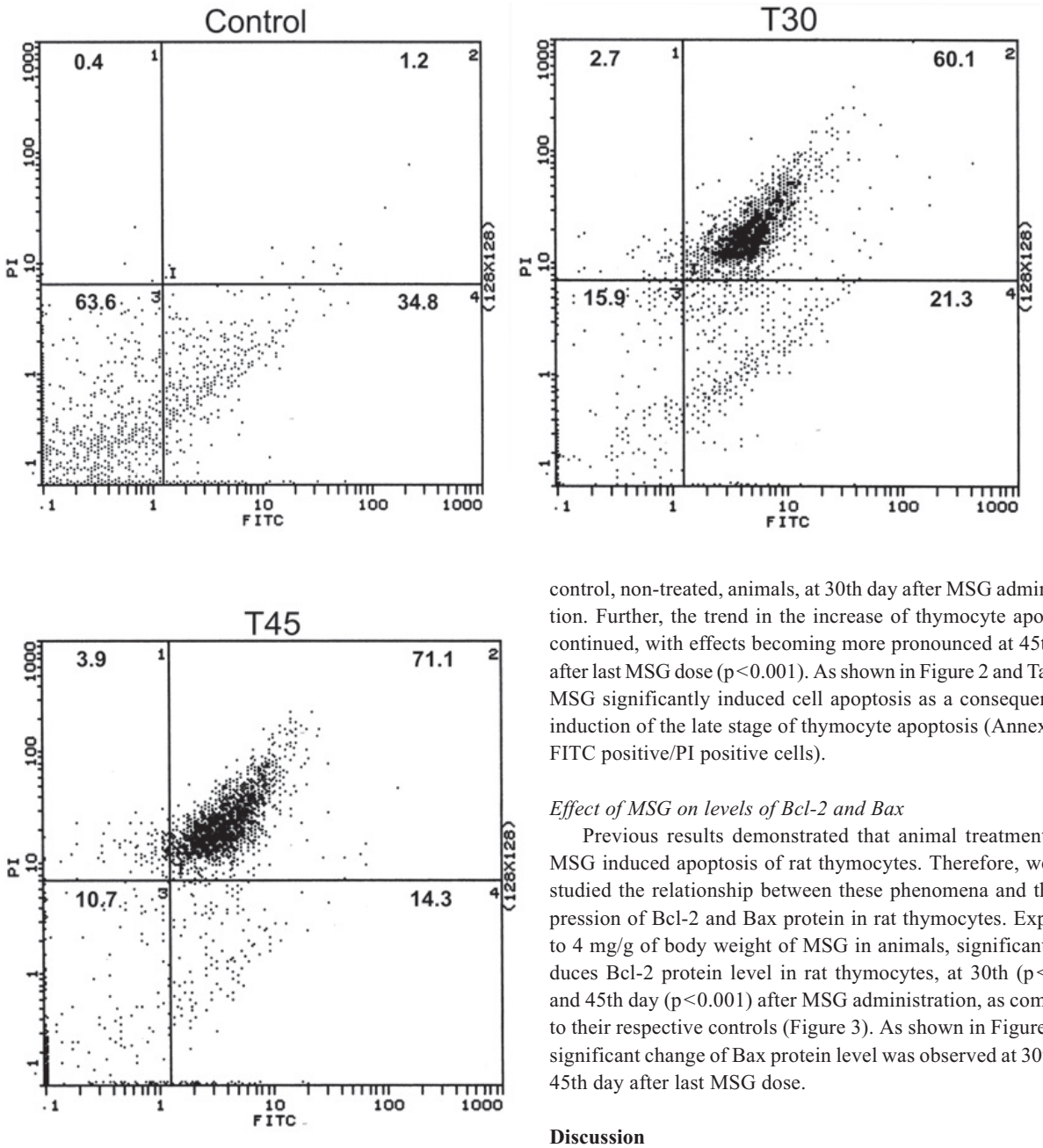

Fig. 2. Effect of MSG treatment on apoptosis of rat thymocytes (flow cytometric analysis). Thymocytes were isolated at 30th and 45th day after MSG administration and then stained with Annexin V-FITC/PI apoptosis detection kit, as described in Material and Methods. Results given in percentage of one representative experiment are presented. 1. Annexin- FITC -/PI + (necrotic cells); 2. Annexin-FITC +/PI + (late phase of apoptosis); 3. Annexin-FITC -/PI - (viable cells); 4. Annexin-FITC +/PI - (early phase of apoptosis).
Abbreviations: T30 – Animals sacrificed at 30th day after glutamate administration, T45 – Animals sacrificed at 45th day after glutamate administration, Control – Animals treated without MSG.

control, non-treated, animals, at 30th day after MSG administration. Further, the trend in the increase of thymocyte apoptosis continued, with effects becoming more pronounced at 45th day after last MSG dose ($p<0.001$). As shown in Figure 2 and Table 1, MSG significantly induced cell apoptosis as a consequence of induction of the late stage of thymocyte apoptosis (Annexin-V-FITC positive/PI positive cells).

Effect of MSG on levels of Bcl-2 and Bax

Previous results demonstrated that animal treatment with MSG induced apoptosis of rat thymocytes. Therefore, we next studied the relationship between these phenomena and the expression of Bcl-2 and Bax protein in rat thymocytes. Exposure to 4 mg/g of body weight of MSG in animals, significantly reduces Bcl-2 protein level in rat thymocytes, at 30th ($p<0.01$) and 45th day ($p<0.001$) after MSG administration, as compared to their respective controls (Figure 3). As shown in Figure 3, no significant change of Bax protein level was observed at 30th and 45th day after last MSG dose.

Discussion

Apoptosis, or programmed cell death, is a naturally occurring cell death process, essential for the normal development and homeostasis of all multicellular organisms. This process plays a very significant role in removing autoreactive or non-functional T cells, during their development within thymus (20). Many interactions occur between microenvironmental cells in the thymus and differentiating thymocytes which are under neuroendocrine control. Intrathymic production of a variety of hormones and neuropeptides modulate intrathymic T cell differentiation, thymic cell proliferation and apoptosis (21).

In the present study, we showed that high MSG concentrations inhibit thymocyte proliferation in response to ConA, at 30th

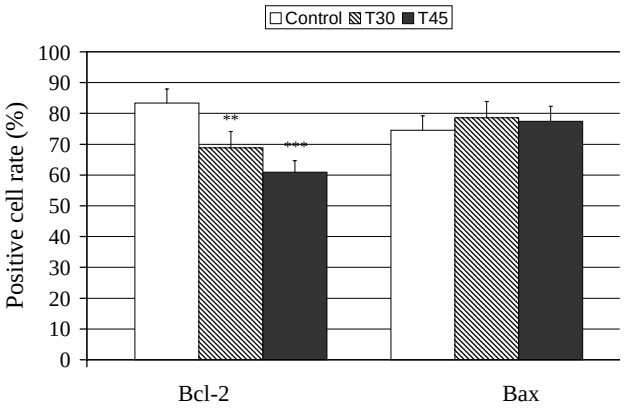

Fig. 3. Flow cytometric analysis of MSG regulation of Bcl-2 and Bax levels (cell positive rate) in rat thymocytes. After isolation, thymocytes were incubated with anti-Bcl-2 and anti-Bax monoclonal antibodies as described in Material and Methods. Cell positive rates were determined by using flow cytometric analysis. Results are given as mean percentage±SD of triplicate samples of one representative experiment (out of three experiments with similar results).
*Abbreviations: T30 – Animals sacrificed at 30th day after glutamate administration, T45 – Animals sacrificed at 45th day after glutamate administration. ** $p<0.01$, *** $p<0.001$ compared to control (non-treated) animals.*

and 45th day after last MSG dose. Evidence for a causal relationship between glutamate concentrations and immunological reactivity has already been reported (22). Recent reports indicated that glutamate inhibited, in a concentration-dependent manner, lymphocyte proliferation (13, 14). This inhibition was probably mediated by the constitutively expressed mGlu5 receptors (23). Rezzani et al showed that mGlu5 receptors are strongly expressed in rat thymocytes (12). Activation of mGlu5 receptors produces increases in intracellular Ca^{2+} (24), which activate a cascade of reactions that play a pivotal role in cell growth, cell differentiation and cell survival (25). Taken together with our results, it appears that activation of glutamate receptors generates an intracellular calcium influx in thymocytes, suggesting that MSG may play a significant role in modulation of thymocyte functions and have important secondary immunological consequences.

Based on the obtained results of thymocyte proliferation, we hypothesized that MSG induced inhibition was due to cell apoptosis. By using Annexin V-FITC/PI staining, our results show that the cells are dying via apoptotic mechanism. Spontaneous thymocyte apoptosis, which was observed in control animals, might be a consequence of rapid apoptosis of thymocytes in vitro. It is believed today that such a spontaneous apoptosis of thymocytes is due to absence of different survival factors provided in vivo by the thymic microenvironment, such as soluble factors and signals, generated through direct cell-cell contacts (26). Furthermore, our findings demonstrate that animal treatment with MSG resulted in increased thymocyte apoptosis at 30th day after last MSG dose. Thymocyte apoptosis, during examination period, continued with increased number of apoptotic and necrotic thymocytes at 45th day after MSG treatment. Obtained results support our hypothesis that MSG induced inhibition of thymocyte proliferation was a consequence of increased thymocyte apoptosis. These findings are in agreement with previous reports, which indicated, that glutamate induced cell death may be the result of apoptosis and necrosis (7, 8). Thymic epithelial cells are target for various neuropeptides and neurotransmitters that are implicated in intercellular communication (21). Storto et al showed the existence of mGlu receptors on mouse thymic stromal cells and thymocytes (11). These findings can suggest possibility that these receptors have a role in the intrathymic lympho-stromal relationships, regulating thymocyte survival and differentiation.

It has been reported that numerous number of genes and their proteins were involved in the process of apoptosis. The roles of these apoptosis related factors may be cell type dependent as well as injury type dependent (27). By using flow cytometric analysis, we studied the changes of the protein level of two important apoptosis related genes (Bcl-2 and Bax protein level) in rat thymocytes. We found that animal treatment with MSG induced a significant decrease of Bcl-2 protein expression, at the end of 30th and 45th day after MSG administration. It appeared that Bcl-2 protein expression was an important apoptosis regulatory factor in MSG induced apoptosis of rat thymocytes. These findings are in agreement with reports demonstrating that glutamate decrease Bcl-2 protein level in different cell type (28), while Bcl-2 overexpression rescues cells from glutamate induced apoptosis (29, 30). Bcl-2 and Bax are members of a family of genes that can regulate the apoptotic process. There are two basic classes of Bcl-2 protein family. One class includes Bcl-2 and Bcl-x proteins which tend to inhibit apoptosis, while the other class is made up of Bax, Bak and Bim proteins, which promote apoptosis (3). Due to the ability of the Bcl-2 family members to form homodimers or heterodimers, these proteins are able to function either independently or together in regulation of apoptosis. Heterodimerization between Bcl-2 and Bax protein is an important determinant of cell survival (27). Previous reports showed that glutamate induced Ca^{2+} influx and disruption of the inner transmembrane potential of the mitochondria, which result in opening the mitochondria permeability transition pore (31, 32). During mitochondrial dysfunction, several essential players of apoptosis, including procaspases, cytochrome C, apoptosis inducing factor and apoptosis protease-activating factor 1 (APAF-1) are released in the cytosol. The complex formed of cytochrome C, APAF-1 and caspase 9 leads up to a chain activation of other caspases, which results in apoptosis (27). Bcl-2 is localized on the cytoplasmic surface of the mitochondrial membrane where it may sense damage to this organelle, act to alter the influx of Ca^{2+} ions and bind proteins (Apaf-1) involved in apoptosis. Bax can antagonize function of Bcl-2 protein through forming homodimers, (able to form membrane pores), which may liberate cytochrome c and start apoptotic process. Also, Bax modulate Bcl-2 protein ability to bind Apaf-1. Unbound Apaf-1 may induce programmed cell death pathway (3, 27). In present study, the glutamate induced down regulation of Bcl-2 and it may disturb

mitochondrial function so that the caspase activating factors were released into cytosol. These findings correlate with increased thymocyte apoptosis at 30th and 45th day after MSG administration. On the other hand, Bax protein levels, at the same time period, were not significantly changed in our study. We propose that the Bcl-2/Bax ratio rather then the Bax level is the important determinant for the induction of apoptosis in thymocytes by MSG. Bax has been reported to be up regulated during apoptosis in several types of cells, together with decrease the Bcl-2 protein level (28). Further, Bax deficiency has been reported to rescue the death of double positive thymocytes in Bcl-2 deficient mice (33). However, there is accumulated evidence suggesting that the levels of Bcl-2 and Bax may influence the sensitivity of cells to the mediators of programmed cell death (8, 34, 35). Cell survival is affected by homodimerization and heterodimerization of Bcl-2 and Bax, as well as by the modulation of Bcl-2/Bax protein ratio (19, 36).

Finally, it can be summarized that the predominant effect of prolonged MSG exposure, in this model of thymocyte activation, concerns to its modulatory influence on thymocyte apoptosis. It seems that the decreased level of Bcl-2 protein and resulting change of Bcl-2/Bax ratio may be an important event, which may cause the activation of apoptotic process. The potential mechanisms involved in glutamate regulation of Bcl-2 and Bax proteins remain to be determined. The experiments are currently in progress.

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