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Effect of Monosodium Glutamate on Apoptosis and Bcl-2/Bax Protein Level in Rat Thymocyte Culture

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Summary

Monosodium glutamate (MSG), the sodium salt of glutamate, is commonly used as a flavor enhancer in modern nutrition. Recent studies have shown the existence of glutamate receptors on lymphocytes, thymocytes and thymic stromal cells. In this study, we evaluated the *in vitro* effect of different MSG concentrations on rat thymocyte apoptosis and expression of two apoptosis-related proteins, Bcl-2 and Bax. Rat thymocytes, obtained from male Wistar rats, were exposed to increasing concentrations of MSG (ranging from 1 mM to 100 mM) for 24 h. 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. Exposure to MSG resulted in a dose-dependent decrease in cell survival (as determined by trypan blue exclusion method). Annexin V-FITC/PI also confirmed that MSG increased, in a dose-dependent manner, apoptotic cell death in rat thymocyte cultures. MSG treatment induced downregulation of Bcl-2 protein, while Bax protein levels were not significantly changed. Our data showed that MSG significantly modulates thymocyte apoptosis rate in cultures. The temporal profile of Bcl-2 and Bax expression after MSG treatment suggests that downregulation of Bcl-2 protein and the resulting change of Bcl-2/Bax protein ratio may be an important event in thymocyte apoptosis triggered by MSG.

Key words

Monosodium glutamate • Thymocytes • Apoptosis • Bcl-2 • Bax

Introduction

Glutamate is an excitatory neurotransmitter in the central nervous system of mammals, where it acts through ionotropic (iGluR) and metabotropic (mGluR) glutamate receptors. iGluR (NMDA, AMPA and kainate receptors) form ion channels permeable to particular cations, while mGluR activate intracellular signaling mechanisms *via* several associated G proteins (Storto *et al.* 2000). On the basis of their sequence homology, pharmacological characteristics and second-messenger coupling, mGluR have been classified into Group I (mGlu1 and mGlu5 receptors), Group II (mGlu2 and mGlu3 receptors) and Group III (mGlu4, mGlu6, mGlu7 and mGlu8 receptors) (Hinoi *et al.* 2004). Recent data showed the expression of glutamate receptors on different non-neuronal cells (Gill and Pulido 2001, Skerry and Genever 2001, Hinoi *et al.* 2004). Among non-neuronal

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cells, iGluR and mGluR have been identified in human lymphocytes (Lombardi *et al.* 2001, Ganor *et al.* 2003, Pacheco *et al.* 2004). Further studies indicated that mGluR also exist in mice (Storto *et al.* 2000) and rat thymocytes (Rezzani *et al.* 2003). Glutamate increased intracellular concentration of calcium ions in human (Lombardi *et al.* 2004) and rodent lymphocytes (Boldyrev *et al.* 2004). However, a precise function of glutamate receptors on lymphocytes is largely unknown.

The neurotoxicity, induced by excessive activation of glutamate receptors, has been associated with diverse neurodegenerative diseases (Gill *et al.* 2000) as well as with the excitotoxicity after ingestion of glutamate in the form of monosodium glutamate (MSG) when consumed in high concentrations (Choudhary *et al.* 1996). The exact mechanism of neuronal cell death, induced by excitotoxins, still remains unknown. However, there is accumulating evidence suggesting that glutamate-induced toxicity can be mediated through necrosis and apoptosis (Martin *et al.* 2000, Ankarcrona *et al.* 1998). Consequences of glutamate release and distribution patterns of its receptors have been mainly studied in the central nervous system but also in several other organs (Storto *et al.* 2001, Gill *et al.* 1999) but not in thymus. Therefore, the current study was designed to examine the *in vitro* effect of MSG on rat thymocyte apoptosis and to answer the question whether this process involves changes in Bcl-2 and Bax protein level.

Methods

Animals

Experiments were performed on adult male Wistar rats (120-140 g), 8-10 weeks old, bred at the Vivarium of the Institute of Biomedical Research, Medical Faculty, Nis, under conventional laboratory conditions. The experimental animals were treated in accordance with national animal protection guidelines.

Materials

Culture medium (CM) was prepared using RPMI 1640 (Sigma, St Louis, Mo., USA), according to the manufacturer's instructions. CM containing 25 mM HEPES, 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml) and 10 % fetal calf serum (FCS). Monosodium glutamate (MSG) was obtained from Fluka Chemika AG (Buchs, Switzerland).

The following monoclonal antibodies were purchased from Immunotech (Marseille, France): mouse anti-rat Bcl-2 (clone 5D4) and goat $F(ab`)_2$ phycoerythrin (PE)-conjugated anti-mouse IgG (H+L). Mouse anti-rat Bax (clone 6A7) monoclonal antibody was obtained from Sigma (St Louis, MO, USA).

Preparation of thymocytes and cell culture

The thymus 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 with increasing concentrations of MSG, ranging from 1 mM to 100 mM. All cultures were treated in triplicates. The thymocytes were cultured for 24 h in an incubator (Assab, Sweden) at 37 °C in an atmosphere of 95 % air and 5 % carbon dioxide.

Determination of cell viability

Thymocytes were cultivated in 96-well flat-bottom plates ($1x10^6$ cells/well; 200 µl) with increasing concentration of MSG (1-100 mM). Cell viability was evaluated after 24 h incubation by the trypan blue exclusion method. The percentages of viable cells were calculated on the basis of total number of cells before cultivation.

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). The staining was performed according to the producer's manual. Five thousand cells per sample were analyzed using an Epics®XL flow cytometer (Coulter, Krefeld, Germany). Annexin V is a Ca^{2+}-dependent phospholipids-binding protein that has a high affinity for phosphatidylserine (PS), translocated from the inner leaflet of the plasma membrane to the outer leaflet in apoptotic cells (Vermes *et al.* 1995). Annexin V-FITC is a sensitive probe for identifying cells that are undergoing apoptosis, because PS exposure occurs early in the apoptotic process (Koopman *et al.* 1994). PI is a non-specific DNA dye that is excluded from living cells with intact plasma membranes but incorporated into nonviable cells (Eray *et al.* 2001). When normal thymocytes are cultured *in vitro*, they undergo spontaneous apoptosis and

Table 1. *In vitro* effect of MSG on rat thymocyte apoptosis and viability.

Culture conditions	Apoptosis (flow cytometry)			Viability (trypan blue exclusion)
	Total (%)	Early (%)	Late (%)	Total (%)
Medium	25.8±3.47	22.3±3	3.5±2.17	67.4±4.2
Medium+1 mM MSG	41.6±7.6*	24.93±3.7	16.7±4.9	55.8±5.2*
Medium+10 mM MSG	54.3±5.9**	19.03±2.7	35.3±7.6	39.2±4.2**
Medium+100 mM MSG	64.6±5.5***	14.7±3.9	50.2±5	26.5±5.1***

Thymocytes ($1x10^6$ cells/well) were cultivated for 24 h with increasing concentrations of MSG. Cell viability was determined by trypan blue exclusion method, whereas apoptosis was measured using flow cytometry as previously described. Total percentages of Annexin-FITC positive cells were considered as total percentage of apoptotic cells. Annexin-FITC +/PI – were cells in the early stage of apoptosis whereas Annexin-FITC +/PI + were cells in the late stage of apoptosis. Values are given as mean percentage ± SD for three separate experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to medium controls.

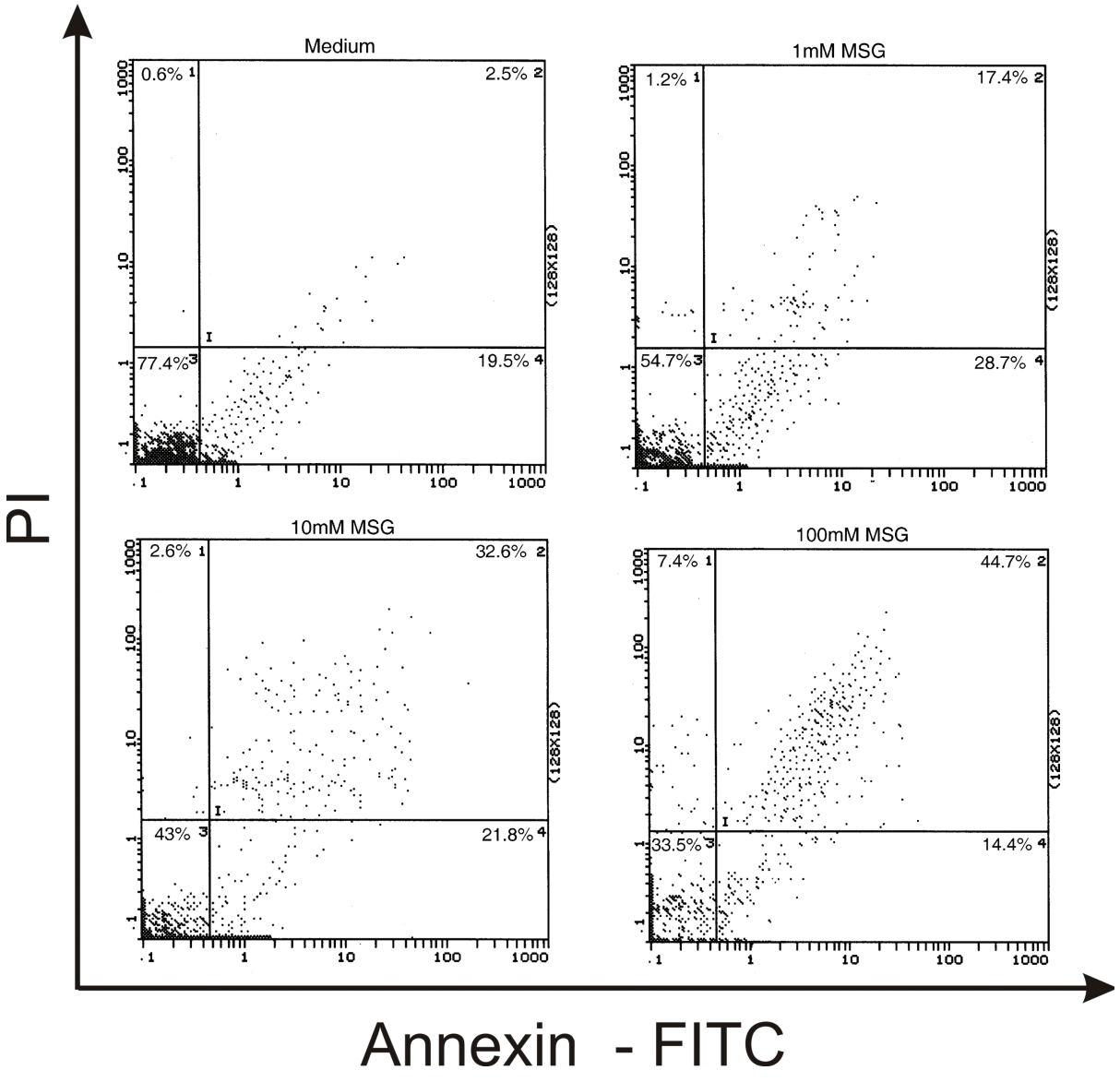

Fig. 1. Effect of MSG on rat thymocyte apoptosis (flow cytometric analysis).

appear initially as Annexin V+/PI– cells. However, with increasing culture duration, the same cells appear as Annexin V+/PI+ cells (Okasha *et al.* 2001). Thus, as single positive populations are considered the early apoptotic (Annexin V+/PI−) or necrotic cells (Annexin V−/PI+), whereas double positive (Annexin V+/PI+) cells are thought to be in a late stage of apoptosis rather than in necrosis (Okasha *et al.* 2001, Vermes *et al.* 1995, Lecoeur *et al.* 2001).

Flow cytometric evaluation of Bcl-2 and Bax levels

The levels Bcl-2 and Bax were measured by flow cytometry as described previously (Antonella *et al.* 1992, Liu and Zhu 1999), with minor modifications. Briefly, thymocytes were cultivated in CM/10 % FCS without or with different concentrations of MSG (ranging from 1 mM to 100 mM) for 24 h. Thereafter the cells were collected, washed twice with in PBS containing 5 % FCS. Permeabilization of thymocytes was done using saponin-based permeabilization reagent IntraPrep™ (Immunotech, Marseille, France), according to the manufacturer instructions. Cells were incubated in the darkness for 45 min at room temperature with anti-rat Bcl-2 monoclonal antibody (final concentration 2 μg/ml) and anti-rat Bax monoclonal antibody (final concentration 10 μg/ml). After incubation, cells were washed twice in PBS containing 5 % FCS and incubated in the darkness, at room temperature, for 30 min with PE-conjugated anti-mouse IgG monoclonal antibody (diluted 1:100). Non-specific binding was detected by the control cells which were incubated with the secondary antibody (PE-conjugated anti-mouse IgG) alone. Labeled cells were fixed in 4 % formalin and analyzed (5000 analyzed cells/per sample) on a flow cytometer.

Statistical analysis

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

Results

MSG induces cytotoxicity in rat thymocytes

To investigate the dose response of MSG, thymocytes were cultured with increasing concentrations of MSG (1-100 mM) for 24 h and assayed for cell viability. Exposure to increasing concentrations of MSG resulted in a dose-dependent decrease in cell viability. A significant increase in cytotoxicity was detected following treatment with 1 mM ($p<0.05$), 10 mM ($p<0.01$) and 100 mM ($p<0.001$) MSG (Table 1).

Effect of MSG on apoptosis rate

To examine whether MSG-induced cytotoxicity involves apoptosis, we evaluated the effect of increasing MSG concentrations on rat thymocyte apoptosis. We showed that MSG, at concentrations ranging from 1 mM to 100 mM, was able to trigger apoptosis of rat thymocytes, as determined by staining with Annexin-V-FITC/PI (Fig. 1). The staining shows a significant dose-dependent increase in the percentage of apoptotic cells in the total population over controls (Table 1). As shown in Figure 1 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 Bcl-2 and Bax levels

Since our previous results demonstrated that *in vitro* treatment with MSG induced apoptosis of rat thymocytes, we studied the relationship between these phenomena and the expression of Bcl-2 and Bax proteins in rat thymocytes. The level of Bcl-2 and Bax in rat thymocytes was determined by flow cytometry, using cells cultured with increasing concentrations (1-100 mM) of MSG for 24 h. As shown in Figure 2, MSG administration induced significant down-regulation of Bcl-2 protein in rat thymocyte cultures. No significant changes in the level of Bax protein in thymocyte cultures were detected at the end of incubation period (Fig. 2).

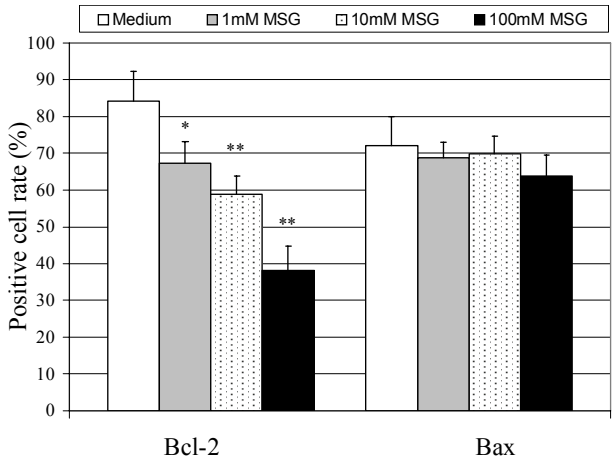

Fig. 2. Flow cytometric analysis of Bcl-2 and Bax levels in rat thymocytes treated with increasing concentrations of MSG.

Discussion

Apoptosis or programmed cell death of developing thymocytes is an active signal-dependent process during which autoreactive or non-functional T cells are eliminated. This process occurs intrathymically during the process of differentiation and maturation of thymocytes (Savino and Dardenne 2000). Thymocytes develop in close apposition to the thymic stromal cells that provide specific microenvironmental signals able to sustain T cell proliferation and maturation (Storto *et al.* 2000). Various hormones, neuropeptides and neurotransmitters originating from neuroendocrine system, as well as those produced intrathymically, modulate intrathymic T cell differentiation, thymic cell proliferation and apoptosis (Savino and Dardenne 2000).

In the present study we showed that MSG was able to induce cytotoxicity in rat thymocyte cultures. By using Annexin V-FITC/PI staining, we have showed that the cells are dying *via* apoptotic mechanism as a result of the MSG-induced cell death. Obtained results support our hypothesis that MSG-induced cytotoxicity was a consequence of increased thymocyte apoptosis. The apoptosis rate of control cultures might be a consequence of rapid apoptosis of thymocytes *in vitro*. It is believed that such a spontaneous apoptosis of thymocytes is due to the absence of different survival factors provided *in vivo* by the thymic microenvironment, such as soluble factors and signals, generated through direct cell-cell contacts (Baumann *et al.* 2000). Furthermore, our findings demonstrate that the exposure to MSG resulted in significantly increased thymocyte apoptosis that was concentration-dependent. These findings correlated with a significant decrease in the proportion of viable cells in cultures, as determined by trypan blue exclusion test. Obtained results are in agreement with previous reports, which indicated that glutamate-induced cell death may be the result of apoptosis and necrosis (Martin *et al.* 2000, Schelman *et al.* 2004), depending on the severity of stimulation (Bonfoco *et al.* 1998). It is well known that interactions between thymocytes and stromal cells are essential for the development of both T cells precursors and thymus stroma (Ritter and Boyd 1993). Thymic epithelial cells are also a target for various neuropeptides and neurotransmitters that are implicated in intercellular communication (Savino and Dardenne 2000). Recent report has shown the existence of mGlu receptors on mouse thymic stromal cells and thymocytes, suggesting a possible role of these receptors in signaling within thymus (Storto *et al.* 2000). Rezzani *et al.* (2003) showed that mGlu5 receptors are strongly expressed in rat thymocytes. Activation of mGlu5 receptors produces increases in intracellular Ca^{2+} (Miglio *et al.* 2005), which activate a cascade of reactions that play a pivotal role in cell growth, differentiation and survival (Bootman *et al.* 2001). Taken together with our results, it appears that the activation of glutamate receptors has a role in the intrathymic lympho-stromal relationships, as well as in modulation of thymocyte functions. The immunological relevance of this modulation remains to be clarified, but we can presume that MSG may play a significant role in regulation of thymocyte survival and differentiation with important secondary immunological consequences.

The bcl-2 family of proto-oncogenes encodes specific proteins, which regulate programmed cell death under different physiological and pathological conditions (Antonsson and Martinou 2000). In the present study, we examined the temporal changes in the protein level of two important apoptosis-related genes (Bcl-2 and Bax) in rat thymocytes subjected to MSG treatment. Using flow cytometric analysis, we found that Bcl-2 protein level is significantly downregulated following increased MSG exposure, suggesting that Bcl-2 protein plays an important role in MSG-initiated apoptosis in rat thymocytes. These observations are in accordance with the findings that glutamate decreases Bcl-2 protein level in different cell type (Peter *et al.* 1997), while Bcl-2 over-expression rescues cells from glutamate-induced apoptosis (Molto *et al.* 2003, Langenau *et al.* 2005). It has been demonstrated that apoptosis is modulated at least in part by the Bcl-2 family of proteins: apoptosis-inhibiting gene products (Bcl-2, Bcl-X_L, Mcl-1, A1, Bcl-w) and apoptosis-accelerating gene products (Bax, Bak, Bcl-X_S, Bim) (Adams and Cory 1998). An important feature of the members of Bcl-2 family is their ability to form homo- as well heterodimers. The heterodimerization between proapoptotic and the antiapoptotic proteins such as Bcl-2/Bax dimerization may be a critical event in regulation of apoptotic cell death (Burlacu 2003). Recent reports showed that glutamate induced Ca^{2+} influx and disruption of the inner transmembrane potential of the mitochondria, which resulted in opening the mitochondria permeability transition pore (Kanki *et al.* 2004, Khodorov *et al.* 2002). When permeability transition pore is out of control, several essential players of apoptosis, including procaspases, cytochrome c, apoptosis-inducing factor and apoptosis protease-activating factor 1 (APAF-1) are

released into the cytosol. They have the ability to activate caspases, which results in apoptosis (Desagher and Martinou 2000). Bcl-2 is localized on the cytoplasmatic face of the mitochondrial outer membrane, endoplasmatic reticulum and nuclear envelope. This protein maintains the membrane integrity of mitochondria by preventing the release of cytochrome c which, together with APAF-1, facilitates the activation of caspase 9 (Burlacu 2003). Bax can antagonize function of Bcl-2 protein through forming homodimers, which may liberate cytochrome c and start apoptotic process. Furthermore, Bax interacts with Bcl-2 to interfere with its ability to anchor APAF-1. Unbound APAF-1 cleaves procaspase 9 into active enzyme, which induces the programmed cell death (Adams and Cory 1998). In our study, the MSG-induced downregulation of Bcl-2 might disturb the control of permeability transition pore opening so that the caspase activating factors were released from mitochondria. These findings correlate with significant dose-dependent increase of cytotoxicity and thymocyte apoptosis. However, Bax protein levels were not significantly changed in our study. We propose that the Bcl-2/Bax ratio rather than the Bax level is the important determinant for the induction of apoptosis in thymocytes by MSG. Bax was found to be upregulated during apoptosis in several types of cells, together with the decrease of the Bcl-2 protein level (Peter *et al.* 1997). Furthermore, Bax deficiency has been reported to rescue the death of double positive thymocytes in Bcl-2-deficient mice (Knudson and Korsmeter 1997). On the other hand, recent reports suggested that the levels of Bcl-2 and Bax may influence the sensitivity of cells to the mediators of programmed cell death (Schelman *et al.* 2004, Bladon and Taylor 2002, Almawi *et al.* 2004). It has been proposed that Bax/Bax homodimer is the dominant regulator of the cell death signal (Sedlack *et al.* 1995) and that the anti-death effect of Bcl-2 is due to its association with Bax, which results in the reduction of the free Bax pool for homodimerization.

In summary, we have shown that upon *in vitro* exposure to MSG rat thymocytes undergo cell death *via* an apoptotic mechanism. The temporal profile of Bcl-2 and Bax expression after MSG treatment suggests that downregulation of Bcl-2 protein and resulting changes of Bcl-2/Bax protein ratio may be an important event in thymocyte apoptosis triggered by MSG. We are currently investigating the *in vitro* response of different thymocyte subsets to MSG exposure in order to determine the cellular basis of its action and signaling mechanisms involved.

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