The effect of monosodium glutamate on rat thymocyte proliferation and Bcl-2/bax protein expression

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Abstract

Introduction: Monosodium glutamate (MSG) is one of the commonest food additives in modern nutrition. Recent studies showed the modulatory effect of MSG on rat thymocyte apoptosis in vivo and under in vitro conditions. However, the influence of MSG on thymocyte proliferation is largely unknown.
Material and methods: We evaluated the effect of increased MSG concentrations on thymocyte proliferation, as well as the expression of their two apoptosis-related proteins, Bcl-2 and Bax. Proliferation was detected by PCNA monoclonal antibody, and cells were analyzed using a flow cytometer. Expression of Bcl-2 and Bax protein were determined with flow cytometry, using respective monoclonal antibodies.
Results: The results obtained in our study demonstrate that MSG significantly decreased thymocyte proliferation, in a dose dependent manner, followed by significantly increased cytotoxicity. The expression of Bcl-2 protein was significantly decreased, while Bax protein expression was not changed in our study.
Conclusions: The results presented in our study indicate that MSG significantly modulated intensity of thymocyte proliferation as a consequence of increased cytotoxicity. The expression of Bcl-2 and Bax proteins suggests that this protein level is an important event in thymocyte cytotoxicity, triggered by MSG.

Key words: monosodium glutamate, proliferation, thymocytes, Bcl-2, Bax.

Introduction

Monosodium glutamate (MSG), the sodium salt of glutamate acid, is commonly used as a flavour enhancer, especially in Chinese and Japanese foods [1]. This amino acid acts at multiple receptor types, divided into metabotropic (mGluR) and ionotropic (iGluR) glutamate receptors [2]. Recent studies showed that excessive activation of glutamate receptors was associated with some neurodegenerative disorders [3, 4]. Olney reported that the subcutaneous injection of MSG could cause brain lesions leading to acute neuronal necrosis in several regions of the developing brain of neonatal mice and acute lesions in the brain of adult mice [5].

Recent studies indicated the existence of mGluR and/or iGlur on many different cells and tissues, including the immune cells [2, 6, 7]. Glutamate receptors were detected on rat [8] and mice thymocytes [9] and human lymphocytes [10]. Our initial studies, in the field of rat thymocytes, showed that increased MSG concentration leads to apoptotic death of rat thymocytes, under in vitro conditions [11] as well as in vivo conditions [12]. However, the influence of MSG on rat thymocytes is largely unknown. Therefore the current study was designed to evaluate the effect of MSG on rat thymocyte

proliferation and to answer the question whether these processes involve changes in Bcl-2 and Bax protein expression.

Material and 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.

Material

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

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

The following monoclonal antibodies were purchased from Immunotech (Marseille, France): Mouse anti-rat PCNA (Cat. No. IM1510), mouse anti-rat Bcl-2 (Cat. No. IM2207) and Goat F(ab`)$_2$ phycoerythrin (PE)-conjugated anti-mouse IgG (H+L) (Cat. No. IM0855). Mouse anti-rat Bax monoclonal antibody (Cat. No. B8429) was obtained from Sigma, St Louis, Mo., USA.

Each group of animals (control and experimental) consisted of three animals and all the experiments were repeated three times.

Preparation of thymocytes and cell culture

Thymocytes were prepared as described previously [13, 14]. Briefly, the thymus was extirpated using sterile technique and placed in CM containing 10% FCS. The thymocytes were released by sliding the thymus along a steel mesh. Cell suspensions were filtered through a sterile nylon filter to remove stroma and then the cells were washed twice with CM containing 10% FCS. The thymocytes were counted and adjusted to a density of 1x10^7 cells/ml. The cells were cultured in 96-well flat-bottom plates (Sarstedt, Newton, USA), containing 100 µl of cell suspension (1x10^6 cells) in each well with increasing concentrations of MSG, ranging from 1 mM to 100 mM. For further evaluation of proliferative activity, the thymocytes were treated with optimal (5 µg/ml) concentration of ConA [15]. All cultures were done in triplicates. The thymocytes were cultured for 24 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]. The proliferative activity of thymocytes was evaluated after 24-hour incubation, by using anti-PCNA monoclonal antibody exactly as described earlier [16] and according to the manufacturer's instructions. The quantity of PCNA-expressing cells detected by flow cytometry could be used as a measure of the total amount of cellular proliferation [16]. Briefly, at the end of the culture period, the cells were collected and washed twice in PBS containing 5% FCS. After that, the cells were fixed and permeabilized in 70% methanol, for 30 min, at -20°C. The alcohol fixatives denature proteins resulting in permeabilization of cells, by extracting phospholipids from the cell membranes [17] The 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). Following incubation, the cells were washed twice and incubated for 45 minutes at room temperature with PE-conjugated anti-mouse IgG (H+L) monoclonal antibody (diluted 1:40). Non-specific binding was detected by the control cells which were incubated with the secondary antibody (PE-conjugated anti-mouse IgG) alone. Labelled cells were analyzed (5000 analyzed cells/per sample) on a flow cytometer (Coulter XL-MCL, Krefeld, Germany).

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) and ConA. Cell viability was evaluated after 24 hours incubation by the trypan blue dye exclusion method. The percentages of viable cells were calculated on the basis of total number of cells before cultivation.

Flow cytometric evaluation of Bcl-2 and Bax levels

The expression of Bcl-2 and Bax were measured by flow cytometry as described previously [18-20], 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 hours. After that, the cells were collected and washed twice with PBS containing 5% FCS. Permeabilization of thymocytes was done using saponin-based permeabilization reagent IntraPrep™ (Immunotech, Marseille, France), according to the manufacturer's instructions. Cells were incubated in the dark for 45 minutes 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 30 minutes in the dark, at room temperature, with PE-conjugated anti-mouse IgG monoclonal antibody

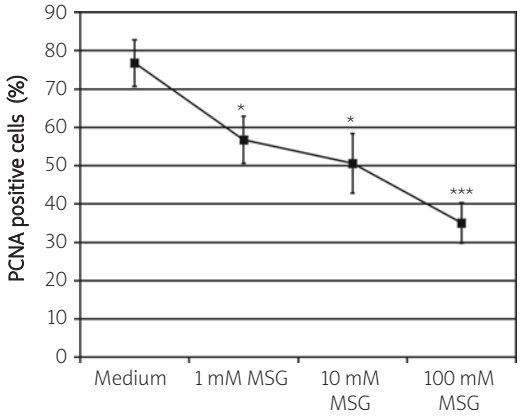

Figure 1. Effect of MSG on rat thymocyte proliferation triggered by ConA. The intensity of thymocyte proliferation was evaluated by measuring the percentage of proliferating cell nuclear antigen (PCNA) positive cells. Values are given as mean percentage ±SD of the three separate experiments

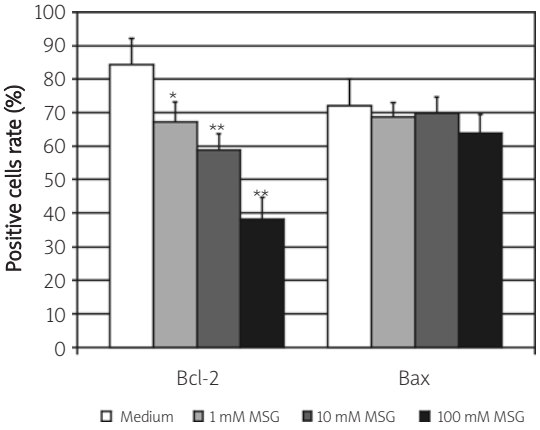

Figure 2. Flow cytometric analysis of Bcl-2 and Bax levels (cell positive rate) in rat thymocytes treated with increasing concentrations of MSG. At the end of the culture period, thymocytes were incubated with anti-Bcl-2 and anti-Bax monoclonal antibodies as described in the material and methods section. Cell positive rates were determined 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); *p<0.05, **p<0.01 compared to medium controls

(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. Labelled cells were analyzed (5000 analyzed cells/per sample) on a flow cytometer.

Statistical analysis

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

Results

Effect of MSG on proliferation of thymocytes stimulated with ConA

To investigate the dose response of MSG on thymocyte proliferation, thymocytes were cultured, for 24 hours, with increasing concentrations of MSG (1-100 mM) and triggered by optimal (5 µg/ml) concentration of ConA. The obtained results, presented in Figure 1, show that MSG administration in vitro significantly decreases thymocyte proliferation, in a dose-dependent manner, as compared to proliferation of thymocytes cultured with CM alone. The most significant decrease (35.11%, p<0.001) in thymocyte proliferation was observed in cultures with the highest MSG concentration (100 mM). As shown in Figure 1, significant decrease of thymocyte proliferation was observed in cultures with 10 mM (50.63%, p<0.5) and 1 mM (56.78%, p<0.5) MSG.

MSG induces cytotoxicity in rat thymocytes

To investigate whether MSG-induced inhibition of proliferation was mediated by increased cytotoxicity, in the next experiments thymocytes were cultured with increasing concentrations of MSG (1-100 mM) for 24 h and assayed by 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 I).

Effect of MSG on expression of Bcl-2 and Bax in thymocyte cultures

Since previous results demonstrated that in vitro treatment with MSG induced cytotoxicity, we next studied the relationship between these phenomena and the expression of Bcl-2 and Bax protein in rat thymocytes. The expression 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, administration of MSG induced significant down-

Table I. In vitro effect of MSG on rat thymocyte viability

Culture conditions	Viability-trypan blue
	Total (%)
Medium	67.36±4.2
+1mM MSG	55.8±5.21*
+10mM MSG	39.16±4.24**
+100mM MSG	26.46±5.06***

*Thymocytes (1x10^6 cells/well) were cultivated for 24 hours with increasing concentrations of MSG and triggered by optimal (5 µg/ml) concentration of ConA. Cell viability was determined by trypan blue dye exclusion method. The percentages of viable cells were calculated on the basis of total number of cells before cultivation. Values are given as mean percentage ±SD for three separate experiments; *p<0.05, **p<0.01, ***p<0.001 compared to medium controls*

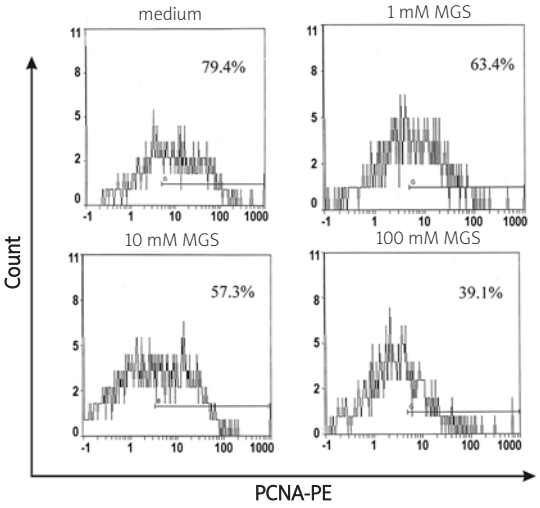

Figure 3. Effect of MSG on the expression of PCNA in cultivated rat thymocytes (flow cytometric analysis). Rat thymocytes were cultivated with increasing concentrations of MSG and optimal concentration of ConA for 24 hours, as described in the material and methods section. The levels of specific fluorescence are indicated by bars. Values (given as a percentage of PCNA positive cells) are presented from one representative experiment

regulation of Bcl-2 protein expression in rat thymocyte cultures. No significant changes in Bax protein expression, in thymocyte cultures, were detected at the end of the incubation period (Figure 3).

Discussion

In the present study, we showed that high MSG concentrations inhibit thymocyte proliferation in response to ConA, in a dose-dependent manner. Evidence for a causal relationship between glutamate concentrations and immunological reactivity has already been reported [21]. These findings are in line with reports indicating that glutamate inhibited, in a concentration-dependent manner, lymphocyte proliferation [10, 22]. It was well documented that this inhibition was probably mediated by the constitutively expressed mGlu5 receptors [23]. Activation of mGlu5 receptors leads to 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. Modulated thymocyte proliferation may lead to misbalance in thymocyte maturation and differentiation, with low numbers of mature T cells and deficiencies in T cell-mediated immunity.

Based on the obtained results of thymocyte proliferation, we hypothesized that MSG-induced inhibition was due to increased cytotoxicity. The obtained results from trypan blue exclusion method support our hypothesis that MSG-induced inhibition of thymocyte proliferation was a consequence of increased cytotoxicity. These findings are in agreement with our previous report, which indicated that MSG induced cell death via an apoptotic mechanism [11]. Also, recent reports showed that glutamate-induced cell death may be the result of apoptosis and necrosis [26, 27]. These findings may suggest a possibility that these receptors have a role in intrathymic lympho-stromal relationships, regulating thymocyte survival and differentiation. Continuous nutritional ingestion of MSG may lead to excessive activation of various glutamate receptors, which may result in increased cytotoxicity, with many immunological disorders.

The Bcl-2 family of proto-oncogenes encodes specific proteins which regulate programmed cell death in different physiological and pathological conditions [28]. By using flow cytometric analysis, we studied the changes in protein expression of two important apoptosis-related genes (Bcl-2 and Bax protein) in rat thymocytes. We found that Bcl-2 protein expression is significantly down-regulated following increased MSG exposure. It appeared that Bcl-2 protein expression was an important apoptosis regulatory factor in MSG-induced apoptosis of rat thymocytes [12, 13, 28]. On the other hand, Bax protein expression was not significantly changed in our study. We propose that the Bcl-2/Bax ratio rather than Bax expression 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 in the Bcl-2 protein level [29]. However, there is growing evidence suggesting that the levels of Bcl-2 and Bax may influence the sensitivity of cells to the mediators of programmed cell death [27, 30, 31].

Conclusions

In summary we have shown that MSG treatment of thymocytes in vitro resulted in decreased proliferation of thymocytes as a consequence of increased cytotoxicity. The expression of Bcl-2 and Bax protein suggests that this protein ratio is an important event in thymocyte cytotoxicity, triggered by MSG.

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