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CLINICAL STUDY

Monosodium glutamate induces apoptosis in naive and memory human B cells

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Abstract: The aim of this study was to establish the existence of mGluR7 in normal B lymphocytes and analyse the effect of monosodium glutamate (MSG) on B cell apoptosis in vitro.
B cells were purified by magnetic cell sorting using anti-CD19-coupled magnetic beads. Cells (10^6/ml) were cultured with increasing MSG concentrations (1–100 mM). Detection of apoptosis by flow cytometry was performed using the Annexin V-FITC/Propidium iodide (PI) apoptosis detection kit. Naïve and memory B cell population were identified by CD27 staining. Expression of GluRs was determined using PCR.
Exposure to increasing MSG concentrations displayed dose dependent effect on B cell viability altogether, ranging from 35 % with 100 mM up to 80 % with 1 mM MSG. Moreover, the number of late apoptotic cells as well as necrotic cells was dose dependant. Both CD27- as well as CD27+ B cells were affected by MSG. Basal expression of GluRs7 was detected in unstimulated B cells.
Glutamate induced apoptosis can be seen in memory as well as naive B cell population and is probably mediated through mGluR7, whose expression in B cells we also confirmed. Our study suggests a new possible mechanism of crosstalk between the nervous and the immune system through glutamate as a potential key mediator *(Fig. 4, Ref. 27)*. Full Text (Free, PDF) *www.bmj.sk.*
Key words: apoptosis, B cells, monosodium glutamate.

Glutamate represents one of the ubiquitous neurotransmitters in the human nervous system and its role in the phenomenon of excitotoxicity is well known. The effects of glutamate are mediated through ionotropic (iGluR) and metabotropic (mGluR) glutamate receptors in the central nervous system. The discovery of glutamate receptors (GluRs) in non-neuronal cells has led to numerous studies which were, regarding immunocompetent cell types, mainly focused on thymocytes and T lymphocytes. Nevertheless, the expression of GluRs has been demonstrated in macrophages (1), dendritic cells (2) as well as in natural killer cells (3). In this research we focused on B lymphocytes, in which GluRs have also been found (4).

The first study of possible glutamatergic mechanisms in human blood lymphocytes was conducted by Kostanyan et al in 1997. Following this, both subclasses of iGluRs – NMDA-activated iGluRs and AMPA-activated iGluRs (GluR3) were detected in human lymphocytes (4, 5) well as group I and III mGluRs (6, 7).

Rush et al discovered that the GRM7 gene (that encodes the mGluR7 receptor) was up-regulated as a result of abnormal methylation of promoters in human CD19 B cells in chronic lymphocytic leukemia (CLL). GRM7 can inhibit cAMP signaling in the induction of apoptosis (8) – group III (mGluR4, mGluR6, mGluR7 and mGluR8) mGluRs are associated with Gi/Go proteins, and their activation inhibits adenylate cyclase activity (9, 10). It has also been shown that in B-CLL, cAMP phosphodiesterases catabolize cAMP to 5' AMP, thus inhibiting apoptosis (11). This inhibition can be reversed by inactivation of phosphodiesterases (12). It has been speculated that GRM7 inhibition of cyclic AMP signaling in the induction of apoptosis creates, as a final effect, the apoptosis resistant phenotype in malignant B cell pull, which currently represents an unsolved therapeutic problem.

Moreover, taking into account the crucial role of apoptosis in the process of development and maturation of B cells (like clonal deletion), the possible modulation of apoptosis becomes important not only as potential glutamate-related therapy of B cell malignancies (application of selective agonists or antagonists) but also for immunomodulatory solutions in autoimmune diseases as well as the understanding of basic regulatory processes during the development of normal, untransformed B cells.

Monosodium glutamate (MSG) is widely used as a food additive and flavour enhancer in both traditional Eastern and modern Western nutrition. Until this study, to our knowledge, the

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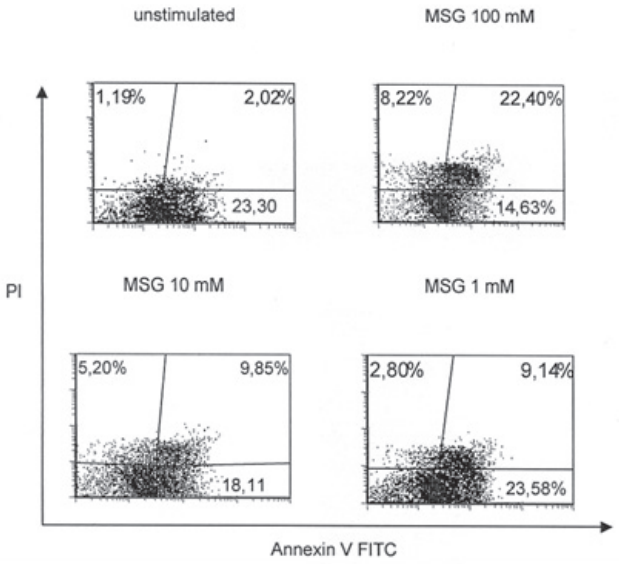

Fig. 1. MSG induces apoptosis of B cells. Isolated CD19+ B cells were cultured in the presence or absence MSG in three different concentrations (100 mM, 10 mM, 1 mM) for 24h. Afterwards Annexin V/PI staining and FACS analyses was performed. Dot blots show a representative of six experiments.

effect of MSG on normal B cells has not been investigated. The MSG induced apoptosis has been proven in thymocytes (13). The aim of this study was to establish the existence of mGluR7 in normal B lymphocytes and analyse the MSG effect on B cell apoptosis *in vitro.*

Material and methods

Blood samples, cell isolation and cell culture

Peripheral blood mononuclear cells (PBMCs) from healthy donors were separated from heparinised blood (30 ml) by density gradient centrifugation. B cells were purified by magnetic cell sorting (MACS) using anti-CD19-coupled magnetic beads (Miltenyi Biotec Gmb, Germany). Cells (10^6/ml) were cultured with increasing MSG concentration (1–100 mM) (Fluka Chemika AG, Switzerland) in flat-bottomed 48-well plates in RPMI 1640 medium supplemented with 10 % heat-inactivated foetal calf serum, glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 ug/ml) (Biochrom KG, Germany). Incubation took place in a water-saturated atmosphere containing 5 % CO_2 at 37 °C. Study was performed according to the declaration of Helsinki.

Determination of cell viability and apoptosis by flow cytometry

Detection of apoptosis by flow cytometry was performed using the Annexin V-FITC/Propidium iodide (PI) apoptosis detection kit (Immunotech, France). The staining was performed according to the manufacturer's manual. Single positive populations are considered as the early apoptotic (Annexin V+/PI) or necrotic cells (Annexin V/PI+), whereas double positive (Annexin V+/PI+) cells represent cells in a late stage of apoptosis (14, 15). Acquisition and analysis were performed on EPICS XL flow cytometer (Coulter Electronics).

RNA extraction, cDNA synthesis and PCR

Total RNA was extracted from purified B cells using RNeasy Mini Kit (Qiagen, Germany) and reverse transcribed by Super-Script (Invitrogen, Germany) according to the manufacturer's guidelines. To analyze the glutamate receptor 7 mRNA transcript specific primer pairs were used cctgggcgttatgacatctt as sense and caatgggcgtgtcattgtag as antisense.

Statistics

The differences between groups were determined by the Mann-Whitney U-test for unpaired data (SPSS 12.0 software, SPSS inc.). Statistically significant differences were considered at p values <0.05.

Results

MSG induces apoptosis in human B cells

In order to investigate the MSG effect on human B cell apoptosis, B cells were cultured with and without increasing concentrations of MSG (1–100 mM) for 24 h. The number of early apoptotic cells, late apoptotic cells and necrotic cells was determined using Annexin-V FITC/PI staining (Fig. 1). MSG titration revealed the highest effect on apoptosis with 100 mM. However, a significant increase in apoptosis was detected using each concentration investigated ($p<0.05$). Exposure to increasing MSG concentrations displayed a clear dose dependant effect on B cell viability altogether (Fig. 2). Moreover, the number of late apoptotic cells as well as necrotic cells was dose dependant, even though the number of early apoptotic cells remained approximately the same (Fig. 3).

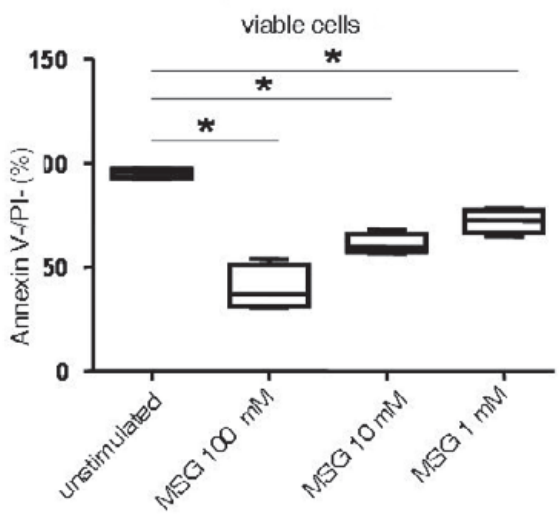

Fig. 2. Viability of MSG treated B cells. Annexin V -/PI- B cells were considered as viable and the percentages from six experiments have been compared. * represents $p<0.05$.

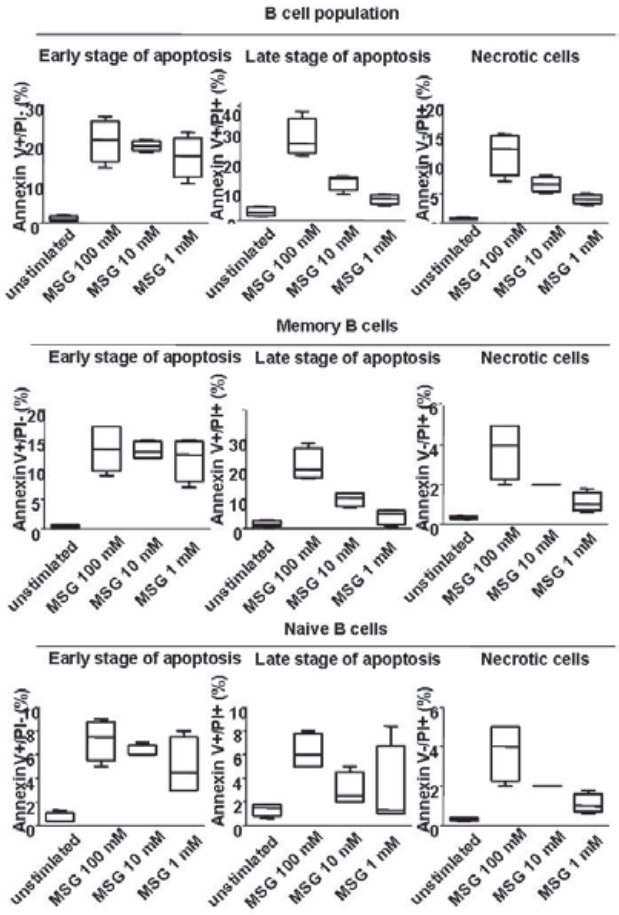

Fig. 3. MSG influence on apoptosis of memory and naive B cells. Isolated CD19+ B cells were cultured in the presence or absence MSG in three different concentrations (100 mM, 10 mM, 1 mM) for 24 h. Surface CD27 staining was used for distinguishing naive and memory B cells. Afterwards Annexin V/PI staining and FACS analyses was performed.

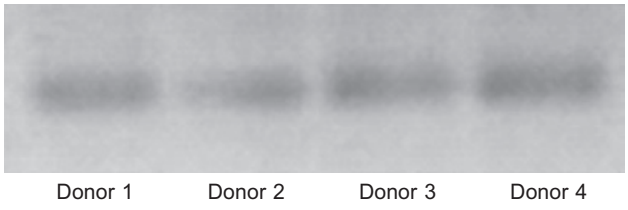

Fig. 4. GRM7 expression in human B cells. PCR product shows expression of GRM7 in four healthy donors.

Naive as well as memory B cells are targets for MSG

To distinguish if MSG induces apoptosis in memory or naive B cells and additional to Annexin-V FITC/PI staining a CD27 surface staining was performed. Both CD27- as well as CD27+ B cells were affected by MSG (Fig. 4). Number of cells in late apoptosis phase as well as necrotic cells was dose dependant of MSG concentration used. However, the number of early apoptotic cells did not differ between memory and naive B cells, what was expectable since it is a very short transient phase.

GRM7 is expressed in human B cells

To gain insight into possible mechanism of MSG effect we analysed the expression of GRM7 mRNA in purified human B cells. Figure 4 shows gel electrophoresis of the PCR product, and demonstrates the basal expression of this receptor in the unstimulated B cells.

Discussion

Apoptosis represents an evolutionarily conserved and highly regulated process which has a tremendous relevance as a form of programmed cell death (PCD) in multicellular organisms. Not only does it represent a basic physiological phenomenon in the processes of tissue development but is also essential for organism homeostasis. Defects in normal programmed cell death mechanisms play a major role in the pathogenesis of an extensive variety of diseases.

In B cell lineage, proper regulation of apoptosis enables homeostasis as well as quality control of the antibody response and tolerance. Recent findings suggest that the extrinsic death receptor pathway is primarily involved in affinity maturation of the antibody response – it prevents activation of irrelevant B cell clones during B cell response to thymus-dependent antigens. On the other hand, the intrinsic death pathway is implicated in homeostasis of the naive, effector, and memory B cell subpopulations (16, 17).

In the present study we showed that MSG was able to induce cytotoxicity in naive and memory human B cells. 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. A clear dose dependant effect on B cell viability was detected. The results obtained in this research are in accordance with previous work by Pavlovic et al regarding MSG apoptotic effect in rat thymocyte culture (13), whose findings demonstrate that exposure to MSG results in significantly increased thymocyte apoptosis that is concentration-dependent. Moreover, it has been shown that glutamate-induced cell death may be the result of apoptosis and necrosis in different neuronal populations *in vivo* as well as *in vitro* (18). These findings indicate that glutamate, one of the principal neurotransmitters in human brain, can also be extremely destructive if present in excess. The described phenomenon of an acute degeneration of neurons caused by glutamate (excitotoxicity) has been implicated in the development of multiple sclerosis (MS) and neurodegenerative diseases as well as in neuron degeneration following head trauma or cerebral ischemia. However, we would like to point out the results of a recent study by Nedergaard et al who in a rat model demonstrated, that the release of glutamate by malignant gliomas is directly related to and facilitates tumor growth *in situ.* Consequent excitotoxic destruction of nontransformed neurons has been a proposed mechanism (19). In the same study, blockage of the NMDA (N-methyl-d-aspartate) glutamate receptor subtype with antagonists MK801 or memantine slowed the growth of gluta-

mate-secreting tumors *in situ*. Moreover, the blockage of NMDA and AMPA (-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) glutamate receptors has recently been reported to decrease the proliferation of various tumors such as colon, adenocarcinoma, breast, astrocytoma and lung carcinoma (20). These findings bring us back to the research done by Rush et al which revealed that the GRM7 gene (that encodes the mGluR7 receptor) was up-regulated as a result of abnormal methylation of promoters in human CD19 B cells in chronic lymphocytic leukaemia (CLL). This up-regulation results in defective cyclic AMP signalling during the initiation phase of apoptosis and creation of apoptosis resistant B cell malignant clone (7). In our opinion, all the facts presented above indicate that further research is needed for the development of potential therapy of B cell tumors through the blockage of glutamate receptors. The basal expression of mGluR7 receptor in human B cells must be taken into account before blockage with appropriate antagonists, due to the potential cytotoxic effect on normal B cells and possibly other immunocompetent cells. An example of the successful and already existing therapy for B cell non-Hodgkin's lymphoma and B cell leukaemia through proapoptotic effect among others, is Rituximab, a chimeric monoclonal antibody which binds to CD20, widely expressed on B-cells. Transformed B cells in CLL are characterized by a relatively low level of CD20 expression; thus, a more efficient therapy would be to combine Rituximab with another therapeutic agent which would recognize and bind to a highly expressed molecule on B cells, with the final result being strong sensitization to apoptosis in malignant B cell clone. The ubiquitous presence of mGluR7 and possibly other glutamate receptor types on B cells could facilitate the creation of appropriate future targeted therapy for B cell malignancies.

B cell targeted depletion has a tremendous potential not only in cancer but also in autoimmune disease therapy - Rituximab in combination with methotrexate has been approved for the treatment of refractory rheumatoid arthritis by the regulatory authorities in both the United States and Europe. Moreover, in an ongoing trial involving more than 100 patients with remitting-relapsing form of MS magnetic resonance imaging has shown that the application of Rituximab significantly reduced nerve damage, giving boost to a novel interest in humoral immunity alterations in MS (21). Another research which emphasizes the potential importance of the future glutamate associated therapy of autoimmune diseases, namely MS, is that of Gilgun-Sherki et al in which it was shown that riluzole, an inhibitor of glutamate transmission, dramatically reduced the clinical severity of MS-like disease in myelin oligodendrocyte glycoprotein (MOG)-induced EAE in mice (22). Kanwar et al in their review article point out the combination of anti-cell adhesion molecules mAb, the AMPA/ /kainate antagonist NBQX, and the NMDA receptor antagonist GPE as a possibly effective therapies for multiple sclerosis (23). In the light of all the facts listed above our discovery of basal mGluR7 expression in human B cells and MSG apoptotic effect on these cells could be the starting point for further research into possible new ways of B cell depletion in MS. On the other hand, a combined effect on different iGluRs, expressed in the cells of the central nervous system, and B cell mGluR7, through the application of selective agonists and antagonists is an alternative therapeutical approach for MS, which should be investigated further.

The results of our research indicate that MSG has a more potent apoptotic effect in naive B cells compared to the memory B cell population. This could indicate a possible selector role of glutamate induced cytotoxicity in the process of clonal deletion and the existence of an alternative pathway of MSG induced apoptosis in naive B cells. Pacheco et al showed that mGluR5 is expressed constitutively in T cells, whereas mGluR1 expression was induced only after formation of the T-cell receptor-CD3 complex. Boldyrev et al demonstrated that activation of murine lymphocyte iGluRs with NMDA increased ROS levels and caused activation of caspase-3 whereas simultaneous treatment of lymphocytes with the iGluR and mGluR agonists, NMDA and L-2-amino-4-phosphonobutyric acid (L-AP4) respectively, caused an opposite effect – a significant reduction of ROS levels (24). Similar relations exist between NMDA-activated iGluRs and group III GluRs in CNS neurons, where simultaneous activation resulted in increased production of ROS and cell death of neurons (25). Likewise, different expression patterns for GluRs in diverse B cell subpopulations could explain the stronger proapoptotic MSG effect found in naive B cells in our study. Furthermore, this implicates a potential immunomodulatory role of glutamate. Precise profiling of glutamate receptor subtypes in naive, effector and memory B cells could be the next step due to the fact that an eminent characteristic of non-neuronal GluR expression is the coexistence of iGluRs and mGluRs, which enables the cross-talk and integration of different signals in the same immunocompetent cell.

The discovery of basal expression of mGluR7 in human B cells becomes even more intriguing if glutamate, being involved in signalling in the immune and nervous systems, is observed not only as an immunotransmitter but also as a neurotransmitter and a potential mediator in complex processes of neuroimmunomodulation. The subclass of glutamate receptors, mGluRs, including mGluR7, are considered to have a primary role in providing a mechanism by which glutamate can finely modulate its activity at the same synapses where it elicits fast synaptic responses through activation of iGluRs (26). Mitsukawa et al have found that group III mGluR subtypes (mGluR4, -6, -7, and -8) have a role in the pathophysiology of stress-related behavioural disorders – anxiety and depression in genetically modified mice and that mGluR7 is an important modulator of the stress response in vivo (27). All this data, together with our identification of mGluR7 in human B cells, could indicate a complex role of glutamate in immunological alterations present in patients with stress-related behavioural disorders – additional analysis of B cell subtypes in these patients and comparison to B cell pull in healthy population is needed to gain a better understanding of the potential neuroimmunomodulatory effect of glutamate.

Conclusion

In conclusion, we have demonstrated that upon *in vitro* exposure to MSG human nad've and memory B cells undergo cell

death *via* an apoptotic mechanism. To further characterize the molecular effects of MSG on human B cells the existence of mGluR7 was analyzed and the basal expression of this receptor in the unstimulated B cells was proven. The next step in our further research would be the blockage of mGluR7 and the analysis of the consequent effects on MSG induced apoptosis in human B cell subpopulations.

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