Modulation of Mast Cell Function by Amino Acids *In vitro*: A Potential Mechanism of Immunonutrition for Wound Healing

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Abstract

Mast cells release important chemical mediators, such as histamine and interleukin (IL)-13, for the regulation of allergic reactions and inflammation. Recently, mast cell activation has been implicated in wound healing. Glutamine (Gln) and Arginine (Arg) are used as "immune nutrients" in severe infections and chronic wounds in malnourished patients, but the potential effect of these amino acids on mast cell activation is unclear. We evaluated the effect of Gln and / or Arg in culture on mast cell function.

The murine mast cell line P815 was cultured in RPMI-1640 medium under different Gln and/or Arg concentrations and IL-6 stimulation. Histamine and IL-13 release into the supernatant was measured using enzyme-linked immunoassays.

As a result, the amino acid concentrations affected cellular viability. Cells cultured in higher Gln concentrations produced a greater histamine response upon IL-6 stimulation while the histamine response in cells cultured in a high Arg concentration was suppressed. IL-13 production in the Gln containing medium was not significantly altered by IL-6 stimulation.

In conclusion, in an inflammatory milieu, Gln and/or Arg concentrations *in situ* may regulate histamine and IL-13 release from mast cells. The appropriate use of functional amino acids as immune nutrients may suppress inflammation and aid in wound healing.

Keywords: Glutamine; Arginine; Mast cells; Histamine; IL-13

Introduction

Mast cells, which are derived from pluripotent stem cells, reside in the submucosa or connective tissue in mammals and play important roles in the innate immune and inflammatory responses, including those involved in allergic reactions. Mast cells contain a number of granules that are rich in chemical mediators, such as histamine and heparin. Mast cells are stimulated to release these mediators crosslinked on the cell surface by immunoglobulin E (IgE), when it has been induced by an antigen. On the other hand, non-IgE-dependent pathways for mast cell activation are also present [1-3]. For example, pro-

inflammatory cytokines such as interleukin (IL)-6 can induce the release of histamine from mast cells, at least *in vitro* [4].

Accumulating evidence suggests a potential enhancing effect of mast cells in the process of wound healing [5-7]. In animal models, mice that lack mast cells demonstrate delayed wound healing compared to their normal littermates [8]. One potential mechanism by which mast cells are involved in wound healing is mast cell-derived histamine, as histamine might promote fibroblast migration [9]. An additional role of mast cells in wound healing might be related to IL-13, which is generally accepted as a T cell-related cytokine. T helper type 2 (Th2) cells are the major source of IL-13, and it activates tissue fibrosis in a variety of diseases by promoting collagen accumulation in fibroblasts and myofibroblasts and regulating inflammation [10-16].

In regards to the relationship between wound healing and immunity, recent developments in "immunonutrition" may contribute to the enhanced healing of chronic wounds such as pressure ulcers [17-20].

Immunonutrition involves nutritional support through the addition of "immunonutrients" such as amino acids (arginine [Arg] and glutamine [Gln]), omega-3 (n-3) fatty acids vitamins or nucleic acids. These nutrients have been shown to have the immune-modulating (or enhancing) effects via several mechanisms, leading to better outcomes after critical illness or surgical operation. For example, Arg and omega-3 fatty acids are suggested to maintain T cell immunity [21], where as Gln is shown to enhance the release of growth hormone and supports protein synthesis [22].

These observations suggest that, considering the potential clinical effect of immunonutrition (in at least a subset of patients), "immune-regulating" nutrients, in particular the amino acids would have some effect also in mast cells in wound *in situ*. In this regard, recently, Lechowski, *et al.* [23] demonstrated that human intestinal mast cells cultured in different culture medium containing different amino acid concentrations (Arg/

Gln) showed decreased IgE-mediated release of leukotrienes and cytokines. The authors concluded that the amino acids exert anti-inflammatory effect on IgE-dependent mast cell activation. The finding provided *in vitro* evidence that Arg and Gln would be key nutrients in mast cell-mediated responses. However, it has not been known how these amino acids might modulate mast cell activation when mast cells were activated in an IgE-independent inflammatory stimulation.

In the present *in vitro* study, we aimed at assessing the potential role of nutrients in mast cell activation by evaluating the effect of the amino acids Gln and Arg in culture milieu on mast cell function with a focus on histamine and IL-13 release.

Materials and Methods

Cell cultures

The mouse mastocytoma cell line, P815, was provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. The cells were cultured at 37°C in an atmosphere of 5% CO₂ in the RPMI-1640 medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) that was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S). The culture medium contained 300 mg/L L-Gln and 200 mg/L L-Arg as its basic composition. The culture medium was regularly changed, approximately twice a week, until reaching confluence. Cells were then subjected to further culture for 24 h in media with different amino acid concentrations. Specifically, the levels of Gln in the medium were adjusted to 500 mg/L and 1000 mg/L, and the levels of Arg were adjusted to 400 mg/L, in the presence of both 0 mg/L and 500 mg/L Gln. The concentrations were achieved using an L-Gln or L-Arg solution (Wako Pure Chemical Industries, Ltd. Osaka, Japan) and non Gln-containing RPMI-1640 (Wako Pure Chemical Industries, Ltd. Osaka, Japan). The other constituents, including FBS and P/S, were not modified.

The cells were subgrouped to be stimulated or not with recombinant murine IL-6 (20 ng/mL) (Peprotech Inc, Rocky Hill, NJ, USA) for 24 h in each medium, and the culture supernatant was collected for analyses. The IL-6 stimulation allowed for focus on the response of the mast cells to inflammatory stimulation rather than an allergen challenge. Previous reports indicate that IL-6 stimulation induces histamine release in P815 cells [4]. The cellular viability and proliferation were assayed using microscopic observation, cell counts were analyzed using trypan blue staining (data not shown), and the MTS assay (CellTiter96 Aqueous Non-Radioactive Cell Proliferation Assay™, Promega Co., Madison, WI, USA) was conducted according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to measure the secreted levels of histamine and IL-13 in the cultured P815 cells. Specifically, after the *in vitro* culture, supernatant were collected and subjected to the ELISA analysis using commercially available enzyme immunoassay

kits (Histamine Enzyme Immunoassay Kit™, SPI Bio Inc., Montigny le Bretonneux, France and Mouse IL-13 Quantikine™, R&D, Minneapolis, MN, USA) according to the manufacturers' instructions. Each experiment was performed in triplicate.

Statistical analyses

The data were calculated and analyzed using Student's *t*-tests or a one-way analysis of variance with Tukey's post hoc tests, as appropriate, using the Ekuseru-Toukei 2010™ software (Social Survey Research Information Co., Ltd., Tokyo, Japan). Differences in the amount of histamine and IL-13 that was secreted were compared between the different conditions within the same amino acid concentration and also between the different concentrations of each amino acid. Comparisons were also conducted between the presence and absence of IL-6 stimulation for each condition. Results with *p* < 0.05 were considered statistically significant.

Results

Amino acid concentrations affect mast cell proliferation

The summary of the initial MTS assay used to test the proliferation of P815 mast cells cultured in different amino acid (Arg and Gln) concentrations is provided in Table 1. Depletion of Gln (in the absence of Arg supplementation) decreased the cellular viability and proliferation, whereas the other conditions did not alter these parameters.

Histamine release from mast cells is regulated by IL-6 and Arg but not Gln

We cultured P815 mast cells in RPMI media with different concentrations of Gln and Arg. The histamine release remained at basal levels under the usual culture condition (i.e., the standard RPMI-1640 containing 300 mg/L of Gln and 200 mg/L of Arg; Figure 1). Variation in only the levels of Gln (0–1,000 mg/L) in

Arg (mg/L)	Gln (mg/L)	IL-6 (ng/mL)	MTS assay OD	Relative value	Histamine nM	Relative value	IL-13 pM	Relative value
200*	0 [#]	0	0.161	0.914	2.16	1.214	31.50	0.857
		20	0.147		2.68		27.00	
	300 [#]	0	0.381	0.806	1.13	2.317	131.39	0.921
		20	0.307		2.69		121.01	
	500 [#]	0	0.239	1.357	1.83	3.280	192.72	1.042
		20	0.324		6.00		200.86	
400*	0 [#]	0	0.242	0.644	13.55	1.411	21.99	0.838
		20	0.156		19.11		18.44	
	300 [#]	0	0.391	0.531	16.86	1.266	106.83	1.004
		20	0.208		21.35		107.31	
	500 [#]	0	0.312	1.383	18.76	1.096	173.97	1.064
		20	0.431		20.56		185.03	

Table 1: Summary of the P815 responses in the presence of varying glutamine (Gln)/arginine (Arg) concentrations and interleukin (IL)-6 stimulation. Results of the MTS assay, histamine enzyme-linked immunosorbent assay (ELISA), and IL-13 ELISA are summarized, and the relative values of the responses to IL-6 (i.e. values of IL-6-stimulated cells/values of non-stimulated cells) are provided.

*, #: compared between groups using a one-way analysis of variance with Tukey's post hoc tests.

Arg = arginine; Gln = glutamine; IL-6 = interleukin-6; IL-13 = interleukin-13

+: cells cultured in the Glu (0) medium had lower viability and the results varied

the RPMI medium did not alter the level of histamine release from the P815 cells ($F(2, 22) = 0.31$, $p = 0.74$). On the other hand, P815 cells significantly increased the release of histamine when stimulated with IL-6 *in vitro* ($F(1, 12) = 14.34$, $p = 0.0026$). The response of the cells to IL-6 differed according to the Gln concentrations in the media with a greater histamine release resulting from IL-6 stimulation in greater Gln concentrations (Figure 1 and Table 1).

Next, we modified the concentrations of both Gln and Arg in the culture media. The summary of the histamine release from the P815 cells in the presence of the different combinations of Arg and Gln concentrations are provided in Figure 2. Compared to those cultured in "normal" Arg (200 mg/L), cells grown in the "high" Arg (400 mg/L) medium released significantly higher levels of histamine ($F(1,11) = 24.26$, $p = 0.0005$), irrespective of the Gln concentrations.

Distinct regulation of IL-13 production from mast cells

We then explored the effect of the amino acid concentration in the medium on the IL-13 production from the mast cells. Figure 3 and Table 1 illustrate the levels of IL-13 secretion in the presence of IL-6 and varying concentrations of Gln. IL-13 production was clearly observed in the P815 cells cultured in high concentrations of Gln (500 and 1,000 mg/L), but not in the media that did not contain Gln (Figure 3). In contrast to histamine, the IL-13 level was not significantly changed by *in vitro* stimulation with IL-6 (Figure 3).

Following this, the production of IL-13 was compared between the different combinations of Arg and Gln concentrations (Figure 4 and Table 1; Figs 3 and Figs 4 represents results from separate experiments). IL-13 production increased in the presence of Gln, regardless of the Arg concentration. More specifically, when the P815 cells were cultured in the RPMI medium with an Arg concentration of 200 mg/L, the IL-13 levels significantly increased with increasing Gln concentrations (0, 300, and 500 mg/L) ($F(2,12) = 7.18$, $p = 0.0089$).

Figure 2: Effect of the combined arginine (Arg) and glutamine (Gln) concentrations in the interleukin (IL)-6-induced histamine release from P815 mast cells.

P815 mast cells were cultured *in vitro* in RPMI culture media containing different levels of Gln and Arg (as indicated). The concentrations of histamine in culture supernatants were then measured using enzyme-linked immunosorbent assay. Data represent mean values ($n = 3$).

Discussion

Our results demonstrate that the *in vitro* culture of mast cells with different Gln and Arg levels results in distinct histamine and IL-13 responses, suggesting that amino acid concentrations may play an important role *in situ* in mast cell activation.

The contribution of amino acids, including Gln and Arg, in "immunonutrition" and wound healing has been widely proposed. For instance, enteral nutrition supplemented with a combination of immunonutrients, including Arg, Gln, omega-3 fatty acids, and nucleotides, has been demonstrated to prevent infectious complications and decrease mortality rates in critically ill and surgical patients compared with standard enteral nutrition [24-26]. In addition, these amino acids have been indicated in the regulation of lymphocyte function [27]; however, their effect on mast cells has remained mostly unclear.

Figure 3: Effect of the glutamine (Gln) concentration on the interleukin (IL)-6-induced IL-13 secretion from P815 mast cells. P815 mast cells were cultured *in vitro* in RPMI culture media containing different levels of Gln (0, 300, 500 or 1,000 mg/L). The concentrations of IL-13 in culture supernatants were then measured using enzyme-linked immunosorbent assay. Data represent mean values (n = 3).

Figure 4: Effect of the combined arginine (Arg) and glutamine (Gln) concentrations on the interleukin (IL)-6-induced IL-13 production from P815 mast cells. P815 mast cells were cultured *in vitro* in RPMI culture media containing different levels of Gln and Arg (as indicated). The concentrations of IL-13 in culture supernatants were then measured using enzyme-linked immunosorbent assay. Data represent mean values (n = 3).

Our present results demonstrate that Gln and Arg may modulate mast cell functions such as degranulation and IL-13 production. Previous studies have indicated that histamine may stimulate fibroblast migration, implicating the role of histamine in wound healing [8-9]. In addition, IL-13 induces collagen synthesis via induction of transforming growth factor- β expression [12,28]. Recent studies have reported the role of IL-13/IL-13R signaling in allergic conditions [29], pulmonary fibrosis [30], and gastroenteritis [31,32]. The pro-fibrotic property of IL-13 also plays a role in fibrosis and remodeling (inflammation and construction of vasculature) of the skin as well as wound healing [33,34]. Considering this, it is likely that amino acids regulate skin lesions, at least in part, by controlling the levels of mediators that are produced from mast cells, such as histamine and IL-13.

Although we did not investigate the role of omega-3 fatty

acids, Park et al. [35] demonstrated that these fatty acids also modulate the Th2 cytokine expression from mast cell lines by regulating the pathways of the transcription factors GATA-1 and/or GATA-2. The authors did not find a significant change in IL-4 and IL-13 expression from the P815 mast cells because the cell line lacked GATA-1 expression; however, their results suggested that the oral administration of diet-derived nutrients (e.g. omega-3 fatty acids in fish oil) might regulate mast cell function *in vivo* [35]. Therefore, it would be interesting to investigate the interaction or cumulative effects of fatty acids and amino acids on mast cell function.

In the present study, we set the concentration of L-Gln in the RPMI culture medium as 0 mg/L, 300 mg/L (2.05 mM; the basic composition of RPMI), 500 mg/L (3.42 mM), and 1,000 mg/L (6.84 mM). As for L-Arg, because Arg-free RPMI was not available, we used concentrations of 200 mg/L (1.15 mM; the basic composition), and 400 mg/L (2.3 mM) by addition of L-Arg to the original medium. These concentrations were higher than the physiological concentrations, as according to Lechowski et al. [23] the physiological serum concentrations of the amino acids are reported to be 0.6 mM for L-Gln and 0.1 mM for L-Arg. Thus our results here may reflect only *in vitro* findings using cultured cell line, however, further *in vivo* studies e.g. by using oral feeding of the immunonutrients to experimental animals would enhance understanding of the mechanism of immunonutrition in mast cell responses. Further, it should be tested another experimental conditions (e.g. different doses of amino acids and cytokine, different culture period, etc), and using another mast cell line including humans, since P815 murine mastocytoma cell line is reported to lack FC ϵ R expression [36] and thus would not reflect the nature of mature human mast cells *in vivo*.

In conclusion, we found that amino acids may play a role in the regulation of mast cell activation in the presence of inflammatory stimulation. Clarification of the role of amino acids and other nutrients in the function of mast cells may provide new avenues for establishing novel therapeutic strategies for a number of inflammatory conditions.

Conclusions

The release of histamine and expression of IL-13 by mast cells in response to inflammatory stimulation may be altered, at least in part, by amino acid concentrations *in vitro*. Regulating amino acids levels may therefore be important for the modification of mast cell function during inflammation as well as in the healing process of chronic wounds.

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