Vol. 204, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1994 Pages 244-251

NITRIC OXIDE-MEDIATED APOPTOSIS IN MURINE MASTOCYTOMA

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Received August 24, 1994

Summary : To investigate how the number of mast cells is controlled, we studied a murine mastocytoma cell line. Based on electron microscopic observation of nuclear condensation and elecrophoretic evidence with DNA fragmentation, these mastocytoma wells were shown to undergo apoptosis. This apoptosis was dependent on the concentrations of serum and L-arginine and was enhanced by TNF-α. We confirmed that apoptosis was mediated by nitric oxide (NO) synthase; inducible NO synthase (iNOS) mRNA was strongly expressed in apoptotic cells, while an inhibitor of NOS, N^G-monomethyl-L-arginine, and dexamethasone prevented apoptosis in addition to inhibiting iNOS mRNA expression. Our results suggest that iNOS expression is very important in regulating the proliferation of mast cells under pathological conditions.

Mast cell precursors circulate in the blood and lymphatics (1), and reach phenotypic maturation within tissues under certain microenvironmental influences (2). It is known that the proliferation and maturation of mast cells are regulated by two factors IL-3 and the ligand of*c-kit*, stem cell factor (SCF) (3,4). Mast cells are rapidly eliminated from inflammatory tissues in recovery from allergenic diseases. Two distinct mechanisms of cell elimination have been identified; necrosis, caused by catastrophic toxic or traumatic event is a passive process and another is apoptosis is an active process of cellular self-destruction with unique morphological and molecular characteristics (5). Under physiological conditions, cell loss most often occurs by apoptosis, an active, programmed process. Recently, Mekori et al (6) reported that murine mast cells underwent apoptosis upon removal of IL-3. Yee et al. (7) also reported that the receptor tyrosine kinase Kit suppressed apoptosis in mast cells.

In this study, we found that murine mastocytoma cell line, p815 cells underwent spontaneously apoptosis with RPMI 1640 complete medium containing 10% fetal bovine serum (FBS). Futher, this apoptosis in mastcytoma cells was mediated by nitric oxide (NO).

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Abbreviations used: NO, nitric oxide; iNOS, inducible NO synthase; FBS, fetal bovine serum; N-MMA, N^G-monomethyl-L-arginine; Dex, dexamethasone.

0006-291X/94 $5.00

NO is a short-lived biological mediator produced by diverse cell types including inflammatory cells and may be cytotoxic at high concentrations (8). NO synthases (NOSs) constitute a family of isozymes that catalyze the oxidation of L-arginine to NO and citrulline (8). NOS is classified into two categories, constitutive (cNOS) and inducible (iNOS). Especially, iNOS has been proposed to have a major influence in the inflammatory process, and its levels of expression are regulated by the inflammatory cytokines including IL-1 and TNF-α. Macrophages, the prototypical effector cells for NO-mediated cytotoxicity, are themselves targets for NO and die prematurely in culture when activated to express iNOS mRNA (9). These may imply the homeostatic mechanism of mast cell number in the tissue. In this paper, we demonstrate that iNOS is produced from mastcytoma cells and apoptosis is induced by iNOS itself.

MATERIALS AND METHODS

Cell culture and treatment The murine mastocytoma cell line, P815 was obtained from the American Type Culture Collection (ATCC, No. TIB64). Cells were maintained in RPMI 1640 containing 10% heat-inactivated bovine serum (FBS) and penicillin G (100IU/ml) in a 5% CO_2 - 95% air atmosphere. Cells were resuspended in six-well ($5x10^5$ cells/2ml) culture plates (Costar, Cambridge, MA, USA) in basic culture medium consisting of RPMI 1640 without L-arginine or FBS. L-arginine (GIBCO, Grand Island, NY, USA), FBS, and NG-monomethyl L-arginine (N-MMA) (GIBCO) were added to the basic culture medium at various concentrations. Cells were then challenged with several reagents: 500U of mouse recombinant (r) TNF-α (specific activity, $2x10^7$ units/mg; Genzyme, Cambridge, MA, USA), 500U of rGM-CSF (specific activity, $3x10^6$ units/mg; Genzyme), 50 ng/ml phorbor 12-myristate 13-acetate (PMA) (Sigma), 250 ng/ml calcium ionophore (CaI) (Sigma), $5x10^{-6}$M eosinophilic cationic protein (ECP) (gift from Dr. Chihara, Kinki University) and $1x10^{-7}$ M dexamethasone (Dex) (Wako Co., Ltd. Osaka, Japan).

MTT cellular proliferation assay and cell viability We used the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cellular proliferation assay (10) to investigate the viability of murine mastocytoma cells. Cells were treated with several reagents in medium containing FBS from 0 to 10% with or without 1mM arginine. Ten microliters of filtered MTT (Sigma) in PBS to a final concentration of 5 μg/μl was added. Cells treated with the reagents were incubated at 37°C for 4 hours (h). One hundred microliters of acidic isopropranol was added and the incubation was extended overnight. Solubilized formazan was quantified and the absorbance ratio (570:630 nm) was determined. Cell viability was determined by light microscopy after staining with 0.02% Trypan blue (Sigma) and was calculated as a percentage of the cell population surviving.

Electron microscopy Cells were harvested by gently scraping from the culture wells and fixed in PBS containing 0.25% glutaraldehyde at pH 7.2 (Sigma). Samples were postfixed in 0.1M cacodylate buffer containing 1% osmium tetroxide and embedded. Sections were stained with uranyl acetate and lead citrate, and examined by transmission electron microscopy. The number of apoptotic cells was determined by their characteristic nuclear morphology resulting from chromatin condensation was then counted.

DNA extraction and gel electrophoresis Cells obtained and treated as described above were lysed with 0.1M NaCl, 10mM Tris-HCl, pH8.0, 5mM EDTA in 0.5% SDS, and incubated overnight at 42°C with proteinase K (500 μg/ml, Sigma). Samples were extracted with an equal volume of phenol:chloroform (1:1) and the total DNA contained in the aqueous phase was precipitated with 1/10 volume of ethanol at -80°C for 20h. DNA pellets were obtained by centrifugation (14000 xg, 15 min.), washed with 70% ethanol, air dried, and resuspended in 200 μl of 10mM Tris-HCl, pH8.0,

containing 1mM EDTA. Samples were then treated with DNAse-free RNAse 10 u/ml (Sigma) for 1h at 37°C. Nucleic acid concentration was determined by measurement of OD260. Electrophoresis was performed using 1% agarose gels at 100V for 1h in the presence of 0.1mg/ml ethidium bromide.

Detection of iNOS mRNA expression using reverse transcripition-polymerase chain reaction (RT-PCR) RT-PCR analysis was performed as described previously (10). Total RNA from the harvest cells was isolated by the acid guanidium-thiocyanate-phenol-chloroform method (12) and 1μg of total RNA was converted to cDNA by reverse transcriptase (Superscript, Gibco). The amplification procedure consisted of 30 cycles (95°C, 1min,; 55°C, 1min.; 72°C, 1min.) with the following oligonucleotide primer sets: iNOS primer: sense 5'-^{2824}GATCAGGAACCTGAAGCCCC^{2845}-3', antisense 5'-^{3398}GCCCTTTTTTGCCCCCATAGG^{3377}-3'. These sequences correspond to iNOS cDNA from the murine macrophage cell line,RAW264.7, as determined previouly (13). Sequences corresponding to the published mouse β-actin cDNA sequence (14) were also used as controls: sense 5'-^{886}TGGAATCCTGTGGCATCCATGAAAC^{910}-3', antisense 5'-^{1234}TAAAACGCAGCTCAGTAACAGTCCG^{1210}-3'. The amplified DNA fragments were as expected, 574bp for iNOS and 359bp for β-actin. Each final PCR product, 10μl, was loaded onto a composite gel containing 1%Nusieve/1%agarose (FMC Co., Rockland, ME, USA), electrophoresed, and visualized by ethidium bromide staining.

Statistical analysis Results are expressed as means ± standard error (SE) of the mean from at least three independent experiments; experiments gave similar results. Data were analyzed by Student's t-test, and $p<0.05$ was considered statistically significant.

RESULTS

Effects of L-arginine and serum on mastocytoma To understand how the number of mast cells is controlled in various pathological processes including mastcytosis and chronic inflammatory conditions, we studied the regulatory mechanism in murine mastocytoma. When mastocytoma cells were cultured with RPMI1640 containing heat-inactivated FBS and L-arginine, the cells grew very rapidly for 2 days after cell seeding under conditions of low cell density. After 3 days, cell proliferation was at high cell density (Fig. 1). Little is known, however, concerning the relationship between apoptosis and cell proliferation. When cells were cultured without L-arginine and FBS, they did not grow and proliferation was inhibited from the first day after culture (Fig. 1, Arg -, Arg +, FBS 0%), confirming that the cells died due to necrosis (data not shown). This obseravation suggests that the change from rapid growth to inhibition of proliferation depends on the concentrations of FBS and L-arginine.

Electron microscopic findings of cell death in mast We found that this arrest of cell growth involved programmed cell death or apoptosis. Morphological evidence of apoptosis came from intranucleosomal DNA fragmentation. The electron micrographs presented in Fig.2 show typical nuclear and cytoplasmic changes detected after 3 days of culture. These changes, which were characteristic of apoptosis, included nuclear and cytoplasmic condensation and persistence of the continuous plasma membrane. Apoptotic findings on day 4 were more marked compared to those on day 3 (Fig. 2, 3 days).

Effects of N-MMA and Dex on induced apoptosis in mastocytoma Time course experiments revealed that no DNA fragmentation within cells occurred up to 2 days after cell seeding

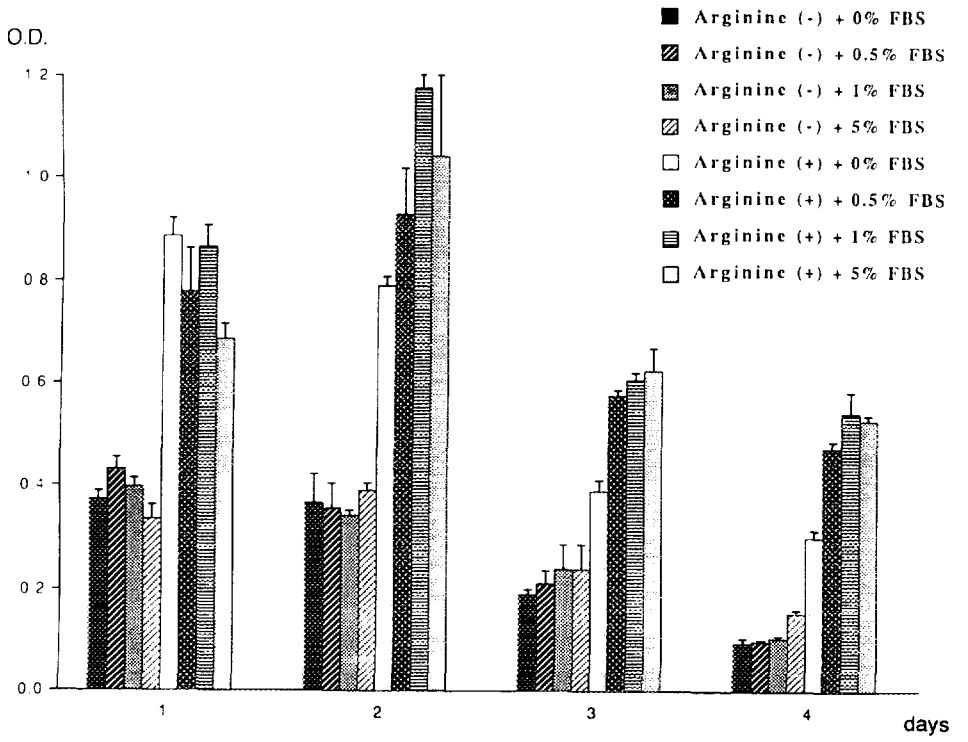

Fig.1. Effects of L-arginine and serum on mastocytoma, estimated by MTT assay

Murine mastocytoma cells (1 x 10^4cells) were seeded into 96-well flat-bottomed culture plates. The cells were incubated with various concentrations of FBS (0-5%), with (Arg+) or without (Arg-) 1mM L-arginine. The MTT assay was performed daily for 4 days. Each point represents the means ± SE of three determinations.

(Fig. 3A). Cells treated with RPMI 1640 containing 10% FBS (Fig. 3B, C: lane 1) and those treated with complete medium plus TNF-α (Fig. 3B, C: lane 2) underwent fragmentation on days 3 and 4, while apoptosis was prevented in cells treated with complete medium plus Dex (Fig. 3C: lane 3), or with complete medium plus Dex+NMA (Fig. 3C: lane 5), or with complete medium plus Dex+TNF-α (Fig. 3C: lane 4). Other reagents tested including GM-CSF, PMA, CaI and ECP failed to affect spontaneous induction of apoptosis (data not shown).

Expression of mRNA iNOS by apoptotic from mastocytoma and inhibitory effects of Dex. As our findings suggested a close relationship between iNOS and apoptosis, we examined the expression of iNOS mRNA in apoptotic cells using RT-PCR. Although no expression of iNOS mRNA was found in non-apoptotic cells on day 2 after cell seeding (Fig. 4A), on day 3 only apoptotic cells treated with complete medium alone or with complete medium plus TNF-α expressed iNOS mRNA (Fig. 4B: lane 1, 2). However, after 4 days of culture, even cells treated with Dex and NMA expressed iNOS mRNA (Fig. 4C: lane 5).

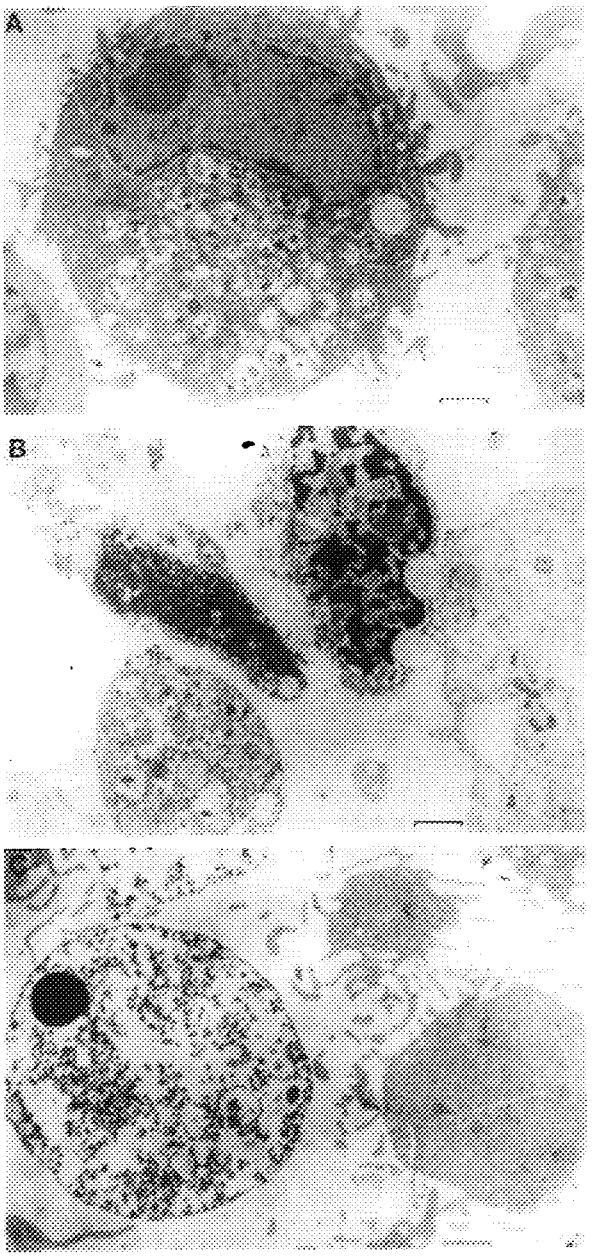

Fig.2. Electron microscopic findings of mastocytoma cell death

Murine mastocytoma cells (5 x10^5 cells/2ml) were cultured in 6-well plates for 4 days in RPMI 1640 containing 10% FBS. After the indicated intervals (A: 2 days after cell seeding, B: 3 days, C: 4 days), cells from each group were fixed in 0.25% glutaraldehyde and postfixed in 1% osmium tetroxide. Thin sections were stained in uranyl acetate and lead citrate and examined by transmission electron microscopy. Size bar=1μm.

DISCUSSION

It has been reported that mouse mast cells from femoral bone marrow of BALB/c mice, which are not identical to mastcytoma cells, underwent apoptosis upon removal of IL-3 (6). Two principal

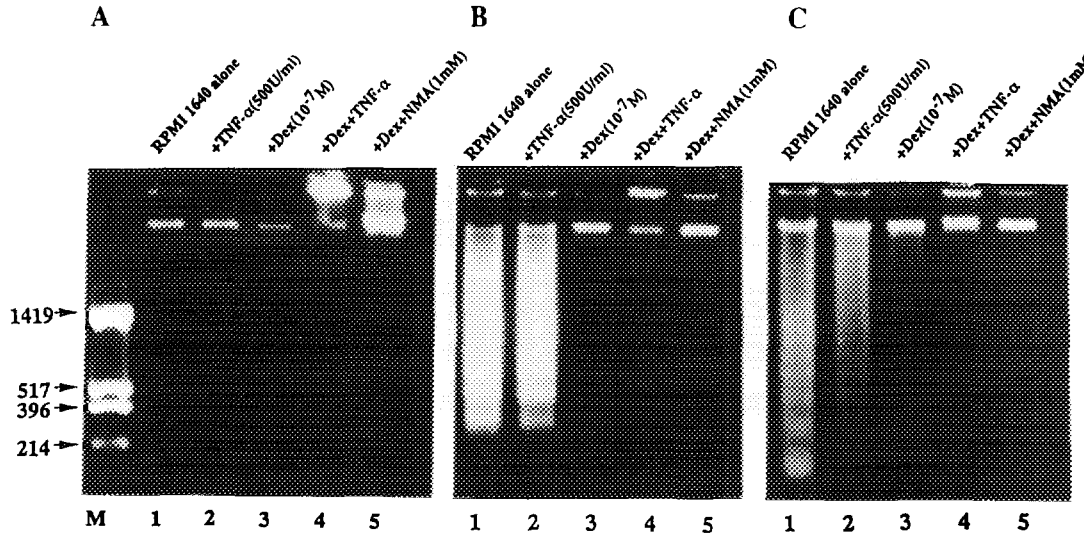

Fig.3. Effects of NMA and Dex on induced apoptosis in mastocytoma cells
DNA fragmentation observed by gel electrophoresis. lane 1: RPMI 1640 containing 10% FBS (complete medium); lane 2:complete medium TNF-α 500U/ml; lane 3: complete medium+10^{-7}M Dex; lane 4: complete medium+TNF-α 500U/ml+10^{-7}M Dex; lane 5: complete medium+10^{-7}M Dex +1mM N-MMA, M: DNA molecular weight markers; PUC 18 plasmid digested by Hinf I. (A: 2 days after cell seeding, B: 3 days, C: 4 days)

cytokines that promote mast cell proliferation have been identified, IL-3 and the c-kit ligand, SCF (3, 4). We demonstrated that the murine mastocytoma cell line, P815 cells also underwent spontaneous apoptosis, although P815 cells were shown to carry a point mutation of c-kit$^{Try\text{-}814}$ considered as a dominant-positive activating mutation (15).

This apoptosis in P815 cells was found with 10% FBS and L-arginine under conditions of high cell density, but the cells grew very rapidly for 2 days at low cell density (Fig.1). Previously, Maeda et al. (16) reported that there may be a cell density-dependent mechanism of apoptosis in hepatocytes. However, little is known concerning the relationship between apoptosis and cell density. We found that P815 cells required L-arginine and could not grow in L-arginine-free medium, while apoptotic cell death after growth was induced in the presence of the high concentrations of L-arginine (Fig. 1). In this study, we conclude that the regulation of the number of P815 cells and their viability was directly controlled by apoptosis mediated through NOS. These alterations were the result of NO production, and this was confirmed by the activation effects in medium containing high concentration of L-arginine and by TNF-α stimulation. An inhibitor of NOS, N-MMA, prevented apoptosis in addition to inhibiting iNOS mRNA expression (Fig. 3, 4). iNOS expression has been reported in macrophages, hepatocytes, pancreatic islet cells, vascular smooth muscle cells, glial cells, retinal epithelial cells and keratinocytes (17). We showed that iNOS in mastcytoma was highly expressed and was the most important regulatory factor of cell number.

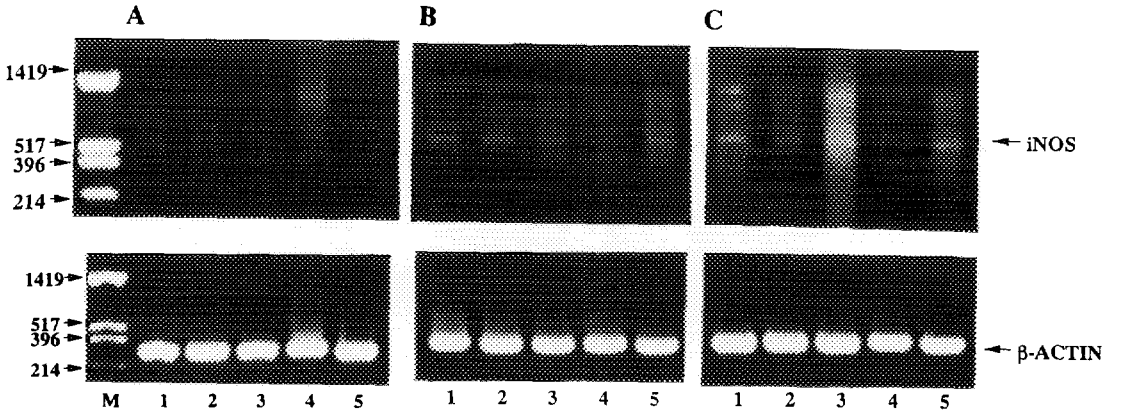

Fig.4. Expression of iNOS mRNA in apoptotic mastcytoma cells and inhibitory effects of Dex

iNOS and β-actin mRNA expression were determined by RT-PCR. The band of specific for iNOS was 574 bp and that for β-actin as the internal control was 359 bp. lane 1: RPMI 1640 containing 10% FBS (complete medium); lane 2:complete medium TNF-α 500U/ml; lane 3: complete medium+10^{-7}M Dex; lane 4: complete medium+TNF-α 500U/ml+10^{-7}M Dex; lane 5: complete medium+10^{-7}M Dex +1mM N-MMA, M: DNA molecular weight markers; PUC 18 plasmid digested by Hinf I. (A: 2 days after cell seeding, B: 3 days, C: 4 days)

NO is produced by the action of at least two different forms of NOS. One type is activated by calcium/calmodulin (Ca/CaM) and is constitutively present in endothelial cells and neurons. The second type is Ca/CaM independent and is only expressed after activation by endotoxin or cytokines (18). Apoptosis in mastocytoma cells was concluded to occur through a Ca/CaM-independent pathway, because TNF-α enhanced the effect, while PMA and CaI stimulation had no effect. It was interesting that Dex was capable of preventing apoptotic cell death in mastocytoma cells, although Dex is known to induce apoptosis in immature thymocytes (19). Our results showed that Dex not only supressed the expression of iNOS mRNA but also prevented apoptosis even in cells stimulated with TNF-α, a known inducer of iNOS. Weshil et al. (20) reported that Dex prevented expression of inflammatory cytokines, including TNF-α. A variety of biological effects have recently been attributed to NO in tumor cells (8). Albina et al. (9) reported that murine peritoneal macrophages showed NO-mediated apoptosis and that glucose starvation and inhibiton of the electron transport chain also resulted in apoptosis. A more direct mechanism for NO-mediated apoptosis has been suggested by Nguyen et al. (21), who demonstrated that NO can deaminate purine and pyrimidine bases in DNA, has been resulting in breakage of DNA strands.

It is important that we understand the mechanism by which mast cells undergo apoptosis, because apoptotic mast cells are eliminated from various tissues. In various pathological conditions and processes including mastocytosis, chronic inflammatory conditions or allergenetic diseases, mast cells must proliferate rapidly and become activated. While NO is stimultanenously synthesized in

mast cells stimulated by inflammatory reagents, most of the cells are then immediately eliminated through a programmed system of cell death after completion of their immediate task. This study suggests that iNOS expression is one of the most important control factors of mast cell proliferation, although many candidates has been proposed as inducers of apoptosis in mast cells.

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