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Regulation of Proliferation of Mouse Bone Marrow-derived Mast Cells by Activated Fibroblasts

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Nitric oxide (NO) is synthesized by various cells involved in inflammatory reactions and may then act on mast cells. In the present work, we attempted to clarify the role of this molecule on the proliferation of mouse bone marrow derived-mast cells (BMMC). Swiss 3T3 fibroblasts produced nitrite (NO_2^-) and nitrate (NO_3^-) upon treatment with interferon γ (IFN-γ). This formation was dependent of *L*-arginine and could be inhibited by the *L*-arginine analogue N^G-monomethyl-*L*-arginine (N^GMMA). The effect of IFN-γ was drastically increased by co-treatment with tumor necrosis factor γ (TNF-γ). BMMC were maintained in vitro for as long as 30 days when cocultured with Swiss 3T3 fibroblasts. Coculture with N^GMMA, significantly increased the number of BMMC. These results indicate that NO involves the inhibition of proliferation of BMMC when cocultured with Swiss 3T3 fibroblasts.

Key words : Nitric oxide, Mouse bone marrow derived-mast cells, Swiss 3T3 fibroblasts, Interferon-γ, Tumor necrosis factor-α

INTRODUCTION

Over the last several years fibroblasts have been shown to be capable of providing a microenvironment in which mast cells can survive, proliferate, and, in some cases, even undergo profound alterations in their phenotype (Levi-Schaffer *et al.*, 1985; Levi-Schaffer *et al.*, 1986; Levi-Schaffer *et al.*, 1987; Levi-Schaffer *et al.*, 1987; Dayton *et al.*, 1988; Fujita *et al.*, 1988; Fujita *et al.*, 1988; Katz *et al.*, 1990). Mast cells develop in media conditioned either by mitogen-stimulated spleen cells, which are rich sources of IL-3, IL-4, and IL-9 (Schrader *et al.*, 1986; Hamaguchi *et al.*, 1987). However, when grown on a feeder layer of mouse 3T3 fibroblasts, such mast cells persist in the absence of IL-3 (Katz *et al.*, 1990). A fibroblast product alternately called steel factor, mast cell growth factor, stem cell factor (SCF), and kit ligand was recently identified that could mimic many of the nurturing effects of fibroblasts on BMMC *in vitro* (Nocka *et al.*, 1990; Tsai *et al.*, 1991). SCF produced by fibroblasts is an important growth and differentiation factor for mast cells (Witte *et al.*, 1990). The addition of SCF to BMMC (50 ng/ml 2-3 times/wk) induced after 28 days the synthesis of more heparin proteaglycans (Tsai *et al.*, 1991). Thus, SCF has been implicated as a major growth and differentiation factor for mast cells. In fact, wild-type fibroblasts can produce a factor that interacts with the c-kit receptor, but Sl/Sl^d fibroblasts do not (Jarboe *et al.*, 1989).

Nitric oxide (NO) produced by activated macrophages is a cytotoxic effector molecule for various cells and bacteria (Hibbs *et al.*, 1984; Hibbs *et al.*, 1987; Stuchr *et al.*, 1989; Liew *et al.*, 1990; Denis *et al.*, 1991). Bidri *et al.* (1995) demonstrated that sodium nitroprusside (SNP) inhibited the proliferation of BMMC at concentrations where cell viability was no affected. They studied the effects of SNP on cyclic nucleotides levels in BMMC. SNP induced a rapid and transient in cGMP level that preceded an accumulation of cAMP in BMMC. They also demonstrated that cyclic nucleotides are closely involved in the inhibition of BMMC proliferation in the presence of SNP. Thus, the current study was designed to determine whether NO could interfere on BMMC proliferation because BMMC was maintained in the BMMC/fibroblast coculture system.

MATERIALS AND METHODS

Materials

Murine recombinant interferon-γ (rIFN-γ; 1×10^6 U/

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mg), recombinant tumor necrosis factor-α (rTNF-α; 1×10^5 U/ml), recombinant interleukin-3 (rIL-3) were purchased from Life Technology (Gaithersburg, MD). Recombinant murine SCF (rmSCF) was a gift from Dr Kitamura Y. (Osaka University, Japan). LiCl, urea, *N*-(1-naphthyl)-ethylenediamine dihydrochloride, sodium nitrite, sulfanilamide, and 2-mercaptoethanol (2-ME) were purchased from Sigma Chemical Co (St. Louis, MO). N^GMMA was purchased from Calbiochem-Behring Corp (La Jolla, CA). Pokeweed-mitogen (PWM), fetal bovine serum (FBS), and other culture reagents were purchased from Life Technologies, and 35-mm and 100-mm diameter petri dishes were purchased from Nunc (Naperville, IL). All reagents and media for tissue culture experiments were tested for their LPS content with the use of a colorimetric *Limulus* amoebocyte lysate assay (detection limit 10 pg/ml; Whittaker Bioproducts, Walkersville, MD).

Mice and cells

Three-month-old $WBB6F_1$-normal mice were sacrificed by decapitation under ether anesthesia. Spleens were removed, and spleen cells were suspended for preparation of both pokeweed mitogen-stimulated spleen cell conditioned medium (PWM-SCM) and BMMC as described previously (Kim *et al.*, 1994). The Swiss 3T3 fibroblast cell line was obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). The Sl/Sl^d fibroblast cell line was a gift from Dr Kitamura Y. (Osaka University, Japan). These two cell lines were adapted to grow in α-minimal essential medium (α-MEM; Flow Laboratories, Irvine, UK) containing 10% fetal calf serum (FCS; Hyclone, Logan Utah, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Establishment of BMMC

PWM-SCM was prepared as described by Kim *et al.* (Kim *et al.*, 1994). Speen cells (2.0×10^6 /ml) were incubated for 5 days in α-MEM containing a 1 : 300 dilution of PWM, 10% FCS, and 10^{-4} mol/L 2-mercaptoethanol. The conditioned medium was centrifuged, filtered through a 0.22-μm filter (Millipore Corp., Bedford, Ma,, USA), and stored at -80°C. PWM-SCM contains at least IL-3, IL-4, and IL-9, and is more effective than rIL-3 for development and maintenance of BMMC (Hamaguchi *et al.*, 1987). Culture flasks containing 2×10^7 spleen cells in 5 ml α-MEM supplemented with 10^{-4} mol/L 2-mercaptoethanol, 10% FCS and 10% PWM-SCM were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Half of the medium was replaced every 3 days, and used for experiments after 21 to 28 days of culture. BMMC harvested from the cultures were > 98% pure as determined by Alcian Blue staining, and resembled the mucosal type of mast cells.

Coculture with fibroblasts

Swiss 3T3 fibroblasts (10^4 cells) were suspended in 2 ml α-MEM supplemented with 10% FCS, and seeded in 35-mm culture dishes. The culture medium was aspirated and replaced with 2 ml of fresh medium every 2 days. After 3 days, the fibroblasts formed a confluent monolayer in each dish containing about 10^6 cells. The medium of the confluent cultures was aspirated, and 10^6 BMMC suspended in 2 ml α-MEM supplemented with 10% FCS was added with 10% PWM-SCM. The medium of BMMC/fibroblast coculture was changed every 2 days as follows: The medium was collected, and the supernatant obtained by centrifugation at 120 g for 5 min was used for sampling. Cells were resuspended in 2 ml of fresh medium, and then returned to the coculture. At various times after the initiation of coculture, the number of mast cells was estimated from the total hemocytometer count and the proportion of mast cells. The proportion of mast cells was determined by staining the cytospin preparations (Shandom Southern, Elliott, IL, USA) with Alcian Blue. During the coculture, the number of fibroblasts did not change.

Measurement of nitrite and nitrate concentration

NO synthesis in cell cultures was measured by a microplate assay method (Ding *et al.*, 1988). To measure nitrite, 100 μl aliquots were removed from conditioned medium and incubated with an equal volume of the Griess reagent [1% sulfanilamide/0.1% *N*-(1-naphtyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4] at room temperature for 10 min. The absorbance at 540 nm was determined in a Titertek Multiskan (Flow Laboratories, North Ryde, Australia). NO_2^- was determined by using sodium nitrite as standard. Cell free medium alone contained 5-8 μM of NO_2^- this value was determined in each experiment and substracted from the value obtained with cells. In some experiments, nitrate was measured by reducing nitrate to nitrite with bacterial nitrate reductase then measuring nitrite by using the Griess reagent.

Preparation of probe

To detect of inducible NO synthase (iNOS) mRNA transcripts, sense and antisense oligonucleotide primers specific for the coding regions of that gene were synthesized by using conventional technology. The following oligonucleotide primers were used: forward primer, 5' -GGCCTTGGCTCCAGCATGTAC-3', 1856 through 1876; reverse primer, 5'-GCTGCCGCTCTCATCCAGAAC-3', 2395 through 2415. The numbers represent the nucleotide numbers on the complementary

strands of iNOS cDNA sequence (Xie *et al.*, 1992). Total cellular RNA (5 μg) from rIFN-γ-and LPS-stimulated macrophages of WBB6F_1-mice was used as a template, and the single-stranded cDNA was synthesized with downstream antisense primers by reverse transcriptase. cDNA was amplified in a 12-μl reaction mixture by using a Dae Han Medical Co. DNA thermal cycler (Seoul, Korea) by using Taq polymerase in 30 cycles of 5 s of denaturation at 94°C, 5 s of annealing at 55°C, and 20 s of synthesis at 72°C. For the analysis of DNA sequence, PCR products were gel purified, treated with T4 polynucleotide kinase, and then with the Klenow fragment of DNA polymerase I. The products were subcloned into the *Eco*RV site of the pBluescript II KS (−) plasmid. One microgram of plasmid DNA was radiolabeled by random priming with [α-^{32}P]dCTP. The resultant specific activity was approximately 1×10^8 cpm/μg and was used at 1×10^7 cpm/blot.

RNA extraction and northern blotting

Total RNA was prepared by using the modified LiCl-urea method (Kim *et al.*, 1994), subjected to electrophoresis in 1.2% agarose-formaldehyde gels, and transferred to nylon membranes by capillary action in 20X SSC (1X SSC=0.15 M NaCl and 0.015 M sodium citrate, pH 7.2). After prehybridization, the filters were hybridized with random [α-32P]dCTP-labeled probes having specific activity of 1 to 5×10^8 cpm/μg in 10% dextran sulfate, 50% formamide, 4X SSC, 1X Denhardt's solution, and 10 μg/ml salmon sperm DNA for 24 hr at 42°C. Then the filters were washed, dried, and examined by autoradiography.

RESULTS

BMMC were cocultured with Swiss 3T3 or Sl/Sl^d fibroblasts in the presence of 10% PWM-SCM. In the *in vitro* BMMC/ fiborblast coculture system, the number of BMMC with Swiss 3T3 fibroblasts was maintained, but the number of BMMC with Sl/Sl^d fibroblasts decreased to about 0.9% of that of BMMC with Swiss 3T3 fibroblasts (Fig. 1).

Coculturing BMMC with Swiss 3T3 or Sl/Sl^d fibroblasts exhibited a significant increase in the levels of supernatants NO, which peaked on day 6 and then maintained to normal steady state levels by day 30 (Fig. 2). There was little difference in NO production between Swiss 3T3 and Sl/Sl^d fibroblasts (Fig. 2).

As shown in Table I, Swiss 3T3 fibroblasts produced NO upon treatment with rIFN-γ was about four-fold enhanced by cotreatment with TNF-α. NO production by the Swiss 3T3 fibroblasts increased from 17 μM in response to rIFN-γ alone to 72 μM in response to rIFN-γ plus TNF-α. Maximal production of NO was observed upon treatment of cells with 50 U/ml IFN-γ in combination with 1000 U/ml of TNF-α. Treatment with TNF-α alone did not stimulate NO formation.

To determine whether the increased NO formation

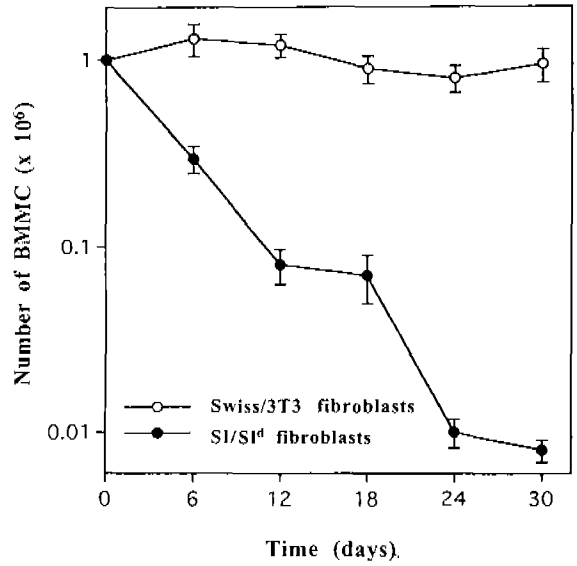

Fig. 1. Number of BMMC at various days after coculture with Swiss/3T3 or Sl/Sld fibroblasts in the presence of PWM-SCM. Culture medium was changed every 2 days as described in the Materials and Methods. The results are shown as means±SEM of 4 dishes.

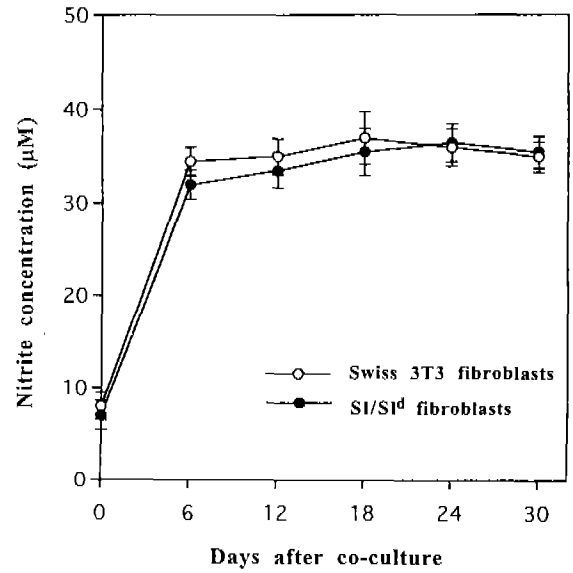

Fig. 2. NO production at various days after coculture with Swiss 3T3 or Sl/Sl^d fibroblasts in the presence of PWM-SCM. To measure nitrite and nitrate, 100 μl aliquots were removed from culture medium. The amount of nitrite and nitrate release was measured by using the method of Griess. Culture medium was changed every 2 days as described in the Materials and Methods. The results are shown as means ±SEM of seven dishes.

Table 1. Synergistic cooperation between rIFN-γ (50 U/ml) and TNF-α (1000 U/ml) to induce NO synthesis in Swiss 3T 3 fibroblasts

Additive		Final Concentration (μM)	
rIFN-γ	TNF-α (1000 U)	NO_2^-	NO_2^- plus NO_3^-
None	None	<8	<8
+	None	17±4*	36±9*
None	+	<11	<11
+	+	72±6**	148±14**

Confluent monolayers were treated with rIFN-γ alone or in combination with TNF-α for 72 hr. NO_2^-, NO_2^- plus NO_3^- was determined in supernatants as detailed in Materials and Methods. Values are the means±SD of six experiments. Student's two tailed *t*-test was used to compare stimulator-induced and spontaneous NO release amount (*p<0.005; **p <0.001).

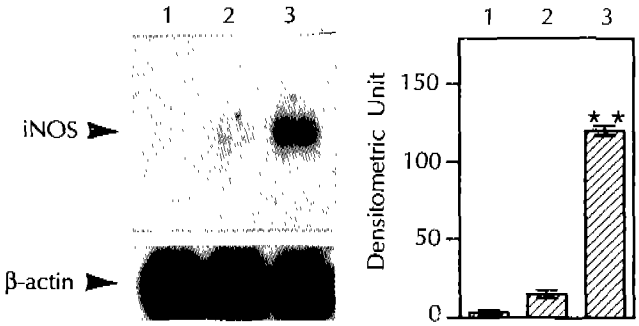

Fig. 3. Induction of the expression of iNOS mRNA during the BMMC/Swiss 3T3 fibroblast coculture. Total RNA was obtained from Swiss 3T3 fibroblasts. Lane 1; TNF-α only ; Lane 2; rIFN-γ only; Lane 3, rIFN-γ plus TNF-α, cultured for 72 hr. Total RNA was prepared according to the modified method described by Kim *et al.* (1994). Twenty micrograms of total cellular RNA was loaded per lane. The blot was hybridized with ^{32}P-labeled cDNA probes for iNOS and exposed to X-ray film for 4 hr. The β-actin probe was used to verify that an equal amount of total RNA (20 μg) was loaded in each lane. Quantitative representation of the data of Northern blots is shown at the right of the figure. Values are the means±SD of four independent experiments. Student's two tailed t-test was used to compare stimulator induced and spontaneous NOS expression levels (**p<0.001).

is correlated with iNOS mRNA levels, we examined iNOS mRNA content. Total RNA was isolated from Swiss 3T3 fibroblasts, separated by denaturing agarose gel electrophoresis, and analyzed by Northern blotting with radiolabeled cDNA that encoded iNOS (Fig. 3). The effect of IFN-α on expression of iNOS gene was markedly enhanced by TNF-α (1000 U/ml).

To test NO-mediated proliferative inhibition, we examined that the production of NO was blocked with N^GMMA. Treatment of N^GMMA (2 mM) during the BMMC/Swiss 3T3 fibroblast coculture induced about 3-fold increase of the number of BMMC (Fig. 4). Cell

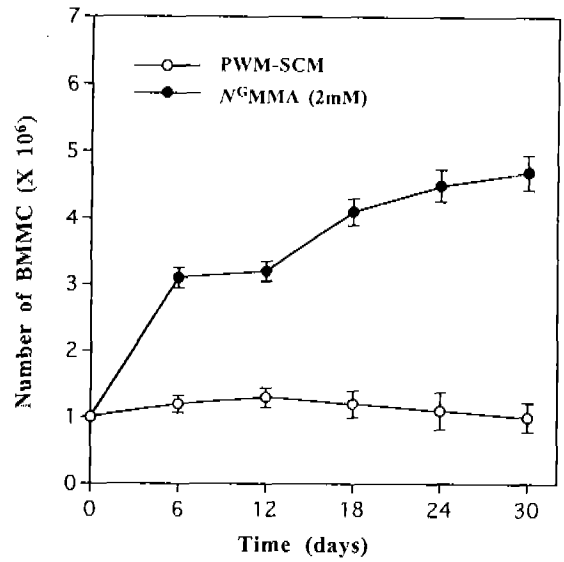

Fig. 4. Effect of N^GMMA in the BMMC/Swiss 3T3 fibroblast coculture system. BMMC/fibroblasts were cultured in the presence of 10% PWM-SCM only and 10% PWM-SCM plus 2 mM N^GMMA. Culture medium was changed every 2 days as discribed in the Materials and Methods. The number of BMMC was determined at various days after the initiation of the BMMC/fibroblast coculture. The number of BMMC was estimated from the total hemocytometer count.

viability was not affected by N^GMMA concentration as assessed by trypan blue exclusion method (Hudson *et al.*, 1976).

To elucidate the disappearance of BMMC in the BMMC /Sl/Sl^d fibroblast coculture system, we treated the rmSCF (50 ng/ml) during the coculture. The rmSCF protected the NO-mediated cytotoxicity (data not shown).

DISCUSSION

The present study indicates that NO inhibited the proliferation of BMMC on BMMC/Swiss 3T3 fibroblast coculture system. The Swiss 3T3 or Sl/Sl^d fibroblasts were capable to produce NO from *L*-arginine upon stimulation either PWM-SCM or rIFN-γ plus TNF-α. This effect of rIFN-γ was enhanced up to three fold by costimulation with TNF-α. TNF-α was a potent second signal for NO formation in rIFN-γ-treated Swiss 3T3 fibroblasts. The Sl/Sl^d fibroblasts could not support BMMC proliferation. These findings suggest that direct interactions between mast cell precursors and fibroblasts may play an important role in development of tissue mast cells in the constitutive steady state, or other inflammatory conditions. NO originate from the oxidation of *L*-arginine to citrulline. This reaction can be inhibited by the *L*-arginine analogue N^GMMA. NO has been identified as a cytotoxic effctor molecule of activated murine macrophages (Hibbs *et al.*, 1987; Stuchr *et al.*, 1989). The cy-

tostatic effect of rIFN-γ in combination with TNF-α on Swiss 3T3 fibroblasts can be attenuated by reducing formation of NO via inhibition of *L*-arginine hydroxylation with N^GMMA, via withdrawal of *L*-arginine. Our findings show that microenvironmental factors play an important role in regulating mast cell numbers by effecting survival.

NOS-dependent cytostasis can explain the action of other growth factors during terminal differentiation in which the initial mitogenic response is replaced by cytostatic phase. It is possible that NO releases from the Swiss 3T3 fibroblasts that produces it to promote cessation of growth in BMMC. Since it is previously reported that SNP induce cyclic nucleotides accumulation in BMMC, and that cGMP accounts for inhibition of mediator release from mast cells (Bidri *et al.*, 1995), we did not examine the cGMP levels. It has been reported that SCF not only can promote the proliferation of either immature or mature mast cells, but also can induce changes in the phenotype of these populations. The mechanisms by which SCF influences mast cell proliferation and maturation remain to be fully elucidated. The effects of SCF on mast cell proliferation *in vitro* require that the *c-kit* ligand interacts with a functionally competent *c-kit* receptor (Nocka *et al.*, 1990). However, the subsequent events leading to mast cell proliferation and/or maturation are not known.

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