*Cytotechnology* **36:** 179–186, 2001.



*Short communication*

# Induction of basophilic and eosinophilic differentiation in the human leukemic cell line KU812

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Received 2 May 2001; accepted 6 August 2001

*Key words:* basophils, eosinophils, Fc$\varepsilon$RI, hydrocortisone, KU812 cells, sodium nitroprusside

## Abstract

We have demonstrated that an immature prebasophilic cell line, KU812 cells can be induced to differentiate into basophil-like cells when cultured with hydrocortisone (HC) with enhanced cell surface expression of Fc$\varepsilon$RI, a high affinity IgE receptor. In this study, we report that sodium nitroprusside (SNP), an intracellular NO donor, also induces cell surface expression of Fc$\varepsilon$RI on KU812 cells. Cell surface Fc$\varepsilon$RI expression was detected in about 20% of KU812 cells treated with SNP for 14 days as well as the cells treated with HC for 7 days, while non-treated KU812 cells did not express Fc$\varepsilon$RI on their cell surface. However, Wright-Giemsa staining and flowcytometry analysis of CD13 and CD15 antigens on HC and SNP treated KU812 cells demonstrated that SNP induced eosinophilic differentiation in KU812 cells differently from HC which induced basophilic differentiation. To further confirm this result, we performed RT-PCR against mRNAs specific for eosinophils, such as eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO). SNP treated KU812 cells but not HC treated cells expressed EDN and EPO mRNA depending upon the induction of differentiation, clearly demonstrating that SNP induces eosinophilic differentiation in KU812 cells. To clarify that different signaling cascades were activated in HC and SNP treated KU812 cells, we analyzed activities of AP-1, NF-AT and NF-$\kappa$B transcription factors by EMSA, which are known to be involved in signal transduction pathways downstream from the Fc$\varepsilon$RI molecule of basophils. All these three transcription factors were activated in HC treated KU812 cells, but not in non-treated and SNP treated KU812 cells. These results indicate that KU812 cells are multi-potent precursor cells which can be induced to differentiate into basophils and eosinophils upon exogenous signals, and that NO is an important factor to decide the eosinophilic differentiation in KU812 cells with enhanced surface expression of Fc$\varepsilon$RI, and further suggest that different signaling cascades can be activated between basophilic and eosinophilic differentiation in KU812 cells.

*Abbreviations:* EDN, eosinophil-derived neurotoxin; EPO, eosinophil peroxidase; HC, hydrocortisone; SNP, sodium nitroprusside.

## Introduction

Mast cells, basophils and eosinophils play central roles in allergic diseases. Cross-linking of cell surface Fc$\varepsilon$RI, a high-affinity IgE receptor, caused by binding of IgE and polyvalent antigens results in degranulation of mast cells or basophils and in release of various inflammatory mediators that trigger allergic inflammation reaction (Gauchat et al., 1993; Yanagihara et al., 1997). On the other hand, the increase in

the number of eosinophils was reported to be closely related to the rise in the concentration of IgE at infection with worm. Then eosinophils were thought to be involved in the IgE-dependent elimination of parasites. Thus, to analyze the mechanisms of degranulation and release of chemical mediators in response to Fc$\varepsilon$RI crosslinking in these cells, and to search for functional substances to inhibit these reactions, we need to clarify the signaling cascades downstream from the Fc$\varepsilon$RI molecule on mast cells, basophils and eosinophils. However, physiologically functional and human origin cell lines are now not available for *in vitro* study. Almost all of studies clarifying the signaling cascades and functionalities of mast cells, basophils and eosinophils are obtained by using the cells of non-human origins. To accurately understand the allergic inflammation reaction in human mediated by mast cells, basophils and eosinophils, we need to use human derived cell lines and to analyze their signaling cascades and functionalities.

Human mast cells and basophils can be established by culturing multi-potent hematopoietic stem cells prepared from human bone marrow and cord blood in the presence of IL-3, stem cell factor and IL-6 (Boyce et al., 1995; Saito et al., 1996; Toru et al., 1996; Nilsson et al., 1994). However, the procedures to establish functional mast cells and basophils are laborious and time-consuming. Establishment of easily available cell lines of functional human mast cells and basophils are desired by many researchers. Commercially available immature prebasophilic cell line, KU812 cells established from peripheral blood of the patient of chronic myeloid leukemia was thought to be a desirable cell line for analysis of human allergic inflammation reaction in that KU812 cells produce histamine and express Fc$\varepsilon$RI $\alpha$ chain mRNA and CD40 mRNA (Valent et al., 1990; Nilsson et al., 1994; Blom et al., 1992; Magnusson et al., 1995). However, KU812 cells express cell surface Fc$\varepsilon$RI at only low level as compared to basophils *in vivo*, thus sufficient induction of differentiation was needed to evaluate signaling cascades and functionalities of basophils by using KU812 cells. We and other groups have demonstrated that KU812 cells can differentiate into functional basophils in response to exogenous signals such as hydrocortisone and IL-4 (Hara et al., 1995, 1998). In this study, we attempted to search for other active substances having an ability to differentiate KU812 cells into basophils expressing high level of functional Fc$\varepsilon$RI on their cell surface, and to clarify the differentiation mechanisms of KU812 cells in response to exogenous signals.

In the course of this study, we found that sodium nitroprusside, reagent known as NO donor, induced KU812 cells to differentiate into eosinophils, indicating that KU812 cells maintain the ability to bi-directionally differentiate into basophils and eosinophils depending upon the exogenous signals.

## Materials and methods

### *Cells and cell culture*

We used an immature prebasophilic cell line, KU812 cells (HSRRB, Japan) established from peripheral blood of the patient of chronic myeloid leukemia in this study. KU812 cells were maintained in RPMI-1640 medium (Gibco BRL, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES and antibiotics (100 U $ml^{-1}$ penicillin G and 100 mg $ml^{-1}$ streptomycin). The cells were passaged every 3–4 days.

### *Induction of differentiation of KU812 cells*

To test for basophilic differentiation, KU812 cells were seeded at the density of $1 \times 10^5$ cells $ml^{-1}$ in 100 mm dishes (Becton Dickinson Labware, Franklin Lake, NJ) containing the medium supplemented with 100 or 1000 nM hydrocortisone (HC) as described previously (Hara et al., 1995). After 4 to 8 days of culture, the cells were collected and applied to phenotypic analysis. To test for eosinophilic differentiation, KU812 cells were cultured in the medium supplemented with 1 pM to 1 nM sodium nitroprusside (SNP; Sigma, St. Louis, MO). After 4 to 14 days of culture, the cells were collected and applied to phenotypic analysis.

### *Analysis of cell surface antigens by flowcytometry*

To detect cell surface Fc$\varepsilon$RI, $1 \times 10^6$ cells were incubated with mouse monoclonal antibody (mAb) specific for human Fc$\varepsilon$RI $\alpha$ chain (CRA-1; Kyokuto, Tokyo, Japan), stained with FITC-labeled goat anti-mouse immunogloblins (TAGO, Burlingame, CA) and analyzed by a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA). Mouse IgG (TAGO) was used as isotype-matched control antibody. To detect cell surface CD13 and CD15 antigens, we used mouse anti-human CD13 mAb (IgG type; YLEM S. R. L., Roma, Italy) and mouse anti-human CD15 mAb (IgM type; Pharmingen, San Diego, CA), respectively. For simultaneous staining of CD13 and CD15

antigens, CD13 antigens were detected by PE-labeled goat anti-mouse IgG antibody (IMMUNOTECH, Marseille, France), and CD15 antigens were detected by FITC-labeled goat anti-mouse IgM antibody (TAGO). To calculate the cells positive for cell surface antigens, the log fluorescence intensities of 95% of the cells stained with the isotype-matched control antibodies were adjusted to be $10^0$ to $10^1$. Then we calculated the number of cells showing over $10^1$ of log fluorescence intensity at each experiment.

*Wright-Giemsa staining*

Wright and Giemsa soln. were purchased from WAKO (Osaka, Japan). Cells fixed onto the slide glasses were incubated in the Wright soln. mixed with the same volume of methanol for 3 min with gentle agitation. After the cells on the slide glasses were washed with water and dried up, cells were incubated in the Giemsa soln. mixed with the same volume of PBS for 20 min with gentle agitation. After washing the cells with water and drying up, stained cells were observed through a microscope. Basophils and eosinophils were reported to be stained as dark red and tango, respectively.

*RT-PCR*

Total RNA was prepared from KU812 cells by using TRIzol reagent (Gibco BRL). cDNA was synthesized from 3 $\mu$g of the total RNA with Superscript II reverse transcriptase (Gibco) by using oligo $(dT)_{20}$ as primer. Each cDNA was served as template for PCR amplification using specific primers. The primer pairs for amplifying mRNAs of Fc$\varepsilon$RI $\alpha$ chain, $\gamma$ chain, tryptase, eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO) were as follows: Fc$\varepsilon$RI $\alpha$ chain (sense) 5'-ATGAAGAAGATGGCTCCTGC-3', Fc$\varepsilon$RI $\alpha$ chain (antisense) 5'-ATTGTGGAACCATTTGGTGG-3', Fc$\varepsilon$RI $\gamma$ chain (sense) 5'-GATGATTCCAGCAGTGGTCTTGCT-3', Fc$\varepsilon$RI $\gamma$ chain (antisense) 5'-TAGGGCCAGCTGGTGTTAATGGCA-3', tryptase (sense) 5'-AGCAAGTGGCCCTGGCAGGTGA-3', tryptase (antisense) 5'-AGAGGAAATGGCGGTGGGAGGC-3', EDN (sense) 5'-CCAGCACATCAATATGACCTCC-3', EDN (antisense) 5'-GTGAACTGGAACCACCGGATA-3', EPO (sense) 5'-CTGCTGGATGCTGCCTACAATT-3', EPO (antisense) 5'-CAAGAGGGGAGAAAGCGATT-3'. PCR was done in the condition of 94 °C, 45 sec, 60 °C, 45 sec and 72 °C, 1.5 min for 31 to 35 cycles. PCR products were resolved in the 2% agarose gel electrophoresis and stained with EtBr.

*Electrophoretic mobility shift assay (EMSA)*

$1 \times 10^8$ cells cultured in the presence of HC or SNP were harvested and pelleted by centrifugation for 5 min at 3000 $\times$ g. Nuclear extracts were prepared according to Dignam et al. (Dignam et al., 1983) with minor modifications. Briefly, after washing the cells with PBS, cells were resuspended in 1 ml of buffer A (10 mM HEPES, pH 7.6, 15 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF (Sigma) and 10 $\mu$g ml$^{-1}$ Leupeptin (Sigma)). After removing soluble fraction by centrifugation at 800 $\times$ g for 1 min at 4 °C, pellets were resuspended in 1 ml of buffer B (buffer A containing 0.2% NP-40). After removing soluble fraction by centrifugation at 800 $\times$ g for 1 min at 4 °C, pellets were resuspended in 1 ml of sucrose buffer (0.25 M sucrose, 10 mM HEPES, pH 7.6, 15 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF and 10 $\mu$g ml$^{-1}$ Leupeptin). After removing the soluble fraction by centrifugation, pellets were resuspended in 20 $\mu$l of buffer D (50 mM HEPES, pH 7.9, 400 mM KCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, 10 $\mu$g ml$^{-1}$ Leupeptin) and incubated for 30 min at 4 °C with agitation. After centrifugation at 600 $\times$ g for 15 min at 4 °C, supernatants were collected and applied to EMSA. Protein concentrations were measured according to the method described by Bradford (Bradford MM 1976) with a BIO RAD Protein Assay kit (Bio-Rad Laboratories, Richmond, CA). Double strand oligonucleotides (40 pmol) containing the NF-$\kappa$B, NF-AT and AP-1 consensus sequences were end-labeled with [$\gamma$-$^{32}$P]dATP (AmershamPharmacia Biotech., Buckinghamshire, UK) and used as probe in EMSA. Sequences of sense strands of these oligonucleotides were as follows; NF-$\kappa$B: 5'-AGTTGAGGGGACTTTCCCAGGC-3', NF-AT: 5'-GATCCGGAGGAAAAACTGTTTCATACAGAAGGCGTG-3' and AP-1: 5'-GATCCGGTTGCTGACTAATTG-3'. Binding reactions (in total 10 $\mu$l) were done by incubating 0.8 $\mu$g of the nuclear extract with [$^{32}$P]-labeled double-strand oligonucleotide probe (1 pmol) in the reaction buffer (10 mM Tris-HCl (pH 7.5), 10 mM KCl, 1 mM DTT, 1 mM EDTA, 12.5% glycerol, 0.1% Triton X-100, 250 $\mu$g ml$^{-1}$ BSA, 50 $\mu$g ml$^{-1}$ poly(dI-dC) poly(dI-dC) (Sigma)) in the presence or absence of competitor for 15 min at room temperature. The resulting DNA-protein com-

plexes were resolved in 5% polyacrylamide gel electrophoresis. Bands were visualized by a radio-image analyzer, BAS1000 (Fuji film, Tokyo, Japan).

## Results and discussion

### *HC and SNP enhanced cell surface FcεRI expression on KU812 cells*

We examined the expression of FcεRI, a high affinity IgE receptor, on untreated KU812 cells by flow cytometry, demonstrating that cell surface expression of FcεRI was very low (< 10%), but that FcεRI accumulated in the cytoplasm of KU812 (> 80%; data not shown). These results suggested that additional signals were required to induce translocation of FcεRI molecule from cytoplasm to cell surface.

In our previous study, we found that hydrocortisone (HC), one of steroid hormones, could enhance the cell surface expression of FcεRI (Hara et al., 1995). Hara, et al. have reported that cell surface expression of FcεRI and cellular histamine content increased by the treatment of IL-4, and that differentiated KU812 cells by the IL-4 treatment released histamine upon IgE-crosslinking (Hara et al., 1998). These results suggest that KU812 cells can differentiate into functional basophils upon exogenous signals. Based upon these studies, we searched for other active substances to induce basophilic differentiation in KU812 cells. Among the reagents tested, sodium nitroprusside (SNP), which is known to be an intracellular NO donor and to have an ability to inhibit growth of cancer cells (Blachier et al., 1996), induced the cell surface expression of FcεRI in KU812 cells (Figure 1). As reported previously, 100 nM of HC enhanced the cell surface expression of FcεRI in KU812 cells after 7 days of culture (about 20%). On the other hand, SNP also enhanced the cell surface expression of FcεRI in KU812 cells. KU812 cells treated with 10 pM of SNP notably increased FcεRI expression after 14 days of culture (about 20%). Although HC treatment was found to induce the enhanced cell surface expression of FcεRI a little faster than SNP treatment in KU812 cells, these results suggest that SNP as well as HC can transmit differentiation signals in KU812 cells.

### *Phenotypic and morphological analysis of HC and SNP-treated KU812 cells*

Both of HC and SNP enhanced the cell surface expression of FcεRI in KU812 cells, then we anticipated that HC and SNP transmit the same differentiation signals in KU812 cells. Here, we firstly analyzed the cell phenotype and morphology of HC and SNP treated KU812 cells by Wright-Giemsa staining. HC treated KU812 cells showed no apparent changes in morphology and most of cells were stained as dark red (data not shown), suggesting that HC treated KU812 cells differentiate into basophil-like cells as reported previosly. However, SNP treated KU812 cells for 4 to 9 days showed eosinophil-like morphology and were stained as tango (Table 1). Especially, 44.1% of SNP treated KU812 cells were judged as eosinophil-like cells when culturing KU812 cells with 0.1 nM SNP for 9 days. These results suggest that SNP transmit different differentiation signals from HC. SNP likely induces eosinophilic differentiation into KU812 cells. We took it into consideration that there were difficulties in discriminating between basophils and eosinophils in Wright-Giemsa staining, then we attempted to characterize HC and SNP treated KU812 cells by other several methods.

*Table 1.* Percentages of eosinophil-like cells in SNP treated KU812 cells

| SNP conc. (nM) | culture period (days) | |
|---|---|---|
| | 4 | 9 |
| 0.1 | 42.9%* | 44.1% |
| 1 | 37.8% | 16.2% |
| 10 | 13.1% | 7.9% |

* Data represent averages of three independent experiments.

### *Surface expressions of CD13 and CD15 antigens on HC and SNP treated KU812 cells*

Basophils and eosinophils can be discriminated by their surface expression of CD antigens. Although basophils express CD13 antigen, but not CD15 antigen, eosinophils express both of CD antigens. By using this criteria, we characterize HC and SNP treated KU812 cells by flowcytometry. Figure 2 shows that HC treated KU812 cells maintained almost the same pattern of cell population of $CD13^+$ and $CD15^+$ cells, namely 94.1% of HC treated KU812 cells express CD13 antigen, but not CD15 antigen. This result suggest that HC treated KU812 cells as well as non-treated KU812 cells showed basophil-like cell surface expression of CD antigens. On the other hand, $CD13^+/CD15^+$ cell population representative of eosinophils increased in SNP treated KU812 cells

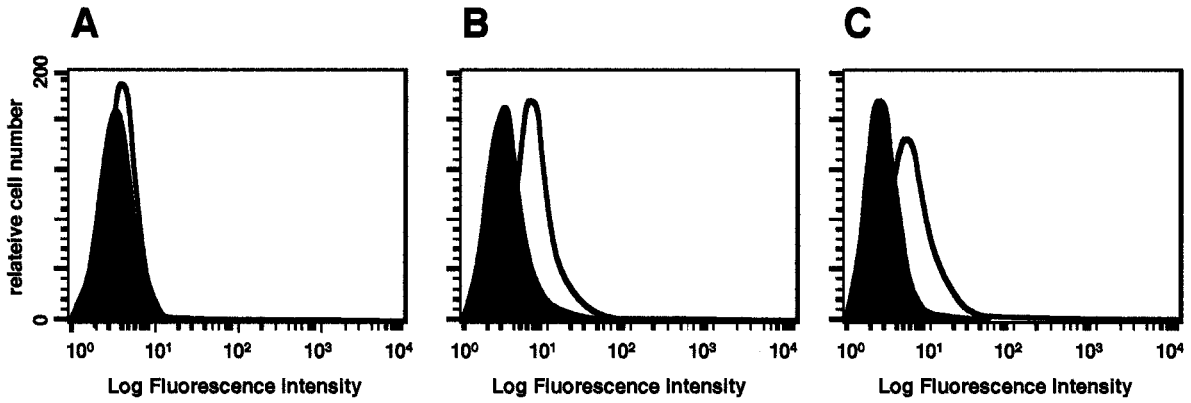


*Figure 1.* Cell surface expression of FcεRI on HC and SNP treated KU812 cells. Cell surface expression of FcεRI on KU812 cells was analyzed by flowcytometry. Cell surface FcεRI was detected by incubating with mouse anti-human FcεRI $\alpha$ chain mAb (CRA-1) and subsequent reacting with FITC-labeled goat anti-mouse immunoglobulins (White). Mouse IgG was used as isotype-macthed control antibody (Black). A, non-treated KU812 cells; B, KU812 cells treated with 100 nM HC for 7 days; C, KU812 cells treated with 10 pM SNP for 14 days.

(15.7%), which corresponds well with the frequency of eosinophils in SNP treated KU812 cells evidenced by the Wright-Giemsa staining (Table 1, 1 nM SNP, 9 days: 16.2%), demonstrating that SNP induces eosinophilic differentiation in KU812 cells differently from HC judging from the cell surface expression of CD13 and CD15 antigens.

*Expression of eosinophils specific mRNA*

To clarify the cell phenotypes of SNP treated KU812 cells by mRNA expression level, we evaluated expressions of mRNAs specific for eosinophils by RT-PCR. We analyzed the expression of mRNA for FcεRI $\alpha$ chain and $\gamma$ chain specific for FcεRI positive cells, tryptase specific for mast cells (Tharp, 1990), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO) specific for eosinophils (Graziano et al., 1989; Abu-Ghazaleh et al., 1989). Firstly we measured mRNA levels for FcεRI $\alpha$ and $\gamma$ chains. Figure 3 showed that FcεRI $\alpha$ chain mRNA was constantly detected in KU812 cells cultured in the presence or absence of HC or SNP, however, a significant increase of FcεRI $\gamma$ chain mRNA expression was observed in KU812 cells cultured in the presence of HC or SNP. These results suggest that the lack of cell surface FcεRI expression in non-treated KU812 cells was due to deficient $\gamma$ chain transcription, and that HC or SNP up-regulated FcεRI $\gamma$ chain expression at transcription level, leading to enhanced cell surface expression of FcεRI. Next, we measured the level of mRNA specific for mast cells. mRNA for tryptase can be detected in non-treated KU812 cells and insufficiently differentiated KU812 cells, suggesting that parental KU812 cells maintain mast cell like phenotypes or have an ability to differentiate into mast cells by exogenous signals. This mast cell like phenotypes were thought to be lost by the induction of differentiation into basophils or eosinophils with HC or SNP, respectively. Finally, we evaluated the levels of mRNAs for EDN and EPO in HC and SNP treated KU812 cells. mRNAs for EDN and EPO can be detected only in SNP treated KU812 cells, clearly demonstrating that SNP induced eosinophilic differentiation in KU812 cells. These results indicate that KU812 cells are multi-potent precursor cells which can be differentiated into mast cells, basophils and eosinophils upon exogenous signals, and further suggest that NO triggers eosinophilic differentiation signals in KU812 cells.

*Signals transmitted in HC and SNP treated KU812 cells*

Considering together with that HC and SNP have different abilities to induce differentiation in KU812 cells, we anticipated that HC and SNP transmit different signals in KU812 cells. Then we analyzed activities of transcription factors in HC and SNP treated KU812 cells by EMSA. Until now, signaling pathways downstream from the FcεRI on basophils have been vigorously investigated (Schroeder and MacGlashan, 1997), but those for eosinophils have been scarce. Then, we analyzed activities of transcription factors involved in signal transduction pathways downstream from FcεRI in basophils. NF-$\kappa$B and AP-1 are known

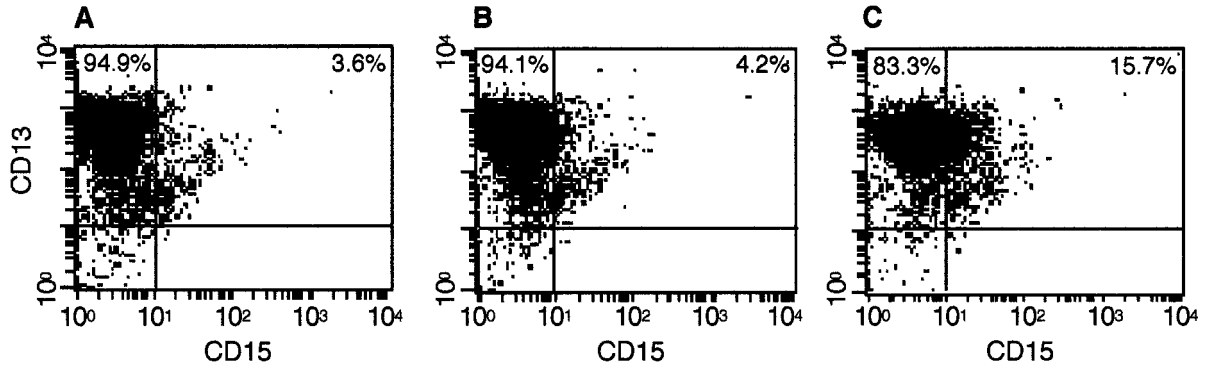


*Figure 2.* Expression of surface marker antigens for basophils and eosinophils in HC and SNP treated KU812 cells. Cell population for $CD13^{+}/CD15^{-}$ (basophils) and $CD13^{+}/CD15^{+}$ (eosinophils) in HC and SNP treated KU812 cells was analyzed by flowcytometry. Cell surface expressions of CD13 and CD15 were detected by simultaneous staining of the antigens. CD13 and CD15 antigens were detected by incubating with mouse anti-human CD13 mAb (IgG type) and mouse anti-human CD15 mAb (IgM type), and subsequent reacting with PE-labeled goat anti-mouse IgG antibody and FITC-labeled goat anti-mouse IgM antibody. A, non-treated KU812 cells; B, KU812 cells treated with 1000 nM HC for 8 days; C, KU812 cells treated with 1 nM SNP for 15 days.

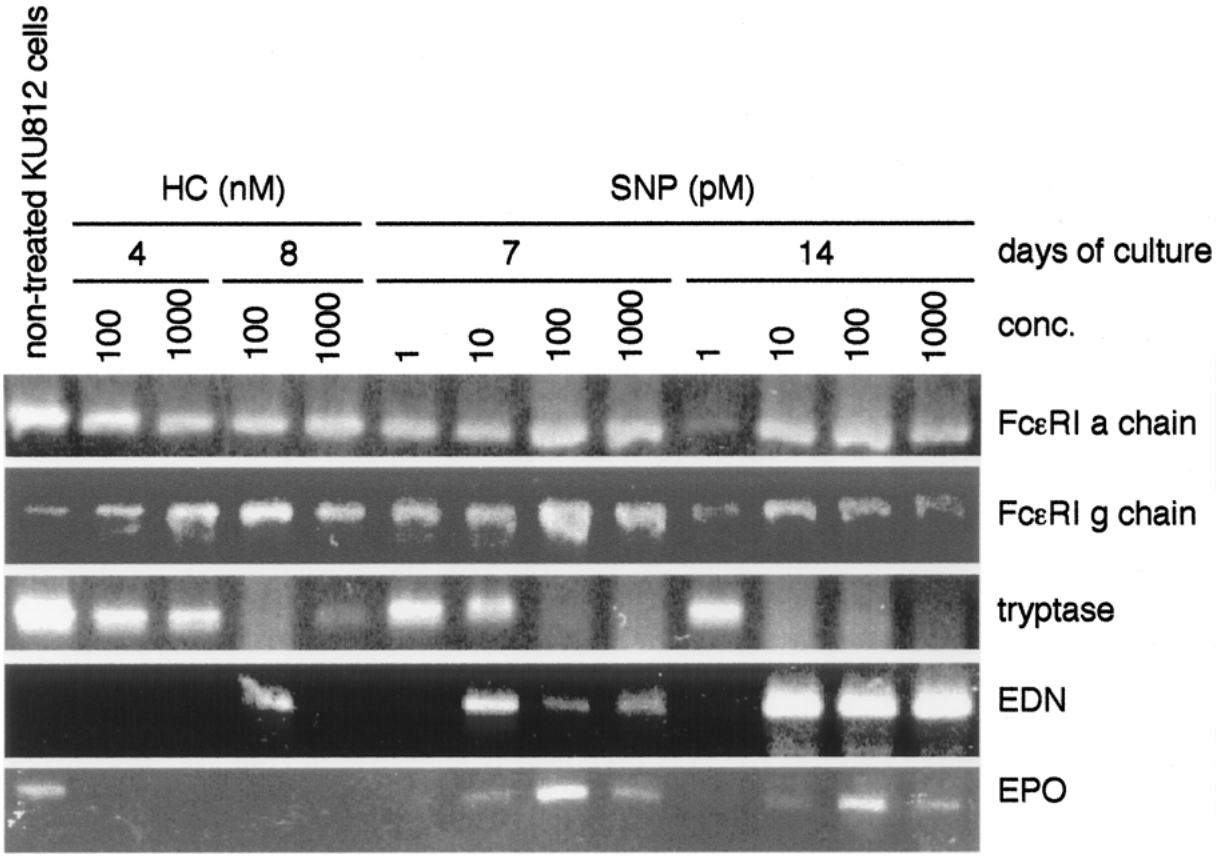


*Figure 3.* mRNA expression in HC and SNP treated KU812 cells Expressions of mRNAs for FcεRI $\alpha$ chain, FcεRI $\gamma$ chain, tryptase, EDN and EPO in HC and SNP treated KU812 cells were analyzed by RT-PCR. Sequences of specific primers and PCR conditions were described in Material and Methods section.

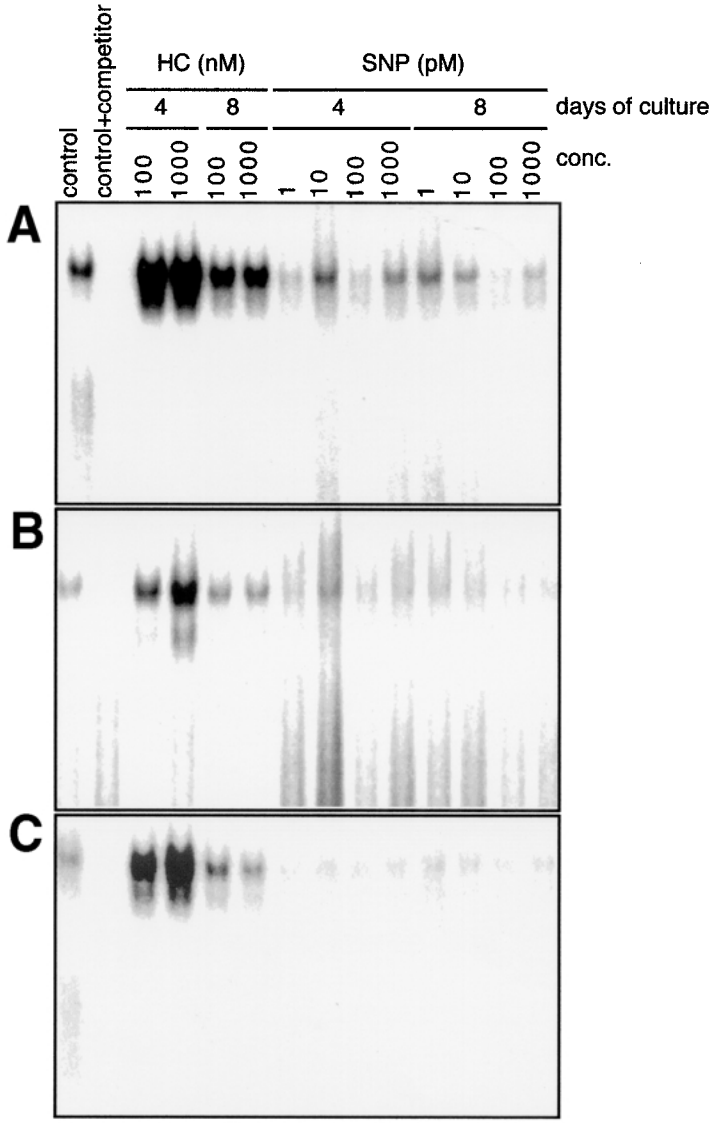


*Figure 4.* Activities of transcription factors in HC and SNP treated KU812 cells. EMSA analysis was done to evaluate activities of transcription factors, AP-1, NF-AT and NF-$\kappa$B in HC and SNP treated KU812 cells. Sequences of probes and condition of binding reaction were described in Materials and Methods section. Non-labeled probes were used as competitors for each experiments. A, AP-1; B, NF-AT; C, NF-$\kappa$B.

to be activated upon Fc$\varepsilon$RI crosslinking and subsequent protein kinase C activation, and NF-AT is known to be activated in response to the increase in cellular calcium concentration following the activation of Fc$\varepsilon$RI. As expected, all transcription factors tested (AP-1, NF-AT and NF-$\kappa$B) were activated in HC treated KU812 cells, but not in non-treated and SNP treated KU812 cells (Figure 4). These results demonstrate that HC and SNP transmit different signals intracellularly, and that HC activates these transcription factors independently from Fc$\varepsilon$RI crosslinking, possibly resulting in basophil-like gene expression and basophilic differentiation. Further studies are needed to identify the transcription factors and signal transduction pathways involved in eosinophilic differentiation in SNP treated KU812 cells.

## Concluding remarks

This study demonstrated for the first time that SNP, the NO donor reagent, induced an immature prebasophilic

cell line of KU812 to differentiate into eosinophils with enhanced Fc$\varepsilon$RI expression. Eosinophils were reported to be a major source of NO-derived oxidants in severe asthma. MacPherson et al. have demonstrated that eosinophils use distinct mechanisms for generating NO-derived oxidants and identifiy EPO as an enzymatic source of nitrating intermediates in eosinophils (MacPherson et al., 2001). This study suggests that NO plays a critical role in maintaining phenotypes and functionalities of eosinophils, and further that NO induces eosinophils-specific gene expression and Fc$\varepsilon$RI expression on their cell surface. Further studies must be needed to clarify the role of NO in maintaining of functionalities of eosinophils, but we conclude here that excess production of NO might lead to activation of eosinophils and occurrence of asthma.

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