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Effects of nerve growth factor (NGF) and other fibroblast-derived growth factors on immature human mast cells (HMC-1)

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SUMMARY

We have previously shown that fibroblast and keratinocyte supernatants up-regulate expression of mast cell characteristics in the human immature mast cell line HMC-1. This effect could not be induced in HMC-1 cells by the well-known mast cell growth factor stem cell factor (SCF), probably due to mutations of the SCF receptor c-Kit in these cells. Here we report the effects of several known fibroblast- and keratinocyte-derived growth factors, namely nerve growth factor (NGF), basic fibroblast growth factor, platelet-derived growth factor and transforming growth factor-β, on mast cell differentiation, using HMC-1 cells as a model. NGF, at 0·1–50 ng/ml concentrations, caused a marked, dose-dependent up-regulation of tryptase, FcεRI and histamine within 10 days of culture, associated with an enhanced expression of mRNA for FcεRI and mast cell tryptase. On restriction analysis, only mast cell β-tryptase, but not α-tryptase, could be demonstrated. Furthermore, the high-affinity NGF receptor (TrkA) was found at both the transcriptional and protein levels, while expression of the low-affinity NGF receptor was detectable at the mRNA level only. None of the other growth factors caused a significant alteration of the mast cell markers studied when added to HMC-1 cells at concentrations known to be biologically active in other culture systems. Immature human mast cells are thus induced to assume a more mature phenotype *in vitro* in response to NGF, most probably via stimulation of the high-affinity NGF receptor expressed on these cells. Besides SCF, NGF should therefore be considered as an additional mast cell growth factor that contributes to human mast cell maturation at tissue sites.

INTRODUCTION

Fibroblasts and keratinocytes are known to produce and release numerous cytokines, including growth factors which influence the proliferation, differentiation and function of mast cells.[1] In man, the only factor produced by both cell types that is known so far to exert these effects on mast cells is the haematopoietic stem cell growth factor (SCF), also named c-Kit ligand or Steel factor.[2–4]

In previous studies on human mast cell differentiation, we have observed that supernatants of a murine fibroblast cell line (L-cells), normal human skin fibroblasts and human keratinocytes are able to up-regulate mast cell characteristics such as FcεRI expression and intracellular tryptase, as well as histamine levels in a human leukaemic cell line (HMC-1), whereas SCF had no such effects in these cells.[5–7] This lack of biological activity is most likely due to a previously described, gain-of-function mutation of the SCF receptor (c-Kit) in these cells.[8] Thus, the effects of the fibroblast and keratinocyte supernatants in these experiments remained unexplained. Other haematopoietic growth factors known to be produced both by fibroblasts and keratinocytes, namely nerve growth factor (NGF), transforming growth factor-β (TGF-β), basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF), were therefore studied here as possible mast cell growth factors in addition to SCF.

NGF, a neurotropic factor with various biological effects also on inflammatory and immune cells,[9–11] was considered to be the most likely candidate mediating human mast cell growth factor activities. In the murine system, this factor has been described to induce degranulation,[12] growth and differentiation from myeloid progenitors,[13] and enhancement of survival and colony formation[14,15] in addition to increased histamine production in cultured human umbilical cord blood cells.[16] NGF is produced by a number of cell types, including lymphocytes,

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Abbreviations: bFGF, basic fibroblast growth factor; FCS, fetal calf serum; FS, fibroblast supernatant; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HS, horse serum; NGF, nerve growth factor; $NGFR_L$, low-affinity NGF-receptor; $NGFR_H$, high-affinity NGF-receptor; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; RT–PCR, reverse transcription–polymerase chain reaction; SCF, stem cell factor; TGF-β, transforming growth factor-; Trk, tyrosine kinase.

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fibroblasts, macrophages, mast cells and keratinocytes,[17–19] and its biological effects are mediated by two distinct proteins, a 140 000 MW high-affinity (p140, gp140, TrkA, $NGFR_H$) and a 75 000 MW low-affinity receptor (p75, gp80, $NGFR_L$).[20] $NGFR_H$ has primarily been identified so far within the central nervous system, but also on blood monocytes, whereas the low-affinity receptor ($NGFR_L$) is found on fibroblasts, keratinocytes, lymphocytes, peripheral neurons and various tumour cells, such as melanoma cells.[17,20–22] Both NGF expression and mast cell numbers have furthermore been found to be increased in the dermis of patients with systemic sclerosis,[23] and a close structural association has been described between nerve fibers and mast cells.[24]

The other growth factors studied here have been shown to induce diverse effects on various myeloid cells, including mast cells.[25–30]

In the present study, the possible effects of NGF, TGF-β, bFGF and PDGF on mast cell tryptase, FcεRI and histamine were studied during a 10-day culture of HMC-1 cells, in comparison to fibroblast supernatants (FS). The data show that only NGF has activities comparable to FS and suggest that the NGF effect on HMC-1 cells is mediated via $NGFR_H$.

MATERIALS AND METHODS

Cells

The human mast cell line HMC-1[31] was kindly provided by Dr J. H. Butterfield, Minneapolis, MN. Cells were routinely cultured in culture flasks with Iscove's medium (GIBCO, Eggenstein, Germany), with 10^{-5} M monothioglycerol (Sigma, Deisenhofen, Germany) and 30% horse serum (HS) (Seromed, Berlin, Germany) added. These culture conditions, designated as control medium, have proven to be superior to cultures of mast cell precursors with fetal calf serum (FCS) in our laboratory and fail to alter HMC-1 function by themselves.[3–6,32]

On the basis of previously published data on its biological activity,[11–13] NGF-β (Boehringer Mannheim, Germany) was added to the basic culture medium at 0·1–50 ng/ml. The NGF concentration in FS, generated as described (10^5 L-cells/ml cultured to confluency) and used routinely at the previously established optimal concentration of 30% for human mast cell differentiation,[32] was 0·25 ng/ml, as determined by commercial enzyme-linked immunosorbent assay (ELISA; Boehringer). Murine NGF has been reported to be active in human cells.[33] TGF-β_1, bFGF and PDGF were studied here in comparison with FS and NGF at a concentration shown to be active in other systems, namely TGF-β_1 at 1 ng/ml,[25] bFGF at 10 ng/ml[29,34] and PDGF at 20 ng/ml[35] (all from PeproTech, London, UK).

Cells were seeded into the different culture media at 1×10^5 cells/ml and fed every other day. Viability of cells was determined by trypan blue exclusion at day 0 and after 10 days of culture and remained above 90% under all culture conditions studied.

Monoclonal antibodies

The monoclonal antibody against mast cell tryptase (AA1)[36] was kindly provided by Dr A. Walls, Southampton, UK, that against the α-chain of the FcεRI (29C6) by Dr J. Hakimi, Nutley, NJ,[37] and that against c-Kit (YB5.B8)[38] by Dr L. Ashman, Adelaide, Australia. The antibody against low-affinity NGF-receptor was purchased from Boehringer, that against the high-affinity NGF-receptor from Santa Cruz Biotechnology (Santa Cruz, CA) and that against desmin (D33) from Dianova, Hamburg, Germany.

Immunocytochemical staining

Immunocytochemistry with all antibodies described above was performed on cytocentrifuge preparations of cells at days 0 and 10 of *in vitro* culture, as previously described, using the alkaline phosphatase–anti-alkaline phosphatase (APAAP) technique.[39] Antibody reactivity was evaluated independently by two observers, counting at least 100 cells, recording distinctively stained cells only, as described before.[40]

Flow cytometric analysis

Fifty thousand cells were incubated for 60 min at 4° with monoclonal antibodies against high- and low-affinity NGF-receptor in 50 l of phosphate-buffered saline (PBS) containing human AB serum (0·1%). After two washes with PBS, cells were incubated for another 45 min at 4° with a 1 : 20 dilution of fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (DAKO, Hamburg, Germany). Following two washes, cells were suspended in 400 l PBS containing 0·1% NaN_3, fixed with 50 l of a 37% formaldehyde solution and were analysed on an EPICS XL cytometa (Coulter, Krefeld, Germany) flow cytometer. Negative controls were performed in the absence of the primary antibody, with mouse IgG and an irrelevant antibody of the same isotype (antidesmin, DAKO). For flow cytometric analysis, the 'Systems II software' (Coulter) was applied.

Tryptase activity

Enzyme activity of mast cell tryptase was measured by cleavage of the peptide Z-Gly-Pro-Arg-pNA (4 mM) in the presence of heparin (5 mg/ml) and α-1-antitrypsin (2 mg/ml) (all from Sigma, St. Louis, MO).[41] Cells were lysed by three cycles of freezing and thawing for determination of intracellular tryptase contents. All values were measured in quadruplicate and data were expressed as mU/10^6 cells.

Histamine contents

Histamine was quantified using an automated fluorometric method, modified after Siraganian.[42] Cells were washed twice in Pipes buffer (Sigma), pH 7·4, containing ethylene diaminetetraacetic acid (EDTA; 0·1 M, Sigma), once in Pipes-containing human serum albumin (Biotest AG, Dreieich, Germany) (20%) and were then resuspended in Pipes buffer containing 0·1 M $CaCl_2$ and 0·5 M $MgCl_2$. For determination of total histamine contents, cells were lysed by adding 500 μl of 2% perchloric acid, as described.[42] Supernatants were kept at −20° until analysis.

Reverse transcription–polymerase chain reaction

Five million cells were lysed and the total RNA was prepared using the RNeasy-total-RNA-kit (Quiagen, Hilden, Germany). Complementary DNA was synthesized by reverse transcription of 5 μl of total RNA, using a cDNA synthesis kit (InVitrogen, Stade, the Netherlands). The following sets of oligonucleotide primers were used to amplify c-DNA (expected fragment length given in parentheses): tryptase: 5′ GGA GCT GGA

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GGA GCC CGT GA and 5′ ACC TGG GTA AGG AAG CAG TGG TG (531 base pairs; bp),[43] FcεRI (5′ CTG TTC TTC GCT TCC AGA TGG CGT and 5′ TAC AGT GTT GAG GGG CTG AG (536 bp),[44] GAPDH: 5′ GAT GAC ATC AAG AAG GTG GTG and 5′ GCT GTA GCC TTC GTT GTC (197 bp),[45] 5′ TGA GTG CTG CAA AGC CTG CAA and 5′ TCT CAT CCT GGT AGT AGC CGT for the low-affinity NGF receptor and 5′ |GGC TCC TCG GGA CTG CGA TG and 5′ CAG GAG AGA GAC TCC AGA GCG for the high-affinity NGF-receptor.[46] Amplification was performed using taq polymerase (GIBCO) over 24–35 cycles with an automated thermal cycler (Perkin Elmer, Langen, Germany). Each cycle consisted of the following steps: denaturation at 94°, annealing at 55°, and extension at 72° for 1 min each. PCR products were analysed by agarose gel electrophoresis and enzymatic digestion, using standard techniques.[47] For semiquantitative RT-PCR, linear correlation of signal intensity for GAPDH was found between 24 and 27 cycles, and for the other markers between 30 and 35 cycles using a video scanner system. This allowed for the demonstration of a clearly enhanced signal, as compared to a barely detectable control signal.

Statistics

Statistical significance was calculated with an unpaired two-tailed *t*-test.

RESULTS

Mast cell tryptase, FcεRI and histamine

NGF, at 10 ng/ml, caused a significant upregulation of intracellular tryptase activity in HMC-1 cells after 10 days of culture, compared to cells kept in control medium (HS alone) ($P<0{\cdot}01$) (Fig. 1a). Values did not differ significantly from those of cells kept in FS during the same time period (Fig. 1a). In contrast, TGF-β, bFGF and PDGF did not significantly increase tryptase levels, compared to controls (Fig. 1a). The up-regulation of intracellular tryptase activity was accompanied by an increased demonstration of the enzyme protein, using immunocytochemical staining of HMC-1 cells with the tryptase-specific antibody AA1. Cells kept in NGF and FS exhibited an increase in number of positive cells (control, 10 ± 5; NGF, 45 ± 10, FS 50 ± 15%) and also in the intensity of staining, whereas no such changes were observed with the other growth factors (not shown).

The NGF-induced increase of intracellular tryptase was dose-dependent, as determined in two separate experiments, with quadruplicate measurements at each time-point. Levels above control culture were detectable already at 0·1 ng/ml (mean value: 78 mU/10^6 cells). Plateau levels, with increases between 45 and 114% above controls, were obtained at concentrations of 10–50 ng/ml (mean maximal values 111–114 mU/10^6 cells).

Another mast cell marker, the α-chain of the high-affinity IgE receptor (FcεRIα), was similarly increased by NGF and FS ($P<0{\cdot}01$), as observed by counting positively stained HMC-1 cells on immunocytochemistry with the antibody 29C6 (Fig. 1b). Furthermore, histamine contents of cells were significantly enhanced by NGF and FS, as compared to controls (Fig. 1c). Once again TGF-β, bFGF, or PDGF caused no significant changes regarding these parameters (Fig. 1b,c).

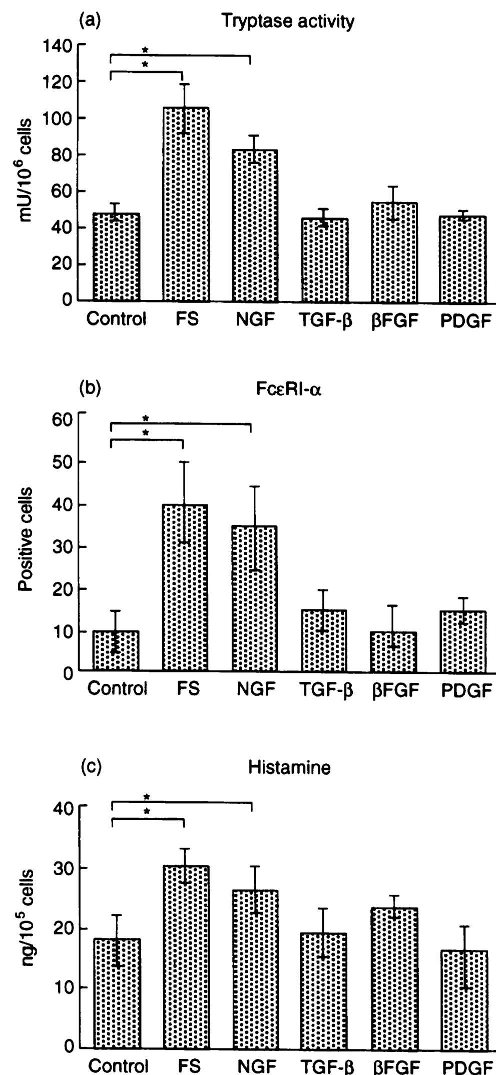

Figure 1. Mast cell characteristics in HMC-1 cells cultured for 10 days in the presence of 30% fibroblast supernatant (FS), different recombinant growth factors, or control medium. The concentrations of the different factors were 10 ng/ml for NGF, 1 ng/ml for TGF-β 10 ng/ml for bFGF and 20 ng/ml for PDGF (means $\pm$ SD of $n=3$ independent experiments). (a) Tryptase activity (mU/10^6 cells); (b) percentage of HMC-1 cells staining for the α-chain of FcεRI (antibody 29C6; immunocytochemistry, APAAP technique); and (c) intracellular histamine contents of HMC-1 cells, measured by spectrofluorometry (ng/10^5 cells). $^*P<0{\cdot}01$, $^{**}P<0{\cdot}05$.

Expression of tryptase- and FcεRIα-specific mRNA

In order to study whether increased expression of specific mast cell markers was regulated at the level of gene transcription, expression of specific mRNA for tryptase and FcεRIα was determined in HMC-1 cells, using semiquantitative RT-PCR with GAPDH for standardization (Fig. 2a). On electrophoretic analysis of PCR products, bands of about 530 base pairs were seen after amplification of cDNA from HMC-1 cells both with the tryptase and the FcεRIα primers. The size of PCR products obtained with the tryptase and FcεRIα primers agreed with the fragment lengths (531 and 536 bp, respectively), predicted from the EMBL data bank. The increased density of bands

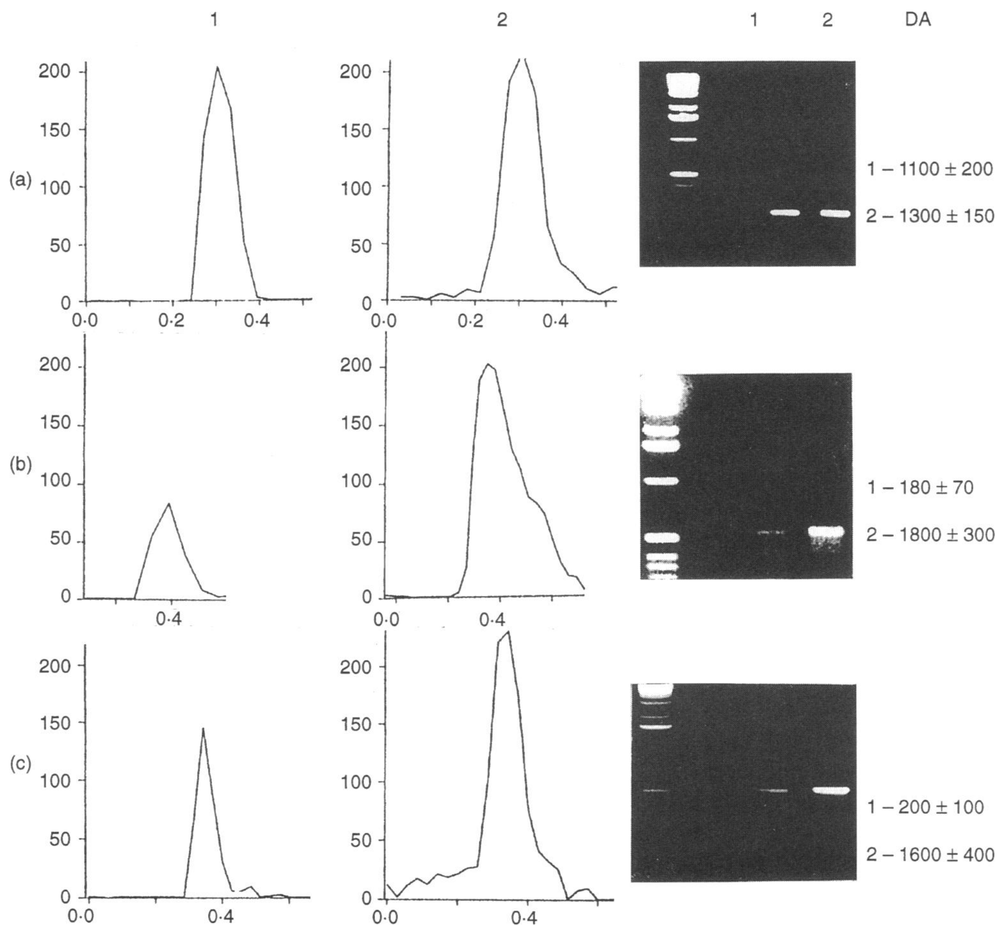

Figure 2. Expression of mRNA for GAPDH (a), tryptase (b) and FcεRIα (c), demonstrated by PCR with cDNA from HMC-1 cells cultured for 10 days under standard conditions (lane 1) or with NGF (10 ng/ml) (lane 2). Left panel, densitometry of signal intensity of lanes 1 and 2, respectively (video scanner analysis) (representative results from one of three experiments); right panel, densitometric analysis (DA) of signal intensity, means ± SD of $n=3$ separate experiments.

obtained from cells cultured in the presence of NGF, 10 ng/ml for 10 days, was confirmed by quantification with a video scanner (Fig. 2b,c).

In order to test whether the increase of HMC-1 tryptase in the presence of NGF was due to up-regulation of expression of mast cell-specific β-tryptase and not to basophil- or monocyte-associated α-tryptase,[48] restriction analysis of PCR amplification products obtained with the tryptase primers was performed using two different enzymes, *Fok*I and *Pst*I. Cleavage with *Fok*I resulted in two (100 bp – not visible in the figure – and 430 bp) bands, and that with *Pst*I in 340 bp and 190 bp bands (Fig. 3). This corresponds to the fragment sizes predicted from the cDNA sequence of β-tryptase (100/431 bp and 338/193 bp, respectively). In the presence of α-tryptase, fragments of 100, 131 and 300 bp would have been generated with *Fok*I, and none with *Pst*I since a *Pst*I restriction site is lacking in an α-tryptase RT-PCR product.[43]

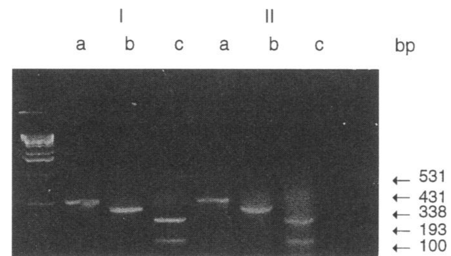

Figure 3. Restriction analysis of PCR products obtained with tryptase oligonucleotide primers and cDNA from HMC-1 cells cultured for 10 days under standard conditions (I) or with NGF (10 ng/ml) (II); (a) uncleaved fragment, (b) digestion with *Fok*I, (c) digestion with *Pst*I. Representative data from one of three experiments.

Expression of NGF receptors

With the clear demonstration of NGF effects on HMC-1 cells, it was of interest to determine the type of receptor involved. As shown in Fig. 4 (lane 4), PCR analysis showed expression of mRNA for the high-affinity receptor ($gp140^{trk}$), with a fragment size of about 260 bp, using a $gp140^{trk}$-specific primer set and cDNA from HMC-1 cells cultured under standard conditions. In contrast, only a barely detectable band was found with primers for the low-affinity receptor $gp80^{LNGFR}$ (expected fragment size: 230 bp) (Fig. 4, lane 2).

These results were confirmed by flow cytometric analysis and immunocytochemistry of NGF receptor protein expression, showing positive staining (80–90% stained cells) with an

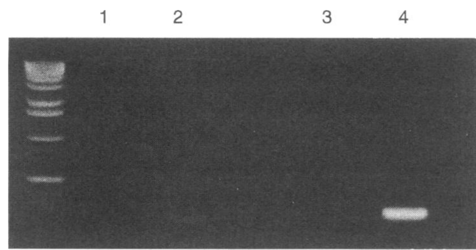

Figure 4. Expression of mRNA for the low-affinity (gp80^{NGFR}) (lane 2) and high-affinity NGF receptor (gp140^{trk}) (lane 4) in HMC-1 cells. PCR was performed with oligonucleotide primers for the respective receptor type on cDNA from cells cultured under standard conditions (lanes 1 and 3, negative controls; representative data from one of three experiments).

antibody against the gp140^{trk}, but not with one against gp80^{LNGFR} (Figs 5 and 6).

DISCUSSION

The present results demonstrate for the first time that NGF can up-regulate specific characteristics of cultured immature human mast cells, showing a dose-dependent increase of intracellular histamine and mast cell β-tryptase levels as well as enhanced expression of FcεRIα. This up-regulation could be shown also at the transcriptional level for tryptase and FcεRIα.

No such effects could be demonstrated with other haematopoietic growth factors studied here which – like NGF and the well-established mast cells growth factor SCF – are known to be released from fibroblasts and keratinocytes. This is in agreement with previous observations where repeated intracutaneous injections of FGF failed to induce a local increase of rat mast cell numbers.[49] Regarding TGF-β, some effects on murine mast cell function, namely induction of chemotaxis,[50] abrogation of antiapoptotic effects of SCF after IL-3 depletion[51] as well as inhibition of IL-3 dependent mast cell proliferation, but not differentiation, have been described in the past.

In contrast to rodents where IL-3 and NGF have been shown to play an important role in the development of mast cells,[13,14] both factors have been found to be ineffective or even inhibitory during SCF-dependent *in vitro* differentiation of human mast cells from haematopoietic progenitors in the bone marrow and the peripheral blood.[52] Instead, NGF stimulated the development of human basophils.[53] Nevertheless, NGF has been shown to affect human mast cell functions in an autocrine manner by inducing mediator release from mature human lung mast cells.[54] The reasons for these divergent findings are unclear but might relate to the different target cells used in culture. Whereas in the studies of Valent *et al.*[52] and Matsuda *et al.*[53] yet undifferentiated mast cell progenitors were cultured in the presence of NGF, we looked for the up-regulation of mast cell markers in a cell line which already expresses a mast cell phenotype, albeit at a low level. NGF might thus exert different biological effects at different stages of mast cell development. An NGF-induced induction of a switch from the mast cell to the basophil phenotype in the HMC-1 cell line studied here is unlikely since restriction analysis of the tryptase–PCR products showed that the increase of tryptase expression induced by NGF was only attributable to mast cell specific β-tryptase and not to α-tryptase, which is the only form of tryptase expressed by basophils.[48,55]

The present data also suggest that the NGF effect on HMC-1 cells is mediated by the high-affinity NGF receptor (TrkA) since this molecule could be demonstrated in the cells at both the protein and mRNA level, whereas expression of the low-affinity NGF receptor was found at the transcriptional level only. These data agree with findings published after submission of this manuscript by Nilsson *et al.*[56] who demonstrated only $NGFR_H$ on HMC-1 and cultured human mast cells at both the protein and mRNA level. If the small amounts of $NGFR_L$ mRNA detected by us should have resulted in

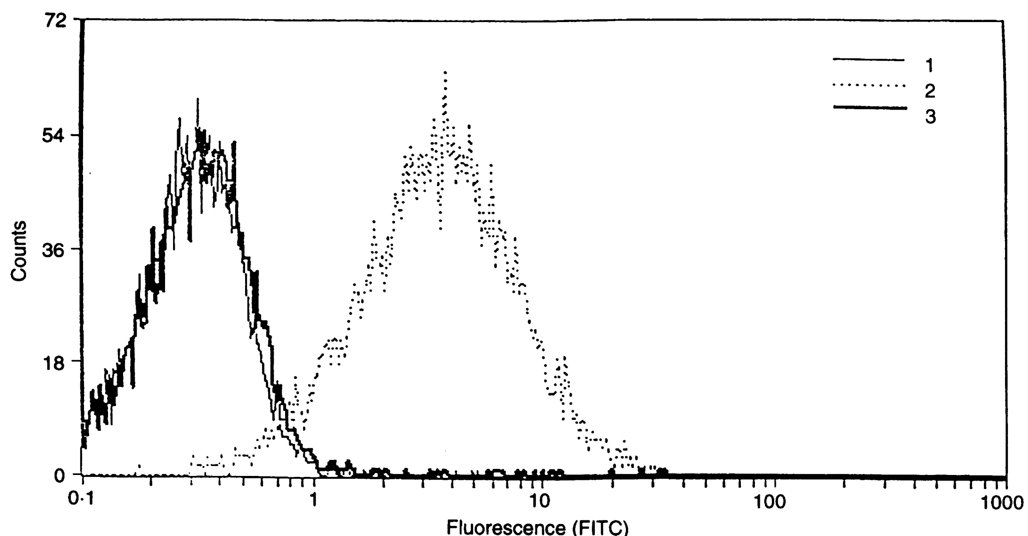

Figure 5. Flow cytometric analysis of HMC-1 cells, cultured under standard conditions, using specific antibodies against the low-affinity (gp80^{NGFR}),[1] the high-affinity NGF receptor (gp140^{trk})[2] and desmin as control.[3] Representative data from one of three experiments.

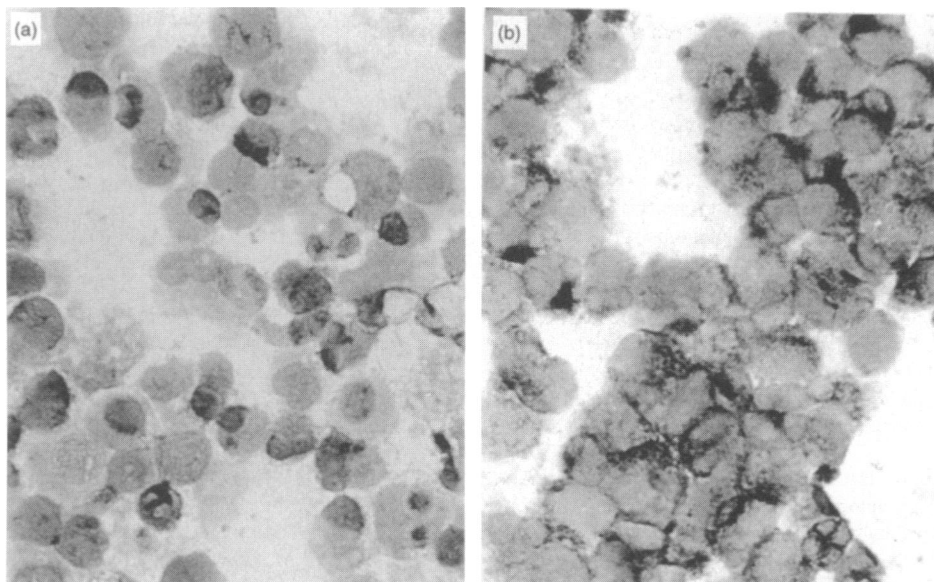

Figure 6. Immunocytochemical staining of HMC-1 cells, cultured under standard conditions, for (a) the low-affinity ($gp80^{LNGFR}$) and (b) the high-affinity NGF receptor ($gp140^{trk}$) (APAAP technique, magnification × 400).

receptor protein production in HMC-1 cells, it is unlikely to be present in more than minor amounts since no $NGFR_L$ was detectable on immunocytochemistry (Fig. 5, 6).

The exact function of the two NGF receptors and their relationship to each other are currently unclear. It has so far been established that TrkA binds NGF with both high and low affinities, whereas $NGFR_L$ binds NGF only with low affinity. In the presence of low NGF concentrations, the rapid binding kinetics of $NGFR_H$ might concentrate NGF in the vicinity of TrkA, and conversely, when NGF is readily available, $NGFR_L$ might sequester an excess of NGF, thus modulating overall NGF-induced TrkA activity.[57,58] NGF binding via TrkA alone is however, apparently sufficient for cell activation, and this also applies for HMC-1 cells.

With the present results as well as data from other laboratories,[53,56] there is increasing evidence that NGF is not only active in murine mast cells and human basophils, but that it also plays an important role in human mast cell differentiation and function. The biological activity of NGF, in both the nervous and immune systems, is all the more interesting in view of increasing recent evidence for a possible link between the two systems, particularly with regard to IgE-binding cells.[10,11,24]

In conclusion, besides SCF, other fibroblast- and keratinocyte-derived factors, like NGF, seem to be able to up-regulate characteristic features of human mast cells. Furthermore, since mast cells themselves are able to produce growth factors like NGF,[18,56] SCF, NGF or other factors in FS might stimulate mast cells to produce additional differentiating factors during culture. Analysis of the *in vitro* interaction of the two human mast cell growth factors clearly identified so far, namely SCF and NGF, and the study of their differential expression in tissues might help to improve our current understanding of the complex network that regulates human mast cell differentiation and function.

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