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Effects of Stem Cell Factor and Other Bone Marrow-derived Growth Factors on the Expression of Adhesion Molecules and Proliferation of Human Neuroblastoma Cells

D. Beck, N. Gross and C. Beretta Brognara

Metastasis in children with neuroblastoma (NB) is a poor prognostic factor despite intensive therapy. In the near future, stem cell factor (SCF) is likely to be used clinically to accelerate bone marrow (BM) recovery after high-dose chemotherapy in patients with advanced NB. The high frequency of BM metastases in NB could be secondary to BM-derived human growth factors (HGF) modulating the adhesion, secondary growth (or both) of circulating metastatic NB cells. To test this hypothesis, we studied the *in vitro* effects on NB cell lines grown in chemically defined medium of SCF, interleukin (IL)-1β, IL-3, IL-6, basic fibroblast growth factor (bFGF), transforming growth factor-β (TGF-β) used alone or in combination. The antigenic expression of NB-associated cell adhesion molecules (CAM) HLA class 1, intercellular CAM-1, neural-CAM and CD44 were assayed by monoclonal antibodies and flow cytometry, and DNA synthesis by ^{3}H-thymidine uptake. The expression of CAM was not modulated by SCF or other HGFs. An increase in thymidine uptake was induced by bFGF alone in IMR-32 cells, while SCF and other HGFs had no notable effect. Our results indicate that SCF and other BM-derived HGFs are unlikely to have a generalised effect on the expression of adhesion molecules by NB cells or proliferation. The clinical administration of recombinant human SCF to children with NB should be safe.

Key words: neuroblastoma, stem cell factor, c-kit ligand, bone marrow metastasis, adhesion molecules, growth factors

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INTRODUCTION

BONE MARROW (BM) metastases are frequent in children with neuroblastoma (NB) at diagnosis or during the course of the disease [1, 2]. These clinical findings suggest that specific factors present in the BM microenvironment could be responsible for the adhesion of metastatic NB cells, their secondary growth or both [3, 4]. Stem cell factor (SCF) and its receptor, a transmembrane tyrosine kinase encoded by the *C-KIT* protooncogene, are highly expressed by cells of the BM stroma [5] or haematopoietic progenitor cells [6] and regulate cell-matrix adhesion [7]. Other human growth factors (HGF) relevant to BM physiology include basic fibroblast growth factor (bFGF), interleukin (IL)-1β, IL-3 and IL-6 [8, 9]. Transforming growth factor-β (TGF-β) has potent positive or negative regulatory properties on haematopoiesis [10].

The complex formed by SCF and *C-KIT* has been suggested to play an important role in homing and chemoattractant mechanisms during mouse neuronal stem cell migration in early development of the nervous system, and in progenitor cell proliferation, differentiation and survival during late development [11]. In the mouse nervous system, an unexpected and complex pattern of expression has been uncovered that suggests the involvement of *C-KIT* and SCF gene products in the organisation of the neural tube, brain and sympathetic nervous system [12].

The proliferative effect of SCF on early BM haematopoietic progenitor cells makes it an attractive growth factor for clinical use [13]. SCF may be useful for accelerating BM recovery after high-dose chemotherapy or in the setting of BM transplantation [14], both modalities presently evaluated in the treatment of children with disseminated NB [1]. It has been shown that haematopoietic growth factors could affect the growth of solid tumours [14].

Therefore, it is important to study the expression of SCF and *C-KIT*, and the functional role of the SCF/*C-KIT* in NB cell lines and tumours. We have previously shown that *C-KIT* is not expressed and has no functional role, while the SCF gene is expressed in the majority of NB cell lines at the mRNA level and released in low concentrations in the spent medium as a soluble factor [15–17]. We now expand these data by presenting the results of experiments aimed at modulating the expression of cell adhesion molecules (CAM) relevant to NB biology and

Correspondence to D. Beck.
All the authors are at the Research Laboratory, Haemato-oncology unit, Paediatric Department, University Hospital CH-1011 Lausanne, Switzerland.

growth kinetics, by addition of SCF or other BM-derived HGF to NB cells grown *in vitro*.

MATERIALS AND METHODS

Cell lines

Eight well established human NB cell lines (LAN-1, LAN-N2, IMR-32, ACN, CA-2-E, SK-N-AS, SK-N-BE, SK-N-SH) were used in this study, all or partially in some experiments [18–20].

Recombinant HGFs and antibodies

The SCF was a gift from Dr Zsebo (Amgen, Thousand Oaks, California, U.S.A.). The other HGFs either have already been used in our laboratory and described previously [18] or were commercially available (Genzyme, Cambridge, Massachusetts, U.S.A.). The following Mabs were used: anti-HLA-ABC, anti-N-CAM (CD56), anti-ICAM (CD54); anti-CD44H, as previously described [19, 20].

Cell cultures

Cell lines were cultured in RPMI 1640 supplemented with 2 mM glutamine, 10% FCS, and gentamicin. Alternatively, cells were grown in N2, a chemically defined medium [21] for 72 h. In some experiments, recombinant HGFs were added to N2 medium at the following final concentrations (determined from preliminary experiments or reported by others): SCF 100 ng/ml, IL-1β 5 U/ml, IL-6 100 U/ml, bFGF 20 ng/ml, IL-3 200 U/ml, G-CSF 50 U/ml, TGFβ 5 ng/ml.

Flow cytometry

Expression of HLA class 1, N-CAM, I-CAM and CD44 molecules was assessed by flow cytometry using either a FACSCAN II (Becton-Dickinson, Mountain View, California, U.S.A.) or a Coulter Profile (Coulter, Hialeah, Florida, U.S.A.) as previously reported [20]. Results were expressed as % of positive cells (after subtraction of % of non-specifically stained cells) and mean relative logarithmic fluorescence light intensity (LFL).

Growth kinetics evaluation by ^{3}H–thymidine uptake

For measurements of DNA synthesis, cells were plated in quadruplicate in 96-well culture plates at 0.5–1 × 10^4 cells/well, and cultured for 48 h in N2; HGFs were added and cultures incubated for another 48 h, then pulsed for 24 h with 1 μCi/well ^{3}H–thymidine ([methyl-^{3}H] thymidine, Amersham). Cells were then briefly incubated in a 1:1 trypsin 2.5%/EDTA 0.5 M PBS solution and harvested using an automatic filter counting system (Inotech, Lansing, Michigan, U.S.A.).

RESULTS

Flow cytometry

The antigenic expression of adhesion molecules was studied in 6 NB cell lines in baseline culture conditions and with the addition of HGF. The results of HLA-class 1 are presented in Table 1. A baseline high expression was found on ACN, SK-N-BE, CA-2-E and SK-N-AS cells, as previously reported [18–20]. The up and down-modulations of the proportion of positive cells induced by the addition of HGF were not accompanied by correspondent changes of the mean LFL, therefore, unlikely to be of biological significance (data not shown). As shown in Table 2, a high baseline expression of N-CAM on SK-N-BE, IMR-32 and LAN-1 cells was detected, confirming a previous report [18]. Again, no biologically significant change was detected after the addition of HGF, as the up-modulation of the percentage of positive ACN cells by IL-6 was not accompanied by a change of the mean LFL. The baseline ICAM-1 expression (Table 3) was high in ACN, intermediate in SK-N-BE and SK-N-AS, and variable (RPMI versus N2 medium) in CA-2-E cells, as previously described [18, 19], and also was not notably modulated by HGF. The results of CD44H expression are summarised in Table 4. The baseline high percentage of positive ACN and CA-2-E cells was in accordance with a previous report [20]. It was variable in SK-N-AS cells and partially down-modulated by SCF, while the proportion of positive SK-N-BE cells was increased by SCF, but again without correspondent changes of mean LFL in both tests.

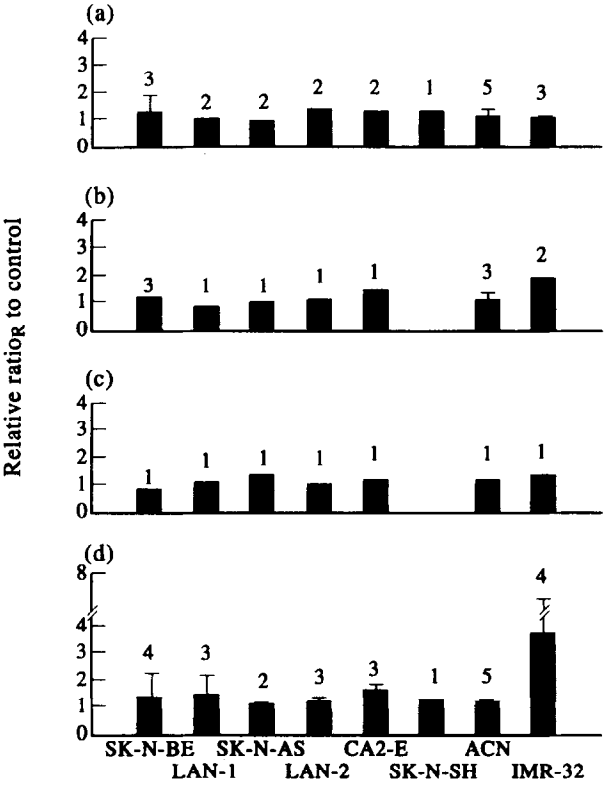

Figure 1. Results of ^{3}H-thymidine uptake assay by 8 NB cell lines following exposure to HGFs, expressed as relative ratio to control values (N2 medium). (a) SCF alone; (b) SCF + bFGF; (c) SCF + bFGF + IL-1β + IL-3 + IL-6 + G-CSF; (d) bFGF alone. The final concentrations of HGF added to N2 culture medium are provided in the text. The numbers above bars are the numbers of experiments. When > 2 experiments were performed, vertical bars represent S.D.

^{3}H-thymidine uptake assay

To further assess the functional role of SCF alone or in combination with other HGFs, we measured the changes of growth kinetics following appropriate cultures, expressed as the relative ratio to control values (Figure 1). In these conditions, SCF did not modulate the *in vitro* growth of the 8 tested NB cell lines (Figures 1a–c). In contrast, bFGF alone increased the uptake of IMR-32 cells (Figure 1d), but this effect was lost when bFGF was used in combination (Figures 1b and c).

DISCUSSION

Our data did not demonstrate any biologically significant change in CAM expression in 6 NB cell lines, nor in cell growth

Table 1. Surface expression of HLA class 1 in neuroblastoma cell lines as detected by flow cytometry

Medium	Cell lines				
	ACN % (*n*)	SK-N-BE % (*n*)	IMR-32 % (*n*)	LAN-1 % (*n*)	SK-N-AS % (*n*)
RPMI + 10% FCS	97 (2)	36 (1)	2.8 (3)	1.7 (2)	80 (2)
N2	95 (2)	32 (2)	3.6 (3)	2 (2)	72 (2)
N2 + SCF	94 (1)	ND	ND	ND	86 (1)
N2 + bFGF	92 (1)	77 (1)	1.4 (1)	2 (1)	84 (1)
N2 + IL-1β	95 (1)	ND	ND	ND	88 (2)
N2 + IL-6	93 (1)	32 (1)	0 (1)	0.8 (1)	74 (1)
N2 + IL-3	97 (1)	ND	6.1 (2)	6.6 (1)	85 (1)
N2 + TGFβ	95 (1)	25 (1)	3.7 (1)	0 (1)	81 (1)

% refers to positive cells, *n* to numbers of experiments. One experiment is one measurement by flow cytometry of the test culture. Mean logarithmic fluorescence light intensity (LFL) was between 2.3 and 15.9. ND, not done.

Table 2. Surface expression of N-CAM in neuroblastoma cell lines as detected by flow cytometry

Medium	Cell lines					
	ACN % (*n*)	SK-N-BE % (*n*)	IMR-32 % (*n*)	LAN-1 % (*n*)	CA-2-E % (*n*)	SK-N-AS % (*n*)
RPMI + 10% FCS	1.2 (3)	46 (3)	84 (5)	88 (3)	11 (2)	9.3 (2)
N2	3.8 (2)	38 (3)	89 (5)	77 (3)	10 (1)	2.1 (2)
N2 + SCF	0.1 (1)	63 (1)	82 (1)	87 (1)	8.1 (1)	0.3 (1)
N2 + bFGF	0.9 (1)	ND	86 (1)	89 (1)	ND	4.4 (1)
N2 + IL-1β	2.2 (1)	64 (1)	80 (1)	88 (1)	11 (1)	5.1 (1)
N2 + IL-6	25.7 (1)	48 (1)	81 (1)	85 (1)	ND	2.2 (1)
N2 + IL-3	1.3 (1)	ND	95 (2)	73 (1)	ND	4.5 (1)
N2 + TGFβ	1.8 (1)	42 (1)	91 (1)	95 (1)	ND	9.5 (1)

% refers to positive cells, *n* to numbers of experiments. One experiment is one measurement by flow cytometry of the test culture. Mean logarithmic fluorescence light intensity (LFL) was between 1.7 and 9.8%. ND, not done.

Table 3. Surface expression of I-CAM in neuroblastoma cell lines as detected by flow cytometry

Medium	ACN % (*n*)	SK-N-BE % (*n*)	IMR-32 % (*n*)	LAN-1 % (*n*)	CA-2-E % (*n*)	SK-N-AS % (*n*)
RPMI + 10% FCS	97 (2)	19 (3)	6.7 (4)	0.3 (3)	7.1 (2)	35 (3)
N2	94 (2)	15 (3)	3.4 (4)	3.3 (3)	22 (1)	28 (3)
N2 + SCF	92 (1)	13 (1)	5.1 (1)	0 (1)	14 (1)	18 (1)
N2 + bFGF	95 (1)	30 (1)	0 (1)	4.3 (1)	ND	56 (1)
N2 + IL-1β	94 (1)	55 (1)	8 (1)	0 (1)	48 (1)	38 (1)
N2 + IL-6	93 (1)	0 (1)	0 (1)	4.2 (1)	ND	36 (1)
N2 + IL-3	97 (1)	ND	3.5 (2)	14 (1)	ND	36 (1)
N2 + TGFβ	94 (1)	10 (1)	3.6 (1)	3.2 (1)	ND	45 (1)

% refers to positive cells, *n* to numbers of experiments. One experiment is one measurement by flow cytometry of the test culture. Mean logarithmic fluorescence light intensity (LFL) was between 6.8 and 10.8. ND, not done.

of the 8 NB cell lines induced by SCF alone or in combination. This confirmed previous results showing a lack of a functional role for SCF in NB cells grown *in vitro*, despite mRNA expression by a majority of these cells and release of soluble SCF molecules in low concentrations in spent media from cultures [16]. The results could be ascribed to the previously described lack of *C-KIT* expression on NB cells [15]. Overall, these results suggest that the constitutive expression of SCF in NB cells reflects genetic regulation associated with arrested differentiation of sympathoadrenal progenitor cells at specific stages of embryonal

Table 4. Surface expression of CD 44 *in neuroblastoma cell lines as detected by flow cytometry*

Medium	Cell lines					
	ACN % (*n*)	SK-N-BE % (*n*)	IMR-32 % (*n*)	LAN-1 % (*n*)	CA-2-E % (*n*)	SK-N-AS % (*n*)
RPMI + 10% FCS	98 (2)	17 (4)	1 (4)	0.2 (3)	95 (2)	78 (3)
N2	95 (2)	19 (4)	1.2 (4)	0 (3)	87 (1)	49 (3)
N2 + SCF	94 (1)	88 (1)	0 (1)	0 (1)	74 (1)	15 (1)
N2 + bFGF	93 (1)	ND	2.1 (1)	3.7 (1)	ND	70 (1)
N2 + IL-1β	95 (1)	21 (1)	5.2 (1)	0 (1)	89 (1)	68 (1)
N2 + IL-6	94 (1)	31 (1)	0 (1)	1.6 (1)	ND	44 (1)
N2 + IL-3	97 (1)	ND	2.1 (2)	0 (1)	ND	63 (1)
N2 + TGFβ	95 (1)	0 (1)	0 (1)	0.1 (1)	ND	64 (1)

% refers to positive cells, *n* to numbers of experiments. One experiment is one measurement by flow cytometry of the test culture. Mean logarithmic fluorescence light intensity (LFL) was between 1.8 and 37.7. ND, not done.

development or neoplastic transformation [17]. SCF does not affect the growth of NB cells nor modulate their expression of biologically or clinically relevant [19, 20] adhesion molecules, thereby playing no role in tumour growth and metastasis, and therefore rhSCF can be safely administered to children with NB within a clinical trial. Similarly, the other BM-derived HGFs used in this study did not appear to influence the biological characteristics of NB cells that were tested, an important finding in view, for example, of the implication of CD44 in the regulation of tumour growth and metastasis [22].

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