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Stem cell factor is not essential for cell survival and proliferation of soft tissue sarcoma of neuroectodermal origin

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ABSTRACT

Background and Objectives. **Stem cell factor (SCF), and its receptor (c-kit) play key roles in the expansion and differentiation of hematopoietic progenitor cells, melanoblasts and primordial germ cells, making it possible that SCF and c-kit are involved in neoplastic processes deriving from these cells. C-kit has been described to be expressed at different levels in neuroblastoma and in soft tissue sarcoma of neuroectodermal origin, and seems to be required for survival processes. In this study we investigate how c-kit expression is regulated and whether a SCF autocrine loop is essential for survival of sarcoma cell lines.**

Design and Methods. **C-kit modulation and internalization was evaluated incubating cells with rhSCF. Cell differentiation and proliferation experiments were performed to test whether c-kit expression is related to cell cycle progression or to differentiation processes. Cell cultures were treated with neutralizing antibody and antisense oligonucleotides in order to assess the possible significance of the SCF autocrine loop.**

Results. In vitro **SCF stimulation induces c-kit down-regulation; this phenomenon could be connected with receptor internalization, and new protein synthesis is necessary for its re-expression. The cell proliferation arrest in G_0/G_1 does not modify c-kit expression while down-regulation of c-kit was demonstrated after cells had been treated with differentiating agents. SCF neutralization does not influence either the S phase or apoptosis in sarcoma cell lines.**

Interpretation and Conclusions. **In sarcoma cell lines, c-kit is regulated by differentiation processes; moreover our results suggest that c-kit activity, but probably not the SCF autocrine loop, is essential for survival of these cell lines.**

Key words: SCF, c-kit, autocrine loop, differentiation

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Primitive neuroectodermal tumors have been shown to cover a wide spectrum of small round cell sarcomas, including Ewing's sarcomas (ES), primitive neuroectodermal tumors (PNET) and neuroblastoma. These tumors are phenotypically related, but with a different grade of neuronal differentiation,[1,2] thus supporting the theory of a common origin of this neoplasm from a primitive cell that is specifically a pluripotential neural crest cell.[3]

Stem cell factor (SCF)and its receptor c-kit have been shown to play major roles in the survival, expansion and differentiation of hematopoietic progenitor cells of various lineages.[4] They have also been demonstrated to play critical roles in cells derived from the neural crest.[5,6] SCF in fact decreases spontaneous apoptosis in neuroblastoma and in soft tissue sarcomas cultured in serum-free medium;[4-7] in addition treatment with c-kit blocking antibody induces a significant apoptosis in neuroblastoma, ES and PNET.[4–7] The role of the SCF/kit pathway has been investigated in different human tumors and an autocrine loop has been assumed in small cell lung carcinoma, in gynecological tumors and in neuroblastoma.[7,8]

Here we investigate the two major questions about the SCF/c-kit pathway in neuroectodermal sarcomas. The first question is whether the presence of an autocrine loop, suggested by the simultaneous expression of c-kit and SCF mRNA,[4] is essential for survival of these malignancies. The second is whether the variability in c-kit expression, demonstrated by low receptor expression in neuroblastoma cell lines[8,9] and an high c-kit expression in ES and PNET, is random or could be related to differentiation as in the case of normal hematopoietic cells.[10]

Design and Methods

Cell lines and tissue samples

Six cell lines were studied: three derived from ES (6647, TC106 and PDE02) and three from PNET (TC32, PDN12 and PDN13). All cell lines were maintained in RPMI 1640 media supplemented with 10% fetal calf serum (FCS) and 0.2 mg/mL of peni-

cillin/streptomycin at 37° in 5% CO_2.[4]

To evaluate the relationship between c-kit expression and proliferative activity, sarcoma cells were cultured for 16h in 24 wells plate with and without aphidicholin (Calbiochem, La Jolla, CA, USA) 11.8 µM and then analyzed for DNA content and surface c-kit expression.

C-kit modulation and internalization

The c-kit modulation and internalization was evaluated by incubating cells with rhSCF (Genzyme, Cambridge, MA, USA) 50 ng/10^5 cells or control medium. Thereafter, cells were washed twice in PBS and analyzed by flow cytometry for surface c-kit expression at various time periods (5-10-30-60-120-240 min). The cells were permeabilized with Fix and Perm according to the manufacturer's protocol[11] (Caltag Laboratories, Burlingame, CA, USA). C-kit evaluation was performed at basal conditions and at 240 min in order to assess possible receptor internalization.

Differentiation experiments

Following the procedure used by Cavazzana *et al.*, cells from all six lines were plated in 75 cm^2 plastic flasks at a density of 5×10^4 cells/mL; N_6-O_2-butyryl-adenosine-3':5'-cyclic monophosphate (c-AMP) (SIGMA), capable of inducing terminal differentiation in these cell lines was used at a concentration of 2.5 mM.[12] The medium was changed every three days; and after three changes the cells were harvested and prepared for flow cytometry and immunocytochemistry. Throughout the experiments, the morphologic response, if any, of the cells to differentiating agents was documented by phase contrast microscopy of viable, unfixed cells.

Cytofluorimetric detection of c-kit receptor

A total of 5×10^5 cells were incubated with the MoAb anti CD117 PE conjugated (Ancell, Corporation, Bayport, USA) at 4°C for 30 min, then washed twice in PBS and analyzed by flow cytometry (Epics-XL, Coulter, Miami, FL, USA). Isotype antibody PE conjugated was used as the control.

To quantify the average number of c-kit receptors per cell, the Quantum™ R-Phycoerythrin-conjugated kit (Flow Cytometry Standard Co., San Juan, Puerto Rico, Valter Occhiena Srl, Turin, Italy) was used. The linear regression equation, correlating the channel number with MESF value, was calculated using specific software (Quickcal v2.0, Flow Cytometry Standard Corp.).[4] The MESF number was calculated on fresh and permeated cells.

Immunocytochemistry

The immunocytochemical analysis, using the avidin-biotin complex immunoperoxidase method was performed on cytospin slides fixed with methanol (10 min –20°) and acetone (5 sec –20°). Experiments were performed as described elsewhere[13] using antibodies against two neural markers; anti-neurofilaments and anti-synaptophysin (Dakopatts, Copenhagen, Denmark).

Propidium iodide staining

A total of 5×10^5 cells were centrifuged and the pellets treated with automated DNA staining kit (DNA-prep) (Coulter). Tubes were placed at 4°C in the dark overnight. The PI fluorescence of individual nuclei was measured by flow cytometry and the results analyzed by multicycle specific software (Phoenix Flow Systems, San Diego, CA, USA).

Evaluation of apoptosis

The cells were fixed by adding formaldehyde (1% in PBS) for 15 min on ice and stored at 4°C in ethanol (70% in PBS). Apoptosis analysis was carried out using a TUNEL technique as described elsewhere.[4] More than 300 cells were double-tested.

Effect of anti-SCF neutralizing antibody on sarcoma cell growth

The polyclonal goat anti-human SCF neutralizing antibody (anti-human SCF R&D Systems Minneapolis MN USA), at a final concentration of 0.4 µg/10^4 cells was added to the cells growing in complete medium. Selection of the appropriate working concentration was based on diluition experiments on the M-07e cell line, demonstrating that 0.4 µg/10^4 cells of anti human SCF are able to inhibit 50% of cell growth. The antibody was added every 72 h and the cells were assayed at 24-72 h and 7 days for percentage in S phase and apoptosis. The experiments were repeated using a normal goat serum as the negative control.

Treatment of cells with antisense oligonucleotide

Four out of five cell lines expressing SCF RNA,[4] ES (6647, PDE02) and PNET (TC32, PDN13) were selected for antisense experiments. The experiments were carried out adopting the specific SCF antisense oligonucleotide kit according to the manufacturer's instructions (antisense sequence GCA AAG CTG TTG TTG C, control sequence GTC CCT ATA CGA ACG; these antisense oligonucleotides were designed and synthesized by Biognostik, Gottingen, Germany).

Uptake studies and cellular growth assays were performed according to methods described previously.[4] Briefly the cells were maintained at 37°C in 5% CO_2, specific SCF antisense oligonucleotides and control were added daily (to make a 2 µM solution). The cells were checked daily to examine the different morphology between antisense treated and control cells; after 7 days 3 wells were counted in a Neubauer's chamber. All assays were performed in triplicate.

Results

C-kit expression

Cell lines were analyzed by flow cytometry before and 5,10, 30, 60, 120 and 240 min after the addition of rhSCF as shown in Figure 1A. At t0 (before addition of rhSCF), all the cell lines were c-kit positive, but with different expression[4] ranging between a minimum of 60732 MESF (TC106-ES) and a maximum

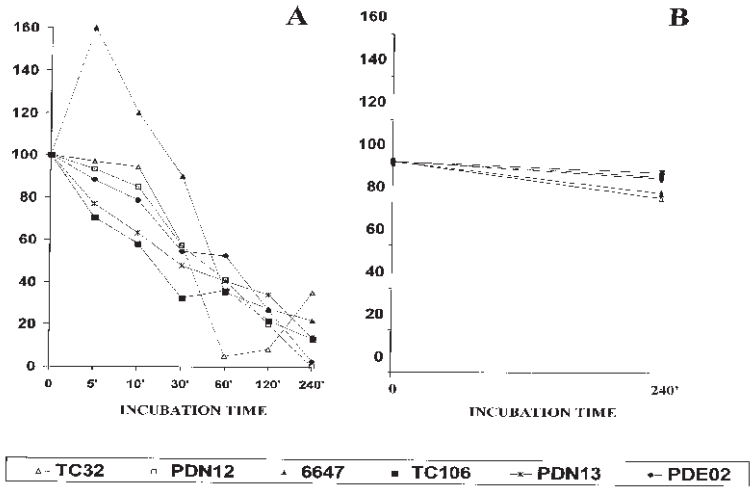

Figure 1.
A) Modulation of surface-expressed c-kit by rhSCF on sarcoma cell lines. Cells were evaluated by flow cytometry before and 5, 10, 30, 60, 120 and 240 minutes after the addition of SCF. The anti-CD117-PE was used to label c-kit; an irrelevant mouse IgG1 was used as a negative control.
B) Internalization of the c-kit receptor. C-kit expression was investigated before and 240 minutes after stimulation with SCF. Cells were treated with a permeabilizing agent, stained with CD117-PE and analyzed by flow cytometry. The percentage of c-kit positive cells exposed to medium alone was normalized to a value of 100 and all other values were normalized to this value to show the trend better.

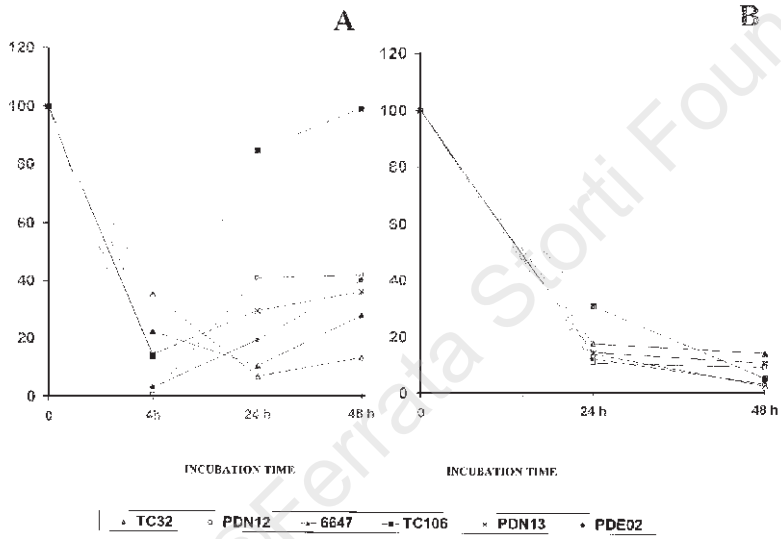

Figure 2.
Inhibition of reappearance of c-kit by cycloheximide. Sarcoma cell lines were provided with SCF (50 ng/10⁴ cells) and diluent control solution (A) or SCF and cycloheximide (5 µM) (B). Flow cytometry analysis was performed at 0, 24h and 48h with anti CD117-PE. Representative data shown are from one of three independent experiments. The percentage of c-kit positive cells exposed to medium alone was normalized to a value of 100 and all other values were normalized to this value to show the trend better.

of 295824 MESF (6647-ES). C-kit expression decreased 5 min after addition of exogenous SCF in 5 out of 6 cell lines, while 6647 (ES) had increased receptor expression after 5 minutes which then began to decrease. The minimum expression of c-kit was found in five out of the 6 cell lines 240 min after stimulation while TC32 (PNET) showed the minimum expression at 60 minutes. An aliquot of cells was analyzed at 240 min after permeabilization; c-kit expression was higher on these cells than in unpermeated cells (Figure 1B), confirming the c-kit internalization.

Effect of cycloheximide on the reappearance of c-kit

To understand whether the reappearance of c-kit depends upon new protein synthesis we treated the cell lines with cycloheximide. Cells were incubated with rhSCF and then 5 µM of cycloheximide or DMSO containing diluent, were added. Flow cytometry evaluation of c-kit was performed at 0, 24 and 48h as shown in Figure 2. The receptor expression increased at 24h and 48h in the presence of diluent control solution (Figure 2A); while c-kit levels

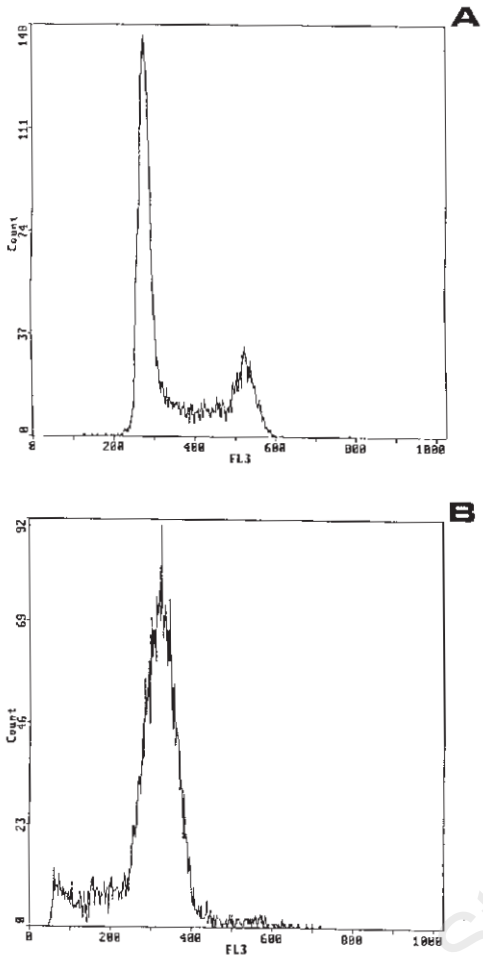

Figure 3. Specific action of aphidicholin on the cell cycle. PDN12 (PNET) cells were cultured for 16h in complete medium (A) or in medium with aphidicholin (11.8 µM) (B). The cytofluorimetric profile of the propidium iodide staining of DNA content, demonstrated the cell cycle block in G_0/G_1 phase.

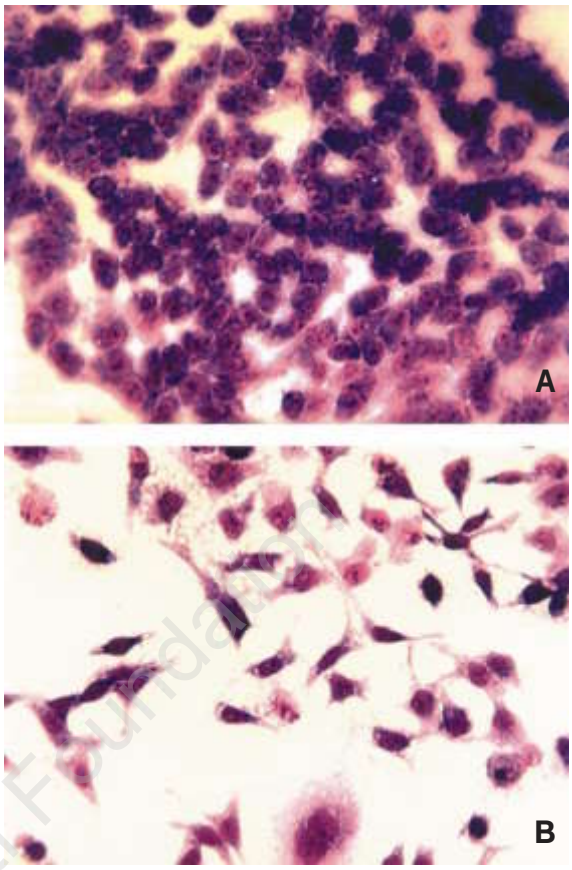

Figure 4. Tissue culture appearance of AMP_c treated tumor cells.
A) 6647 (ES) cell line in standard culture conditions;
B) 6647 (ES) cell line treated with dibutyrylcyclic AMP in complete medium condition. Cell bodies are interconnected by numerous neuritic processes. Cells were stained with May-Grünwald-Giemsa dye.

remained down-regulated in the culture to which 5 µM of cycloheximide were added (Figure 2B).

C-kit expression and cell proliferation

The 6 cell lines were treated with aphidicolin to evaluate whether the cell proliferation might have a role on the c-kit expression. After 16h of incubation the DNA analysis demonstrated a block of cell cycle progression in G_0/G_1 phase (Figure 3). No significant changes in the c-kit surface expression were observed (Figure 5A).

Exposure of sarcoma cell lines to differentiating agent

Treatment of sarcoma cell lines for 10 days with or without AMP_c was performed to evaluate the influence of *in vitro* differentiation processes on c-kit expression. After treatment, possible neuronal differentiation was screened for by direct microscopy and immunocytochemistry, using specific antibodies against neurofilaments and synaptophysin. The six cell lines, negative for the neuronal markers in basal culture conditions appeared to be morphologically differentiated after incubation with AMP_c (a representative case is shown in Figure 4); moreover, 2 of the six cell lines became positive for neurofilaments.

All the 6 cell lines showed a marked reduction of c-kit expression ranging from 18% in TC32 (PNET) to 92% in TC106 (ES) (Figure 5B).

S phase and apoptosis after exposure to anti SCF neutralizing monoclonal antibody

The role that exogenous SCF plays in the survival and proliferation processes of the cell lines was assessed by treating culture with neutralizing SCF polyclonal antibody for 24 and 72h and 7 days.

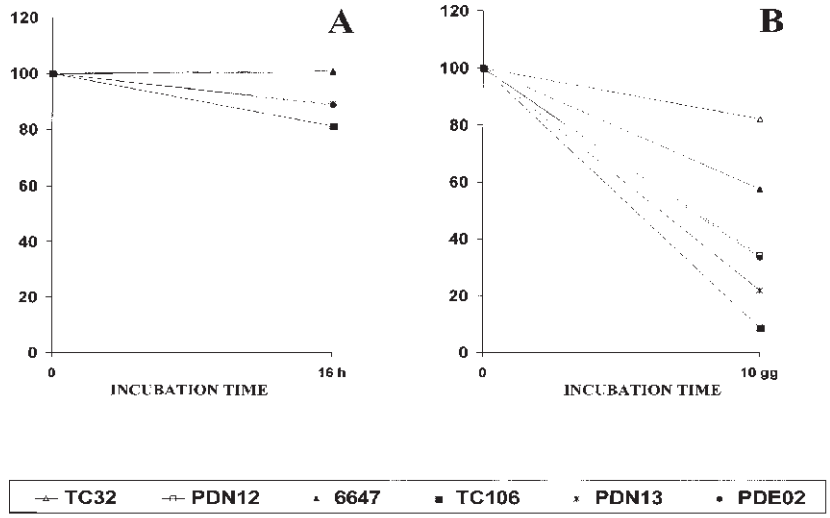

Figure 5. Relationship between c-kit and proliferation or differentiation processes. Sarcoma cell lines were incubated for 16h with aphidicholin (11.8 µM) to block the cell cycle (A) or with AMPc (2.5 µM) for 10 days to induce neural differentiation (B). Flow cytometry was performed at the indicated times with the anti-CD117-PE. Each experiment was performed in duplicate (mean of two reproducible experiments shown). The percentage of c-kit positive cells exposed to medium alone was normalized to a value of 100 and all other values were normalized to this value to show the trend better.

Table 1. Effect of anti-SCF neutralizing antibody on percentage of cells in S phase. Cells of 6 sarcoma cell lines were treated with anti-SCF neutralizing antibody 0.4 µg / 10^4 cells for 24h and 72h, and 7 days and S phase was evaluated after DNA staining with propidium iodide. Each experiment was performed in duplicate (mean of two reproducible experiments).

	% in S phase			
	0	*24h*	*72h*	*7 days*
PDN12	24	21	31	21
PDN13	30.6	32	26	25
PDE02	31	25	32	25
6647	47.5	45.3	34.5	44.8
TC32	22.3	26.6	24.9	25.6
TC106	24.2	27.6	20.4	20.3

Table 2. Effect of SCF neutralizing antibody on apoptosis of sarcoma cell lines. TdT assay was performed on cells treated for 24h and 72h and 7 days with anti-SCF neutralizing antibody 0.4 µg / 10^4 cells. Each experiment was performed in duplicate (mean of two reproducible experiments).

	Apoptosis			
	0	*24h*	*72h*	*7 days*
PDN12	0.4	0.5	0.4	0.5
PDN13	10.3	12	11	11.5
PDE02	0.76	0.7	0.8	0.8
6647	7.2	6.6	7.5	7.5
TC32	4	5	6	6.1
TC106	10.2	11.3	12	11.5

Tables 1 and 2 show that no significant modifications occurred in the percentage of cells in S phase and apoptosis after their neutralizing treatment.

Treatment of sarcoma lines with specific SCF antisense oligonucleotides

To determine whether specific SCF mRNA was present in the sarcoma lines, cells were treated with specific SCF FITC conjugated antisense oligonucleotides.

The four cell lines tested took up the oligonucleotides after treatment; the fluorescent pattern was already evident in the cell cytoplasm 1h after addition of the oligonucleotides and remained evident for 48h (Figure 6), demonstrating the presence of the specific SCF mRNA, evidenced by RT-PCR in these cell lines.[4]

The effect of specific oligonucleotides on cell growth was studied on four cell lines treated or not treated with sense or antisense oligonucleotides. The oligonucleotide activity was tested in the TC32 cell line and in different neuroblastoma cell lines, capable of releasing SCF into the supernatants.[4-8] The oligonucleotides were effective at inhibiting the endogenous production of SCF to a great extent (data not shown) but no significant reduction of cell growth was evident at 7 days (Figure 7). The study was performed for 15 days on the PDN13 cell line demonstrating no noticeable difference in the absolute number of cells even after a longer incubation (data not shown).

Simultaneous treatment with antisense oligonucleotides and anti-SCF neutralizing antibody

To test the effect of simultaneous neutralization of endogenous and exogenous SCF we incubated one cell line (TC32-PNET) with specific oligonucleotides and with SCF blocking antibody. The TC32 cell line

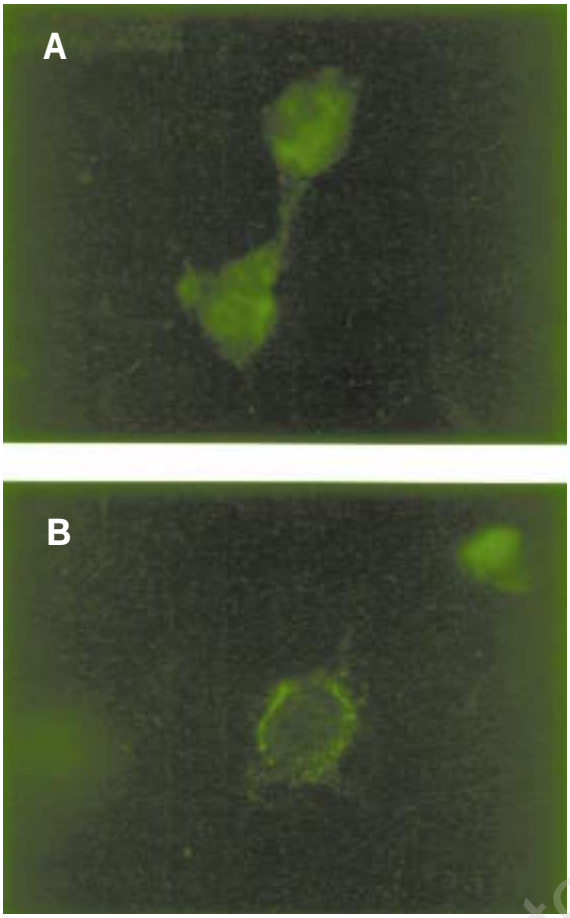

Figure 6. Uptake of specific SCF antisense oligonucleotides. 6647 (ES) cells were treated for 48, 24, 8, 4, 2, 1 or 0 hours with specific SCF FITC-conjugated antisense oligonucleotides. (A) t0 control cells, (B) fluorescent pattern after 48h of incubation

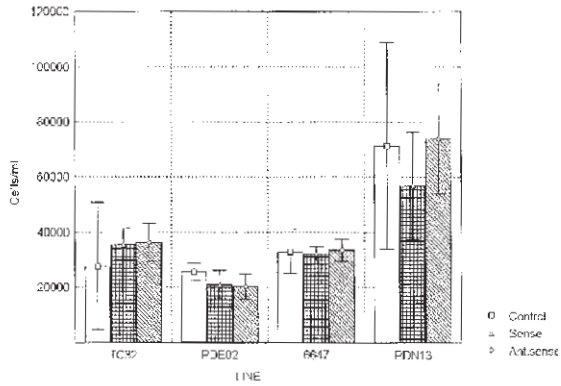

Figure 7. Cell growth inhibition by SCF antisense oligonucleotides. Untreated and treated (sense ▽ or antisense ◊ oligonucleotides 2 µM) cells of 4 lines, TC32-PDN13 (PNET) and 6647-PDE02 (ES), were incubated for 7 days at 37°C in 5%CO_2. Cell counting was performed as a direct indicator of the proliferative activity (mean of three different experiments shown).

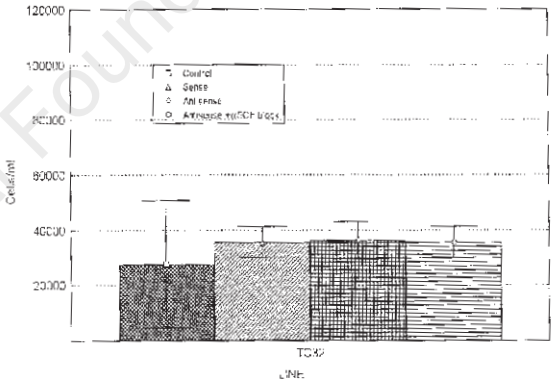

Figure 8. Cell growth inhibition by SCF antisense oligonucleotides and SCF neutralizing antibody. Absolute number of TC32 (PNET) cells after treatment with ▽ sense oligonucleotides (2 µM / day), ◊ antisense oligonucleotides (2 µM / day), ○ antisense oligos (2 µM / day) plus anti-SCF neutralizing antibody (0.4 µg / 10^4 cells every 72h) for 7 days at 37°C in 5% CO_2. Mean of three different experiments shown.

was chosen because it is the only one able to release SCF into the supernatant.[4] Cell growth was not influenced by this treatment, showing that neither self production of SCF nor its exogenous administration is essential for survival and proliferation of these neoplastic cell lines (Figure 8).

Discussion

Previous studies have demonstrated that cells of neuroectodermal origin exhibit c-kit;[4-8] we demonstrate here that the receptor behavior is in line with that of cells of different origins. Flow cytometry analysis reveals that SCF treatment of sarcoma cell lines induces a swift (5 minutes) reduction of the surface receptor with a nadir at 4h after the growth factor administration. Evaluation of c-kit after cell treatment with a permeabilizing agent showed no changes in the total amount of protein, indicating that down-regulation of surface c-kit expression depends on internalization. The reappearance of surface c-kit is detectable 24h after exposure to rhSCF and the recovery requires new protein synthesis; in fact the receptor does not reappear if cycloheximide is added to the culture. These data confirm the results obtained using fetal liver mast cells[14] and hematopoietic cells,[15,16] demonstrating a common mechanism in the receptor modulation unrelated to the origin of the c-kit expressing cells.

Our report highlights, for the first time, that there is no relationship between cell cycle progression and c-kit display in sarcoma cells; in fact aphidicholin treatment, which blocks cells in G_0/G_1, phase did not modify the receptor density.

Cavazzana *et al.* demonstrated that AMP_c induces neural differentiation in ES cell lines;[12] we used this approach to evaluate the possible role of differentiation processes on c-kit expression in sarcoma cell lines. The six cell lines showed morphologic evidence of neural differentiation after 9 days of treatment; in addition neurofilaments, absent in untreated cells, were demonstrated in some of the cell lines (2 out of 6). The differentiated cells showed a marked decrease of c-kit expression, supporting the hypothesis that neoplastic cells of neuroectodermal origin regulate c-kit in the same way that hematopoietic cells do: in fact in these cells c-kit expression decreases according to differentiation.[10] Differentiated sarcoma cells show a clear neural pattern[12] and the surface c-kit expression is similar to that already demonstrated in neuroblastoma cell lines[7] (more differentiated neoplasm of common neuroectodermal origin).

It has been hypothesized that there is an autocrine loop in some neoplastic cells as well as in sarcoma and neuroblastoma cell lines. In fact RT-PCR shows simultaneous expression of RNA for c-kit and SCF in the majority of the cell lines and biopsy samples;[4,7-9] moreover the addition of exogenous SCF to serum-free cultures reduces spontaneous apoptosis while programmed cell death is induced by receptor functional block.[4] Experiments performed with neutralizing antibodies, antisense oligonucleotides and both, seem to indicate that SCF is not essential for survival of sarcoma cell lines; in fact endogenous or exogenous neutralization did not affect cell growth or apoptosis in complete medium. On the other hand a part of the autocrine loop could be retained for incomplete endogenous SCF inhibition. Receptor block obtained with neutralizing antibody or antisense oligonucleotide has been demonstrated to be able to induce relevant apoptosis.[4]

These results could indicate that the c-kit natural ligand plays an important but not essential role in these cell lines in standard culture conditions. The hypothesis that a mutation in the kinase domain of the receptor could confer constitutive activation is not reliable; in fact, in serum-free conditions, the receptor is down-phosphorylated and phosphorylation rapidly increases after ligand interaction.[4] Studies of the biochemical interaction involved in signaling from cytokine receptors suggest that common patterns of transcriptional factor,[17] transducing molecules[18-22] and protein kinase[20-22] are shared. Moreover it has been reported that receptor-associated proteins, such as the Jak family members, are involved in the early transduction events elicited by both tyrosine and non-tyrosine kinase receptor.[23]

These findings, in conjunction with our data, raise the intriguing possibility that another non-specific substrate could play a role in the c-kit receptor transduction pathway even in the absence of exogenous or endogenous SCF.

In conclusion we provide direct evidence that c-kit behaves in the same way in neuroectodermal sarcoma cell lines as in hematopoietic cells, and that its expression is mainly related to differentiation processes and not to cell proliferation. Even if c-kit interaction with its specific ligand is able to reduce apoptosis in serum-free conditions, SCF, unlike its receptor, appears not to be essential for survival in non-deprived culture conditions.

In accordance with these results we could hypothesize that c-kit functional integrity is necessary for cell survival and that an alternative transduction pathway could be triggered even in the exogenous absence of its specific ligand.

Contribution and Acknowledgments

ER: mainly responsible for managing the experiments and writing the paper. NB: key role in carrying out the experiments. AP: differentiation experiments. SV: statistical precessing of results. LCdM: experimental design and critical revision of contents. EM: final approval of the version to be published. GB: conception, design, analysis, interpretation of data and final approval of the work. The order in which the authors' names appear was chosen according to the importance of their practical and intellectual contribution to the study, with the exception of the last name, to which the major importance is to be attributed.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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