Flt-3 and Its Ligand Are Expressed in Neural Crest–Derived Tumors and Promote Survival and Proliferation of Their Cell Lines

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SUMMARY: Flt-3 ligand (FL) is a cytokine that promotes the survival, proliferation, and differentiation of hematopoietic progenitors in synergy with other growth factors, such as stem cell factor. Previously we have demonstrated that stem cell factor and its receptor c-kit are expressed in neural crest–derived tumor cells and that a c-kit block induces their apoptosis. Here we have evaluated the expression of flt-3 and its ligand in 12 neuroectodermal tumor cell lines from neuroblastoma (NB), neuroepithelioma (NE), Ewing sarcoma (ES), and peripheral neuroectodermal tumor (PNET) and in 38 biopsies: 19 from NB and 19 from ES and PNET. RT-PCR demonstrated the expression of flt-3 and FL in all lines. Coexpression was observed in 42% of NB and in 74% of ES and PNET biopsies. Flow cytometry confirmed the presence of membrane and cytoplasmic flt-3 and membrane FL in all lines, whereas soluble FL protein was not measurable in their supernatants. Microphysiometric demonstration of acidification of the medium provided evidence of the specific response of cell lines to FL stimulation. Specific flt-3 phosphorylation after FL treatment was also demonstrated by Western blotting analysis. In cells growing in RPMI plus 1% fetal calf serum, FL revealed a significant proliferating activity, more evident in NB and NE lines (mean increase of viable cells, $73 \pm 26\%$ after 1 day). Treatment with flt-3 antisense oligonucleotides significantly inhibited cell growth. FL also displayed an antiapoptotic activity: after a 12-hour culture in the presence of 0.1% fetal calf serum, FL caused a 50% reduction of apoptotic cells. These results provide further evidence that neuroectodermal and hematopoietic cells share common regulatory pathways, and could be of interest in the clinical management of neuroectodermal tumors. (*Lab Invest* 2001, 81:1025–1037).

Flt-3 is a transmembrane glycoprotein. Like the stem cell factor (SCF) receptor (c-kit) and those for M-CSF (c-fms/FMS) and PDGF (PDGFRA and PDGFRB), it belongs to the tyrosine kinase receptor class III (Lyman et al, 1993; Rosnet and Birnbaum, 1993; Rosnet et al, 1991; Shibuya et al, 1990). Flt-3 is expressed on human hematopoietic progenitors, immature thymocytes, monocytes, acute myelogenous leukemia (AML) and B cell precursors acute lymphoblastic leukemia (ALL), mouse fetal liver, and various adult mouse tissues, such as brain and cerebellum (Birg et al 1992; Drexler et al 1999; Rosnet et al, 1993). Its ligand, flt-3 ligand (FL), is a cytokine that promotes the proliferation, survival, and differentiation of hematopoietic progenitors (Broxmeyer et al, 1995; Hannum et al, 1994; Lyman et al, 1994; Rasko et al, 1995; Vanu et al, 1999; Zeigler et al, 1994) in synergy with other growth factors, namely SCF, IL-3, IL-6, IL-11, IL-12, and GM-CSF (Drouet et al, 1999; Jacobsen et al, 1995). In association with G-CSF, FL mobilizes stem

and progenitor cells to peripheral blood (Molineux et al, 1997; Papayannopoulou et al, 1997). It plays an important role in lymphopoiesis (in synergy with IL-7) and in the production of dendritic cells (Hunte et al, 1996; Ohm et al, 1999; Ray et al, 1996). All of these biological functions make FL potentially useful in various clinical settings (Lyman, 1998).

SCF and c-kit (Bernstein et al, 1991; Yarden et al, 1987; Zsebo et al, 1990) are involved in development of the peripheral nervous system and are expressed in neural crest–derived tumor cells (Cohen et al, 1994). c-kit block induces neuroectodermal tumor cell apoptosis (Ricotti et al, 1998, 1999; Timeus et al, 1997). Neural and hematopoietic cells are closely correlated and “hematopoietic” growth factors such as FL are active in the generation of neural cells from bone marrow mesenchymal stem cells (Reyes and Verfaillie, 1999).

SCF and FL synergize in the regulation of hemopoiesis (Lyman and Jacobsen, 1998). The role of FL/flt-3 alone and its possible cooperation with SCF/c-kit in the biology of neuroectodermal tumors are investigated in this study.

Results

RT-PCR analysis demonstrated the expression of flt-3 and FL RNA in all 12 lines (Fig. 1). Flt-3 RNA was

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Figure 1.

RT-PCR analysis of flt-3 and Flt-3 ligand (FL) expression in 12 neuroblastoma (NB), neuroepithelioma (NE), Ewing sarcoma (ES), and peripheral neuroectodermal tumor (PNET) cell lines. The specific flt-3 207 bp and the FL 235 bp fragments are visible in all lines. MW: molecular weight marker. Lane 1: negative control (K-562 for flt-3 and M07-E for FL). Lane 2: positive control (HL60 for flt-3 and 5637 for FL). Lanes 3 to 14: CHP-100, AF8, IMR-5, SK-N-SH, SJ-N-KP, SK-N-BE, 6647, TC32, TC106, PDN13, PDE02, PDN12. Lane 15: no DNA.

expressed in 14 of 19 neuroblastoma (NB) and 17 of 19 Ewing sarcoma (ES) and peripheral neuroectodermal tumor (PNET) biopsies; FL was expressed in 9 of 19 NB and in 14 of 19 ES and PNET biopsies. Flt-3/FL coexpression was observed in 42% of NB biopsies and in 74% of ES and PNET biopsies (Table 1, Fig. 2).

Flow cytometry showed the presence of membrane and cytoplasmic flt-3 and membrane FL in all lines (Fig. 3), whereas soluble FL protein (evaluated by ELISA) was not measurable. Cell lines responded to FL stimulation by acidifying the medium in a concentration-dependent manner, as demonstrated by the cytosensor (Fig. 4); the peak rate was reached after about 2 minutes at 2,500 ng/10⁶ cells FL concentration.

Inhibition of the receptor-response by pretreating the FL preparation with a suitable concentration of specific FL-neutralizing antibody provided the evidence for cytokine/receptor-specific activity (Fig. 5). Specific flt-3 phosphorylation after FL treatment was demonstrated by using Western blotting analysis on flt-3 immunoprecipitates (Fig. 6).

When the lines were cultured in medium supplemented with 1% fetal calf serum (FCS), the presence of FL significantly ($p < 0.005$) increased the number of viable cells (evaluated by trypan blue exclusion) after 1 to 3 days of culture (Table 2, Fig. 7). This activity was more evident in NB and NE lines (mean increase vs controls, 73 ± 26% after one day and 50 ± 23% after 3 days). The association FL + SCF showed an additive effect. Treatment with flt-3 and c-kit antisense oligonucleotides specifically inhibited cell growth from the

sixth day. After a 9-day culture in the presence of flt-3 antisense, cellular flt-3 expression decreased (data not shown) and there was a 45% specific reduction of viable cells (Table 3, Fig. 8).

FL showed a significant antiapoptotic activity in the experiments at 0.1% FCS: after a 12-hour culture, FL caused a 50% reduction of apoptotic cells, as demonstrated by flow cytometry for annexin V (Table 4, Fig. 9). SCF showed a similar behavior. FL + SCF had the same antiapoptotic effect as the single cytokines.

Discussion

FL and SCF are cytokines that play a crucial role in regulation of the early phases of hemopoiesis. They protect stem cells from apoptosis and, in cooperation with other growth factors, promote the proliferation of hemopoietic progenitors. Their activities on these progenitors mostly overlap, but are often synergic. Many data point to the existence of a relatively early progenitor coexpressing c-kit and flt-3. Hematopoietic and nervous systems have a similar differentiation pathway from a stem cell to various final elements. Recent data suggest the existence of a common stem cell that can alternatively differentiate to the hematopoietic or nervous direction: donor-derived hematopoietic cells develop in sublethally irradiated mice transplanted with neural stem cells (Bjornson et al, 1999) and marrow cells differentiate into microglia and macroglia (Eglitis and Mezey, 1997). We have previously demonstrated that the system SCF/c-kit is expressed in primitive neuroectodermal tumors and exerts an in

Table 1. RT PCR Analysis of FL and flt-3 Expression in NB, NE, ES and PNET Tumor Cell Lines and Biopsies

	FL+ flt-3+	FL+ flt-3-	FL- flt-3+	FL- flt-3-
NB and NE lines (6)	6	0	0	0
ES and PNET lines (6)	6	0	0	0
NB biopsies (19)	8	1	6	4
ES and PNET biopsies (19)	14	0	3	2

FL, Flt-3 ligand; NB, neuroblastoma; NE, neuroepithelioma; ES, Ewing sarcoma; PNET, peripheral neuroectodermal tumor.

Figure 2.

RT-PCR analysis of flt-3 and FL expression in 19 biopsies from NB and NE (A) and 19 biopsies from ES and PNET (B) cell lines. The specific flt-3 207 bp fragment is visible in 14 biopsies in A and 17 biopsies in B. The specific FL 235 bp fragment is visible in 9 biopsies in A and 14 biopsies in B. Amplification of β -actin mRNA was used as a control and yielded a 506 bp product.

vitro proliferative and antiapoptotic activity on their cell lines.

We now show that neural crest–derived tumors express both flt-3 and FL m-RNA. Their lines also express membrane and cytoplasmic flt-3 protein and FL protein in a membrane-bound form. This is probably the first demonstration that flt-3 and its ligand are expressed in neural crest–derived tumor cell lines. PCR performed on biopsies substantiates our results by giving them biological significance and ruling out aberrations of cell lines.

The expression of FL/flt-3 and SCF/c-kit in neural crest–derived tumor cells and their proliferative and antiapoptotic activity provide further evidence that hematopoietic and neural progenitors share common regulatory systems (Quesenberry et al, 1999; Sieber-Blum, 1998). Whereas in neuroectodermal tumor cell lines, c-kit expression decreases according to differentiation, as in the hemopoietic progenitors, and is lowest in the more differentiated neuroblastoma lines (Ricotti et al, 1998; Timeus et al, 1997), flt-3 expression is similar in ES, PNET, and neuroblastoma lines and is thus unrelated to differentiation of neural progenitors.

Proliferation and apoptosis experiments demonstrated a functional role of FL/flt-3 in the regulation of these tumor lines. In cultures with a suboptimal FCS

concentration (1%), addition of FL significantly increased the number of viable cells from the first day. The increase was higher after 1 than after 3 days, probably because of the increasing number of cells per well. In these experimental conditions, FL showed a direct proliferating activity, whereas its antiapoptotic effect was negligible (not shown). Confirmation of the role of flt-3 in the proliferation of neuroectodermal tumor cells was provided by the demonstration that flt-3 antisense oligonucleotides significantly reduced the number of viable cells from the sixth culture day. In the experiments with very low FCS concentration (0.1%), FL also showed a significant antiapoptotic activity, like SCF, with a 50% reduction of apoptotic cells after a 12-hour culture.

As previously discussed, SCF and FL often show a synergic activity on the hematopoietic progenitors. In the neuroectodermal tumor cell lines, their association displayed an additive rather than a synergic effect in the proliferation assays, whereas its antiapoptotic effect was the same as that of the single cytokines. This may reflect the existence of a threshold of maximal antiapoptotic activity. Similar results were observed when cultures were carried out in the presence of antisense oligonucleotides and in cytosensor experiments in which flt-3 + SCF caused the same acidification as that of the single cytokines (not shown).

The in vitro proliferative and antiapoptotic activity of FL/flt-3 was negligible in standard culture conditions (10% FCS) in which other factors presumably hold cell viability and proliferation at an optimal level.

FL protein was only present in a membrane-bound form in the cell lines and its soluble form was undetectable in their supernatants; this could be attributed to a rapid self-utilization of FL from cells or to a very low FL level in the supernatants, not detected by our method. FL/flt-3 coexpression in most lines and tumors points to an autocrine loop mediated by cell-to-cell interactions. This mechanism could be more substantial in ES and PNET than in NB and NE (FL/flt-3 coexpression in 74% and 42% of biopsies, respectively) and certainly plays a favorable role in tumor proliferation. Only further experiments on normal human tissues can clarify whether it is a causal factor in tumor transformation.

All biopsies were obtained from stage III and IV tumors. The prognostic value of FL/flt-3 expression is under investigation.

In conclusion, our data demonstrate that FL and flt-3 are expressed in neuroectodermal tumors and in neuroblastoma where they exert a biological activity by promoting the survival and proliferation of tumor cells. The clinical usefulness of these data needs further investigation. The fact that hematopoietic growth factors stimulate neuroectodermal tumor cells provides further evidence of a common ancestral precursor for neuroectodermal and hematopoietic cells. FL mobilizes hematopoietic progenitors and promotes dendritic cell production and is therefore potentially suitable for cancer therapy. Our data suggest that its

Figure 3.

Flow cytometry evaluation of flt-3 and FL expression in SJ-N-KP (NB), PDN12 (PNET), PDE02 (ES) cell lines. For surface flt-3 detection, the anti-flt-3 PE-conjugated BV10A4H2 MoAb was used. For the detection of intracellular flt-3, cells were first treated with the Fix and Perm Cell permeabilization kit. Surface FL expression was investigated by indirect flow cytometry using the anti-FL 40406.111 MoAb as primary antibody and the goat antimouse F(ab)2 FITC-conjugated MoAb as secondary antibody. The K-562 and M07-E lines were used as negative controls (CL) for flt-3 and FL, respectively. A, Surface flt-3. B, flt-3 expression after cell wall permeabilization. C, Surface FL.

clinical use in neural crest–derived tumors ought to be cautiously evaluated.

Materials and Methods

Cell Lines

Twelve tumor cell lines of neuroectodermal origin: six NB and NE lines (IMR-5, SK-N-SH, SK-N-BE, AF8, SJ-N-KP, CHP-100), three ES lines (6647, TC106, PDE02), and three PNET lines (PDN12, PDN13, TC32) (Biedler et al, 1973, 1978; Pagani et al, 1998; Rocchi et al, 1987; Schlesinger et al, 1976; Thiele et al, 1987) were cultured in RPMI 1640 (GIBCO, Grand Island, New York) plus 10%, 1%, or 0.1% FCS (Irvine, Santa Ana, California). The TC32 cell line was used only for molecular studies. As controls, the M-07E line (Avanzi et al, 1990) was maintained in Iscove's modified Dulbecco's medium (GIBCO) supplemented with 5% FCS and rhGM-CSF (Sandoz, Basel, Switzerland) 150 U/ml, and K-562, 5637, and HL60 lines were cultured in RPMI 1640 plus 10% FCS.

Supernatants for ELISA were obtained by culture at 5×10^4 cells/ml. The conditioned medium was removed after 72 hours, centrifuged, filtered, and assayed.

Biopsies

Nineteen biopsies from stage III and IV NB (kindly provided by Dr. G. P. Tonini, Genova) and 19 biopsies from ES and PNET (all with the chimeric fusion gene product EWS/FLI1, kindly provided by Dr. E. Frascella, Padova) were analyzed. All diagnoses were confirmed by a reference team of Italian Pediatric Hematology and Oncology Association (AIEOP) pathologists.

RT-PCR Analysis

Total RNA was extracted by the guanidine salts and phenol-chloroform method (Ultraspec RNA; Biotec

Figure 4.

Cytosoft rate window demonstrating the dose-response curve of SJ-N-KP cells exposed to FL. Concentrations (in ng/10⁶ cells): 0 (orange), 1.25 (dark blue), 12.5 (red), 125 (green), 1,250 (black), 2,500 (light blue), 5,000 (purple). Acidification peaked at 2,500 ng FL/10⁶ cells. The arrow indicates the time at which growth factor was added. Time: minutes, 1 μ V/sec = 1 millipH/minute.

Laboratories, Houston, Texas). cDNA was prepared from 2 μ g of total RNA, using poly-T as primer and MMLV reverse transcriptase (GeneAmp RNA PCR Kit; Perkin Elmer, Foster City, California). PCR was performed in a Perkin Elmer GeneAmp PCR System 9700, using one-fourth of the reverse transcription product and 25 pmol of each oligonucleotide as primers. After one denaturation step at 96° C for 3 minutes, 35 cycles of amplification were performed: denaturation at 94° C for 1 minute, annealing at 65° C or 69° C (for flt-3 and FL, respectively) for 1 minute, synthesis at 72° C for 1 minute, and extension at 72° C for 5 minutes. Oligonucleotides for flt-3 expression analysis were: sense primer 5'-CAAGTGC-TGTGCATACAATTCCC-3' (positions 1536–1558 on the

sequence), antisense primer 5'-ACCTGTACCATCT-GTAGCTGG-3' (positions 1722–1742) (Rosnet et al, 1993); oligonucleotides for FL were: sense primer 5'-TGTCAGCCCCGACTCCTCAACC-3' (positions 555–575) and antisense primer 5'-AGTGCTCCACAAGCAG-CAGGT-3' (positions 769–789) (Lisovsky et al, 1996). To check cDNA quality, beta-actin was amplified using sense primer 5'-CCACTGGCATCGTGATGGACT-3' and antisense primer 5'-TCCTTCTGCATCCTGTGCGC-3' (Ponte et al, 1984).

One-tenth of the amplified products was run on 2% agarose gels in 1 $\times$ Tris borate buffer and visualized with ethidium bromide. The M07-E and K-562 lines were used as negative controls for FL and flt-3, respectively. 5637 and HL60 were the positive controls.

Figure 5.

Cytosoft rate window demonstrating the real-time response of SJ-N-KP cells to a 6-minute exposure to 2,500 ng FL ($\circ$)/ 10^6 cells, 2,500 ng anti-FL neutralizing antibody ($\triangle$)/ 10^6 cells, FL plus anti-FL ($\triangle$). Medium alone, ($\bullet$). The arrow indicates the time at which growth factor and neutralizing antibody were added. The peak reached after 2 minutes is followed by a rapid return to the baseline despite the continuing exposure of cells to the growth factor. Time: minutes, $1 \mu\text{V}/\text{sec} = 1 \text{ millipH}/\text{minute}$.

Enzyme Immunoassay for Soluble FL Protein

Soluble FL protein was determined according to the manufacturer's instructions by ELISA (Quantikine; R & D Systems, Minneapolis, Minnesota) in conditioned medium of NB, NE, ES, and PNET lines cultured for 72 hours. Briefly, 96-well microtiter plates coated with a murine monoclonal antibody against FL were used. One hundred microliters of assay diluent were added to each well, then FL standards and samples ($50 \mu\text{l}$) were added and incubated for 2 hours at room temperature. After washing, an enzyme-linked polyclonal antibody specific for FL ($200 \mu\text{l}$) was added for 1.5 hours. Two hundred microliters of peroxidase substrate were added and incubated for 20 minutes at room temperature. Absorbance was read at 450 nm with a Microplate Reader (Bio-Rad Laboratories, Milan, Italy). The minimum detectable concentration of FL was 7 pg/ml. The control was RPMI 1640 medium.

Flow Cytometry Evaluation of Flt-3 and FL Expression

Cell lines were tested for the presence of surface flt-3 and FL and cytoplasmic flt-3. Scraped cells (2×10^5) were incubated with $5 \mu\text{l}$ anti-flt-3 PE-conjugated BV10A4H2 (Caltag, San Francisco, California) for 20 minutes at 4°C , then washed with PBS plus 1% w/v bovine serum albumin before analysis with a flow cytometer EPICS XL (Coulter, Miami Lake, Florida) equipped with an argon laser. Isotype PE conjugate was used as negative control. To detect cytoplasmic

Figure 6.

A, FL-induced tyrosine phosphorylation of flt3. SJNKP cells were incubated for 10 minutes with $2.5 \mu\text{g}$ FL/ 10^6 cells, lysed, and immunoprecipitated with Flt3 antiserum. Proteins were resolved by 7.5% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antiphosphotyrosine antibody. The molecular mass of protein standards is indicated in kDa. 1, Untreated cells; 2, FL-treated cells. B, Flt3 immunoblot. The immunoblot from A was stripped and reprobed with an anti-flt3 polyclonal antibody.

flt-3, cells were incubated according to the manufacturer's instructions in $100 \mu\text{l}$ fixing solution (Fix and Perm Cell permeabilization kit; Caltag) for 15 minutes at room temperature, washed in PBS, resuspended in permeabilizing solution in the presence of $5 \mu\text{l}$ PE-conjugated BV10A4H2, incubated for 15 minutes at room temperature, and then washed and analyzed as described. Cord blood CD34+ cells and K-562 cells were used as the positive and negative control, respectively. Surface FL expression was investigated by indirect flow cytometry using anti-FL 40406.111 MoAb (R & D Systems, Milan, Italy) as primary antibody and goat antimouse F(ab)2 FITC-conjugated MoAb (Technogenetics, Milan, Italy) as secondary antibody. The M07-E line was used as negative control.

Cytosensor Experiments

An innovative instrument, the microphysiometer "Cytosensor" (Molecular Devices, Sunnyvale, California), which measures the ligand/receptor reaction from the output of acid from cells (McConnell et al 1992; Salon and Owicki, 1994), was used to study the response to FL stimulation of SJ-N-KP and PDN12 cell lines. Cells

Table 2. Percentage Increase of Viable Cells over Basal Values in Cultures^a

	FL 24 h	SCF 24 h	FL+SCF 24 h	FL 48 h	SCF 48 h	FL+SCF 48 h	FL 72 h	SCF 72 h	FL+SCF 72 h
AF-8	67	76	251	34	29	57	48	34	77
SJ-N-KP	85	113	236	148	145	235	86	65	122
IMR-5	110	85	165	52	67	128	34	36	60
CHP-100	83	59	175	68	75	130	69	80	122
SK-N-BE	52	58	135	36	42	75	32	15	58
SK-N-SH	38	26	95	52	73	133	29	50	94
TC106	29	13	75	40	56	63	19	27	47
PDN13	58	50	113	39	63	108	37	16	55
6647	65	65	155	26	57	103	21	10	60
PDE02	50	38	96	39	53	110	42	52	92
PDN12	57	74	148	33	30	66	54	38	84
m ± SD	63 ± 23	60 ± 28	149 ± 56	51 ± 34	63 ± 31	119 ± 50	43 ± 20	38 ± 22	79 ± 26

SCF, stem cell factor.

^a Cultures carried out in the presence of FCS 1% with the addition of 50 ng/mL FL, 50 ng/mL SCF, 50 ng/mL FL+50 ng/mL SCF for 24, 48, and 72 hours.All increases were significant, $p < 0.005$.

in RPMI 1640 were seeded into cytosensor capsule cups 18 hours before the experiment. The capsules were then transferred into a silicon-containing sensor chamber. The instrument measures the rate of output of acid from cells (acidification rate); the fluid flow is periodically halted to allow a build-up of acid metabolites in the chamber. Determination of the baseline acidification rate from eight replicate chambers after normalizing data was followed by introduction of the effector agent, and its effect on the acidification rate was monitored. The FL working concentration was calculated from a dose-response curve in which the cells were incubated for 6 minutes with 0, 1.25, 12.5, 125, 1,250, 2,500, and 5,000 ng FL/ 10^6 cells. Acidification peaked with FL 2500 ng/ 10^6 cells. This concentration was used to demonstrate ligand-receptor specificity by adding an anti-FL neutralizing antibody (R & D Systems) at 25,000 ng/ 10^6 cells. Synergy between FL and SCF was evaluated in other experiments that compared SCF (500 ng/ 10^6 cells) with FL + SCF.

Proliferation Assays

NB, NE, ES, and PNET cell lines were cultured (2×10^4 /ml) in RPMI 1640 plus 1% FCS with or without 50 ng/ml recombinant human FL, 50 ng/ml recombinant human SCF, 50 ng/ml FL + 50 ng/ml SCF (R and D Systems), alone and associated with 0.5 μ g/ml anti-FL (40406.111 MoAb, R and D Systems) and 20 μ g/ml anti-SCF (goat IgG polyclonal Ab; R & D Systems) neutralizing antibodies. Cultures were also performed in the presence of 50 ng/ml IL-2, as negative control. Cytokines and antibodies were added every day. After 24, 48, and 72 hours, cells were trypsinized and viable cells were counted by trypan blue exclusion in a Neubauer chamber. All assays were performed in triplicate. The increase in the number of cells was determined by comparison with day zero.

In further experiments, cells cultured in RPMI 1640 + 10% FCS were exposed to phosphorothioate anti-

sense oligonucleotide for flt-3, c-kit, flt-3+c-kit (4 μ M; TIB MOLBIOL, Genova, Italy; flt-3 antisense sequence: 5'- CXTXgX CXgXCX CXAXAX CXgXCX CX-CXgX CXAXT; c-kit antisense sequence: 5'- gXCXCX gXCXgX AXgXCX gXCXCX TXCXTX CXAXT; X= phosphorothioate modification), according to previously described methods (Ricotti et al, 1998), for 3, 6, and 9 days. The uptake of FITC-conjugated antisense oligonucleotides had been previously determined. An example is shown in Figure 10. Antisense oligonucleotides were added to cultures every day. Cultures with sense and randomized-sequence phosphorothioate oligonucleotides were also performed. The K562 line was used as negative control. After 3, 6, and 9 days, cells were trypsinized and viable cells were counted by trypan blue exclusion. The increase in the number of viable cells was calculated by comparison with day zero. The specific inhibition of cell growth was determined as the difference between cell growth inhibition caused by antisense and sense oligonucleotides.

Evaluation of Apoptosis

Apoptosis of NB, NE, ES, and PNET cells cultured (2×10^4 /ml) in RPMI 1640 plus 0.1% FCS with or without 50 ng/ml FL, 50 ng/ml SCF, 50 ng/ml FL + 50 ng/ml SCF for 72 hours was evaluated by flow cytometry, using annexin V-FITC conjugates (Apoptosis Detection Kits; R and D Systems), according to the manufacturer's instructions. Briefly, cells were first washed with PBS containing calcium and then incubated for 15 minutes at room temperature with 0.025 μ g annexin V-FITC and 0.5 μ g propidium iodide before analysis with a EPICS XL2 flow cytometer. All of the experiments were performed in triplicate. Cytospin preparations were stained with May Grunwald-Giemsa for morphologic evaluation of apoptotic cells.

Immunoprecipitation Studies

The ability of FL to induce protein tyrosine phosphorylation in the SJ-N-KP cell line was determined. Cells

Figure 7.

Proliferation assays in SJ-N-KP, PDE02, and PDN12 lines. Cells were cultured for 24, 48, and 72 hours in RPMI 1640 plus 1% FCS in the presence or absence of 50 ng/ml FL, 50 ng/ml SCF, 50 ng/ml FL + 50 ng/ml SCF, and 50 ng/ml IL-2 as negative control. After trypsinization, viable cells were counted by trypan blue exclusion in a Neubauer chamber. Y axis, cell number; CL, control.

were resuspended in RPMI medium supplemented with 0.1% FCS, incubated for 24 hours at 37° C in 5% CO₂, then untreated and FL-treated cells (10 minutes with 50 ng/2 × 10⁴ cells) were rapidly pelleted and resuspended in lysis buffer (10 mM Tris-HCl, pH 8.6, 1.5 mM MgCl₂, 0.14 M NaCl, 1% NP40, 2 mM phenylmethylsulfonylfluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mg/ml pepstatin A). Lysates were incubated for a minimum of 30 minutes on ice, frozen,

thawed, and centrifuged at 13,000 rpm at 4° C for 20 minutes. Immunoprecipitation of flt-3 was performed on the clarified supernatant with a rabbit polyclonal antihuman flt-3 (Santa Cruz Biotechnology, Santa Cruz, California) after 1 hour of rotation at 4° C. Protein A-Agarose (Santa Cruz Biotechnology) was added and the rotation was prolonged for 16 hours at 4° C. Immunoprecipitates were then washed twice with lysis buffer, once with lysis buffer without NP-40, and once

Table 3. Proliferation Assays in Neuroectodermal Tumor Cell Lines^a

	R	flt-3 S	flt-3 A	c-kit S	c-kit A	S+S	A+A
D3 NB&NE	84	86	68	86	70	93	69
D3 ES	86	84	62	90	65	75	56
D3 PNET	91	95	84	90	79	88	72
D3 K562 ^b	84	88	94	88	96	99	96
D6 NB&NE	88	81	61	79	66	81	57
D6 ES	80	75	44	86	59	78	42
D6 PNET	92	86	34	83	37	78	40
D6 K562 ^b	90	98	94	98	100	93	100
D9 NB&NE	81	78	54	80	53	82	47
D9 ES	90	87	24	83	28	72	16
D9 PNET	85	88	39	89	36	80	39
D9 K562 ^b	89	87	95	92	92	89	92

R, randomized-sequence oligonucleotide; S, sense oligonucleotide; A, antisense oligonucleotide; S+S, flt-3 + c-kit sense oligonucleotide; A+A, flt-3 + c-kit antisense oligonucleotide.

^a Tumor cell lines were cultured in RPMI + 10% FCS plus 4 μ M randomized-sequence phosphorothioate oligonucleotide, flt-3, and c-kit sense or antisense oligonucleotides. Cultures were evaluated after 3 days (D3), 6 days (D6) and 9 days (D9). Values express the percentage of increase of viable cells in comparison to basal values. NB&NE, ES, PNET: mean of increase of viable cells respectively in NB and NE, ES, and PNET lines.

^b The K562 line was used as negative control.

Figure 8.

Proliferation assays in NB&NE, ES, and PNET lines after 3-, 6-, and 9-day cultures in RPMI 1640 with 10% FCS (CL, control) or RPMI 1640 with 10% FCS plus 4 μ M phosphorothioate antisense oligonucleotide for flt-3 (flt-3 A), sense oligonucleotides for flt-3 (flt-3 S), and randomized-sequence phosphorothioate oligonucleotides (R). The oligonucleotides were added to cultures every day. After trypsinization, viable cells were counted by trypan blue exclusion in a Neubauer chamber. Data express the mean of the results of the experiments performed with five NB and NE lines, three ES lines, and three PNET lines. The K562 line was used as negative control. Y axis, cell number.

Table 4. Percentage of Apoptotic Cells after 12-Hour Culture with Various Supplements

	0.1% FCS ^a	FL ^b	SCF ^c	FL+SCF ^d
SJ-N-KP	43.9	19.4	14.1	17.3
PDN12	78.8	43.6	53.8	47.3
6647	28.0	9.5	7.4	5.6
TC106	31.1	15.9	23.5	22.1
SK-N-BE	30.2	19.5	19.8	19.6
CHP100	34.5	13.8	14.6	14.3
IMR-5	61.7	37.8	36.2	40.5
AF8	57.9	26.8	28.2	30.8
PDN13	31.2	20.9	13.3	15.3
SK-N-SH	42.9	22.5	24.1	20.8
PDE02	36.4	17.4	17.5	13.3
m ± SD	43.3 ± 16.2	22.4 ± 10.1**	22.9 ± 12.9**	22.5 ± 12.4**

FCS, fetal calf serum.

^a 0.1% FCS alone.^b 0.1% FCS plus 50 ng/ml FL.^c 50 ng/ml SCF.^d 50 ng/ml FL + 50 ng/ml SCF.** Significantly different from control cultures, $p < 0.005$.**A** **B** **Figure 9.**

Flow cytometry analysis demonstrating the antiapoptotic effect of 50 ng/ml FL on SJ-N-KP line cultured for 12 hours in RPMI 1640 + 0.1% FCS (B) compared with control culture (RPMI 1640+0.1% FCS) (A). Gate B (upper gate): viable cells; gate C (lower gate): not viable cells. Dual-color analysis detects early apoptotic cells (annexin V+/propidium iodide-) and late apoptotic cells (annexin V+/propidium iodide+). Numbers in each panel show the percentage of apoptotic cells. Gate A = B + C.

with Tris-HCl 50 mM, pH 6.5. Samples then underwent elution from protein A with sodium dodecyl sulfate sample buffer. The resulting protein was subjected to 7.5% SDS-PAGE with the PhastSystem (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Protein was transferred electrophoretically to nitrocellulose (Hybond ECL; Amersham) with Phast-

System. The filter was incubated with a 3% bovine albumin (Sigma, St. Louis, Missouri) blocking solution in TBS-Tween (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) overnight at 4° C. Antiphosphotyrosine antiserum 4G10 (Upstate Biotechnology, Lake Placid, New York) was added at the same solution and incubation was carried out for 2 hours at room tem-

Figure 10.

Cytospin demonstrating the oligonucleotide uptake of the SJ-N-KP cell line cultured in RPMI 1640 + 10% FCS for 48 hours with 4 μ M FITC phosphorothioate antisense oligonucleotide for flt-3.

perature. The filter was then washed three times (10 minutes each) with TBS-Tween and reacted for 1 hour at room temperature with antimouse horseradish peroxidase-conjugated antibody (Amersham). The filter was again washed as above and visualized with the ECL Western blotting analysis system (Amersham). The nitrocellulose filter was stripped of antibody by 200 mM glycine, pH 2.2, SDS 0.1%, Tween-20 1%, for 2 hours, then reprobed with anti flt-3 polyclonal antibody (Santa Cruz Biotechnology).

Statistical Analysis

Statistics were obtained with Student's paired *t* test.

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