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Insulin-like Growth Factor-I-dependent Growth and *in vitro* Chemosensitivity of Ewing's Sarcoma and Peripheral Primitive Neuroectodermal Tumour Cell Lines

Susanne Hofbauer, Gerhard Hamilton, Gerhard Theyer, Karin Wollmann and Franz Gabor

Serum-free growth of Ewing's sarcoma (ES) and primitive peripheral neuroectodermal tumour (pPNET) cell lines was achieved by supplementing a basal medium with insulin-like growth factor-I (IGF-I). These cultures were used to investigate the sensitivity of 3 ES (EW-2, RD-ES, SK-ES-1) and 3 pPNET (SIM-1, KAL, SAL) cell lines to a panel of anti-tumour agents in short-term (48-h) proliferation assays. Of the four cytostatic drugs included in the currently used multi-drug regimens, cyclophosphamide, doxorubicin and actinomycin-D inhibit the proliferation of the cell lines with high efficacy, whereas the vinca alkaloids were less effective. Cisplatin, etoposide, mitomycin-C and mitoxanthrone were also found to have a high inhibitory activity in this *in vitro* ES/pPNET system. The most remarkable effect was observed for cytosine arabinoside (ARA-C), which gave a half-maximal inhibition at drug concentrations approximately 5000 times below the clinical peak plasma concentrations (250 μg/ml). The ARA-C sensitivity of ES and pPNET cell lines is comparable with the established ARA-C sensitivities of leukaemia-derived cells. The different ES and pPNET cell lines showed a rather uniform response to the different cytostatic drugs with decreased sensitivity of individual pPNET cell lines to vinblastin, ARA-C and mitoxanthrone. Modulation of the IGF-I/IGF-I receptor/IGF-I binding protein system, which seems to constitute an important stimulator of cell growth in neuroectoderm-derived or -related tumours, can be used to enhance the drug sensitivity of the tumour cells *in vivo* or in *in vitro* therapeutic procedures. According to our results, serum-free conditions for autologous bone marrow purification are expected to result in significantly increased chemosensitivity of ES and pPNET cells in response to anthracyclines and cisplatin.
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INTRODUCTION

EWING'S SARCOMA (ES), the second most frequent bone tumour in childhood, represents a undifferentiated small round-cell tumour of uncertain origin, expressing neuroectodermal markers and a unique t(11;22) (q24;q12) reciprocal translocation or a deletion of the long arm of chromosome 22, del(22) (q12) [1]. Recently, with the help of the HBA-71 monoclonal antibody [2], high levels of MIC2, a pseudoautosomal human gene product involved in T-lymphocyte cell adhesion, has been found in ES and primitive peripheral neuroectodermal tumours (pPNET) [3]. The ES-related peripheral (extracranial) pPNET are small round-cell malignancies of presumed neural crest origin, which share, among other characteristics, the chromosomal reciprocal translocation t(11;22) and the expression of high levels of MIC2/HBA-71-antigen with ES [3, 4]. The HBA-71-antibody inhibits the growth of ES and pPNET cell lines by interfering with the action of insulin-like growth factor-I (IGF-I), which seems to function as specific growth factor for both tumours [5]. The recognition of pPNET as a distinct entity is based on

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intricate ultastructural, immunocytochemical features and the apparently distinct clinical profile of the patients [6, 7].

The disease-free survival of the ES patients has been improved significantly by adding a four-drug regimen of chemotherapy (cyclophosphamide, doxorubicin, actinomycin-D and vincristine) to the surgical and radiotherapeutic local treatment, but remains poor for patients with systemic metastasis [8, 9]. The poor survival rate of pPNET patients is associated with large burdens of disseminated tumours making complete surgical excision impossible [10]. The small number of patients treated at individual centres and the lack of a uniform treatment approach has precluded conclusions about drug efficacy and possible heterogeneity of pPNET. New modalities of treatment are clearly needed to improve the low disease-free survival rates (30–60% for 3 years) and to avoid the side-effects of an aggressive multidrug chemotherapy for these patients, which results in severe toxicity in up to 60% of the patients [8].

In this study we have established short-term serum-free cultures of ES and pPNET cell lines, using IGF-I as specific growth factor, and tested the ability of different antitumour agents to inhibit cellular proliferation. The efficacies of different drugs either clinically applied in these patients or of potential use were compared for the ES and pPNET cell lines.

MATERIALS AND METHODS

Cell lines

The ES cell lines EW-2, RD-ES, SK-ES-1 and the pPNET cell lines SIM-1, KAL and SAL were used in the present study [2, 5]. The lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS: Flow, Irvine, UK), 4 mmol/l glutamine and 75 μg/ml gentamycin (37°C, 5% CO_2). Viability was tested by ethidium bromide/acridine orange staining and found to exceed 95%.

Serum-free short-term cultures

The cells were washed with RPMI-1640 medium without supplements and transferred to RPMI-1640 supplemented with 5 μg/ml transferrin, insulin/IGF-I (Boehringer Mannheim, FRG) and 1×10^{-5}mol/l selenite. The cells were kept in wells of microtitre plates (96 wells; 5×10^4 cells/well; 100 μl) under tissue culture conditions (5% CO_2, 37°C) for 48 h and labelled with 37 kBq [^{3}H]thymidine (in 20 μl medium) for the final 18 h. All determinations were done in triplicate. Incorporated radioactivity was measured by cell harvesting (Skatron, Lier, Norway) and scintillation counting.

Chemosensitivity testing

100 μl cultures of the different cell lines were set up as described above and two-fold dilutions of the respective drugs in serum-free or complete medium added in 100 μl aliquots. The complete medium for drug testing consisted of serum-free medium supplemented with 10% dialysed (phosphate buffered saline, cut-off 8000 D) FBS. For cyclophosphamide, the cultures were supplemented with a liver cell suspension from mice. The proliferation of the cells was measured by thymidine incorporation as above (incorporation rate for controls: 5×10^4–3×10^5 cpm/culture). All drugs, with the exception of mitoxanthrone (Cynamid, Wolfratshausen, FRG), were obtained from Sigma. Each drug was tested in two independent sets of experiments for the individual cell lines, measuring over a range of 10 two-fold dilution steps in triplicate cultures in serum-free or complete medium respectively.

RESULTS

Serum-free growth of ES and pPNET cell lines

The function of IGF-I as specific growth factor for ES and pPNET cell lines has been demonstrated previously [5]. Figure 1 shows the quantitative dependence of the proliferation of these cell lines upon insulin (Fig. 1a) or IGF-I (Fig. 1b) supplements. Half-maximal stimulation is achieved with 5–13 ng/ml IGF-I (Fig. 1b), which can be replaced by either low (< 1 ng/ml for EW-2 and SIM-1) or unphysiologically high levels (up to 1 μg/ml for KAL) of insulin. The SK-ES-1 cell line shows a half-maximal growth rate at 7.5 ng/ml IGF-I (data not shown; effect of insulin not determined). The different cell lines can be cultured at reduced growth rates (approximately −50% compared with FBS-supplemented complete medium) in IGF-I medium supplemented with 10 μg/ml bovine serum albumin for several months (manuscript in preparation).

Drug sensitivity profiles of individual cell lines

The typical effects of a panel of cytostatic drugs on the proliferation of the representative KAL pPNET cell line in serum-free IGF-I medium (25 ng/ml final concentration of IGF-I) is shown in Fig. 2. All drugs were continuously present in the assay system for the whole incubation period of 48 h. The thymidine incorporation into control cultures is set at 100% and the incorporation rates measured are proportional to the number of duplicating cells. The two-fold dilutions of the different cytostatic drugs are aligned according to the achievable peak plasma concentrations (PC) in clinical application (PC values used here are given in Table 1). At these concentrations anthracyclines (ADM, mitoxanthrone), cisplatin (CDDP), mitomycin-C

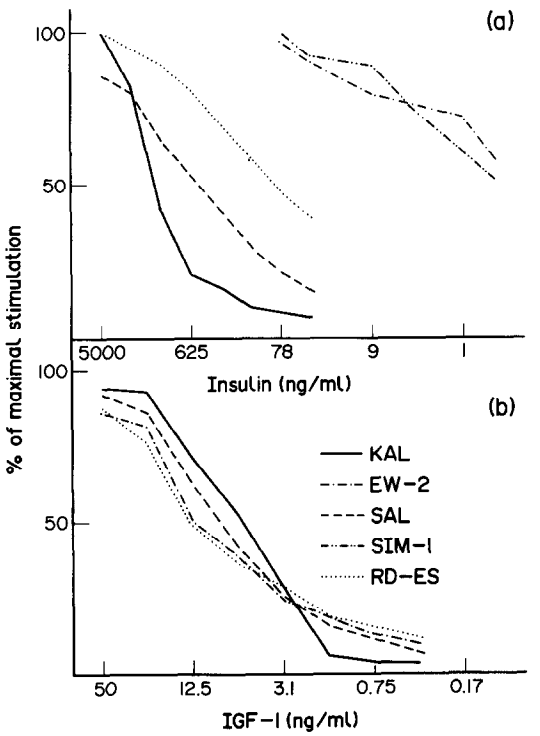

Fig. 1. Effects of insulin (a) and IGF-I (b) on the proliferative activity of ES and pPNET cell lines in short-term serum-free tissue cultures (48 h). The cells were kept in RPMI-1640 medium supplemented with 5 μg/ml transferrin, 1 mmol/l glutamine, selenite and two-fold dilutions of insulin and IGF-I, respectively (thymidine incorporation assay; control cultures supplemented with 5% fetal bovine serum = 100%).

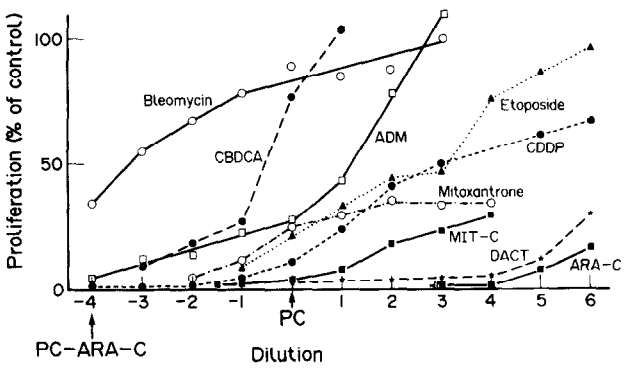

Fig. 2. Chemosensitivity profile of the pPNET cell line KAL shown relatively to the achievable peak plasma concentration (PC) of the cytostatic drugs. Proliferation of the cells was measured by a thymidine incorporation assay (control with drugs omitted = 100%). The individual drugs, bleomycin, carboplatin (CBDCA), doxorubicin (ADM), etoposide, cisplatin (CDDP), mitoxanthrone, mitomycin (MIT-C), actinomycin-D (DACT) and cytosine arabinoside (ARA-C) were added in two-fold dilution steps. For ARA-C the maximal peak plasma concentration is located at dilution −4.

and D-actinomycin inhibit the growth of the KAL cell line by > 70%. For cyclophosphamide no linear concentration/response relationship was observed, since high drug concentrations inhibited the activation of the drug by the liver cells. Carboplatin (CBDCA) and bleomycin showed low activity at the relevant drug concentrations. The cells exhibit a marked sensitivity to the action of the ARA-C, since the PC value for this drug is shifted to dilution −4 to fit into the diagram. The results of ARA-C treatment reflect not simply an interference with the thymidine incorporation assay, since cell counting and viability testing of ARA-C-treated cultures revealed a true loss of viable tumour cells (data not shown).

Chemosensitivity of ES and pPNET cell lines

The drug concentrations, which inhibit the proliferation of the individual cell lines to 50% (IC_{50}), were calculated from the individual thymidine incorporation – drug concentration relationships and the mean values (SD) calculated for the three ES and three pPNET cell lines, respectively (Table 1). The combined results for ES and pPNET cell lines as well as the clinically relevant *in vivo* peak plasma concentrations are shown for comparison. Statistically significant differences ($P < 0.05$; F-test) for the drug sensitivities of individual ES and pPNET cell lines were observed for vinblastine, mitoxanthrone and ARA-C, with apparently lower drug sensitivities in the group of pPNET cell lines (Table 1). ARA-C has an IC_{50} approximately 5000 times below the clinically peak plasma concentration. Partial inhibition of the proliferative activity of ES and pPNET cell lines was observed in response to interferon-alfa (IFN-α): the growth of ES lines was inhibited to [mean (SD)] 33 (8)% with 115 (118) U IFN-α and the inhibitory effect was 40 (22.5)% for the pPNET cell lines with 460 (470) U/ml in serum-free culture.

Effects of FBS on the drug sensitivity of ES and pPNET cell lines

The IC_{50} for the different drugs investigated here were determined for four cell lines (EW-2, RD-ES, SIM-1 and KAL) in complete medium, containing dialysed FBS, and compared with the corresponding values obtained in IGF-I-supplemented serum-free medium. Drugs with significantly different IC_{50} values (*t*-test, $P < 0.05$) in complete and serum-free medium are listed in Table 2. Group I drugs (doxorubicin, CDDP and mitoxanthrone) were less effective in serum-supplemented medium (3.7–7.8-fold increase in IC_{50}), whereas group II drugs (vincristine, mitomycin-C and actinomycin D) showed higher activity in complete medium (1.5–2-fold decrease in IC_{50}).

DISCUSSION

ES and pPNET are two closely related small round-cell tumours with poor prognosis, especially if a high tumour load or distant metastases are present. The disease-free survival rates for more than 3 years are 30–60% in ES and approximately 20% in pPNET, respectively, under combined surgery/radiotherapy and multicycle aggressive chemotherapy [8–10]. ES is radiosensitive and therefore conventional radiotherapy plays a major role in the local tumour control, whereas in disseminated pPNET radiotherapy seems to have no curative effect. Most multiple drug regimens include cyclophosphamide, vincristine, dactino-

Table 1.

Drug	IC_{50} (μg/ml) ES cell lines	pPNET cell lines	Combined lines	Peak plasma concentrations (μg/ml)
Cyclophosphamide	2.7 (0.9)	1.3 (0.45)	2.0 (0.9)	30
Doxorubicin*	0.52 (0.17)	0.63 (0.6)	0.57 (0.4)	0.4
Actinomycin-D*	6 (2.8)	4.5 (3.5)	5.2 (2.9)	—
Vincristine	74 (13)	39 (11.2)	56.2 (21.5)	0.5
Cisplatin	0.78 (0.58)	0.23 (0.14)	0.51 (0.48)	2.5
Carboplatin	7.2 (3.6)	12.5 (5.2)	9.8 (4.9)	7.0
Methotrexate	400 (73)	45.6 (20.4)	210 (191)	2.75
Etoposide	4.3 (2.7)	9.7 (6.7)	7.5 (5.8)	30
Vinblastine	26.2 (1.8)	30.0 (10.9)**	28.5 (8)	1
Mitomycin-C*	60 (28)	60 (70)	60 (50)	1.5
Mitoxanthrone*	1.5 (0.7)	47 (37)**	29 (36)	0.1–1
Bleomycin	46.7 (5.8)	26.0 (1.4)	38.4 (12)	4
ARA-C*	60 (50)	20 (1)**	40 (4)	250

Values of cell lines are expressed as mean (SD).

*IC_{50} in ng/ml.

**Statistically significant differences ($P < 0.05$; F-test).

Table 2. Significant changes of the IC_{50} of specific cytostatic drugs in response to the addition of fetal bovine serum to the chemosensitivity assay system of ES and pPNET cell lines

	IC_{50} culture conditions	
	Fetal bovine serum (ng/ml)	Serum-free (ng/ml)
Group I		
Doxorubicin	1.83 (0.49)	0.495 (0.12)
Cisplatin*	2.3 (0.44)	0.55 (0.5)
Mitoxanthrone	186 (72)	24 (3.6)
Group II		
Vincristine*	25.5 (4.4)	45.8 (13.6)
Mitomycin-C	25.0 (9.1)	45.2 (3.8)
Actinomycin-D	2.68 (1.2)	4.7 (1.64)

Values are expressed as mean (SD).
Cell lines are EW-2, RD-ES, SIM-1 and KAL.
*IC_{50} in μ/ml.

mycin and doxorubicin. In the multicenter Cooperative Ewing's Sarcoma Studies (IESS, CESS) treatment consists of several weeks cycles of the four-drug chemotherapy (VACA) followed by surgical resection if possible, local radiation and additional cycles of chemotherapy [8, 9]. From the results of clinical studies, single-agent chemosensitivities of 60% in response to cyclophosphamide, 55% for doxorubicin, 60% for dactinomycin and 20% for vincristine have been reported for ES [12]. Other drugs such as CDDP [13] and teniposide [14], cytosine arabinoside, methotrexate, etoposide and melphalan have been used in small trials with promising result [15]. Refractory or pelvic sarcomas have been treated with chemotherapy/total body irradiation and autologous bone marrow reconstitution with limited success [16, 17].

Refinement and individualisation of the current treatment strategies may further increase disease-free survival rates, but new therapeutic modalities are necessary to obtain greater complete tumour control in ES and pPNET in addition to reduced side-effects. Characterisation of the specific growth factor requirements of these related tumors as well as the introduction of new effective cytostatic drugs may help to improve the therapeutic regimen. In a previous study we have shown that IGF-I is a specific factor supporting the proliferation of ES and pPNET cell lines. These findings were extended in this study by quantitative experiments showing that the half-maximal stimulatory concentrations of IGF-I are 5–15 ng/ml for the ES/pPNET cell lines and that this growth factor can be replaced by physiological amounts of insulin for certain cell lines. These results are in good agreement with a recent report describing the specific expression of IGF-I, IGF-I binding protein-2 and IGF-I receptor mRNA in cells with the t(11;22) chromosomal translocation, characteristic for ES and pPNET [18]. However, our results show rapid decrease in cellular proliferation in non-supplemented medium and do not support the role of IGF-I as an autocrine growth factor for these cells [5]. Growth inhibition is achieved by antibodies interfering indirectly (HBA-71) or directly (alpha IR3) with the IGF-I/IGF-I receptor/IGF-I binding protein system and, since IGF-I is available in recombinant form for clinical trials, it may be used to recruit proliferating tumour cells for chemotherapeutic treatment with cell cycle phase-specific drugs. The IGF-I system, as described for the human central nervous system, seems to play an important role in the growth of peripheral neuroectoderm-derived tumours and the related ES [19].

The results from our chemosensitivity testing confirm the efficacy of cyclophosphamide, dactinomycin and doxorubicin in inhibiting the growth of ES and pPNET tumour cells. Doxorubicin dose intensity was reported to represent the most important therapeutic factor in ES and osteogenic sarcoma in a recent meta-analysis of relevant clinical trials [20]. Proliferation of the cells is also inhibited by mitoxanthrone, etoposide, CDDP and mitomycin-C at drug concentrations which are usually observed in patients. The relative ineffectiveness of the vinca alkaloids correlates with the low response rate *in vivo* (20%; [12]). The most remarkable result is the extreme sensitivity of the ES and pPNET cell lines to ARA-C, which resulted in almost complete inhibition of the proliferation in drug concentrations far below clinically peak plasma concentrations. ARA-C is most commonly used in acute leukaemia and lymphomas of adults, but rarely for children with solid tumours [21]. The inhibitory ARA-C concentrations in ES/pPNET are lower or comparable with the drug levels which suppress the growth of leukaemia-derived cell lines *in vitro* (10^{-6}–10^{-5} mol/l [22]). Recently, the toxicity of high-dose cytosine-arabinoside was tested in a clinical study in advanced childhood tumours resistent to conventional therapy and found to be manageable [23]. Our data, as well as *in vitro* data from brain tumour cell lines [24] demonstrate the extraordinary chemosensitivity of neuroectodermal-derived cells to the action of ARA-C, which should be exploited further in clinical trials in combination with other drugs such as CDDP and etoposide (PAE), investigated in recurrent brain tumours [25].

The three ES (SK-ES-1, EW-2, RD-ES) and three pPNET (SIM-1, KAL, SAL) cell lines used here, established independently in culture from tumour biopsies prior to, or after chemotherapy, responded very homogeneously to the different cytostatic drugs, with decreased sensitivity of individual pPNET cell lines to vinblastin, ARA-C and mitoxanthrone. A similar trend was observed for doxorubicin, but did not reach statistical significance. Therefore, differences in clinical responses of ES and pPNET are expected to be only partially due to differing chemosensitivities in addition to larger contributions of tumour localisation, size, spreading and variations in diagnostic and therapeutic procedures to the poorer clinical results in pPNET. Serum-free conditions were found to increase the sensitivity of ES and pPNET cells to anthracyclines and CDDP significantly and may, therefore, result in improved tumour cell killing in *in vitro* autologous bone purging procedures, which have failed so far to prolong the disease-free survival rates in advanced ES and pPNET.

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In vitro Effect of Suramin on Lung Tumour Cells

István Á. Mórocz, Béatrice Lauber, Doris Schmitter and Rolf A. Stahel

In the search for new therapeutic concepts in lung cancer chemotherapy, suramin, a potential anticancer drug which evades multidrug resistance, was tested *in vitro* on 25 lung-derived cell lines, either non-tumorigenic cells, or established cell lines from five different tumour types. Suramin treatment resulted in a time- and dose-dependent decrease in [^{3}H]thymidine incorporation, except in one adenocarcinoma cell line where DNA synthesis was highly stimulated. [^{3}H]Leucine incorporation was less affected, indicating that suramin acted cytostatically rather than cytotoxically. Our results show that suramin affected DNA synthesis of the different types of lung derived cells, including non-tumorigenic and tumour cell lines, to a similar extent.
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INTRODUCTION

SURAMIN HAS a multitude of diverse properties and has been used in the treatment of trypanosomiasis (sleeping disease) since the early 1920s. The antitumoral activity of suramin described a few years ago [1] is either mediated by obstructing extracellular growth factor-receptor interaction [2], or by direct intracellular interference with enzymes essential for growth [3]. Clinical studies show that the antitumoral effect of suramin in a variety of neoplasms is accompanied by severe side-effects [4] due to a plasma half-life of above 50 days [5] and a wide spectrum of biological interactions.

With the exception of small cell lung cancer (SCLC), the results of chemotherapy in inoperable lung neoplasms are marginal, mainly because of primary or secondary multidrug resistance [6]. The cytostatic action of suramin, in particular its interference with growth factors, is mediated by mechanisms which evade multidrug resistance. Lung tumours are known to produce a variety of growth factors [7], some acting in an autocrine manner [8, 9]. Considering these facts, we evaluated whether suramin might be a promising drug for lung cancer treatment by investigating the effect of suramin *in vitro* on

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