

Detection and treatment of minimal residual disease in high-risk neuroblastoma

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Abstract: Intensive, myeloablative therapy supported by autologous hematopoietic stem-cell transplantation (AHSCT) has improved the outcome for children with high-risk neuroblastoma. However, >50% of patients develop recurrent neuroblastoma, often from minimal residual disease (MRD). Immunocytological and reverse transcriptase polymerase chain reaction (RT-PCR) for genes highly expressed in neuroblastoma both can detect small amounts of MRD in blood and bone marrow, and detection of MRD at certain levels during therapy has prognostic value. Radionuclide scans using meta-iodobenzaguanidine (MIBG) imaging allows sensitive detection of neuroblastoma in patients, but whether or not all MIBG-positive disease detected after AHSCT will progress remains to be defined and is complicated by use of post-AHSCT therapy. Selective removal of tumor cells from marrow or blood stem cells harvested for AHSCT could decrease recurrence by preventing infusion of tumorigenic cells with AHSCT. Treating MRD after AHSCT with the differentiation-inducing retinoid 13-cis-retinoic acid significantly improved EFS of high-risk neuroblastoma patients. Randomized clinical trials in the Children's Oncology Group are testing the value of purging blood stem cells and also whether post-AHSCT therapy with an anti-GD2 monoclonal antibody (combined with cytokines) improves outcome over use of 13-cis-retinoic acid alone. New approaches to treating neuroblastoma MRD that are in early clinical trials include the cytotoxic retinoid fenretinide and the hu14.18-IL2 immunocytokine. It is anticipated that testing novel approaches to treating neuroblastoma MRD will be the subject of future phase-III randomized trials.

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The potential reasons for therapy failures in high-risk neuroblastoma patients include tumor cells developing resistance to chemotherapy (1, 2) and tumor cells surviving chemotherapy in 'sanctuary' sites with poor oxygenation or poor

drug penetration (3). Increasing doses of drugs to myeloablative levels has been successfully employed in high-risk neuroblastoma to overcome tumor-cell drug resistance in many patients and to increase drug penetration (4–6). Myeloablative therapy was shown in a prospective randomized phase-III trial to achieve a higher EFS than did three cycles of intensive, submyeloablative consolidation chemotherapy (6). While use of AHSCT to restore hematopoiesis has allowed widespread use of myeloablative therapy, autologous hematopoietic stem cells carry with them the risk of infusing tumor cells that can re-establish the disease after myeloablative therapy (4, 7). Even if no tumor cells were infused with AHSCT, undetectable MRD could persist after myeloablative therapy and lead to

Abbreviations: 13-cis-RA, 13-cis-retinoic acid; ABMT, autologous bone marrow transplantation; AHSCT, autologous hematopoietic stem cell transplant; CCG, Children's Cancer Group; COG, Children's Oncology Group; EFS, event-free survival; ENSG, European Neuroblastoma Study Group; GM-CSF, granulocyte macrophage colony stimulating factor; IL-2, interleukin 2; MIBG, metaiodobenzaguanidine; MRD, minimal residual disease; MTD, maximally tolerated dose; PBSC, peripheral blood stem cells; PET, positron emission tomography; RT-PCR, reverse-transcription polymerase chain reaction; TH, tyrosine hydroxylase.

disease recurrence. Here we will review methods for detecting MRD (in hematopoietic stem cells harvested for AHSCT and in patients) and therapeutic approaches that may decrease the chances of disease recurrence from MRD in neuroblastoma patients.

Radionucleotide scanning with MIBG for sensitive detection of neuroblastoma

Scintigraphy with radiolabeled MIBG, which is concentrated in tumor tissue of about 90% of neuroblastoma patients, can provide a highly sensitive and specific means of detecting tumor in patients (8–12). Most centers employ ^{123}I -MIBG for imaging because of its superior results compared with ^{131}I -MIBG (13). Use of SPECT with ^{123}I -MIBG has been reported to improve sensitivity (14).

MIBG uptake appears to occur also in well-differentiated tumors, so the detection of MIBG uptake does not necessarily provide evidence of active, malignant neuroblastoma (15). PET scanning with fluorine-18 fluorodeoxyglucose has been reported to provide data equivalent to MIBG (16). It is not clear that PET scanning could replace MIBG and its role may be to provide adjunctive information in selected cases. Although data support the use of MIBG scanning at diagnosis and during induction therapy as a prognostic indicator (8), the significance of MIBG-positive lesions detected soon after completing myeloablative therapy, especially in patients receiving maintenance therapy with agents such as 13-cis-retinoic acid (13-cis-RA), remains to be defined.

Detecting MRD in bone marrow and blood

Detection of MRD in bone marrow or blood from neuroblastoma patients has been reported using immunocytology (7, 17, 18), immunofluorescence (19), flow cytometry (20), and RT-PCR for various gene products expressed selectively in neuroblastoma relative to blood or marrow, including PGP 9.5 (21), TH (22–27), GAGE (18, 28), GD2 synthase (29, 30), and chromogranin A (31). In general, immunocytology can detect one tumor cell in 100 000 to 1 million hematopoietic cells (depending on the number of cells analyzed), while RT-PCR methods detect one tumor cell per 10^6 – 10^7 hematopoietic cells.

Different groups of investigators have reported that sensitive detection of neuroblastoma cells in bone marrow, blood, or PBSC during or after therapy correlated with a significantly worse outcome (7, 17, 27, 32, 33). Two of these studies examined bone marrow during therapy, one

study utilized immunocytology (7), while another study employed detection of TH gene expression by RT-PCR (27). Both of these studies showed that persistence of detectable tumor in marrow after completion of induction chemotherapy was associated with a lower EFS. Another study showed that detection of GAGE expression by RT-PCR in bone marrow 24 months after completion of intensive chemotherapy combined with anti-GD2 antibody therapy correlated with poor EFS (28). Like TH, detection of GD2 synthase by RT-PCR has been shown to be a sensitive and specific method for detecting neuroblastoma in blood or bone marrow (29, 30), and detection of GD2 synthase by RT-PCR prior to, or persistence after, therapy with the 3F8 monoclonal antibody was associated with a worse outcome (34). Another study showed that detection of TH by RT-PCR in blood obtained at diagnosis correlated with lower event-free and overall survival (32). Two different groups have reported an apparent decrease in survival was associated with detection of TH by RT-PCR in peripheral blood stem cell harvests from small numbers of patients (35, 36). Detection of tumor in blood by immunocytology at diagnosis was also significantly associated with decreased EFS (7).

Therapies used after completion of myeloablative therapy could decrease the significance of MRD detected prior to completion of therapy, especially in the case of therapies employed after MRD detection is performed. Indeed, in the CCG study that examined the relationship between MRD detection by immunocytology and outcome, MRD detection in marrow harvested for AHSCT in patients who were randomized to not receive 13-cis-RA showed a significant correlation of positive immunocytology in the marrow harvest with a poor EFS. However, this was not the case for patients randomized to receive 13-cis-RA (7). The latter observation suggests that post-AHSCT therapy (such as with 13-cis-RA) may decrease the chances for tumor progression from small amounts of tumor infused at time of AHSCT. Other differences in therapeutic approach (such as purging of tumor cells from bone marrow or PBSC) could also influence the impact of MRD that persists in bone marrow during induction therapy.

In contrast to measuring MRD during therapy, detection of tumor cells in bone marrow 2 years after completion of therapy may be less influenced by different therapeutic strategies that occur since the initial study. Thus, RT-PCR of post-treatment bone marrows is being explored as a means of predicting eventual treatment failure in neuroblastoma (28). However, detec-

tion of a positive signal by RT-PCR in bone marrow has the potential to detect mature (possibly non-malignant) residual tumor cells. As the prognostic value of post-therapy RT-PCR has only been reported for patients who had not been treated with the differentiation inducer 13-cis-RA (28), additional studies of patients treated with current therapy (i.e. myeloablative therapy followed by 13-cis-RA) will be needed to determine if RT-PCR analysis of post-therapy bone marrow aspirates can identify those neuroblastoma patients who will develop late relapses.

Because of the confounding variables that can potentially influence the impact on outcome of detecting MRD in bone marrow during or after therapy, large and well-controlled clinical trials will be needed to establish the prognostic significance of MRD detection. Careful evaluation of the impact of MRD in bone marrow and blood before, during, and after therapy is an integral part of both the ongoing high-risk neuroblastoma phase-III trials in the COG. These two trials are (i) A3973, a randomized comparison of purged to non-purged PBSC used in AHSCT and (ii) ANBL0032, a randomized comparison of post-AHSCT therapy with 13-cis-RA vs. anti-GD2 antibody + cytokines followed by 13-cis-RA. Detection of MRD in the COG studies will be performed at various times during therapy, and will be performed by both immunocytology (7, 17) and RT-PCR for multiple gene products (21, 24, 25, 29). The large number of uniformly treated patients that will be studied in the COG phase-III trials will provide the statistical power needed to define the prognostic value of detecting persistent MRD in marrow during therapy and after completion of therapy.

Should persistent MRD in bone marrow be confirmed to predict a poor clinical outcome, then a logical next question will be what should be done to improve therapy? A number of novel approaches to treating neuroblastoma are being tested in clinical and preclinical studies, including therapies suitable for use after AHSCT that could eliminate MRD not effectively treated with 13-cis-RA, such as monoclonal antibodies (commonly used with cytokines) (37–42) and the retinoid fenretinide (43–45). Developing markers to identify those stage 4 neuroblastoma patients over 1 yr of age at diagnosis who are at high-risk of progressive disease with current therapy will facilitate design of clinical trials aimed at testing such new approaches. Indeed, the use of MRD detection in bone marrow may not only allow stratifying patients for such studies, but will

provide a means for assessing the effect of novel therapies against MRD, without having to wait for the larger tumor burden necessary to quantify tumor by routine clinical methods.

Purging of neuroblastoma from hematopoietic stem cells

MRD leading to recurrence after AHSCT could come from tumor cells that survive the myeloablative therapy, or from tumor cells that were infused with the autologous stem cells. The ability of small amounts of neuroblastoma cells present in bone marrow to cause tumor recurrence was established using gene-marking techniques (46). The potential for infusing tumor cells with autologous bone marrow is considerable, with about 30% of patients having tumor detectable by immunocytology in bone marrow harvested for AHSCT (7). Several different methods to purge neuroblastoma cells from marrow have been developed, but the most widely clinically applied purging methods have used monoclonal antibodies. A single monoclonal antibody has been used with complement for purging (47, 48). Two different sets of monoclonal antibodies have been employed as 'cocktails' with magnetic beads to purge neuroblastoma from bone marrow (4, 49–53).

To support clinical trials of AHSCT carried out within the CCG, a central reference laboratory was established for purging. Purging was carried out using a cocktail of monoclonal antibodies that could remove three to four logs of neuroblastoma cells with only minimal effect on myeloid progenitors (4, 51). A schematic outlining the purging procedure is shown in Fig. 1. The CCG purging method was tested in clinical trials and was demonstrated to be able to remove tumor cells detectable by immunocytology without causing any significant delay in marrow engraftment over what was expected for unpurged bone marrow (5, 54, 55). A comparison carried out between patients undergoing HSCT with allogeneic marrow or with purged autologous bone marrow showed no significant difference in tumor recurrence or survival (55). This same purging method was used to support the CCG-3891 phase-III trial that compared (on a randomized basis) myeloablative therapy supported with purged autologous marrow vs. consolidation with three cycles of intensive non-myeloablative therapy (6). All patients randomized to receive purged AHSCT in the CCG-3891 trial received marrow that had no detectable tumor by immunocytology, although 30% of those patients had tumor detected at time

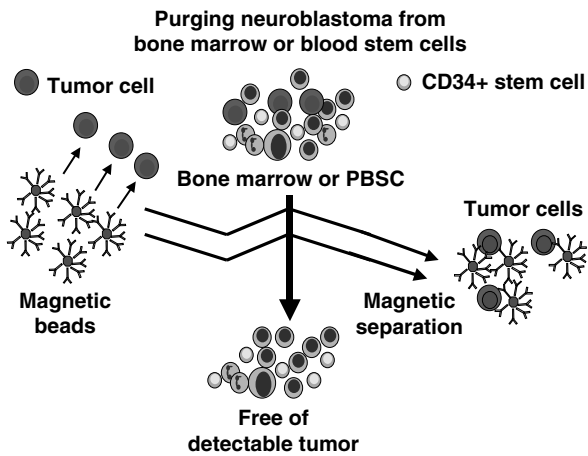


Fig. 1. Approach to using monoclonal antibodies linked to magnetic beads for purging neuroblastoma cells from bone marrow or PBSC. Prior to magnetic separation of bone marrow erythrocytes and cell clumps (along with some tumor cells) are removed by sedimentation in hetastarch and filtration (4, 55, 91). To employ this method for PBSC, phagocytes are first removed using carbonyl iron and magnetic separation, concentrating the PBSC so that purging with magnetic beads is feasible. Multiple monoclonal antibodies are conjugated directly to magnetic microspheres via goat-anti-mouse antisera. The antibody-armed magnetic immunobeads bind to neuroblastoma cells, but not to marrow progenitors, allowing selective removal of tumor from marrow or PBSC without decreasing the ability of the stem cell product to restore hematopoiesis (4, 51, 55, 91).

of marrow harvest (6, 7). Patients randomized to purged AHSCT on CCG-3891 had a superior EFS compared with patients randomized to non-meloablative therapy.

The incidence and amount of neuroblastoma cells contaminating blood is lower than that of bone marrow (7). However, PBSC collections still can contain neuroblastoma cells, (35, 56–61) although the frequency and amount of contamination appears to be lower than that of bone marrow (24). Because AHSCT is now most frequently performed using PBSC, the method used for purging of bone marrow in CCG studies has been adapted to purge PBSC. A phase-III randomized trial comparing outcome for patients receiving AHSCT with unpurged vs. purged PBSC (A3973) is currently ongoing in the COG.

Methods other than magnetic beads have been employed for purging of bone marrow from neuroblastoma patients, such as use of a monoclonal antibody and complement (47). Selection for CD34+ cells has also been employed as a means of reducing the potential for infusing neuroblastoma cells during AHSCT. However, neuroblastoma cells are not infrequently detected in PBSC even after CD34+ selection (56–64), perhaps because of expression of the CD34 antigen on some neuroblastomas (65, 66). In

addition, CD34+ selection may increase the risk from complications such as lymphoproliferative disease (67) and viral illness (68, 69).

Treating MRD in neuroblastoma after AHSCT

Even for patients receiving purged AHSCT, relapse because of tumor cells in the stem cell product that escape purging is possible, as is recurrent disease from tumor cells that survive myeloblastic therapy in the body. Patients who have undergone AHSCT are unable to tolerate additional marrow-toxic chemotherapy soon after myeloablative therapy, and low doses of cytotoxic therapy that such patients might tolerate are unlikely to have significant anti-tumor activity. This has led to an interest in developing anti-neuroblastoma agents with minimal marrow toxicity that could be employed shortly after AHSCT. In addition to having minimal hematopoietic toxicity, another desirable characteristic of drugs to use against MRD in post-AHSCT neuroblastoma patients would be a mechanism of action with the potential for non-crossresistance with the types of cytotoxic therapy employed during induction and myeloablative consolidation therapy. We will briefly review two such forms of therapy that have completed or are undergoing clinical trials, the differentiation inducer 13-cis-RA and anti-GD2 monoclonal antibodies used in combination with cytokines. We will also review newer approaches to treating MRD in neuroblastoma that are currently in early clinical trials such as fenretinide (a cytotoxic synthetic retinoid) and the hu14.18-IL2 immunocytokine.

Treating neuroblastoma MRD with high dose, pulse, 13-cis-RA

Model studies with a human neuroblastoma cell line that showed induction of morphological differentiation, sustained growth arrest, and down-regulation of MYCN expression were achieved *in vitro* with sequential 2 wk courses of 5 μM 13-cis-RA (43). Based on the *in vitro* model studies, a phase-I trial was undertaken to dose-escalate 13-cis-RA given in 2 wk courses, with the goal being to obtain drug levels > 5 μM in patients who had recently completed myeloablative therapy supported by BMT. Using the intermittent schedule, 13-cis-RA was dose-escalated to a MTD of 160 mg/m²/day in post-BMT patients, with the dose-limiting toxicity being hypercalcemia (70). The mean peak drug level at the MTD for 13-cis-RA were 7 μM (70, 71), exceeding the target level of 5 μM , and as mean trough levels were found to be 4 μM , and drug

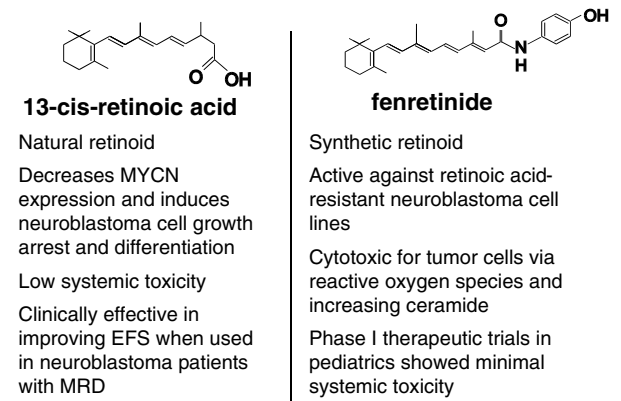


Fig. 2. Structures and characteristics of the retinoids 13-cis-retinoic acid and fenretinide. Clinical trials have established a role for 13-cis-retinoic acid in treating neuroblastoma MRD (6, 43, 91), while fenretinide is still under investigation (43, 91).

was given every 12 h, 13-cis-RA levels in patients closely approximated the *in vitro* model system. Complete responses were seen in the phase-I trial in four of the 10 patients with measurable disease after myeloablative therapy and bone marrow transplantation, and two of those four patients entered a prolonged remission (> 2 yr) (70). The latter observation suggested that high-dose, pulse 13-cis-RA might delay or prevent tumor recurrence in high-risk neuroblastoma patients, if given to patients in a setting of MRD after completion of myeloablative therapy. The structure and a summary of characteristics for 13-cis-RA (isoretinoin) are shown in Fig. 2.

13-cis-RA after completion of chemotherapy for high-risk neuroblastoma

CCG-3891 was a phase-III trial was conducted from January of 1991 to April of 1996 that entered a total of 560 patients, 539 who were eligible after review (6). Patients received induction chemotherapy regimen using cyclophosphamide, doxorubicin, cisplatin, and etoposide, during which marrow harvest and purging, and surgical resection, was accomplished. Patients were initially randomized to either myeloablative therapy employing melphalan, carboplatin, etoposide, and total body irradiation, supported with magnetic immunobead-purged autologous marrow transplant (ABMT), or to three cycles of intensive non-myeloablative therapy utilizing cisplatin, etoposide, doxorubicin, and ifosfamide/mesna. A second randomization (quasi-factorial design) assigned patients who completed either myeloablative or non-myeloablative consolidation therapy to either no further therapy or to receive 13-cis-RA at 160 mg/m²/day

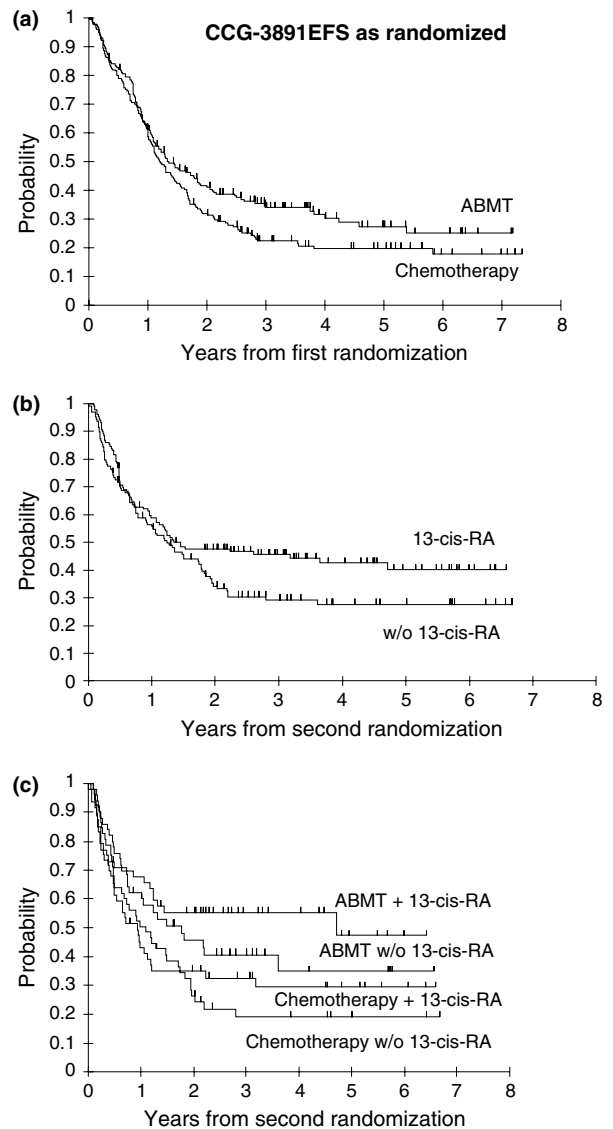


Fig. 3. Event-free survival for CCG-3891 showing the primary randomization between non-myeloablative therapy and ABMT (a) 13-cis-RA vs. no further therapy; (b) the four groups created by the quasi-factorial design and (c) the latter curves are limited to only those patients completing both randomizations. All curves are shown from time of randomization (6).

(divided and given bid) for 2 wk each month over a 6 month period. Patients who had documented active tumor by biopsy at the end of consolidation were non-randomly assigned to receive 13-cis-RA.

Intent-to-treat (as randomized) EFS data for CCG-3891 are shown in Fig. 3. There were 190 patients randomized to consolidation chemotherapy and 189 to myeloablative therapy/ABMT, and subsequently 130 were randomized to receive 13-cis-RA, while 128 patients were randomized to no further therapy. There were 37 patients

non-randomly assigned to 13-cis-RA for proven residual tumor and 24 patients who refused the second randomization, four of whom chose to receive 13-cis-RA. The first randomization showed that ABMT achieved a significantly higher EFS from time of first randomization of $34 \pm 4\%$ compared with $22 \pm 4\%$ for those randomized to consolidation chemotherapy ($p = 0.034$). The 3-yr EFS (intent to treat analysis) from the time of second randomization for patients randomized to 13-cis-RA was $46 \pm 6\%$, significantly better than the 3-yr EFS of $29 \pm 5\%$ for those randomized to no further therapy ($p = 0.027$). The effect of 13-cis-RA was most pronounced in a setting of truly MRD, as subset analysis showed the most significant effect for 13-cis-RA in stage IV patients was for those who achieved initial complete remission. The positive benefit of 13-cis-RA for those patients with MRD was not seen for children who were non-randomly assigned to 13-cis-RA for histologically proven residual disease, as this latter group showed a 3-yr EFS of $12 \pm 6\%$.

Because of the two randomizations in the CCG-3891 study, one can examine the apparent EFS for the four different treatment groups created, although small group size of each of these four treatment groups limits statistical power and the two different randomization time-points precludes a formal analysis (Fig. 3c). Treatment with 13-cis-RA appeared to be beneficial both for patients who received either ABMT or non-myeloablative chemotherapy. The 3-yr EFS from time of second randomization in patients undergoing both randomizations was higher for ABMT and 13-cis-RA ($55 \pm 10\%$), compared with ABMT alone ($41 \pm 10\%$). The 3-yr EFS for chemotherapy and 13-cis-RA was $33 \pm 7\%$, compared with chemotherapy alone ($19 \pm 7\%$; $p = 0.17$). It is likely that myeloablative therapy was most effective in reducing disease burden prior to 13-cis-RA therapy. The outcome for patients treated with both ABMT and 13-cis-RA, taken together with the poor survival of patients who had documented active disease at the time of beginning 13-cis-RA therapy, emphasizes that the optimal application for 13-cis-RA is in a setting of MRD (6).

In 1989 the ENSG initiated a randomized trial of 13-cis-RA vs. no further therapy in children with advanced neuroblastoma who achieved remission after high-dose therapy (72). When this trial was initiated, the dose escalation results with high-dose, pulsed 13-cis-RA (70) had not yet been published, so patients randomized to 13-cis-RA on the ENSG study were given a

single daily dose of 0.75 mg/kg ($22.5 \text{ mg/m}^2/\text{day}$) continuously for 4 yr or until relapse. Approximately 175 children were entered into the study with 88 patients randomized to receive 13-cis-RA (3 yr EFS = 37%) and 87 patients were randomized to placebo (3 yr EFS = 42%). No advantage in EFS was shown in this trial for children randomized to receive low dose, continuous 13-cis-RA. These results contrast with the improved survival seen with high-dose, pulse 13-cis-RA (6) and indicate the need for utilizing adequate dose levels and optimal dosing schedules to achieve pharmacologically efficacious drug levels when employing retinoids as anti-cancer agents.

Antibody and cytokines for treating neuroblastoma MRD

Monoclonal antibodies offer the potential for anti-tumor activity with minimal toxicity because of their selective binding to tumor cells as opposed to normal cells. The disialoganglioside G_{D2} is highly expressed on neuroblastoma cells and monoclonal antibodies that recognize G_{D2} have been developed for clinical use in neuroblastoma and melanoma (37, 39–42). The major toxicity from anti- G_{D2} antibody therapy is neurogenic pain, thought to be due to binding of antibody to normal nervous tissue. Responses to anti- G_{D2} antibodies used alone, or in combination with cytokines, have been reported. However, most responses have been seen in bone marrow and the activity of antibody therapy against mass-disease is uncertain. The latter observation, combined with the lack of hematopoietic toxicity, and the need to administer antibodies at a time of immune suppression, has led to a focus on use of anti- G_{D2} antibody therapy against MRD soon after myeloablative therapy. Monoclonal antibodies to G_{D2} currently in clinical trials include the murine antibody 3F8 (40) and the chimeric antibody ch14.18 (derived from the murine 14G.2A antibody) (39–42).

Another approach to stimulating the immune system to attack neuroblastoma MRD is to administer cytokines. The latter approach has been for the most part limited to use of IL-2 (74–76), which has been tolerated, but has not demonstrated anti-tumor responses. Combinations of anti- G_{D2} antibody with GM-CSF (39, 42) or with IL-2 (38, 73) were demonstrated to be tolerable and active in phase-I and II clinical trials. These latter data have led to the development of an ongoing phase III randomized trial (ANBL0032) by the COG which is comparing post-AHSCT therapy with ch14.18 + GM-CSF (or with IL-2 in alternating courses) followed by

Reynolds

13-cis-RA, to post-AHSCT therapy with 13-cis-RA alone.

New approaches to antibody therapy that are in early clinical trials are focused on trying to enhance the anti-tumor activity of anti- G_{D2} monoclonal antibodies. Preclinical studies have shown that β -glucan enhances the anti-tumor activity of the 3F8 antibody via iC3b receptors on leukocytes (77), and 3F8 + β -glucan is currently being tested in a phase-I trial. A fusion protein (immunocytokine) combining the ch14.18 anti- G_{D2} antibody with IL-2 has shown activity in preclinical studies (78, 79). For clinical trials, a humanized version of the antibody has been employed to form the immunocytokine hu14.18-IL2 (80). The hu14.18-IL2 immunocytokine has completed a phase-I trial in the COG and a phase-II study is in preparation (Paul Sondel, personal communication). Depending on activity and tolerability of these new approaches to enhancing anti- G_{D2} antibodies, future clinical trials targeting post-AHSCT MRD in neuroblastoma may employ one or more of these new approaches.

Vaccine approaches

A number of strategies to try and harness the immune system against neuroblastoma are in various stages of development from laboratory studies to clinical trials. Vaccine strategies include cytokine-transduced live neuroblastoma cells, dendritic cells, anti-idiotypic monoclonal antibodies, and DNA vaccines (81–83). Of these, the use of cytokine-transduced tumor cells has the largest clinical experience to date (81). Vaccines will likely need to be used against MRD in patients with sufficient immunological capacity to mount an anti-tumor response. Thus, defining activity will be very difficult without conducting a randomized clinical trial. Production of the biological reagents in sufficient quantities for a randomized trial is a barrier that will have to be overcome for the vaccines.

^{131}I -MIBG

As residual disease after therapy can be detected with MIBG, treating such disease with ^{131}I -MIBG (if verified by biopsy to be active tumor) is being actively explored (84). Caution is warranted if considering use of ^{131}I -MIBG for therapy of undetectable disease or MIBG-positive lesions without biopsy confirmation, especially soon after myeloablative therapy. The natural history of MIBG-positive lesions detected soon after myeloablative therapy remains to be defined, and there are toxic effects of

^{131}I -MIBG on bone marrow, including the potential for inducing secondary malignancies (85, 86).

Fenretinide

A synthetic retinoid made in the late 1960s, N-(4-hydroxyphenyl) retinamide or fenretinide (4-HPR) has been reported to inhibit the growth of neuroblastoma cell lines *in vitro* with 1–10 μM concentrations in a dose-dependent manner and 4-HPR was highly active against retinoic-acid resistant neuroblastoma cell lines at 5 to 10 μM drug levels (87). In contrast to 13-cis-RA and all-trans-retinoic acid (ATRA), 4-HPR does not induce maturational changes, but is cytotoxic, causing both apoptosis and necrosis (88). Toxicity of 4-HPR in chemoprevention clinical trials has been minimal and no hematologic toxicity has been reported, with the major clinical toxicity of 4-HPR being decreased night vision, because of decreased plasma retinol levels (43). In pediatrics, fenretinide has been well tolerated (44, 89), and the MTD of oral 4-HPR given for 7 days, every 3 wk that is currently used for the COG phase-II study is 2475 mg/m²/day, which achieved 4-HPR plasma levels of 6–10 μM with minimal systemic toxicity (44). Figure 2 shows the structure of 4-HPR and summarizes the characteristics of the drug.

Clinical data indicate that many neuroblastomas are resistant, or develop resistance during therapy, to 13-cis-RA. (6). As 4-HPR has been shown to achieve multi-log cytotoxicity in neuroblastoma cell lines resistant to ATRA and 13-cis-RA (87), if suitable 4-HPR plasma levels can be achieved clinically with tolerable toxicity, 4-HPR could be effective against 13-cis-RA-resistant neuroblastomas. Resistance to 13-cis-RA in neuroblastoma cell lines appears to involve selection for increased expression of MYCN or c-myc, and such retinoic acid-resistant neuroblastoma cell lines are collaterally hypersensitive to 4-HPR (87). Because of the potential for collateral sensitivity to 4-HPR in 13-cis-RA-resistant neuroblastoma, the sequential use of 13-cis-RA, followed by 4-HPR, could be an especially effective approach to treating MRD in neuroblastoma patients after myeloablative therapy.

The mechanism by which 4-HPR achieves anti-tumor cytotoxicity is not completely understood, but current data indicate that more than a single mechanism is involved. Recent studies have shown that 4-HPR stimulated large increases of ceramide in neuroblastoma cell lines, which is likely one of the mechanisms by which anti-tumor cytotoxicity is achieved with 4-HPR (88).

Future clinical trials may employ 4-HPR in combination with agents that modulate ceramide metabolism so as to increase the anti-tumor activity of 4-HPR (45). An example of the latter approach is to combine 4-HPR with agents that inhibit glucosylceramide synthase/1-O-acylceramide synthase or sphingosine kinase, or use of safinol (L-threo-dihydrosphingosine) (45, 90). Such agents, which modulate ceramide metabolism and/or action, can significantly increase 4-HPR anti-tumor activity in preclinical studies at levels that are minimally toxic to normal fibroblasts or bone marrow myeloid progenitors (CFU-GM) (90). Thus, 4-HPR combined with inhibitors of ceramide metabolism have features that suggest possible utility as agents to treat neuroblastoma MRD.

One problem with fenretinide is the need for large doses of the drug to achieve the drug levels predicted by preclinical studies to be effective. The currently available oral dose form is poorly bioavailable and difficult to administer to small children. New oral and intravenous formulations of fenretinide have been developed and are entering clinical trials in 2004.

Conclusions

Intensive, myeloablative therapy has been shown to improve outcome for high-risk neuroblastoma patients in pilot studies and in a prospectively randomized phase-III clinical trial (CCG-3891). Many of the patients in CCG-3891 underwent a subsequent randomization to receive post-AHSCT 13-cis-RA vs. no further therapy, and patients randomized to 13-cis-RA had a significantly better EFS, thus demonstrating 13-cis-RA was active against neuroblastoma MRD. In addition, the CCG-3891 phase-III trial employed an effective multi-modality purging of marrow for all patients, which minimized the chances of infusing tumor cells with the bone marrow, and all infused bone marrows were free of tumor by immunocytology.

It is clear that the outcome of patients undergoing myeloablative therapy was improved by retinoid therapy of MRD. The role that small numbers of neuroblastoma cells, which may potentially be infused with stem cells, play in causing tumor recurrence remains controversial. Gene marking data suggest strongly that such neuroblastoma cells infused at time of AHSCT can often be tumorigenic and can lead to recurrent disease, perhaps even in a setting of patients who otherwise may not have tumor

recurrence. However, PBSC appear to have a smaller tumor burden than bone marrow, and the potential for tumor recurrence from PBSC is unknown, although many investigators have demonstrated neuroblastoma cells in PBSC collections. An ongoing phase-III randomized trial (A3973) in the COG seeks to determine if neuroblastoma cells in PBSC do contribute to relapse and if purging of PBSC preparations can improve EFS. The recent improvements in sensitivity for detection of neuroblastoma in marrow or PBSC using RT-PCR will facilitate analysis of the A3973 study, and may potentially be useful as a prognostic indicator as well.

Regardless of whether neuroblastoma MRD arrives in the patient via stem-cell infusion, or is derived from tumor cells that survive the myeloablative therapy, recurrence from MRD remains a significant problem. One approach being tested in clinical trials is to determine if post-AHSCT therapy with an anti-GD2 monoclonal antibody + cytokines (GM-CSF, IL-2, or both) can improve outcome over treating MRD with 13-cis-RA alone. Because the majority of patients who are treated with post-AHSCT therapy have no detectable disease, the only definitive way to determine the potential contribution of monoclonal antibody therapy in the post-AHSCT setting is to conduct a randomized trial. The COG is currently conducting such a (ANBL0032) phase-III trial.

Additional therapies are being developed to treat MRD after myeloablative therapy in neuroblastoma. Like 13-cis-RA and anti-GD2 monoclonal antibodies, these new approaches to treating MRD will likely require testing in a randomized trial in order to determine their value (or lack of value) in treating high-risk neuroblastoma. A variety of pharmacological and immunological approaches are being studied in the laboratory and in early clinical trials. One of these new approaches, the cytotoxic retinoid fenretinide, is currently being tested in a COG phase-II study, and more bioavailable formulations of fenretinide are in phase-I clinical trials. Another approach, the hu14.18-IL2 immunocytokine has completed phase-I testing and a phase-II trial is planned to begin in 2004. The phase-II studies of hu14.18-IL2 and of fenretinide both include cohorts of patients with minimal, but detectable disease (i.e. patients with tumor detected only in bone marrow or by MIBG imaging). Results from these clinical trials will help guide selection of the agent (or agents) to test in future randomized trials seeking to improve neuroblastoma EFS by treating MRD.

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