科技资料

Experimental Hematology Today-1989

N.C. Gorin L. Douay
Editors

Experimental Hematology Today—1989

Selected Papers from the 18th Annual Meeting of the International Society for Experimental Hematology, July 16–20, 1989, Paris, France

With 57 Illustrations

Springer-Verlag
New York Berlin Heidelberg
London Paris Tokyo Hong Kong

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Library of Congress number 79-641222.

Printed on acid-free paper.

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Camera-ready copy provided by the editors.
Printed and bound by Edwards Brothers, Ann Arbor, Michigan.
Printed in the United States of America.

9 8 7 6 5 4 3 2 1

ISBN 0-387-97246-3 Springer-Verlag New York Berlin Heidelberg
ISBN 3-540-97246-3 Springer-Verlag Berlin Heidelberg New York

Preface

The 18th annual meeting of the International Society for Experimental Hematology took place in Paris on July 16–20, 1989. A thousand participants attended and 589 papers were presented, a record over the previous years. *Experimental Hematology Today – 1989* cannot be considered as fully representative either of the meeting or even of the status of research in our field. It only represents a subjective selection of a few papers taken from a large mass of outstanding contributions.

Manuscripts in this book have been chosen from the group that received the highest score from the scientific committee and finally selected by the editors of the book. The book is divided in 4 parts:

Present aspects of transplantation of stem cells
Control of hemopoiesis
Hemopoiesis in malignancies
From molecular biology to gene transfer.

The present yearbook of experimental hematology somehow is a testimony of the activity of our society, and I believe that it has a place of value in all scientific libraries.

N.C. Gorin
Chairman of the ISEH '89 Meeting

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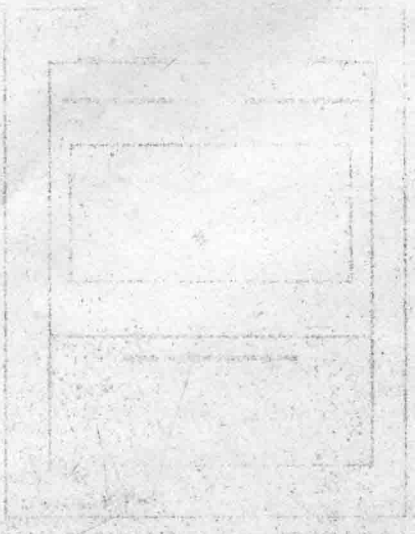

1 The Role of Autologous Bone Marrow Transplantation in Acute Leukemia

Karel A. Dicke, M.J. Dicke-Evinger, J. Spinolo, and V. Spencer

TREATMENT OF ACUTE LEUKEMIA

With conventional dose chemotherapy, the prognosis of adult acute leukemia varies with the morphological type from 25% long-term disease-free survival (DFS) in acute myelogenous leukemia (AML) to 40% in acute lymphocytic leukemia (ALL). For this reason, we would like to discuss the role of autologous bone marrow transplantation (ABMT) separately for each disease modality.

ACUTE MYELOGENOUS LEUKEMIA (AML)

From published data there is evidence in support of AML as a clonal disease. The leukemic cell population appears to escape the normal growth regulatory mechanism at either the stem cell level or at a more differentiated level such as the myeloblast or promyelocyte and begin proliferating in an uncontrolled manner. Such unresponsiveness to normal growth regulatory mechanisms is intrinsic to the leukemic cell and is most likely not due simply to a change in the feedback regulatory system of the cell. Numerous chromosomal abnormalities have been described for AML many of which involve specific gene rearrangements or deletions. Based on such chromosomal markers, prognostic factors have been identified to predict the rate as well as the durability of response to therapy. One such model is that described by Keating (1). This model predicts the probability of achieving remission (PR) and the probability of staying in remission for one year (PCR1). Using this model we are able to predict that the remission durability in patients with the -5, -7 chromosome deletion is short (PCR1 <0.6 or 60%) whereas inversion 16 or translocation 15,17 have a high probability of staying in remission for 1 year (PCR1 >0.6 or 60%). The DFS in patients with an inversion 16 or a 15,17 translocation in the leukemic cell clone is approximately 40% whereas the majority of the patients with the -5, -7 leukemic cell population survive less than a year. Variability in prognosis is a pitfall for any treatment modality to be tested on its effectiveness since patient selection may heavily influence the outcome of the study. To avoid the influence of patient selection, such studies must either be performed separately for each leukemic subpopulation or be performed under strict randomization.

AUTO-BMT IN AML CR1

For the reasons mentioned above, the European ABMT data in AML collected by Gorin (2) have restricted value. The patients described were transplanted in numerous institutions and the criteria for patient entry at these various centers are not known. Gorin has reported a 32% long-term survival in AML patients transplanted in CR1 with unpurged marrow harvested within six months after achieving CR. Patients treated with cyclophosphamide and total body irradiation (TBI) rescued with marrow cleansed in vitro with mafosfamide had a 56% three year DFS. When comparing the treatment of these two patient groups, purging may appear the difference in the ability to achieve increased DFS, however, the time of marrow harvest may also influence the prognosis. As a result, multivariate analysis of the data is necessary to evaluate purging.

Lowenberg reported the first randomized study in CR1 AML comparing conventional dose therapy to remission intensification with cyclophosphamide + TBI and unfractionated marrow, shortly after consolidation treatment (3). Randomization was done immediately after achieving complete remission. Statistically the outcome of these two groups were not significantly different. Time of marrow harvest was early after remission induction (within 2-3 months) and the intensity of the consolidation program was moderate — most likely leaving significant numbers of leukemic cells in the marrow at the time of harvest.

cells. Due to minimal cell loss with this technique, a combination with chemopurge is possible without significantly compromising the hematopoietic restoration potential of the sample. Whereas patients with good prognosis in AML may only need in vivo purging, our recommendations in ALL are a double in vitro purge. Effective intensification and consolidation programs for in vivo purging do not exist yet. It is likely that a different mode of treatment is necessary for this disease. Not only are residual cells in the harvest marrow biologically significant, but also leukemic cells escaping the conditioning regimen. Escape may not be due to lack of sensitivity but to the sanctuary sites of ALL cells.

Results in CR2, CR3 are poor. The best data are published by the Minnesota group in 22 children treated with Cy + TBI and monoclonal antibody purged marrow (8). The inversion rate was 31%. Since this has not been compared with conventional chemotherapy results, interpretation is limited.

In conclusion, the role of ABMT in ALL is unclear. The results seem less favorable than in AML. A continuing relapse rate is noted after BMT so that more intensive and longer therapy might be necessary. In vitro purging with a combination of antibody purge and chemopurge might be effective. Studies for conclusive evidence need to be undertaken.

EFFICACY OF PURGING

As mentioned earlier it is unclear if the purging of bone marrow from leukemic cells is beneficial to the long term survival of AML patients undergoing ABMT. The biological significance of such procedures will depend in part on the role these re-infused leukemic cells play in the recurrence of disease after transplantation. The fewer leukemic cells to escape the transplantation conditioning regimen, the more significant the leukemic cell population in the graft will be. It is likely, then, that the biological significance of purging will be greater in first rather than in second or subsequent remissions. A significant drawback in the answering of this question has been the inability to monitor the assumed removal of these residual leukemic cells. There are two major constraints in the detection of minimal numbers (<1%) of tumor cells: 1) the lack of technology available with sufficient sensitivity and 2) identification of specific tumor markers.

When testing leukemic cell separation techniques on untreated or relapsed leukemic patients, it is possible to monitor the separations with little difficulty using standard techniques such as morphology, cytochemistry, cytogenetics and the in vitro colony formation assays. None of these assays are specific and sensitive enough to detect the small numbers of leukemic cells which may be present in remission marrow. Since the purpose of the above mentioned separation techniques is to be used for remission marrow, such detection methods leave us to do these separations blindly. For the past decade considerable effort has been made to use molecular technology to resolve these problems.

Since these tumor cells are part of a heterogeneous population of cells, the use of Southern and/or Northern blotting techniques have not enabled us to detect fewer than 1% contaminating tumor cells unless pre-selection is used through which our sensitivity increases to 0.1% at best. From estimates of the leukemic cell population in remission marrow, the frequency of the leukemic cell population is one leukemic cell in 20,000 cells, or 0.005%, which is far below the detection limits of the techniques discussed above. Therefore, these assays cannot be used to monitor tumor cell separation techniques in remission marrow.

ABERRANT GENE EXPRESSION IN LEUKEMIA

There are numerous reports of aberrant proto-oncogene expression in tumor cells and more specifically in AML (9-17). The importance of these genes in normal cellular proliferation and differentiation has become well accepted; many of these genes appear to be related to either growth factors and their receptors, or involved in the signal pathway (18). We have found that several of the proto-oncogenes analyzed by Northern blot hybridization were expressed in hematologically normal bone marrow (15), supporting the concept that these genes do play a role in adult hematopoietic metabolism. There is growing evidence that qualitative and quantitative changes in proto-oncogene expression may be associated with neoplasia (19).

The hematopoietic system has proven attractive for these studies since several proto-oncogenes are located at the breakpoint of chromosomal translocations or inversions specifically associated with various leukemias (20). Perhaps this leads to the deregulated expression of these genes. Furthermore, the enhanced expression of cellular oncogenes have been consistently observed in neoplastic hematopoietic cells. Several groups in addition to ourselves have identified two genes, MYC and SIS, to be present at abnormally high levels in the peripheral blood and bone marrow cells of untreated and/or relapsed AML patients (21-26). By Northern and dot blotting analyses we were unable to detect this abnormality in the bone marrow cells of any AML patient clinically classified as in remission. Since the limitations of these techniques are in the range of 1-5% contamination of leukemic cells in the samples tested, any abnormality present at lower levels would remain undetectable.

DETECTION OF LEUKEMIC CELLS BY RNA-IN SITU HYBRIDIZATION

Our approach to this problem has been the identification of an abnormality which is detectable at the single cell level. We have reported the development of an extremely

Our study of ABMT in CR1 AML was originally designed as a randomized study comparing intensification of remission with ABMT versus conventional dose therapy (4). In the beginning of the study, however, the patients refused randomization so that patients were entered in the ABMT arm on a voluntary basis. The treatment schedule is outlined in Table 1. The time interval between onset of CR and marrow harvest was -3 months, and between CR and ABMT, six months. Remission was induced by amsacrine, ARA-C, vincristine and prednisone, intensification occurred by HD ARA-C 12-15 gm/m^2 and amsacrine, 250-350 ug/m^2. To harvest sufficient numbers of marrow cells, patients were treated after intensification with three courses of AD-OAP followed by Cytoxan: 6 g/m^2, BCNU: 300 ug/m^2, and VP-16: 750 mg/m^2 (CBV). The CBV treatment schema is depicted in Table 2. Two days after the last dose of VP-16, unpurged marrow was infused. After hematopoietic recovery, 3-6 courses of AD-OAP/AMSA-OAP were administered. The results of 18 patients are documented in Table 3. Ten out of 18 patients (56%) are alive and in CCR; the shortest remission is three years, the longest six years. Eight patients relapsed with a median remission time of 19 months (range 7-22 months). It appeared that of the 12 patients with favorable prognostic factors (PCR1 $\geq$ 0.6) eight are still in CCR (67%) whereas only two out of six patients (33%) with unfavorable factors (PCR1 $\leq$ 0.6) are still in CCR.

Our data are comparable to the European data published by Gorin (2) reporting a 56% DFS in patients treated with Cy +TBI and purged marrow. Although we have not used any in vitro manipulation of the marrow, the intensification with ARA-C and AMSA prior to harvest may have acted as an in vivo purge rendering the leukemic cell burden to a minimal level. We do not expect CBV alone to be more anti-leukemic than Cy + TBI; however, with the addition of CBV alone two months after treatment with HD ARA-C and AMSA, CBV might be strongly anti-leukemic. AMSA, ARA-C combination might be very effective as observed in the BAVC regimen (BCNU, ARA-C, VP-16,). A 75% DFS (median follow-up two years) has been reported with this regimen in CR2 by the Rome group (5).

Like the European data, the interpretation of our results is limited due to possible selection of patients. Promising as they may be, a randomized study to prove the biological significance is necessary.

AUTO-BMT IN AML CR2

The interpretation of transplantation results in second remission, as in first remission, is open to question. The biological effect of a procedure can be measured by comparing the length of remission after ABMT with the duration of remission preceding those remissions in which BMT occurred. When the transplantation remission is longer than the previous CR this procedure may have changed the natural history of the disease. The inversion rate with conventional dose chemotherapy is 10-20% as published by Keating et al (1). Wiernick et al (6) reported a 30% DFS and inversion rate in second remission with multiple pulses of HD ARA-C and mitoxantrone. The transplantation data reported by us, Gorin and Santos are no different. Santos reported a 25-30% inversion rate and contributed this to in vitro purging with 4-hydroperoxy-cyclophosphamide (4HC). However, in the light of the data published by Keating and Wiernick, those data are not significantly better so that no conclusions concerning the efficacy of purging can be drawn. In addition, the transplantation data in CR2 reported by our group are not significantly better than conventional chemotherapy. The only transplantation data more favorable than the conventional chemotherapy data are those reported by the Rome group using the BAVC regimen with an inversion rate of 50%.

The question of whether or not purging has a biological effect still remains unanswered. It is likely that a biological effect can only be expected when the leukemic cell population escaping the conditioning regimen is low. Primarily, this occurs in CR1 where the leukemic cell population is least resistant to cytoreductive therapy. Systematic studies to solve this question have not been done.

In conclusion, in AML the question of ABMT still remains unresolved. Studies in CR1 are underway to prove its efficacy. The role of purging is controversial; especially in CR2 with potentially high leukemic burden after cytoreductive therapy the significance of purging may remain questionable. We recommend a randomized study in CR1 with in vivo purged marrow in the good and intermediate prognostic patients, and a one arm study with in vitro and in vivo purged marrow in bad prognostic patients.

ACUTE LYMPHOCYTIC LEUKEMIA

The European ABMT data in adult ALL in CR1 reported by Gorin (2) are equivalent to the conventional chemotherapy data of the German Cooperative Group published by Hoelzer et al., reporting close to a 40%, five year DFS (7). As in AML, prognostic factors need to be taken into account before any interpretation of data can be done. In our studies the ALL data look less favorable to draw definite conclusions than the AML CR1 data although the median follow up of 31 months is relatively short. Fifteen of the 28 patients are still in CCR, however, it looks as if a plateau of the curve has not been reached. A continuous relapse rate is noted in contrast with AML.

It may well be that in ALL more than one intensification is necessary in addition to longer post transplant chemotherapy. Potential elimination of ALL cells from the graft without loss of hematopoietic stem cells might be possible based on antibody separation technology, since ALL cells differ in phenotype from early progenitor

sensitive and rapid RNA-in situ technology which permits the detection of specific mRNAs within individual cells (27). This technology enables us to identify cells with the abnormal expression of any marker gene selected at a level of 1/50,000 cells. Using this RNA-in situ hybridization methodology, we have identified the abnormally high expression of two genes, MYC and SIS, which occur in approximately 90% of untreated and relapsed AML patients (26). MYC, a nuclear oncogene, has a major role in the regulatory pathway of normal cellular proliferation. In AML, it has been reported to be over-expressed at both the mRNA and protein levels (26,28,29,30). The second gene, SIS, encodes for a protein homologous to the beta-chain of platelet-derived growth factor (31,32). PDGF has been reported to stimulate expression of genes including MYC in fibroblasts (33,34).

Since the expression of these genes is associated with cellular proliferation, one could postulate that the elevated expression of MYC in AML could be a reflection of an increased proliferative potential no different than what would be present in normal hematopoietic cells during rapid proliferation. In a recent study in our laboratory (35) we have focused on the expression of MYC and SIS in normal bone marrow cells at a time of rapid proliferation to determine if their expression levels approach the high level found in the leukemic cells.

Bone marrow cells of 18 breast cancer patients were examined in a longitudinal study with several time points before and after BMT. In all cases, bone marrow was determined to be free of tumor involvement by morphology on aspiration. For each of these patients, both pre and post ABMT samples were obtained. The pre-transplant sample was used as the baseline standard against which post-transplant specimens were compared (i.e., each patient was his own control). In addition, bone marrow samples from normal donors were utilized as independent controls. Of the 18 patients examined, 15 expressed MYC and SIS within normal limits and three had a small population of cells present which had an elevated expression of either MYC or SIS.

After high dose chemotherapy with CVP or MVT and ABMT, samples were aspirated when the patient's peripheral white blood count indicated bone marrow regeneration with increasing monocytes and a total white blood count ≥800. In not one case did the immature hematopoietic cells proliferating in the bone marrow after transplantation express MYC or SIS at the high levels found in AML. We did find an increase in the number of cells present expressing normal levels of MYC implying, perhaps, that a greater proportion of the population was actively proliferating during bone marrow regeneration.

The high levels of MYC and SIS mRNA found in these cells cannot simply be attributed to the proliferative capacity of these cells or to the presence of normal, immature hematopoietic cells. As one line of evidence that the cells we examined actually belong to the leukemic cell compartment, comparisons were made with the percentage of blast cells determined morphologically in these AML patients. The presence of such abnormal cells, as defined by gene expression, in these AML patients correlated well with the percentage of blast cells determined morphologically. As shown in Table 4, the percentage of cells over-expressing either MYC or SIS at least equals, and often exceeds, the number of blast cells present in the marrow.

In addition, we have identified the presence of such abnormal cells in AML patients studied shortly after remission induction, but these cells are often present at a much lower frequency than that found in either untreated or relapse AML (Table 5). Several, but not every patient, in which we found this abnormal group of cells have relapsed; in contrast, none of the patients whose bone marrow cells were found to be normal in their expression of MYC and/or SIS have relapsed since this study was completed. These results led us to question whether the presence of such an abnormal population of cells could be predictive of early relapse in acute leukemia.

To help us determine the significance of this abnormal cell population in the eventual clinical stability of the AML patient, we have also examined bone marrow cells of 10 AML patients who are long term survivors after BMT. The median CR duration at the time of examination for this group was 38 months with the individual remissions ranging from 14-78 months.

The presence of an abnormal cell population expressing MYC at high levels similar to that found in AML short term remission patients, does not occur in this patient group. However, in 3/10 patients a high level of SIS expression alone was present in a variable percentage of cells, occasionally as high as 80%. At this time, none of the three patients identified with this abnormality at the RNA level have been classified as having a recurrence of leukemic cells in the bone marrow by conventional morphological criteria.

MONITORING OF PURGING PROCEDURES

It is unclear if the purging of bone marrow of leukemic cells is beneficial to the long term survival of AML patients undergoing ABMT. As discussed earlier, a significant drawback in the answering of this question has been the inability to monitor the assumed removal of these residual leukemic cells. We have begun to apply our RNA-in situ hybridization technique to this problem. Since we find such high levels of MYC to be present in AML patient bone marrow, we were interested to see if these cells are removed during a purging procedure using monoclonal

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