Correlation of Proliferative and Clonogenic Tumor Cells in Multiple Myeloma

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ABSTRACT

To expand on the findings from previous clinical trials that the growth of residual tumor is increased at a predictable time following initial drug administration, malignant plasma cells from bone marrows of patients with multiple myeloma (MM) were examined for changes in proliferation and clonogenicity induced in vivo by cyclophosphamide and in vitro by drug-induced humoral stimulatory activity. Peak plasma cell \(^{3}H\)thymidine labeling index (LI) occurred predictably following drug and paralleled changes in agar colony formation by marrow cells obtained during therapy. Colony-forming capacity of pretreatment MM marrow populations was enhanced when those cells were cultured with humoral stimulatory activity, similar to the increased colony formation detected in Day 9 postcyclophosphamide marrows at the time of peak plasma cell LI.

To further define a relationship between proliferative plasma cells and colony-forming tumor cells, MM marrows were fractionated by sedimentation on an isokinetic gradient. Enrichment of a proliferative tumor cell cohort was achieved, evidenced by \(^{3}H\)thymidine LI. Colony-forming cells were also enriched by isokinetic gradient sedimentation, and agar colony formation by MM marrow cell fractions correlated with the kinetic characteristics of the isolated subpopulations. These studies of whole and fractionated human MM marrow cell populations suggest that the kinetically active cells which are induced to proliferate in vivo and in vitro are closely related to the clonogenic tumor cells which produce colonies in agar and which, like those cells measured by \(^{3}H\)thymidine LI, respond to growth stimulation by drug-induced humoral stimulatory activity.

INTRODUCTION

The timing of sequential chemotherapy of hematopoietic cancers is based on drug-induced cell kinetic perturbations in DNA synthesis and concomitant induction of humoral stimulation that promotes tumor growth (1, 2, 8, 10, 18). Serial in vivo studies in rodent and human acute leukemias (1, 2, 18) and humans with MM \(^{3}\) (8, 10) have demonstrated that maximal growth of residual tumor cells occurs at a predictable time after initial drug administration and coincides with detection of peak drug-induced HSA. In vitro chemotherapy models have demonstrated the proliferative effect of this drug-induced HSA on malignant marrow cells (1, 7, 8, 10) and the enhanced sensitivity of those HSA-induced proliferating tumor populations to cycle-active antitumor agents (7, 10). A clinical trial of timed sequential chemotherapy in poor-risk CY-refractory patients with CY administration on Day 1 followed by Adriamycin given on Day 9 at the time of predicted peak drug-induced HSA and malignant plasma cell growth yielded a 67% objective response rate and a significant prolongation of survival, supporting the contention that the activity of cycle-dependent Adriamycin was enhanced when given at the time of increased tumor proliferation (10).

MM, like other hematological neoplasms, contains proliferating and nonproliferative normal and malignant cells (4, 5, 8, 10). The tumor population is also morphologically heterogeneous with possible correlations between proliferative, morphological, and clonogenic subpopulations (5, 6, 13, 16, 17). The self-renewing, clonogenic tumor population is the cell cohort that must be therapeutically attacked in order to achieve significant clinical remission and prolong duration of survival. Therefore, it is important to determine if the induced in vivo regrowth of tumor, measured by proliferation assays, reflects changes in the clonogenic tumor fraction. These studies detail the direct correlations of proliferative changes measured by \(^{3}H\)dThd LI and clonogenic capacity in whole human MM marrow induced in vivo by drug and in vitro by HSA. Further, this clonogenic tumor population can be separated from the underlying clonogenic normal population by IGS (9, 14, 15), with correlation between proliferation and clonogenicity of these subpopulations detected.

MATERIALS AND METHODS

Bone Marrow Cells. Bone marrow cells were obtained for IGS by routine needle aspirations from 12 patients with poor-risk MM (4, 5) who had >70% malignant plasma cells in their marrow, as judged morphologically by multiple bone marrow aspirates and biopsies. No marrow-depressing therapy had been administered within 4 to 6 weeks of study. Ten of these MM patients had marrow aspirated prior to and at intervals following therapy with CY, 2400 mg/m\(^2\) (10) for LI and clonogenicity assays. Aspirated marrow cells were collected in RPMI 1640 and monodispersed through a 25 gauge needle. Bone marrow cell suspensions from each patient were prepared and handled individually.

Sera Collection. Sera were collected prior to and throughout the therapeutic course, as described previously (1, 8, 10). Pooled drug-induced HSA was obtained from patients with MM after treatment with CY, as described previously (8, 10), assayed to determine activity, and used in agar cultures of pretreatment (Day 1) MM marrows.

IGS. This method utilizes a continuous gradient of low-density Ficoll and low centrifugal force to sediment cells at a constant velocity related to both the diameter and density of the specific cell type (14, 15). Linear density gradients of sterile Ficoll (average molecular weight, 400,000; Pharmacia Fine Chemicals, Piscataway, NJ) in RPMI 1640, supplemented with 25 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer to ensure pH stability, were constructed as detailed previously (9). Final gradient density varied from 2.7% to 1.0% of the sample gradient interface 13.7 cm from the center of revolution to 5.5% Ficoll at the gradient-cushion interface (9, 14, 15). Solutions of Ficoll in medium were sterilized prior to use.

\(^{3}\)This work was supported in part by USPHS Grant CA-06973.

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\(^{3}\)The abbreviations used are MM, multiple myeloma; HSA, humoral stimulatory activity; CY, cyclophosphamide; \(^{3}H\)dThd, tritiated thymidine; LI, labeling index; IGS, isokinetic gradient sedimentation; NCC, nucleated cell counts; CPU, colony-forming units; G-M, granulocyte-macrophage.

Received December 27, 1983; accepted June 6, 1984.

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Monodispersed MM bone marrow cell suspensions obtained from patients prior to therapy (107 ± 5 (S.E.) × 10^6 cells in 5 ml RPMI 1640) were layered over the gradient, which was then centrifuged at 4°C using a swinging bucket rotor (DuPont Instruments, Newtown, CT) at 96 × g for 8 min with gradual acceleration to the desired speed during the first 3 min. The time and speed of centrifugation for optimal cell migration and separation on the gradient were determined by cell fraction counts and differential morphology in control experiments (5, 15). Following centrifugation, the gradient was collected in 3-ml fractions, after an initial 5-ml fraction (equaling the volume of added cells) was obtained and discarded by displacement with a 50% sucrose solution. Refractive indices were measured on all gradient fractions with a Bausch and Lomb refractometer and confirmed linearity of the gradient. Each 3-ml cell fraction was centrifuged at 800 rpm for 5 min to remove Ficoll and refractive and differential morphology in control experiments (9, 15). Following centrifugation, the gradient was collected in 3-mi fractions, after an initial 5 min) onto duplicate slides coated with gelatin. Autoradiographs were prepared with Kodak NTB-2 photographic emulsion exposed for 19 days, developed, and stained with buffered Wright’s solution. The [%] dThd LI was determined by counting the numbers of malignant plasma cells per 1000 that contained 5 or more grains overlying the nucleus. Background labeling was estimated by the number of grains present in a cell-free area equivalent to the area of representative cell nuclei. Multinucleated plasma cells were counted as one nucleus when one or all nuclei were labeled. Greater than 90% of all cells scored contained approximately 25 grains. Results are reported as percentage of labeled plasma cells. Our S.E. for this method is ±1%.

**Agar Assay for CFU.** Cell suspensions from patients with >70% malignant marrow plasma cells were studied prior to and following fractionation for their abilities to form MM and/or normal G-M colonies during 21 days in agar culture. Whole MM marrow suspensions and cells from pooled postgradient centrifugation fractions were cultured at final concentrations of 5 × 10^6 cells in quadruplicate 35- × 10-mm Falcon Petri sterile tissue culture dishes in 1 ml of 0.3% Bacto-Difco and again in CMRL 1066 medium supplemented with penicillin (100 units/ml), streptomycin (2 mg/ml), 2 mM glutamine, asparagine (0.6 mg/ml), DEAE-dextran (0.5 mg/ml), and 15% normal human serum (11), autologous serum, or pooled drug-induced HSA (8, 10). All media were further supplemented with 20% autologous serum, or pooled drug-induced HSA (8, 10).

**Table 1**

<table>
<thead>
<tr>
<th>Day of CY therapy</th>
<th>[%] dThd LI</th>
<th>Colony formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.6 ± 1.6^a (2.2–13.5)^e</td>
<td>16.5 ± 6.8^d (0–45)</td>
</tr>
<tr>
<td>5</td>
<td>5.2 ± 1.6 (1.8–8.5)</td>
<td>11.0 ± 2.6^e (4–19)</td>
</tr>
<tr>
<td>9</td>
<td>17.8 ± 2.9 (8.1–31.6)</td>
<td>54.3 ± 6.5^h (36–85)</td>
</tr>
<tr>
<td>14</td>
<td>8.5 ± 1.8 (4.4–14.8)</td>
<td>8.3 ± 3.9^f (2–20)</td>
</tr>
</tbody>
</table>

^a Percentage of labeled plasma cells, 10 individual marrows.
^d Mean ± S.E.
^e Numbers in parentheses, range for individual marrows.
^f Colonies/5 × 10^6 cells plated, 10 individual MM marrows.
^g Colonies/5 × 10^6 cells plated, 5 individual MM marrows.

**Table 2**

<table>
<thead>
<tr>
<th>Day of bone marrow aspiration and CY pretreatment serum</th>
<th>Autologous pretreatment serum</th>
<th>Pooled drug-induced HSA</th>
<th>Autologous serum, Day 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14.8 ± 6.5^i (0–31)^f</td>
<td>42.7 ± 8.1 (12–82)</td>
<td>54.3 ± 6.5 (36–85)</td>
</tr>
<tr>
<td>9</td>
<td>16.4 ± 4.1 (5–26)</td>
<td>49.1 ± 10.4 (25–77)</td>
<td>54.3 ± 6.5 (36–85)</td>
</tr>
</tbody>
</table>

^i Mean ± S.E. of colonies/5 × 10^6 cells plated.
^f Numbers in parentheses, range for individual marrows.

**Table 3**

<table>
<thead>
<tr>
<th>IGS of MM marrows</th>
<th>NCC × 10^6</th>
<th>[%] dThd LI</th>
<th>Colony formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole marrow</td>
<td>102 ± 2.5^j</td>
<td>5.7 ± 1.2^k</td>
<td>18.2 ± 4.5^l (7.5–27.5)</td>
</tr>
<tr>
<td>Pooled IGS Fraction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4–6</td>
<td>23.5 ± 3.1 (15.5–27.5)</td>
<td>2.5 ± 0.6 (1.4–4.3)</td>
<td>13.2 ± 3.7 (6.1–26.0)</td>
</tr>
<tr>
<td>7 + 8</td>
<td>13.7 ± 1.5 (11.4–18.3)</td>
<td>9.7 ± 1.4 (6.5–14.3)</td>
<td>32.1 ± 4.4 (20.7–42.5)</td>
</tr>
<tr>
<td>9 + 10</td>
<td>10.4 ± 1.9 (6.2–15.6)</td>
<td>12.1 ± 0.8 (9.2–13.4)</td>
<td>28.5 ± 6.1 (17.5–47)</td>
</tr>
<tr>
<td>11 + 12</td>
<td>6.3 ± 1.3 (3.3–9.6)</td>
<td>6.2 ± 0.8 (4.3–8.4)</td>
<td>14.2 ± 3.3 (2.5–23.0)</td>
</tr>
<tr>
<td>13 + 14</td>
<td>5.6 ± 1.4 (2.7–9.3)</td>
<td>3.8 ± 0.5 (2.1–5.2)</td>
<td>10.9 ± 3.3 (2.0–19.5)</td>
</tr>
</tbody>
</table>

^j Mean ± S.E.
^k Percentage of labeled plasma cells.
^l Number of colonies/5 × 10^6 cells plated.
^m Numbers in parentheses, range for individual marrows.
supplemented with 10% colony-stimulating activity elaborated by the human monocytoid GCT cell line (Grand Island Biological Co.) (3), the colony-stimulating activity deemed to be required for proliferation and maturation of normal CFUGM in agar (12) but its role in MM colony formation being as yet uncertain (6). Agar cultures were maintained at 37° in a humidified atmosphere containing 7% CO2 and scored for numbers of colonies formed (a colony containing >50 cells) at intervals between Days 7 and 21 of culture, using a dissecting microscope at x 25.

To determine the numbers of CFLUn versus the numbers of CFUm in whole and fractionated MM marrow cell suspensions, all individual colonies were removed from each agar plate by gentle capillary pipeting under microscopic observation at intervals throughout the culture period. Each colony was transferred to glass slides and stained with 0.5% orcein in 60% acetic acid or buffered Wright's stain to evaluate the cell types present in individual colonies.

RESULTS

CY-induced perturbations in [3H]dThd LI and agar clonogenicity of malignant marrow plasma cells from 10 MM patients are depicted in Chart 1 and Table 1. The [3H]dThd LI and clonogenic assays were performed in the presence of autologous serum obtained at the same time as the marrow specimen in order to approximate the in vivo environment and thus parallel ongoing in vivo cell growth milieu. The LI of each marrow population (Chart 1) increased markedly (p < 0.001) by Day 9 after CY, returning toward base line thereafter. Agar colony formation (Table 1) was detected in 7 of 10 marrow suspensions cultured pretreatment (Day 1) in autologous MM serum with prominent variability of clonogenicity for the individual marrows (mean, 16.5 ± 6.8 colonies; range, 0 to 45). Marrow cells from 5 patients obtained Day 9 following CY were cultured in autologous Day 9 serum, predicted to contain maximal levels of drug-induced HSA (10). Increased colony formation by these Day 9 post-CY residual marrow tumor cell populations relative to day 1 pretreatment marrow clonogenic capacity paralleled the drug-induced kinetic perturbations, as determined by plasma cell LI. The effects of autologous Day 1 pretreatment sera on proliferative Day 9 post-CY marrow clonogenicity and the effects of pooled drug-induced HSA on both Day 1 pretreatment and Day 9 MM populations were assessed (Table 2). Pretreatment sera inhibited Day 9 marrow cell colony formation (16.4 ± 4.1 colonies) relative to the effects of autologous Day 9 sera (54.3 ± 6.5 colonies). In contrast, pooled HSA stimulated Day 1 marrow clonogenic capacity, with pretreatment cells from each MM marrow forming colonies and with enhanced clonogenicity detected in these stimulated populations (42.7 ± 8.1 colonies; range, 12 to 82). This enhanced colony formation by pretreatment MM marrow cells cultured in HSA was similar to the colony formation detected for Day 9 post-CY populations cultured in either autologous Day 9 serum or pooled drug-induced HSA.

The results of IGS of marrow cells from the individual pretreatment MM cell suspensions are depicted in Table 3. Following centrifugation, Fractions 4 to 6 contained maximal total NCC, while plasma cells having peak [3H]dThd LI migrated to Fractions 9 plus 10, demonstrating enrichment of a proliferative tumor cell cohort. Agar colony formation was determined for whole and fractionated cell suspensions from 5 of these MM populations. Peak colony formation in each MM marrow occurred in cells sedimenting in the lighter regions of the gradient. Peak migration of CFUgm corresponded to the sedimentation profile of the
proliferative plasma cell cohort, with maximal migration of clonogenic tumor cells occurring just prior to or concomitantly with tumor cells having peak LI. Although individual fractionated MM marrow suspensions behaved somewhat differently (Table 4), 2- to 3-fold enrichment of CFU_LMM relative to the respective unfractio-
nated marrow populations was detected in the lighter density fractions. In contrast, a 3- to 4-fold enrichment of CFU_LMM was detected in pooled Fractions 11 plus 12 and 13 plus 14, corresponding to the peak migration of proliferative granulocytes (9). Coisolation and coenrichment of both clonogenic and kinetically active subpopulations relative to the unfractio- nated marrow were achieved.

**DISCUSSION**

These studies demonstrate that the *in vivo* drug-induced perturbation in MM marrow proliferation, as measured by [3H]dThd LI, is paralleled by changes detected in the clonogenic capacity of those marrow cells in patients receiving CY. The enhanced colony formation of the residual tumor population obtained Day 9 after CY is also similar to the increased clonogenicity of pretreatment MM marrow populations cultured in pooled drug-

induced HSA relative to cells maintained in autologous pretreat-

ment serum. These results using the clonogenic agar assay are further similar to our previous findings, which demonstrate in-

creased proliferation of malignant marrow plasma cells cultured in HSA, as measured in short-term liquid culture by a [3H]dThd incorporation assay, *in vitro*. [3H]dThd LI, and tumor cell counts (8, 10), and suggest a direct relationship of tumor growth and humoral stimulation.

The correlation of overall MM marrow proliferation and clono-

genicity is further substantiated when these MM marrow prolif-
eration and clonogenicity is further substantiated when these MM marrow populations are fractionated by IGS with purification and enrichment of a proliferative tumor cell cohort which is separable from normal hematopoietic precursors (9). Our studies suggest that the kinetically active cells in MM marrows that are induced to proliferate *in vivo* by drug or *in vitro* by drug-induced HSA are closely related to the clonogenic tumor cells which produce colonies in agar. These malignant clonogenic cells, moreover, respond *in vitro* to HSA. From these data, we postu-
late that timed sequential chemotherapy, in which the adminis-

tration of the second drug in sequence is timed to coincide with maximal residual tumor proliferation and HSA induction following initial drug therapy (1, 2, 10, 18), exerts maximal antitumor effect against the self-renewing tumor cell fraction as measured by [3H] dThd LI and CFU_LMM. Our data further suggest that the normal underlying CFU_LMM present in MM demonstrate increased growth once separated from the proliferative, clonogenic MM subpopu-

lation, perhaps because the inhibitory effects of tumor on the normal self-renewing compartment have been removed (8, 10).

These studies demonstrate that clonogenic tumor cells respond to humoral factors induced in the host by the initial drug in sequence and support the empirically developed timed sequence of drugs based on growth kinetic perturbations (1, 2, 7, 8, 10, 18), with therapeutic advantage in the patient with tumor-sup-

pressed underlying normal CFU_LMM when cycle-active drugs are administered at the time of peak residual tumor clonogenicity.

**ACKNOWLEDGMENTS**

We wish to thank Barbara Chian and Wanda Novak for their secretarial expertise.

**REFERENCES**


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Cancer Res 1984;44:4197-4200.

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