Modulation of Marrow Proliferation and Chemosensitivity by Tumor-produced Cytokines from Syngeneic Pancreatic Tumor Lines

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ABSTRACT

Purpose: A dynamic process exists in which hematopoietic progenitor and stromal cells interact to maintain normal hematopoiesis or to adjust to hematopoietic needs under “stress” situations. The effect that tumor-produced growth factors have on hematopoiesis has not been addressed. We postulate that an excess of tumor-produced stimulatory or inhibitory cytokines could impact marrow proliferation and sensitivity to cytotoxic agents.

Methods: We used two tumor lines (TGP47 and TGP51) taken from a panel of syngeneic murine pancreatic carcinomas, in which each produces a unique array of cytokines, and evaluated their effect in vitro on marrow proliferation and chemosensitivity.

Results: TGP51- and TGP47-conditioned medium increased [3H]thymidine incorporation into cultured marrow cells by ~12-fold and 4.8-fold, respectively. The percent of cells in the S + G2-M phases of the cell cycle increased by 110% (TGP51) and 44% (TGP47), and the MCF for proliferating cell nuclear antigen expression increased by 104% (TGP51) and 45% (TGP47). Marrow proliferation of untreated cells could be reduced by interleukin 6 but not by granulocyte macrophage colony-stimulating factor neutralization. Conditioned medium-induced stimulation was unchanged by either interleukin 6 or granulocyte macrophage colony-stimulating factor α. FLT3-Lα reduced marrow proliferation induced by TGP51 medium. Addition of FLT3-L to TGP47 medium additionally enhanced the marrow proliferation. Antitumor necrosis factor α additionally increased marrow proliferation induced by TGP47 and TGP51 conditioned medium, whereas addition of tumor necrosis factor α reduced marrow proliferation associated with TGP51 medium. The TGP51-induced increase in marrow proliferation resulted in increased marrow chemosensitivity to three myelosuppressive drugs: doxorubicin, cyclophosphamide, and CPT-11, decreasing the IC50 by 46%, 38%, and 95%, respectively.

Conclusion: Tumor-produced cytokines can affect marrow proliferative activity and, thus, chemosensitivity to three distinct classes of chemotherapeutics.

INTRODUCTION

The hematopoietic system is a rapidly proliferating organ that maintains normal peripheral blood cell counts and must respond to stress situations such as infection, blood loss, and therapy-induced myelosuppression (1–3). The steady state production of blood cells depends in part on the interaction between hematopoietic stem/progenitor cells and the different components of the marrow microenvironment. These components include stromal cells (e.g., fibroblasts, macrophages, endothelial cells, and adipocytes), accessory cells (T lymphocytes, monocytes), and their products (extracellular matrix proteins like collagen, laminin, and fibronectin, and cell-secreted cytokines). Positive and negative regulation of hematopoiesis occurs by direct interaction (cell-to-cell contact) or by indirect contact with secreted regulatory molecules (cytokines; Refs. 4, 5). Cytokines trigger the quiescent CFU-S component of the stem cell compartment into active cell cycling until normal bone marrow cellularity is achieved (6, 7).

Under the permissive influence of these early and late-acting hematopoietic cytokines, differentiation and proliferation of multiple lineages from pluripotent stem cells is induced (8), resulting in maintenance of the steady state between production and consumption of mature blood cells (9). Under stressful conditions, (e.g., radiation, chemotherapy, blood loss, infection, or inflammation), they play a major role in cellular adaptation processes (3). They trigger quiescent CFU-S of the stem cell compartment into active cell cycling until normal bone marrow cellularity is restored (6). For example, in one study, serum G-CSF was inversely related to leukocyte blood counts in patients treated with chemotherapy. After chemotherapy, as blood counts declined, G-CSF peaked from undetectable levels (<20 pg/ml) to 1000–2000 pg/ml, and as blood counts were restored to normal, G-CSF fell back to undetectable levels (10).
in serum cytokines correlates with the severity of myelosuppression (11). In addition to published findings on the stimulatory cytokines, there is also important information emerging on the inhibitory cytokines. Negative regulation of myelopoiesis occurs through several inhibitory cytokines, most notably MIP-1α (12, 13) and TGF-β3 (14) acting on CD34+CD38− cells (15). A 10-ng dose of MIP-1α results in a 98% decrease in S phase CFU-S in vivo. Combinations of two cytokines have a synergistic effect (16, 17).

Eighteen years ago, it was reported that hematopoietic abnormalities resulted from the presence of a solid tumor (18). The hematopoietic system in tumor-bearing mice was more radiosensitive than in mice without tumors. In the presence of tumor, CFUs were suppressed after radiation therapy, and bone marrow repopulation was inhibited. The mechanism governing these observations could not be explained. Over the last few months, several reports have shown that many nonhematopoietic tumors have the ability to produce multiple cytokines in vivo. Approximately 80–100% of bladder, kidney, pancreas, prostate, cervical, endometrial, and ovarian cancers produce significant amounts of GM-CSF, IL-6, and M-CSF. Other cytokines produced in some but not all tumors include G-CSF, SCF, TNF-α, TGF-β, IL-8, and IL-10 (19–22). These cytokines may act as autocrine growth factors, regulating proliferation and migration of endothelial, tumor, and immune cells. The type of cytokines produced varies as a function of the type of cancer and stage of disease (21–23). We questioned whether tumor-produced cytokines could influence marrow physiology, i.e., cell proliferation and cell sensitivity to chemotherapy. Using a syngeneic mouse pancreatic tumor system, we address the biological effects of tumor-produced cytokines on bone marrow.

MATERIALS AND METHODS

Cell Lines. Six pancreatic tumors derived from Tg(Ela-1-SV40)Bri18 transgenic mice (24) were generously provided by Dr. Olive Pettengill, Dartmouth Medical Center, Hanover, NH. These six murine TGP lines were developed by introducing viral or human oncogenes under the control of a pancreas-specific elastase-1 promoter and SV-40 early tumor antigen into 129/SvJ mice (24). These six transgenic mice were then crossed with 129/SvJ mice to produce the TGP lines. The TGP cell lines were collected, cell debris pelleted, and cytokines in RPMI 1640 with 10% FBS, 1% glutamine, 1% nonessential amino acids, and 1% penstrep.

Cytokine Quantitation. Medium from semiconfluent TGP cell lines was collected, cell debris pelleted, and cytokines in the conditioned medium measured by ELISA kits from Endogen (IL-1β, IL-3, IL-6, GM-CSF, IFN-γ, TNF-α) and R&D Systems (G-CSF and FLT3-L). All of the assay kits have high sensitivity (approximately 3–15 pg/ml), are specific, and show no cross-reactivity with any other murine or human cytokines. Measurements were made in triplicate and the study repeated twice from different generations of TGP growing cells.

Intracellular Cytokine Expression. Cultured cells were washed in cold fluorescence-activated cell sorter buffer (Dulbecco’s PBS, 1% FBS, and 0.01% sodium azide), and 10 ng/mL were resuspended in Cytofix buffer (2014KZ; PharMingen, San Diego, CA) and incubated on ice for 30 min. Fixed cells were pelleted and then incubated in 250 µl Perm/Wash buffer (phycoerythrin 2091KZ; PharMingen) on ice for 20 min followed by a 45-min incubation with biotinylated antimonu SCF Ab. Cells were washed and incubated for 45 on ice in the dark with streptavidin-PE. Cells are washed and incubated for 12 weeks in 1 ml fluorescence-activated cell sorter buffer, and the percentage of positive cells and MCF were determined by flow cytometric analysis.

Marrow Proliferation Assay. Murine femoral bone marrow cells were flushed from young mice (6–12 weeks in age), counted, and seeded in triplicate into 96-well round-bottomed culture plates. One of four concentrations ranging from 50,000 to 400,000 cells/well was seeded in 10 ng/mL of neutralizing Ab on ice for 20 min followed by a 45-min incubation with biotinylated antimonu SCF Ab. Cells were washed and incubated for 45 on ice in the dark with streptavidin-PE. Cells are washed and incubated for 12 weeks in 1 ml fluorescence-activated cell sorter buffer, and the percentage of positive cells and MCF were determined by flow cytometric analysis.

PCNA Expression. PCNA is essential for cellular DNA synthesis and has been used as a proliferation marker for both hematopoiesis and tumor growth. It is expressed in proliferating cells in the late G1 phase and early during the S phase of the cell cycle.
cycle (25). Femoral marrow was collected at 10 a.m.; the cells were washed and incubated with FITC-conjugated anti-PCNA (Clone PC10; PharMingen). The percentage of PCNA-positive cells was determined. Appropriate negative and positive controls were used. Samples were run in triplicate, and the study was repeated twice.

**Cell Cycle Analysis.** Washed femoral marrow was fixed with drop-by-drop addition of 1 ml ice-cold 70% ethanol, covered with paraffin, and stored overnight. Cells are pelleted and washed with permeabilizing buffer (6.05 g Tris buffer, 49.5 ml dH2O, 4.5 g NaCl, 73.5 mg CaCl2, 23.8 mg MgCl2, and 5 ml NP40). One ml of a 0.5 M SYTOX Green [1 M 5 mm SYTOX Green (frozen, −20°C) + 9 ml permeabilizing buffer + 1 ml 1000 Kunitz RNase A] is added and the sample is incubated at room temperature for 15 min with gentle rocking. Cells are pelleted (3000 rpm × 10 min) and resuspended in 1 ml of sheath fluid with 1% formalin.

**MTT Cytotoxicity Assay.** The assay was done according to the method of Mosmann (26). Cells are plated at 10^4/well in a 96-well dish in 150 μl medium and incubated for 24 h in a humidified chamber. Three drugs were evaluated: doxorubicin (S phase-specific topoisomerase II inhibitor), cyclophosphamide (noncycle active alkylating agent), and CPT11 (Camptosar; S phase-specific topoisomerase I inhibitor), which induce mild, moderate, and high myelosuppressive responses, respectively. Drugs were added at various concentrations in 150 μl and incubated for 3 days. The medium was removed, and 0.5 mg/ml MTT (100 μl) was added to each well and incubated for 2–4 h at 37°C. Acid-isopropanol (0.4 N HCl in isopropanol) was added to each well (100 μl) and mixed to dissolve purple crystals. The plate is read at 570 nm. Data were converted to percentage of control (no drug). The results of percentage of survival versus log of the drug concentration are plotted to obtain the IC50.

**RESULTS**

<table>
<thead>
<tr>
<th>Tumor line</th>
<th>GM-CSF (pg/ml)</th>
<th>G-CSF (pg/ml)</th>
<th>FLT3-L (pg/ml)</th>
<th>IL-3 (pg/ml)</th>
<th>IL-6 (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
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<tr>
<td>TGP47</td>
<td>187.4</td>
<td>37.97</td>
<td>11.82</td>
<td>2.5</td>
<td>0</td>
<td>58.54</td>
<td>32.7</td>
<td>142.71</td>
</tr>
<tr>
<td>TGP48</td>
<td>0</td>
<td>6.59</td>
<td>30.26</td>
<td>1.8</td>
<td>65.7</td>
<td>170.44</td>
<td>9.9</td>
<td>140.25</td>
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<tr>
<td>TGP49</td>
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<td>1.9</td>
<td>0</td>
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<td>8.5</td>
<td>140.25</td>
</tr>
<tr>
<td>TGP50</td>
<td>0</td>
<td>0</td>
<td>46.80</td>
<td>2.5</td>
<td>115.0</td>
<td>76.56</td>
<td>14.0</td>
<td>151.30</td>
</tr>
<tr>
<td>TGP51</td>
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<td>0</td>
<td>46.80</td>
<td>2.7</td>
<td>40.7</td>
<td>76.56</td>
<td>100.9</td>
<td>147.61</td>
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<tr>
<td>TGP54</td>
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<td>0</td>
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</table>

**Table 1**  Cytokine production of murine TGP tumor lines

The expression of IL-3 and IFN-γ was similar between the various lines. IL-1β was not expressed by any of the lines (results not shown). The TGP47 line produced substantial GM-CSF (187 pg/ml) and G-CSF (37.97 pg/ml), and the TGP49 and TGP51 lines produced large amounts of IL-6 (250 and 115 pg/ml, respectively) and FLT3-L (87.5 and 46.8 pg/ml), but TGP-51 also produced some GM-CSF and had very low amounts of the constitutive inhibitory cytokine, TNF-α (17 pg/ml) compared with the TGP49 line (122.2 pg/ml). Expression of one additional stimulatory cytokine, SCF, was performed by flow cytometry, and the results are summarized in Table 2. TGP48 and TGP54 demonstrated the highest percentage of positive cells (43.7% and 44.96%, respectively), and TGP48 had the highest MCF (496.61).

To select the best model(s) to evaluate the effect of tumor cytokine production on hematopoiesis, we evaluated in vitro marrow proliferation ([3H]thymidine incorporation) in the presence of medium from cytokine-producing lines (Fig. 1). TGP51-conditioned medium increased marrow proliferation 12-fold, as demonstrated by a shift in marrow cell cpm from 172 ± 68 to 2068 ± 228 when 400,000 cells were plated (P < 0.001). TGP47 medium increased [3H]thymidine uptake by 4.8-fold (704 ± 55 cpm), whereas TGP49 and TGP50 medium stimulated uptake by 4.5- (601 ± 38 cpm) and 2.4-fold (404 ± 31 cpm), respectively. The results suggest that high levels of stimulatory cytokines coupled with low levels of TNF-α result in an increase in marrow proliferation (e.g., TGP51). These same stimulatory cytokines appear to be counterbalanced by high TNF-α (e.g., TGP49).

To additionally demonstrate that tumor-produced cytokines affect marrow proliferation, we evaluated cell cycle kinetics (Fig. 2, top) and the expression (MCF) of PCNA (Fig. 2, bottom) in femoral marrow cells grown for 48 h in control medium or in conditioned medium from the TGP47 or TGP51 pancreatic tumor lines. The percentage of marrow cells in the S or G2-M phases increased from 4.86 ± 0.6% in untreated cells to 6.98 ± 0.6 (P < 0.02) when grown in TGP47-conditioned medium to 10.25 ± 0.4% (P < 0.001) when grown in TGP51-conditioned medium. MCF increased from 92.7 ± 16.0 in untreated marrow cells to 134.2 ± 15.8 (P < 0.05) in TGP47-cultured cells, and to 188.8 ± 19.5 in TGP51-cultured cells.
Tumor Cytokines and Marrow Proliferation

Fig. 1 Tumor-produced conditioned medium increases marrow proliferation in vitro. Marrow proliferation measured by [3H]thymidine incorporation (50,000 to 400,000 plated marrow cells) after a 72-h incubation in unconditioned medium or conditioned medium from various pancreatic tumor lines with different patterns of cytokine production. Results represent the mean of triplicates; bars, ±SD.

(P < 0.01). These studies are in agreement with the thymidine incorporation studies presented in Fig. 1.

Because circadian fluctuations in bone marrow DNA synthesis, CFU-GM activity, cell number, and cytokine production exist, we assessed the effect of conditioned medium on marrow cells collected at three different times of the day: 3 HALO (9 a.m.), 12 HALO (6 p.m.), and 18 HALO (12 a.m.). For all four of the tumor lines studied, the greatest increase in marrow proliferation occurred at 18 HALO and the least at 3 HALO (Fig. 3). For example, for TGP-51 cultured marrow cells, [3H]thymidine incorporation increased 5.2 ± 0.5-fold at 3 HALO, 7.5 ± 0.5-fold at 12 HALO, and 9.6 ± 1.1-fold at 18 HALO (P < 0.01) between 3 and 18 HALO and between 12- and 3 HALO, and P < 0.05 between 12 HALO and 18 HALO). Similar results were obtained for marrow cells grown in TGP47-, TGP48-, and TGP50-conditioned medium.

To establish which cytokines found in the conditioned medium were responsible for the observed changes in marrow proliferation, we performed several studies in which cytokine effects were neutralized by addition of specific Ab or exogenous cytokine was added directly to marrow cells grown in unconditioned medium or conditioned medium from various pancreatic tumor lines, normal mouse marrow proliferation by 28% (P < 0.02), whereas addition of FLT3-L to TGP47 medium resulted in a 2.17-fold increase in proliferation above TGP47 (P < 0.01). Addition of TNF-α to TGP51 medium reduced marrow proliferation induced by the conditioned medium by 27% (P < 0.01), and anti-TNF-α increased marrow proliferation of TGP47 and TGP49 medium by 1.86-fold (P < 0.01) and 1.78-fold (P < 0.02), respectively (Fig. 5).

To ascertain whether increased marrow proliferation induced by tumor-conditioned medium influences chemosensitivity, we determined the IC50 of doxorubicin, cyclophosphamide, and CPT-11 on marrow cells when grown in the presence of medium without cytokines or in the presence of conditioned medium containing tumor-produced cytokines (TGP51) for 3 days. The results of these assays are shown in Fig. 6. The IC50 for doxorubicin decreases from 0.24 ± 0.07 μg for untreated cells to 0.13 ± 0.05 μg for TGP51-treated cells (P < 0.02). For cyclophosphamide, the untreated IC50 was 5.38 ± 1.50 μg, and the TGP51-treated IC50 was 3.33 ± 0.88 μg (P < 0.01). The difference in the IC50 between untreated and TGP51-treated marrow cells exposed to the most myelosuppressive drug, CPT-11, was the most significant (P < 0.001), decreasing the IC50 from 425.3 ± 105.7 μg to 23.2 ± 10.3 μg. These results clearly demonstrate that the increase in marrow proliferation associated with tumor-produced cytokines translates into a significant increase in marrow chemosensitivity.

DISCUSSION

Chemotherapy-induced myelosuppression is the most common dose-limiting and potentially fatal complication of cancer treatment (1, 2, 29). Drug-induced hematopoietic toxicity is a frequent reason for curtailing high-dose chemotherapy in cancer patients (30). The grade of toxicity resulting from a fixed dose can vary substantially within the patient population. We have recently completed a thorough statistical analysis of the interacting factors potentially affecting hematological toxicity (31). Both univariate and multivariate analyses have confirmed the
significance of absorbed red marrow dose as the single most important factor affecting hematological toxicity after radioimmunotherapy. Baseline peripheral blood cell counts and presence of multiple bone marrow metastases were also significant factors affecting hematological toxicity. Aside from these three predictive factors, there were still many patients who experienced greater toxicity and many patients who experienced less toxicity than would have been predicted. Another critical factor regulating the magnitude of toxicity, which to date has not been addressed, is the effect that tumor-produced growth factors have on the marrow microenvironment. We postulated that differences in the expression of various tumor-produced cytokines might impact on the magnitude, duration, and slope of recovery after therapy-induced myelosuppression. Tumor production of excess stimulatory cytokines (e.g., G-CSF, GM-CSF, IL-3, IL-6, SCF, and FLT3L) would result in more active marrow proliferation, and the same dose of a therapeutic would induce more myelotoxicity. Similarly, a greater tumor production of inhibitory cytokines (e.g., MIP-1α, TGF-β3, TNF-α, and IFN-γ) would have the reverse effect.

Support for the idea that cytokines from distal sites to marrow or spleen affect hematopoiesis comes from the field of inflammation. Several older papers report that infection or inflammation induces changes in hematopoiesis (32, 33). Total number of peripheral nucleated cells, and marrow stem cells, stromal cells, and erythroblasts were increased in mice experiencing chronic inflammation (34). These findings can now be explained by changes in the cytokine milieu associated with the expression of inflammatory mediators like IL-1β, TNF-α, and IFN-γ (35, 36). It is well established that cytokine expression in a particular tissue can be modified in response to exposure to other cytokines, e.g., TGF-β or IL-4 can modulate tumor SCF production (37, 38).

To address the question of the effect of tumor-produced cytokines on marrow activity and marrow chemosensitivity, we chose an in vitro model to establish a proof or principle. Each of the TGP cell lines (24) studied produces a unique panel of stimulatory and inhibitory cytokines. Conditioned medium for all of the TGP lines enhanced marrow proliferation, although TGP51 medium resulted in significantly greater stimulation of marrow cells. Interestingly, the magnitude of stimulation in marrow proliferation varies depending on the time of day that the marrow is harvested and placed into culture suggesting that the expression of receptor(s) needed to respond to the active component(s) in conditioned medium fluctuates. This is not surprising because circadian fluctuations in cell number, cell activity, cytokine production, immunomodulating hormone production, and cellular and humoral activities are known to occur (39–42). In humans, bone marrow DNA synthesis and myeloid progenitor cell (CFU-GM) activity is at a maximum at 0–8 HALO (7:00 p.m. to 3:00 a.m.) and at a minimum at 12–20 HALO (7:00 p.m. to 3:00 a.m.). Peak lymphocyte counts occurs at 17–19 HALO (12:00–2:00 a.m.) in both mice and humans (43). Recently, it was noted that the response of CFU-GM to CSFs (IL-3, GM-CSF, and G-CSF) is also governed by circadian rhythms, with peak stimulation occurring at 3 HALO for all of the factors (44). We have also observed that marrow cytokine expression varies with the time of day, as does the expression of various cytokine receptors (45, 46). Thus, the net influence of tumor-produced cytokines on marrow activity has greater impact at night in mice than during the day. Because mice are nocturnal, the reverse may prove to be true in humans.

Because each tumor line produces an array of cytokines, we attempted to determine which of the many expressed cytokines was most critical for increasing. These studies were not intended to identify all of the tumor-produced cytokines responsible for hematopoietic changes. Rather, it was designed to demonstrate for the first time that cytokines synthesized by tumor cells could significantly impact the marrow environment and could be a source for differences in myelotoxicity in response to a given therapeutic. On the basis of the profiles of expression (Tables 1 and 2) and the induced proliferation (Fig. 1), we selected three stimulatory cytokines (GM-CSF, IL-6, and FLT3L) for neutralization studies. We found that GM-CSF, a later acting stimulatory factor, was not essential for either baseline proliferation or tumor-cytokine-induced stimulation of marrow. Blocking of IL-6 resulted in a small reduction in baseline

Fig. 3 Time of marrow harvest influences baseline marrow proliferation but not condition-induced proliferation. Marrow proliferation ([3H]thymidine incorporation) into cultured marrow cells (300,000/well) collected at 3, 12, and 18 HALO (9 a.m., 6 p.m., and 12 a.m.) in unconditioned medium or conditioned medium from TGP47, TGP48, TGP50, and TGP51 tumor lines. Results represent the mean of triplicate values; bars, ±SD.

Fig. 4 Anti-IL-6 neutralizes part of baseline marrow proliferation but not conditioned-medium induced proliferation. The effect of neutralization of IL-6 (3.6 μg/well) or GM-CSF (0.52 μg/ml), or antibodies to both cytokines on untreated- and TGP51 tumor-produced cytokine effects on marrow proliferation is depicted. Isolated murine femoral marrow (250,000 cells/well) were cultured for 3 days with medium or TGP1-conditioned medium with or without neutralizing antibodies. [3H]Thymidine (1 μCi) was added to each well on day 3 and incubated for 24 h. Cells were harvested and counted. The results represent the mean of quadruplicate wells; bars, ±SD.
proliferation but did not block proliferation in response to conditioned medium. FLT3-L, a relatively new stromal cell-produced positive stimulatory cytokine (47, 48), is thought to act at a very early stage before the separation of myeloid and megakaryocytic lineages. Addition of FLT3-L enhanced marrow proliferation induced by condition medium, whereas neutralization of FLT3-L reduced stimulation by conditioned medium. It is also possible that the balance of positive and negative regulators determines differences in induction of marrow activation. FLT3-L, a relatively new stromal cell-produced positive stimulatory cytokine (47, 48), is thought to be neutralized by an Ab. Negative regulation of myelopoiesis is also controlled through other inhibitory cytokines, most notably MIP-1α (12, 13) and TGF-β3 (14, 17). Future work will require that expression of these cytokines in tumor-conditioned medium is also quantitated.

We have not yet attempted to identify lineage-specific effects or specific changes within the hematopoietic hierarchy. Nor is it known whether the effect on marrow-proliferative activity by tumor-produced cytokines is direct, indirect, or both. Tumor-produced cytokines may bind directly to receptors found on marrow cells. Alternatively, a sizeable body of literature demonstrates that exogenous cytokines can affect expression of adhesion molecules on marrow stromal fibroblasts (50–52), on hematopoietic cells (53), and on progenitor cells (54–56). Exogenous cytokines can also influence secretion of cytokines by human marrow cells and expression of cytokine receptors by marrow cells (57–60). The specific mechanism(s) by which tumor-produced exogenous cytokines affect marrow proliferation remains to be determined. Clonal analysis of defined populations of primitive and committed progenitor cells is essential for future studies.

The increase in marrow proliferation exposed to conditioned medium translated to significant differences in chemosensitivity. A downshift in the IC₅₀ was observed for all three of the drugs studied with the greatest difference occurring when the most cytotoxic drug (CPT-11) was used. An important consideration when performing these studies is the age of the animal when the marrow is harvested. Two isolated studies were done with marrow from older mice (9–12 months), and the conditioned medium was not able to reduce the IC₅₀ values for either doxorubicin or cyclophosphamide (results not shown). Age-related changes in the proliferation of marrow, telomere length, and in cytokine secretion by marrow have been reported (61–63). However, when marrow was collected from young animals the trend was constant; a lower dose of the drug in more active marrow produced the same toxicity as a high dose of the drug in more quiescent marrow.

Although the production of cytokines by tumor cells has long been established, this is the first time that a connection has been made between the tumor biological phenomenon and the clinical concern of variation in myelosuppressive responses to chemotherapy. These studies have been generated from an in vitro model, yet the striking results provide strong support for the next phase of studies, i.e., translation of the in vitro findings to in vivo preclinical models. We have grown TGP51 tumors in mice and measured the presence of greater levels of several cytokines in the serum from these mice with only moderate tumor load compared with nontumor-bearing mice. We are now ready to address the biological significance that these circulating tumor-produced cytokines have on marrow biology. In addition to studies on magnitude and duration of myelosuppression and myelorecovery from cytotoxic therapy, we will

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also explore the impact of tumor-produced cytokines on stem cell transplantation technology; i.e., mobilization, engraftment, and differentiation of transplanted stem cells. These studies should help explain the differences in the grade of toxicity in response to cytotoxic therapy observed in patients receiving similar doses of myelosuppressive therapy. It may also eventually permit the sorting of patients into subpopulations that are likely to experience different myelosuppressive responses and myelorecovery profiles in response to therapy.

We would like to emphasize that this research fits well with decades of work demonstrating changes in hematopoiesis (e.g., abnormal total and differential blood cell counts and splenomegaly) as tumor burden increases. The contribution is this paper on the influence of tumor-elaborated cytokines on the marrow environment need to be evaluated in the context of other hematopoietic perturbations such as cytokine cascades initiated by the endogenous hematopoietic cells of the host reacting to the presence of a tumor, or metabolites produced by live or dying tumor cells.

In summary, we have used an in vitro system to demonstrate that tumor-produced cytokines can affect marrow proliferative activity and, thus, chemosensitivity to three distinct classes of chemotherapeutics. Certain cytokines are more important at affecting marrow activity and, indeed, the balance of positive and negative regulators are an important consideration.

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