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LONG TERM HEMATOPOEITIC DAMAGE AFTER CHEMOTHERAPY AND CYTOKINE

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1. ABSTRACT

Cancer chemotherapy causes severe damage to hematopoietic stem cells in both experimental animals and humans. While all levels of differentiation may be impacted, the most pivotal target of damage is the most primitive hematopoietic stem cell, PHSC. This cell not only suffers defective repopulating activity but also is quantitatively depleted. The causes of this damage are not clear. Severe possible explanations for this damage are discussed. They include: ineffective stromal support of stem cell function and reproduction; residual DNA damage preventing replication; accelerated cycling; and decreased responsiveness to normal physiologic growth stimuli. Efforts at chemoprotection, including manipulation of glutathione or aldehyde dehydrogenase levels, cytostatic peptides, immunomodulatory chemicals and cytokines are detailed. In particular, concern has been raised regarding potential deleterious consequences of combined chemotherapy-cytokine use, but substantiation of the cited data is warranted.

2. INTRODUCTION

Dose intensification of chemotherapeutic agents has been proposed as a way to overcome tumor cellular resistance to chemotherapy and to improve long-term survival. However, the short-term dose-limiting effect of chemotherapy is most often myelosuppression, and its consequences --- consequences readily dealt with by skilled and selective use of transfusional and antibiotic therapy. More difficult to predict and perhaps more difficult to treat are the long-term residual hematologic complications of chemotherapy. These include marrow hypoplasia or aplasia, which have as a clinical consequence life-threatening infection or prolonged transfusional dependency.

Upon searching for an animal model for chronic aplastic marrow failure or aplastic anemia, Morley and Blake discovered in 1974 that mice receiving extended use busulfan developed late marrow aplasia and failure, with 80% of the animals eventually dying from this complications' effects, despite earlier normal peripheral blood findings (1). That such a defect arose primarily from damage to hematopoietic stem cells was evident after normal, unmanipulated and transplanted marrow cells corrected the hematopoietic defect in busulfan treated mice (2). Several years later Botnick, Hannon, and Hellman observed hematopoietic failure after exposure of mice to alkylating agents, phenylalanine mustard, busulfan and 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU) (3, 4). They ascertained that this effect was a differential one, varying with the use of different drugs and possibly affecting differing stem cell compartments (5).

Clinical examples of drug-induced hematopoietic failure do exist but fortunately are rare. This failure to detect stem cell defects after chemotherapy use in human may result from several factors:

1. There are no reliable assays for measuring in humans the *in vivo* function of the most primitive hematopoietic stem cell (PHSC), --- certainly, no assay comparable to murine assays such as serial bone marrow transplantation, limiting dilution assay or competitive repopulation (6-8). The lack of such assays makes the detection of subtle or not so subtle changes in proliferative performance of these earliest stem cells difficult. Accordingly, the choice of assay used to study the problem of residual hematopoietic defect assumes a pivotal importance. Conflicting results can be seen when hematopoietic defects are defined by different *in vitro* assays.

2. While murine data have been found to have excellent correlation with human data (9), the disparate life-spans of these two animal species may mean that intervals between the treatment period or cessation of treatment, and eventual development of late-term hematopoietic defect may span decades in humans.

3. Lastly, such a defect is probably not always readily evident in humans unless periods of hematopoietic stress such as intervening myelosuppression, infection and drugs including additional chemotherapy intervene.

Still, examples of deleterious hematopoietic stem cell effect are striking when they do occur. Cumulative bone marrow toxicity was first observed in the 1970's. This toxicity was manifested as delayed and prolonged myelosuppression occurring 3 to 5 weeks after drug administration in 42% of patients who received multiple doses of lomustine (MeCCNU) (10). In a similar experience, Osband *et al* reported that 7 of 17 patients treated by them with MeCCNU eventually went on to have severe, protracted hematologic compromise which included aplasia (11). Since then, there have been other reports of long-term chemotherapy-related marrow damage after the use of other chemotherapeutic agents, whether given alone or in combination with others: adriamycin with cyclophosphamide (12); a combined regimen of cyclophosphamide, methotrexate and 5-fluorouracil (12), mitoxantrone (13), and other regimens (14-17).

Inferential or less direct evidence for chemotherapy-induced stem cell defect can also be seen through the experiences of autologous transplantation. Of 7 patients with myeloid leukemia who received re-infusions of marrow that had been purged *ex vivo* with the cyclophosphamide derivative, 4-hydroperoxycyclophosphamide, at doses of 120 micrograms/ml, 3 became persistently aplastic (18). This implies that stem cell damage was incurred after exposure to the drug but prior to infusion of marrow. Other investigators have also accrued data which allow them to reach the same conclusion: that prior and prolonged drug exposure, as well as the amount of high-dose exposure of stem cells to cytotoxic chemotherapy, is proportionately related to defects in marrow proliferative potential (19, 20).

3. THE TARGETED CELL

The mechanisms of chemotherapy-induced hematopoietic damage are unclear. The severest toxicities of many chemotherapeutic agents are especially seen after cell-cycle dependent drugs are used and take place in tissues or organs and portions of cells which are cycling. Such cells are most likely to have the highest cellular turnover, e.g. gastrointestinal mucosa or skin. One would then similarly presume that the primary target cells of cytotoxic agents would be committed, rapidly cycling progenitors within the myeloid and lymphoid compartments. Then, one would see the induction of proliferation and forced differentiation of more primitive or less committed cellular populations. So, one would not necessarily or naturally assume that the primary target for these cytotoxic agents would be the most primitive hematopoietic stem cells, PHSC.

An elusive cellular population, PHSC are usually quiescent and have been estimated to comprise no more than 0.001% to 0.05% of the total bone marrow (21). It is thought that as few as 10% of these cells, once isolated, could provide for life-long marrow reconstitution (22). Previously, the most widely used assays were *in vitro* colony assays which only had the capacity to assess function of committed progenitor populations (23) or the colony-forming spleen (CFU-S) assay (24), which measures the pluripotent myeloid precursor cells that are active in the first two weeks after transplantation. In fact, by using CFU-S concentrations many investigators have arrived at gross over-estimations of PHSC numbers, or actual predicted values (21, 25-27). However, with the advent of the more sensitive and accurate assessment of long-term functional capacities of PHSC offered by the competitive repopulation assay (8), it is possible to measure the relative marrow repopulating abilities of mixtures of cells from mice of differing genotypes and to detect even subtle differences in the ability of PHSC to reconstitute marrow over large fractions of a mouse's life-span.

That the selective target of cytotoxic agents is the PHSC has been amply demonstrated. Two studies, in particular, are cited that stress this. In the first study, Neben *et al* tested the drugs, cytosine arabinoside (ARA-C), cisplatin (cis-DDP), cyclophosphamide (CTX), BCNU and busulfan for their effect on PHSC. They made the following observations: marrow previously exposed to all the above drugs had a drug-specific reduction in competitive repopulating ability ranging from 4-100 fold and seen up to 10 months after exposure (28). Despite this decline in marrow self-renewal capacity, marrow CFU-S content was not significantly decreased and remained at/or near normal levels (28). Gardner *et al* also assessed permanent damage to PHSC after ARA-C, CTX, 5 fluorouracil (5FU), vincristine and actimomycin D, using the competitive repopulation assay (29). Effects on PHSC were compared to those on CFU-S and colony-forming-units granulocyte-macrophage (CFU-GM) and colony forming units-erythroid (CFU-E). Again, PHSC suffered irreparable, profound damage after all chemotherapy at the doses used, except ARA-C and 5FU, when the latter was given in a single dose of 150 mg/kg. The *in vitro* assays, CFU-GM or CFU-E and the *in vivo* assay CFU-S, failed to predict the degree of damage incurred; neither CFU-S or CFU-E concentrations were altered and only CTX effected a reduction in concentrations of CFU-GM.

The defect seen in PHSC proves to be not just a qualitative one. Experiments have been performed to determine whether the PHSC defect could also be quantitative. Marrow was given limited, or more extensive exposure to commonly used chemotherapeutic agents (30). The competitive re-population assay was combined with simple statistical analyses to assess PHSC numbers: equivalent precursor number = (Mean)(100-Mean)/covariance, where mean = average of 2 sample means, $P_L + P_E$, and covariance = $SD_L \times SD_E \times r$

(lymphocyte: erythrocyte Pearson correlation coefficient or $L:E_r$ [21]). Precursor concentrations were determined by dividing the equivalent precursor number by the number of cells injected. A decline in repopulating units (RU) of cells was noted. (RU is an artificial determination which includes all cells responsible for re-population regardless of stage of differentiation: RU = (%) (number of 10^5 competitor cells used)/(100 - %), where % = percentage donor cell type and 1 RU = repopulating ability of 10^5 untreated competitor marrow cells [21]). PHSC numbers were preferentially impacted by cytotoxic drugs such as CTX, or ARA-C at relatively high doses (30). Declines in PHSC concentrations and absolute numbers to <50% of control values were noted. Even when the repopulating ability of PHSC was not significantly altered, ---as in the case of vincristine or vinblastine---, drastic declines in PHSC concentrations or numbers per donor mouse could be observed. Such data support the hypothesis of the Hayflick theory that suggests that all stem cells, including PHSC, are present in finite numbers and have limited proliferative or self-renewal capacity (31). If circumstances, e.g. cytotoxic agent exposure, were to supervene, perhaps forcing stem cells through excessive cycling, then exhaustion of the stem cell pool would occur, leading to the clinical effects of marrow hypoplasia.

4. PATHOPHYSIOLOGY EXPLORED

4.1. Chemotherapy effect on stroma

The effects of chemotherapy on the hematopoietic microenvironment or stroma have not been as extensively studied as the effects on hematopoietic stem cells. It is well established that marrow fibrosis can be seen in individuals undergoing cancer therapy with drugs (32). Some of this change can perhaps be attributed to hematopathology resulting from the underlying disease (33), since myelofibrosis is sometimes a reactive phenomenon associated with drugs, malignancy, or other infiltrative processes. Histologic examination of marrow biopsies has revealed that distinctive morphologic changes occur in various cellular components of stroma after chemotherapy. Edema, sinusoidal dilation, and large unilocular fat cells that have developed from multilocular preadipocytes are observed after chemotherapy (34).

Since hematopoiesis requires some degree of cell-cell contact for its regulation or control to be effective, it is then possible that alterations of matrix and adhesion molecules after chemotherapy could make the microenvironment less receptive to the stem cells which would otherwise nest within this environment prior to proliferating. That such disruption is possible is indicated from data originating from several investigative groups. A reduced capacity for hematopoietic support has been reported after *in vitro* exposure of marrow stroma to VP16 (35) and busulfan (36). Gibson and colleagues detected reduced levels of vascular cell adhesion molecule-1 (VCAM-1) on marrow stromal cells after exposure to VP16.

In one study, pure mouse stromal cell cultures were treated with BCNU prior to seeding with hematopoietic stem cells (36). While the seeded cultures were indistinguishable from cultures established with untreated control cells, cells grafted onto the chemotherapy-treated stroma were abnormal, revealing swelling of the endoplasmic reticulum that could have been indicative of stromal injury. In another study, already-formed stromal monolayers were also treated with BCNU and later seeded with hematopoietic stem cells (37). Cultures were then examined for total adherent and non-adherent cell and granulocyte-macrophage colony-forming cell (GM-CFC) numbers. BCNU treatment resulted in significant reduction in non-adherent GM-CFC numbers. However, these adverse stromal effects occurring after chemotherapy exposure appeared to be reversible. If stem cell seeding of stromal layers was delayed after BCNU, no deleterious effects on GM-CFC numbers was observed.

In experiments utilizing human stem cells, marrow was removed from patients who had received extensive chemotherapy. Only a small percentage (13%) of marrow that was previously exposed to chemotherapy and placed in long-term culture to evaluate stromal quality and function proved capable of forming complete, confluent stromal layers (38). Yet failure to form complete stromal layers did not prevent hematopoietic support, since hematopoietic reconstitution still occurred uneventfully after transplantation. Clearly, the extent or even presence of stromal damage after chemotherapy and its significance need to be more fully assessed.

4.2. Ineffective DNA repair

Progression through the cell cycle, under normal circumstances, is tightly controlled. Any deviation from the usual sequence of events could lead to permanent cellular damage, even death, of the cell and any resulting progeny (40-43). If cell cycle length were drastically shortened, as might occur with chemotherapy-induced cycling, then it is possible that there would be insufficient time for repair of cellular or DNA damage caused by chemotherapeutic agents, especially those whose use leads to disruption of DNA metabolism.

Does evidence for residual DNA damage in the cellular genome long after the drug has been employed exist? The chemotherapeutic agents most extensively studied for their residual effects on DNA are the alkylating agents such as cisplatin or cyclophosphamide. After use of cisplatin or its analogue, carboplatin, DNA single-stranded breaks were noted after a one-hour exposure (44). Such breaks were repaired quickly and were eventually eliminated, but inter-strand crosslinks persisted at low levels for 8 or more hours. DNA-protein cross-linking was present 24 hours later and adduct formation, especially that of the Pt-GGadduct, persisted at 48 hours (44).

With cyclophosphamide, reversible and irreversible binding to cellular protein is observed within one hour of exposure to the drug, and can persist for several days after drug use (45). Lesions induced by alkylation of the N7 position of guanine persist up to 48 hours after exposure. (46).

Admittedly, most damage resulting from exposure to chemotherapy, UV- or gamma-irradiation (or other insult) is repaired. However, several studies have documented proof of late residual DNA damage. There is murine data indicating the persistence of DNA mutations at an increased frequency in bone marrow 10 days after treatment of lambda lacZ transgenic mice with high-dose procarbazine (47), a regimen designed to cause depletion of the repair enzyme, O^6-alkylguanine-DNA-alkyl-transferase (AGT). Although both liver and bone marrow had simultaneous accumulation of high levels of the DNA mutation, repair of liver DNA took place while residual DNA damage persisted in the marrow (47). DNA adduct dosimetry was also measured by Fong *et al* in rats and pigs after extended usage of procarbazine (48). They discovered that repair of a promutagenic lesion, O^6-methylguanine, was efficiently carried out in most tissues. However, still detectable levels of O^6-methylguanine were reported in pig leukocytes after 4 weeks of therapy, a finding which correlated with low levels of repair enzyme in not only leukocytes, but also lymph nodes and brains of treated animals.

Clinical investigations have included a study by Tice *et al* who, by using single cell gel electrophoresis, were able to identify significant, variable increases in DNA damage in peripheral blood lymphocytes from patients who had received cyclophosphamide and cisplatin in high doses for treatment of breast cancer (49). Increased levels of DNA damage were absent in most patients, indicating probable adequate repair in the majority of individuals, but cells having damaged DNA were still detectable in some patients long after treatment. Similar data were obtained by another group of investigators, who found that the elution rate of DNA from patients' mononuclear cells was increased after exposure to alkylating agents, proof that DNA strand breakage or other damage had occurred (50). Interestingly, in some individuals' cells, such an increase in elution rate was apparent even before current drug exposure had taken place, suggesting that cells which had been previously exposed to cytotoxic therapy had persistent although, otherwise, subtle DNA damage.

Sister chromatid exchanges (SCE), polycyclic aromatic hydrocarbon-DNA adducts and acrolein adducts have also been measured; these abnormalities were increased in lymphocytes of patients who had received cyclophosphamide, after successive courses of treatment (51,52). The frequency of SCE is correlative with the dose of drug used and can still be seen 21 days after treatment (52). Platinum-DNA adducts were present in autopsy tissue of patients receiving cisplatin up to 15 months after the drug was last administered (53). Also, fifteen cancer patient treated with combination chemotherapy, including ifosfamide and cyclophosphamide, were found to have an increased frequency of mutations in the HPRT gene, as well as increases in detection of micronuclei, for periods of up to 490 days after stoppage of chemotherapy (54). Ifosfamide and cyclophosphamide were most damaging to DNA, while equivocal findings were seen with regards to the ability of adriamycin and bleomycin to induce DNA damage.

Despite this latter data, anthracycline-associated free-radical formation results in DNA base modifications which, while usually repaired within 24 hours of drug exposure, can still persist, since repair of these modified bases is not universal, leaving persistent changes which have been held to be possibly premutagenic (55). Other drugs implicated as causes of permanent DNA damage include 5-azacytidine which causes a persistent hypomethylated state and diminishes replicative potential (56) and; epipodophyllotoxins which through illegitimate recombination cause permanent changes in the genetic makeup of cells that later are manifest as leukemo-or tumorigenic effect (57, 58). Other drugs have been reported to cause permanent DNA defects that have in turn led to chromosomal aberrations in bone marrow cells and spermatogonia, and may be the underlying cause of mitotic gene conversion, mutation and later carcinogenesis (59, 60). Therefore, long-lasting DNA damage as a potential cause for hematopoietic deficit, as well as carcinogenesis, is not specific to alkylating agents and needs to be studied after use of other drugs.

It is uncertain why such damage would persist. A distinct repair bias can exist for transcriptionally active genes, a preferentially transcribed strand, or certain genes which correlate with a specific stage of differentiation (61-63). An example of this bias is the preferential repair of DNA after induction of pyrimidine dimer or intra-strand adduct by cisplatin (62). For instance, cis-DDP lesions are repaired far less efficiently than trans-DDP lesions, while intra-strand cross-links d(GpG) are handled very poorly. Or, there may be a bias for repair of certain genes which correlate with a specific stage of differentiation (61). Thus, it is conceivable that not all cells meet cellular differentiative criteria or that not all DNA conforms to transcriptional requirements necessary for repair activity to be carried to completion, especially if such damage is extensive. Then, too, long-lasting damage to DNA may result from inactivation of DNA polymerase or damage to other parts of the repair system.

There is other evidence--- inferential and incomplete but present---- for the importance of DNA repair (or the lack thereof) as a causative factor in residual hematopoietic defect after alkylating agents. In humans, very low levels of DNA repair enzymes responsible for mismatched correction repair of the alkylation or methylation damage of guanine after exposure to alkylating agents, are present in hematopoietic cells (64). DNA containing the sequence for repair enzyme was transfected into bone marrow (and cell line) cells by Jelinek *et al* (65), and Maze and his colleagues (66). Elevated enzyme activity, expressed ≥4 months after transfection, conferred chemoprotection against the cytotoxic effects of the drugs on transfected cells and allowed recovery of colony-forming activity to near-control levels. The transfer of the repair gene into marrow stem cells led to modest resistance to BCNU's deleterious effects, as determined by lesser reductions in marrow and spleen cellularity, lower mortality rate and *in vitro* survival of high-proliferative-potential colony-forming cells (HPP-CFC) and colony-forming cells (CFC) (66). Unfortunately, as previously

stated, *in vitro* data have certain important deficiencies and long-term, *in vivo* data are needed. Transgenic mice bearing this enzyme's gene exist and express high levels of enzyme but have, to our knowledge, been examined mainly for their susceptibility to tumorigenesis (67, 68).

4.3. Decreased responsiveness to normal physiologic growth stimuli

High levels of cytokines are detectable in the serum of patients after chemotherapy (69). Baiocchi and others have reported that patients' own bone marrow responds poorly to exogenous cytokine or to colony-stimulating activity in serum if exposed previously to chemotherapy, producing fewer CFU-GM than would ordinarily be expected (69). Such experience either implies there are fewer stem cells that are readily available to respond to growth factors, or there is a type of "end-organ failure" in which changes in stem cell behavioral characteristics have occurred which make the cell more resistant or less responsive to normal levels of cytokine or growth factor. Such changes in stem cells, whether PHSC or other early progenitor, could include declines in cytokine gene expression, cytokine receptor levels, changes in receptor binding affinity or in transcription factor or oncogene induction, all of which could affect individual cellular growth and proliferation.

4.4. Accelerated cycling at the expense of self-renewal

One alternative hypothesis that could account for the stem cell depletion observed after chemotherapy can be posed: temporary exhaustion of neutrophils and of late myeloid progenitor cell pools may occur after chemotherapy. PHSC would then be forced to terminally differentiate at the expense of maintaining a pool of quiescent pluripotential progenitors necessary for self-regeneration and later re-population of those same differentiated precursor stores. The PHSC compartment would then suffer excessive stress. If this hypothesis were true, then agents capable of preventing cellular turnover or cycling of relatively committed progenitors (and indirectly of PHSC) should allow PHSC to be spared.

Several groups of investigators have identified naturally occurring peptides and other substances which act as negative regulators of hematopoietic stem cells at various stages of differentiation (70). These regulators include the hematoregulatory peptide, pEEDCK (Glu-Glu-Asp-Cys-Lys) (71, 72) and the tetrapeptide, acetyl-N-Ser-Asp-Lys-Pro (AcSDKP). PEEDCK has the capability of inhibiting myelopoiesis and bears a striking similarity to a 5-amino acid sequence in the effector domain of the alpha-subunit of inhibitory G-proteins (73). While the dimerized peptide indirectly stimulates hematopoiesis *in vitro* (74), Paukovits and others have used the monomeric peptide to protect hematopoietic progenitors from damaging chemotherapeutic effect (75). Noting that the peptide appeared to be cytostatic towards CFU-S, perhaps having a physiologic role in the maintenance of low levels of CFU-S proliferation and the ability of pEEDCK to prevent ARA-C-induced cycling of CFU-S, he and co-investigators administered peptide to mice which then were given ARA-C and nitrogen-mustard (75). Mice receiving both peptide and cytotoxic agent endured far less severe neutropenia and had preservation or enhancement of progenitor cell activity at the CFU-S and CFU-GM differentiative level.

AcSDKP inhibits the entry of CFU-S into the S phase of the cell cycle, and directly prevents colony-formation of purified human $CD34^{+}HLA\text{-}DR^{high}$ and $CD34^{++}HLA\text{-}DR^{low}$ cells (76). Investigators used the peptide to protect normal hematopoietic stem cells against the deleterious effects of cytotoxic therapy. AcSDKP was observed to protect CFU-S after treatment with ARA-C (77). The fact that the peptide can act selectively on normal marrow progenitors but has no effect on leukemic cells appears to be an added advantage to its use (78).

Similar presumptive chemoprotective properties have been noted with other stem-cell negative regulatory substances. The cytokine, murine macrophage inhibitory protein-1alpha (MIP1alpha) reversibly inhibits proliferation of CFU-S and other hematopoietic progenitors (79). The ability of the human homologue of this protein, rhMIP1alpha to confer protection on hematopoietic progenitors was tested by Dunlop and others (85). They found that MIP1alpha acted preferentially on less mature CFU-S, inducing quiescence, or suppression of proliferation. After ARA-C administration, multiple injections of rhMIP1α provided significant protection to CFU-S, resulting in enhanced recovery of peripheral blood neutrophil counts and improved CFU numbers, as well.

Data from Kriegler *et al* has purported that dexamethasone, brought about protection of hematopoietic progenitors, as determined by *in vitro* assays (81). Such protection was afforded only after repeated doses of 5-fluorouracil but not after cyclophosphamide and seemed to be preferentially offered to the earliest hematopoietic progenitors studied (HPP-CFC-1>HPP-CFC-2>GM-CFC) in a dose-and schedule-dependent fashion (81). By effecting cell-cycle arrest, dexamethasone appeared to prevent cell cycling and damage at a time when chemotherapeutic agents were present at their most toxic levels.

Unfortunately, few studies have been performed on the progenitor subset of greatest interest (and importance). It is not known whether such protection offered by these substances can be extended to PHSC . To show such protection would be the real test of the validity of the hypothesis of “cycling vs. preservation”. Preliminary data from our laboratory has suggested that CFU-S only are preserved through use of dexamethasome before 5-fluorouracil. Unfortunately, no similar protection for PHSC has been evident (unpublished). Further studies are underway to conclusively prove effect or lack thereof.

5. CHEMOPROTECTION

From the first realization of the severity and potential impact of the latent, adverse hematologic consequences of chemotherapy, the search was on for a means of protecting PHSC from these cytotoxic effects. Human hematopoietic cells bear high levels of the enzyme, aldehyde dehydrogenase (82). This enzyme is responsible

for inactivation of cyclophosphamide and is implicated in the conferral of resistance against this drug in murine tumors and normal cells (83-86). Cell survival of marrow cells appears to be directly related to intracellular aldehyde dehydrogenase levels in human leukemia cell lines (85), but no attempts to verify benefits of elevated enzymatic levels in mouse or man have been made, to our knowledge.

Alternatively, glutathione (GSH) levels have been directly correlative with the ability of cells to detoxify a number of different drugs. For example, GSH, or GSH peroxidase or catalase may detoxify agents like anthracyclines, such as adriamycin which act through the production of free radicals or activated oxygen species (87-90). GSH is also involved in the detoxification of melphalan, an alkylating agent, perhaps through direct interaction with melphalan, with subsequent production of drug-thiol adducts, although this mechanism remains speculative (94). Again, *in vivo* evidence of the effectiveness of GSH manipulation in protecting PHSC from chemotherapy-induced toxicity does not exist.

The putative protective effect of inhibitory or negative regulatory peptides and cytokines, such as MIP1alpha has been discussed above. However, other investigators have turned their attention to pharmaceutical alternatives. One of these is amifostine, a thiol-containing compound, first noted to protect normal tissues from irradiation-related damage (91). Its mechanism of action is like that previously discussed for GSH. It acts as a scavenger of oxygen-free radicals and directly binds to and detoxifies alkylating agents, preventing in the case of cis-DDP, the formation of DNA adducts---fortunately, an effect which seems to target preferentially normal cells (92).

Even norepinephrine has been examined for its ability to protect hematopoiesis from chemotherapy effect. Maestroni and co-investigators, in noting that marrow cells have alpha-1-adrenoreceptors on their surfaces, gave norepinephrine to mice that received carboplatin (93). Protection was evident at the level of CFU-GM; survival of mice receiving norepinephrine and carboplatin was superior to survival of mice receiving the cytotoxic agent alone (77% vs. 30%, respectively). Administration of the alpha-1-adrenoreceptor antagonist, prazosin, counteracted the chemoprotection observed with norepinephrine.

Kalechman *et al* described protection of both PHSC and stroma with the use of an immunomodulatory compound, ammonium trichloro(dioxyethylene-O-O')tellurate (AS101) (94). The protection seen with AS101 presumably is mediated by endogenously secreted cytokines, e.g. interleukin-1 (IL-1) or -6 (IL-6), tumor necrosis factor (TNF) or stem cell factor (SCF) (95). Use of neutralizing antibodies against these cytokines resulted in abolition of the chemoprotective properties of AS101.

A number of cytokines, including IL-1, SCF, IL-6, MIP1-alpha, TNF-alpha, interferon-gamma (IFN-gamma), interleukin-11 (IL-11), have been reported to protect hematopoietic progenitors against the myelotoxic effects of chemotherapy (96-110). One possible explanation of any putative chemoprotection observed with the use of cytokines together with chemotherapy could be suppression of chemotherapy-associated apoptosis in hematopoietic stem cells (107-109). Alternatively, cytokines which act as negative regulatory influences, e.g. TNF-alpha or IFN-gamma could prevent cycling of cells prior to their exposure to chemotherapeutic drugs, thus preventing damage. DeHaan *et al* have proposed yet another possible explanation (110). They hypothesized that through the induction of active cell cycling, with ensuing increases in both primitive and committed hematopoietic precursors, the normal physiologic feedback loop regulating hematopoiesis could be taken advantage of. Eventually, cycling activity would cease after chemotherapy use, especially for drugs which were cell-cycle specific, in this instance 5-fluorouracil. This prevention of cycling would then act as a preventive for hematopoietic damage (110).

Most evidence for chemoprotection by cytokines stems from an observation of improved survival rates, superior LD_{50} and LD_{90}, accelerated marrow and peripheral blood neutrophil recovery and reduced episodes of acute toxicity. The experience with IL-1 attests to this; few murine tests have attempted to look at repopulating activity after IL-1 or other cytokines, in relation to cytotoxic drug use. Exceptions to this general rule do exist, however. For instance, Gardner investigated the ability of IFN-gamma, given as a single dose, to protect PHSC using the competitive repopulating assay (106). Addition of IFN-gamma to the treatment with cyclophosphamide improved repopulating ability of PHSC, with repopulating ability of treated marrow returning to levels comparable to control marrow.

Hornung and Longo tested various combinations of cyclophosphamide, IL-1 and granulocyte-macrophage colony-stimulating factor (GM-CSF), using serial transplantation to examine marrow repopulating ability (120). Over a 12 week period, biweekly injections of cyclophosphamide with or without cytokines were administered to mice. Bone marrow cells of mice so treated were subjected to 3 serial transfers. After the third transfer, survival of mice was significantly lower in mice treated with CTX alone, as were bone marrow cell numbers and CFU-C content. Alarmingly, these parameters declined even more dramatically with the addition of GM- or G-CSF to the treatment regimen. If IL-1 were administered prior to drug and GM-(or granulocyte (-G)) CSF, restoration of function was noted. Incidentally, IL-1 pre-administration by itself appeared to offer little protection and may even have proven deleterious. Confirmation of these results is, of course, greatly needed. Cytokines are increasingly being used in clinical oncologic settings in an attempt to ameliorate the myelosuppressive effects of chemotherapy. Because of the constraints of methodology, clinical correlation has been lacking. Schwartz and others attempted to assess the clinical efficacy of combined chemotherapy/cytokine usage (5-fluorouracil, leucovorin, doxorubicin and CTX [FLAC] and PIXY321, a synthetic cytokine resulting from the fusion of

the GM-CSF and interleukin-3 (IL-3) genes). Their assessment, limited by the use of *in vitro* assays and thus restricted to measurement of only the more committed progenitors, was that cytokine did not relieve the suppression or toxicity of chemotherapy for either $CD34^+$ cells or committed progenitors (112).

Experimental variance may lead to differences in cytokine effect, i.e. if there is chemoprotection or not, since the timing of administration may give starkly contrasting results. As an example, SCF while reported as chemoprotective has been observed to be extremely toxic to PHSC, if administered prior to chemotherapy (113), while IL-1 may fail to protect PHSC adequately unless given at least 20-24 hours before cytotoxic drug administration (103).

If cytokines are found to have deleterious effects on PHSC, this conclusion would have significant implications for the clinician. As stated, no significant clinical damage is apparent, as of this writing, but the usage of cytokines is still relatively recent and unfortunately without controlled trial or full knowledge of the hematologic sequelae (124). Further investigation is necessary to elucidate their role and ultimate effect.

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