Variations in Radiation Sensitivity and Repair Among Different Hematopoietic Stem Cell Subsets Following Fractionated Irradiation

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The radiation dose-survival of various hematopoietic cell subsets in murine bone marrow (BM) was determined in the cobblestone area forming cell (CAFC) assay under conditions of single-, split-, and multiple-dose irradiation. A greater recovery in cell survival with decreasing dose per fraction, or increasing fraction number, was observed for primitive CAFC day-28 and day-35 than for CAFC day-6 and day-12 (colony-forming unit (CFU)-granulocyte macrophage and CFU-spleen day-12 equivalents). Linear quadratic (LQ) model analysis of CAFC survival data provided an estimate of the α/β ratio that is an inverse index of the fractionation effect and is known to be lower for late than for acutely responding tissues. This analysis gave decreasing α/β ratios with increasing primitiveness of the CAFC subset. These values were found to be comparatively low (about 4 Gy) for CAFC day-28 and day-35 and are in agreement with previous studies on long-term repopulation in vivo. In contrast, α/β ratios of CAFC day-6 and day-12 were relatively high (above 6 Gy) and are consistent with values obtained from acute marrow failure. Delayed harvesting of BM after a single dose of 6 Gy showed little evidence of proliferative repopulation over 1 week and hence the differential dose-sparing effect of fractionation among the CAFC subsets appears to be mostly attributable to the influence of sublethal damage repair. These results require a reevaluation of previous notions of marrow stem cell radiosensitivity and repair based on acute marrow lethality (LD50,30) or spleen colony (CFU-S) data, especially when applied to fractionated total body irradiation effects on long-term repopulating stem cells in a BM transplant setting.

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MANY MURINE STUDIES, dealing either with manipulations of the donor bone marrow cell (BMC) inoculum or with cytotoxic effects of different host conditioning agents, have shown how the level and timing of blood reconstitution after BM transplantation (BMT) is determined by functionally distinct stem cell subsets within a hematopoietic hierarchy. In this respect the evaluation of blood chimerism using the Gpi-1 congenic marker has provided valuable information on long-term repopulation in vivo arising from the primitive stem cell pool of high self-renewal. Variations in both the host total body irradiation (TBI) and the donor BMC dose has enabled the construction of dose-response curves at 5 months post-BMT corresponding to stable engraftment of stem cells with long-term repopulating ability (LTRA). These dose-response curves provided evidence for a greater radioresistance and fractionation dose-sparing of LTRA cells than their colony-forming unit–spleen (CFU-S)–like descendants.

There is a wealth of data to show that tissues which manifest an early response to radiation damage may be distinguished from those that respond late. This distinction is based on the ratio α/β of the linear quadratic (LQ) radiation response model: α/β is smaller for late-responding than for acutely responding tissues. Therefore, it is interesting that the LQ model analysis of the fractionation sensitivity of long-term BM engraftment has given a low α/β ratio (≤2 Gy) in keeping with that of a late-responding tissue. In an attempt to explain postirradiation reconstitution on the basis of the survival of different stem cell subsets after irradiation in vivo, we have used the cobblestone area forming cell (CAFC) assay. This assay allows frequency analysis of a series of stem cell subsets on the basis of differences in their stromal-dependent repopulating ability in vitro. The correspondence between the CAFC day-types and other assays has been confirmed in terms of variations in radiation sensitivity (D0) and in split-dose recovery (D0-D1). The aim of the present study is to investigate CAFC survival following more comprehensive fractionation schemes and to obtain certain radiation repair parameters (eg, α/β ratios) based on the LQ model for comparison with LTRA in vivo as reported recently by van Os et al.

MATERIALS AND METHODS

Animals and Irradiations

Male C57BL/6Jico (B6) mice (IffaCredo, L'Abeeles, France) were used at 14 to 15 weeks of age. TBI was delivered to groups of 3 to 4 mice using a 15Co-gamma irradiation unit (Siemens Gammatron 3) at a dose-rate of 35.0 to 36.6 Gy/min using fractionation schemes with intervals of 6 hours (split dose and 1.2 Gy per fraction) or alternate 6- and 18-hour intervals (2 Gy per fraction) as described in our recent study on BMT recipients.

Determination of Hematopoietic Subset Frequencies In Vitro (CAFC assay)

At 20 hours after the end of radiation treatment (fractionation experiment) or 20 hours, 4 days, 7 days, and 28 days after 6 Gy (delayed harvesting experiment) femoral BMCs were procured, pooled from groups of 3 to 4 mice, and plated at limiting dilutions (12 dilutions with 15 wells per dilution step) on pre-established and irradiated (20 Gy) BM stromal cultures as previously described. These cultures provided growth of hematopoietic precursors under the stromal layer giving the appearance of cobblestones on phase contrast microscopy. The CAFC content per femur was then determined for each radiation dose group at the indicated times after overlay. This in vitro system provided an estimate of the surviving...
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Fig 1. Relationship between the femoral content of different CAFC subsets measured at different times in long-term BM cultures and other assays of hematopoietic progenitor and stem cell function. A number of studies involving physical separation of donor BM cells or recipient pre-treatment with various cytotoxic agents have shown how CAFCs appearing at around days 6, 12, and beyond 28 days correlate with CFU-GM, day-12 CFU-S, and pre-CFU-S (narrow repopulating ability [MRA] and LTRA, respectively. The vertical arrows indicate the times of CAFC assay used in the present study.

Fractionation of a spectrum of CAFC day-types that correspond to CFU-granulocyte macrophage (CFU-GM) (CAFC day 6), CFU-S day 12 (CAFC day 10-12), and the primitive stem cells with long-term repopulating ability (CAFC day 28-35) as illustrated in Fig 1.

Analysis of CAFC Radiation Dose Survival and Estimation of LQ Parameters

CAFC survival per femur was determined in a limiting dilution analysis, whereby the numbers of clonogens of various types were tracked roughly proportionately with proportions of femur placed into wells, such that absence or presence of a colony in a well was a binary endpoint and likelihood analysis was appropriate. In the analyses (estimation of dose-response parameters) the distribution of CAFC clones followed single-hit kinetics.

Estimation of cell survival. Let \( f \) = number of femurs/well, \( k \) = number of clonogens/femur, and \( pe(d) \) = plating efficiency at dose \( d \). Then the average number of survivors/well = \( f k p e(d) \), and the probability \( p \) of a negative well is correspondingly given by \( p = \exp[-f k p e(d)] \) with the usual randomness assumptions. For convenience in the likelihood estimations we use the equivalent expression

\[
p = \exp[-\ln(f) + \ln(k) + \ln(pe(d))]
\]

for \( i = 1, 2 \), with \( \beta_1 = \ln(k) + \ln(pe(0)) \) and \( \beta_2 = \ln(k) + \ln(pe(d)) \). Maximum likelihood estimates of the parameters \( \beta_i \) are then obtained using the complementary log-log link function as shown above. Calculations are performed pairwise, with data for each nonzero dose coupled with controls. For each dose \( d \) log survival is given by \( \ln S = \ln(pe(d)/pe(0)) = \ln(k) + \ln(pe(d)/pe(0)) = \beta_1 - \beta_2 + \ln pe(0) + \beta_2 \). Confidence limits are estimated by error propagation from \( \text{var} \ln S = \text{var} \beta_1 + \text{var} \beta_2 - 2 \text{cov} \beta_1 \beta_2 \). The covariance was <10^-10 in these analyses.

Dose-response fractionation parameters. The effects of fractionation on recovery of CAFC survival were interpreted in terms of the LQ model given by

\[
\ln S = -(\alpha D + \beta D^2)
\]

where it may be assumed that irreparable and repairable components of radiation damage are represented by the \( \alpha \) and \( \beta \) terms, respectively. The \( \alpha/\beta \) ratio has the dimensions of dose and indicates the dose at which cell kill from the linear low-dose term is equalled by cell kill from the dose-squared term. Tissues of low \( \alpha/\beta \) ratios have a large capacity for dose-sparing from fractionated or low dose-rate irradiations.

As it is shown that CAFC plating efficiency is proportional to clonogenic survival, then \( pe(d) = \text{const.} S, \ln(k,pe(d)) = \ln(k,\text{const.} S) = \ln(k,\text{constant}) + \ln S \). Therefore, the likelihood model is given (according to the complete-repair LQ model) by

\[
p = \exp[-\ln(\beta_1 + \ln f - \beta_2 D - \beta_3 D^2)]
\]

with \( D = \text{total dose} \) and \( n = \text{number of fractions} \), and the parameters of the LQ model identified by \( \alpha = \beta_1 \) and \( \beta = \beta_2 \). If it can be assumed that the proportionality constant \( \sim 1 \), then \( \beta_1 = \ln \text{clonogen number} \). The number of clonogens tracked proportionately with the proportion of femur placed into a well as tested by replacing \( \ln f \) by \( \beta_1 \ln f \), and seeing whether 95% confidence limits for \( \beta_1 \) include the value 1. Differences between treatment groups were considered to be significant when the 95% confidence limits were not overlapping.

RESULTS

Effect of Fractionated Irradiation

Radiation cell survival curves were made for all CAFC day-types and fractionation schedules and fitted using the likelihood model described above. Survival curves for single dose, split dose, 2 Gy per fraction, and 1.2 Gy per fraction for four CAFC subsets measured at 6, 12, 20, and 35 days in culture are presented in Fig 2. Although the CAFC day-6 type appeared to be the most resistant to single radiation doses, it showed a relatively small increase in slope upon dose fractionation. The CAFC subset appearing later at day-12 was more radiosensitive and showed a very small effect of fractionation. The increasing primitiveness of the CAFC subsets from day 12 to day 35 was clearly accompanied by an increasing dose-sparing effect of fractionated irradiation.

Application of direct analysis according to the LQ model of cell survival yielded repair parameters that varied among the different CAFC day-types. Figure 3 displays the \( \alpha, \beta \), and \( \alpha/\beta \) estimates for comparison with those obtained from LTRA in vivo. This shows a dramatic decrease in the \( \alpha/\beta \) parameter with increasing CAFC primitiveness, i.e., ranging from values of more than 6 Gy for CAFC day-6 and day-12 to less than 6 Gy for CAFC day-28 and day-35 (Fig 3A). The low \( \alpha/\beta \) value for late-developing CAFCs was accompanied by changes in both the \( \alpha \) and \( \beta \) constants (Fig 3, B and C). Overlapping 95% confidence limits indicate that these estimates for the 35 day-type were not statistically different from the \( \alpha/\beta \), \( \alpha \), and \( \beta \) values for LTRA as measured from direct analysis of donor BM engraftment data.

Effect of Delayed Marrow Harvesting After 6 Gy

Because the multiple fractionated irradiation courses necessitated overall treatment times of up to 3.25 days, it was considered appropriate to investigate any possible proliferative recovery of CAFC survival by extending the time of marrow harvest beyond 20 hours to 4, 7, and 28 days after
DISCUSSION

Our determination of BM CAFC survival after multiple fractionated irradiation schemes has enabled us to resolve the behavior of different hematopoietic subsets in terms of their radiation sensitivity and repair. This approach also allowed certain repair parameters in the LQ-model of cell survival (e.g., $\alpha/\beta$ values) to be derived for comparison with other end-points or tissues. The results obtained from extending the time to marrow harvesting imply a negligible effect of CAFC repopulation during the period of fractionated irradiation (<1 week) and hence the recovery of cell survival appears to be confined to the process of sub-lethal damage repair between fractions. A similar conclusion was reached from the relatively constant levels of donor stem cell engraftment after recipient irradiation with extended interfraction intervals or after delayed BMT.\textsuperscript{12}

The early arising CAFC day-6 subset, corresponding to the CFU-GM population, were the most resistant to single radiation doses, confirming previous cell survival studies on different hematopoietic subpopulations.\textsuperscript{13,14} Despite its high radiosensitivity, CAFC day 6 exhibited a comparatively low capacity to recover during fractionated irradiations. The lower radioresistance and poor repair capacity of the CAFC day-12 subset is generally consistent with results obtained from numerous fractionation and low dose-rate irradiation studies on CFU-S survival.\textsuperscript{15,16,21} Reevaluation of fractionation data for CFU-S survival using the LQ-model indicates a high $\alpha/\beta$ ratio of about 20 Gy.\textsuperscript{22} A relatively high $\alpha/\beta$ value of around 8 Gy is found from analysis of acute marrow lethality data (LD<sub>50,30</sub>) where overall treatment times were kept within 1 week.\textsuperscript{23-26} These values are comparable with the $\alpha/\beta$ ratios determined from CAFC day-6 to day-20 cell survival (see Fig 3).

The most remarkable finding in the present study was the large dose-sparing effects of fractionation on late-appearing CAFC subsets (day-28 and day-35) corresponding to the ancestral pre-CFU-S population. This effect appears to follow a trend according to stages of cell development along the hematopoietic hierarchy but it is not directly related to their differences in sensitivity toward single-dose irradiation. The increasing sparing effect of dose-fractionation with CAFC primitiveness was clearly illustrated by a dramatic
Fig 3. Similarity of radiation dose-response parameters for LTRA in vivo to late-developing but not early developing CAFC subsets. The $\frac{\alpha}{\beta}$ (A), $\alpha$ (B), and $\beta$ (C) estimates with 95% confidence intervals are plotted as a function of CAFC day-type. The shaded area represents the 95% confidence limits for these parameters of LTRA in vivo obtained from reanalysis of the long-term donor marrow engraftment data from van Os et al using equivalent regimens of fractionated irradiation and the direct analysis method.

Fig 4. Surviving fraction of CAFC day-types harvested from femoral BM at 20 hours (●), 4 days (◆), 7 days (▲), and 28 days (▼) after a single radiation dose of 6 Gy. The error bars represent the 95% confidence limits.
decrease in the α/β ratio reaching a level that lies close to that previously obtained from the LTRA end-point in a BM chimera model (see Fig 3A). The similarity between the cell survival curves of the most primitive CAFC subset and the dose-response curves corresponding to recipient stem cells with LTRA is illustrated by the comparison given in Fig 5. Therefore, it is postulated that the CAFC day-28 to day-35 subtype represents the host target cell population responsible for in vivo long-term donor-type engraftment after BMT whereas earlier developing CAFCs are probably better related to hematopoietic repopulation in the short-term. Such relationships agree with recent CAFC and chimera studies on the differential toxicity of chemotherapeutic compounds. The selective depletion of CAFC day-6 to day-20 type populations after 5-fluorouracil (5-FU) treatment is of particular interest in view of the well-known toxic effects of this agent against cycling cells. In this case the high cycling activity of committed progenitors appears to be associated with the higher α/β ratios as previously hypothesized for various cell-renewal systems.

The general classification of different tissue systems in terms of their proliferative organization and α/β ratio needs reappraisal in the light of the present results on distinct stem cell subsets in BM. This tissue has long been placed in a category associated with a typical acute radiation response and high α/β. However, the existence of hematopoietic subpopulations with a wide range of α/β estimates may necessitate a reclassification of the hematopoietic system into a mixture of cell types that have different radiobiologic properties. In this case, cycling CFU-S-like cells produce a characteristic early response of acute marrow failure whereas the more primitive quiescent LTRA stem cell may be better regarded as a discrete slow renewing sub-compartment. The noncycling status of LTRA cells under steady-state conditions probably allows for more efficient repair and confers the low α/β ratio that is commonly associated with late radiation responses. Therefore, the maturation of these cells into CFU-S-like descendents represents a critical transition point separating two important normal tissue reactions but within one hierarchical cell system. The advent of recent stem cell isolation techniques now opens up the opportunity for further molecular-orientated investigations aimed at resolving the mechanisms of radiation repair. Furthermore, the recent development of an analogous CAFC assay system for human BM may prove valuable in extending these radiobiologic studies to BMT patients for closer comparisons with clinical responses.

The large capacity of late-appearing CAFC subsets (equivalent to LTRA stem cells) to repair radiation damage can pose a problem for attempts to improve TBI conditioning in the clinic. When radiation dose fractionation is applied in an effort to reduce nonhematologic toxicity, especially in the lungs, the improved survival of primitive host stem cells may reduce the conditioning effect and thereby reduce the extent of hematopoietic engraftment on behalf of composite stem cells from the donor. In addition, if the heterogeneity of the stem cell compartment also applies to leukemic cell populations in a single patient, differences in radiation damage repair during fractionated TBI therapy may as well exist and lead to a discrepancy between actual relapse rates and predictions based on measurable leukemic blast cell survival. Finally, the concepts developed here raise the question as to whether other normal or malignant tissues contain elusive resting stem cell populations with similar radiobiologic properties to the hematopoietic LTRA stem cell.

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