

A Hematopoietic Stromal Lesion Associated with Fractionated Radiotherapy (FxRT): Time- and Dose-effects

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Abstract. *Earlier, we reported that the local exposure of femoral bone marrow to a clinically-relevant course of fractionated radiotherapy [FxRT; 2.0 Gy (q24h x 5)→74 Gy] resulted in the delayed appearance of a stromal lesion which temporally appeared after exposures to a threshold dose of 20 Gy FxRT. To further define this threshold dose, the temporal recovery of blood-forming elements ("cobblestone area" forming colonies; CAFC_{7-28d}) and the microenvironmental stroma (long-term bone marrow cultures; LTBM) of the marrow was examined over a 17-week period following 10 and 30 Gy FxRT. After a subthreshold dose of 10 Gy, each of the 4 CAFC subpopulations were significantly dampened, with recovery initiated within a 6-week interval of 10 Gy of FxRT. Above the threshold dose (30 Gy FxRT), the CAFC subpopulations were again reduced to a level similar to that observed with 10 Gy FxRT. However, after exposures to 30 Gy FxRT, CAFC recovery was significantly well beyond the 6-week interval observed with a 10 Gy Dose of FxRT. Similarly, cell production in LTBM prepared from marrow exposed to either 10 or 30 Gy FxRT was significantly dampened for at least 3 weeks following the FxRT. Moreover, while cell production in LTBM derived from marrow exposed to 10 Gy was eventually restored to normal, the dampened cell production observed in LTBM prepared after 30 Gy FxRT persisted for a period in excess of 17 weeks. Collectively, these observations provide additional support to our earlier observation suggesting that FxRT generates two forms of dose-dependent damage in the marrow: the first an early lesion*

arising in the blood-forming CAFC subpopulations; the second form, a delayed lesion that involves the persistent expression of a dysfunctional microenvironmental phenotype, appearing to disrupt the normal regulation of hematopoietic stem cell (HSC) proliferation and differentiation of the HSC during the recovery process.

While hematopoiesis occurs at a multiplicity of sites in the mammalian body, on an individual basis, many of these sites reside outside the radiation field during conventional radiotherapy (xRT). Nonetheless, the bone marrow is still viewed as a radiation dose-limiting tissue and, as a result, continues to be the subject of considerable interest and investigation. Collectively, studies have given rise to the generally-accepted premise that the hematopoietic response to xRT represents the net result of interactions occurring between the blood-forming (stem/progenitor) cell and the hematopoietic inductive microenvironmental (stromal) compartments of the marrow (1, 2). Unlike its stromal counterpart, the blood-forming compartment is seen as an overlapping age-generation hierarchy (3), where cells, arranged from the most primitive to the most mature hematopoietic stem cell (HSC) and progenitor cell subpopulations, demonstrate a progressive decrease in radiosensitivity with increasing HSC maturation (4, 5). Since it was originally proposed, additional support for this hierarchy has been provided by the identification of a corresponding set of temporally-linked HSC that arise as stromal-cell-dependent "cobblestone area" forming cell (CAFC) colonies (6-8). In conjunction with a syngeneic chimeric mouse engraftment model, the CAFC assay, which has also been used to define HSC radiosensitivity, has demonstrated α/β parameters for early and late radiation responses of short-term repopulating (STR) and long-term repopulating (LTR) HSC (5).

In addition to these blood-forming cells, the hematopoietic inductive microenvironment (HIM) or "stroma" also contributes, albeit more indirectly, to the hematopoietic response to xRT. Originally recognized by

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Table I. Characteristics of the stem cell and stromal cell subpopulations of the bone marrow in animals receiving FxRT.

FxRT	CAFC per femur** (% of age-matched control)				LTBMC cell production*** (x 10 ⁶)
	7d	14d	21d	28d	
Control*	100.00	100.00	100.00	100.00	19.87±6.71
10 Gy	0.89±1.01	1.23±0.208	3.87±3.56	29.36±8.32	18.79±2.51
30 Gy	1.38±0.23	6.39±2.41	7.32±3.08	14.05±4.33	5.12±1.08
50 Gy	2.09±0.01	5.05±0.01	9.02±2.12	15.22±2.98	1.27±0.99
74 Gy	2.56±0.98	5.67±1.52	9.87±3.02	19.06±4.98	1.30±0.79
121d post 74 Gy	4.39±0.16	11.33±3.07	37.97±5.33	51.22±8.96	2.06±1.05
231d post 74 Gy	15.32±3.87	22.09±8.09	79.59±11.59	91.35±6.31	1.51±1.01

* - Age matched control mice receiving sham irradiation.

** - Data from 5-6 individual mice 24 h post FxRT and expressed as the mean% control (± SEM).

***-Accumulated cell production over a 9-week period in 5-6 LTBMC per animal.

the radiosensitivity of a stromal-fibroblast progenitor (the CFU_f), and characterized by a broad shoulder and elevated D₀ values (9, 10), this CFU_f, not unlike the LTR HSC, demonstrates an appreciable dose-rate sparing effect in response to both *in vivo* and *in vitro* abbreviated courses of fractionated xRT (FxRT; 10, 11). More recently, HIM radiosensitivity also has been assessed using the *in vitro* long-term bone marrow culture (LTBMC) where, following irradiation *in situ*, the maintenance of hematopoietic cell production within the LTBMC for extended intervals is significantly compromised (12-14). Furthermore, the adherent cellular layer of the LTBMC is currently viewed as being responsible for the expression of regulatory hematopoietic factors and adhesion factors, which are believed to represent hematopoietic "niches" for the HSC (13). Moreover, LTBMC studies have led to the recognition of two forms of radiation-induced damage to the HIM; one that affects the proliferative potential of the "stromal" cells (*vis-à-vis*, the *ex vivo* establishment of the adherent layer of the LTBMC), and a second that restricts the functional integrity of the HIM to support hematopoietic activity (13, 14). Of interest, these two forms of damage also have been distinguished by their respective radiosensitivities; (a) the proliferative potential of stromal cells being exquisitely more sensitive to radiation damage than the "relatively" radioresistant supportive function of the HIM (13), and (b) the expression of proliferative damage appearing to be inversely proportional to the time between exposure and analysis and, as a result, suggestive of significant potentially lethal damage (14).

Unfortunately, our understanding of the hematopoietic response to xRT has been derived, for the most part, from studies involving single doses or very abbreviated courses of

FxRT. More recently, however, this laboratory has reported on the kinetics of radiation damage expression in both the HSC compartments and HIM of murine marrow exposed to FxRT [2.0 Gy (q24h x 5)→74 Gy] modeled after a more conventional course of clinical FxRT (15). A comparison between the response of the blood-forming HSC (CAFC) and supportive stromal (LTBMC) compartments of the marrow to FxRT was consistent with the generation of a dose-dependent lesion in the HIM; a lesion that persisted long after HSC recovery was observed in the marrow. In this report, we provide additional support for the generation of this "stromal lesion" and further define the kinetics of the hematopoietic response to accumulating FxRT dose levels on either side of the FxRT-induced lesion's threshold dose of 20 Gy.

Materials and Methods

Experimental design. A course of fractionated [2.0 Gy (q24h x 5)→74 Gy] pelvic xRT simulating a clinical course of fractionated wide-field pelvic xRT in the C57Bl/6 mouse has been described (15,16). For the present studies, the femur was included in the FxRT field. Animals, randomly assigned to the experimental groups, received either 10 Gy or 30 Gy of accumulated FxRT starting on day 0. At designated times as described in the results, a minimum of 5-7 mice per group were randomly sacrificed by cervical dislocation (under anesthesia) to determine the hematopoietic recovery within a single femur. The data from individual mice were collated and comparisons made between irradiated and control animals using SAS 5.0 software to generate Pearson correlation coefficients. A *p*-value of <0.05 was considered to represent a significant difference between matched pairs. These experiments were carried out in accordance with protocols sanctioned by the Animal Care and Use Committee at East Carolina University and the provisions of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals, U.S.A.*

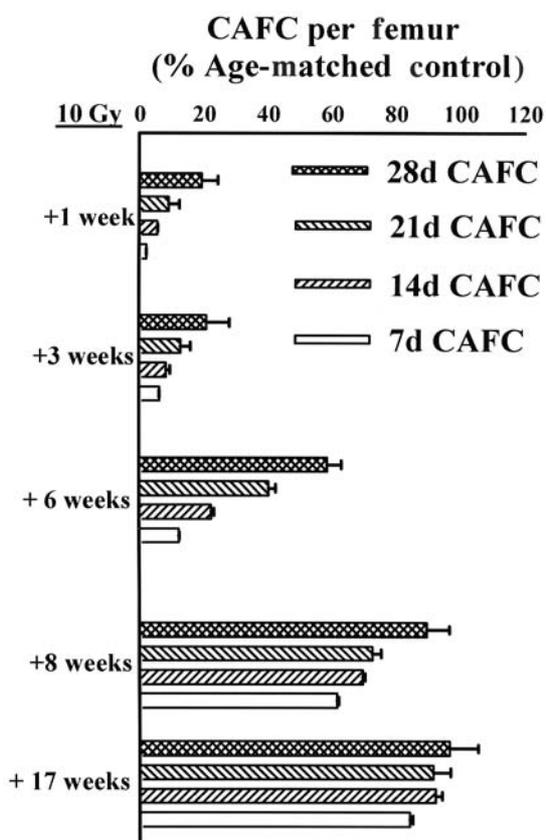


Figure 1. The temporal response of CAFC subpopulations in femoral marrow exposed to an accumulated FxRT dose of 10 Gy. CAFC_{7, 14, 21, 28d} frequency was determined in femoral marrow at intervals from 1-17 weeks after exposure to 10 Gy of FxRT. The data is presented as a percent of non-irradiated, age-matched C57Bl/6 control mice. Each data point was derived from the mean (\pm SEM) CAFC frequency observed in the femurs of 4-6 individual animals. Significant differences between experimental groups amounting to $p \leq 0.05$ are reported in the text.

Animals and xRT treatment. Eight-week-old C57Bl/6 mice, obtained from the NIH (Charles River) animal resources program and housed in microisolators with sterile food and acidified water provided *ad libitum*, were used during this study. For FxRT, air-breathing mice were positioned individually in a tight-fitting, leucite restraining jig with the tails immobilized to prevent forward movement. While restrained, the animals were covered with a 6-mm thickness of lead, cut to shield the entire animal except for a dorsal anterior port running 2.4 cm (field size of 20 x 24) from the anus to the cecum. For the present studies, the field size was extended so that one femur would also receive a full dose of FxRT Gy. The inclusion of the femur (or femur + tibia for LTBM) in the field was confirmed by X-ray radiography at the TSD. Confirmation of field-dose uniformity, lead shielding attenuation (>98%) and tissue volume-dose distribution was documented using TLD dosimetry on age-matched cadavers. FxRT was delivered from a Stabiplan at a dose rate of 82 c Gy/min with physical factors of 250 KvP, 15 mA, 2 mm Cu filtration and a TSD of 32 cm.

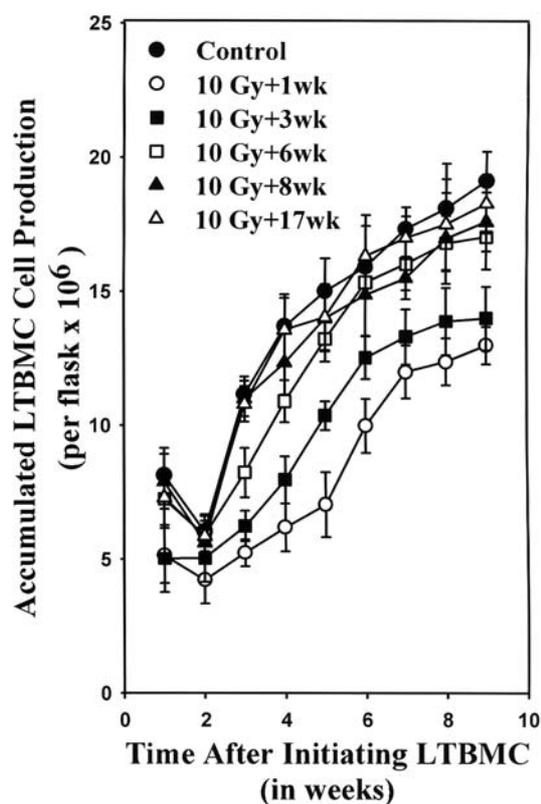


Figure 2. Accumulated cell production over a 9-week interval by LTBM established from the marrow of animals receiving local exposure to an accumulated FxRT dose of 10 Gy: The effect of time after exposure. Animals received an accumulated dose of 10 Gy of FxRT and LTBM were established at a time equivalent to 1-17 weeks after exposure. Accumulated cell production by the individual LTBM was assessed over a 9-week interval and the data presented as the mean (\pm SEM) accumulated cell production by a minimum of 5 LTBM per experimental group. Significant differences between experimental groups amounting to $p \leq 0.05$ are reported in the text.

Hematopoietic recovery. A single cell suspension of femoral hematopoietic cells was obtained as previously described (15, 17). The hematopoietic response to FxRT in the marrow of control and irradiated mice were assessed using the CAFC and LTBM assays. **CAFC assay.** Stem and progenitor cell frequency were determined in the femurs of individual mice using *in vitro* limiting dilution CAFC analysis, as previously described (15, 18). Briefly, adherent layers of the murine stromal-cell FBMD-1 line were prepared by incubating 1×10^4 cells, in gelatin-coated 96-well flat-bottom microtiter plates, in 0.2 ml of DMEM-10% fetal calf serum, at 33°C in 5% CO₂ in humidified air for 24 hours. Serial 2-fold dilutions of fresh bone marrow cells ($8 \times 10^4 \rightarrow 1.5 \times 10^2$ cells/well, 20 wells/dilution) from individual murine femurs were then plated over the stromal layer and the cultures incubated at 33°C in 5% CO₂ in humidified air. The CAFC cultures were refed every 7 days with Fishers medium supplemented with 10% horse, 10% BCS and 10^{-6} M hydrocortisone-21-hemisuccinate. Individual cultures were removed from the incubator and, using an inverted

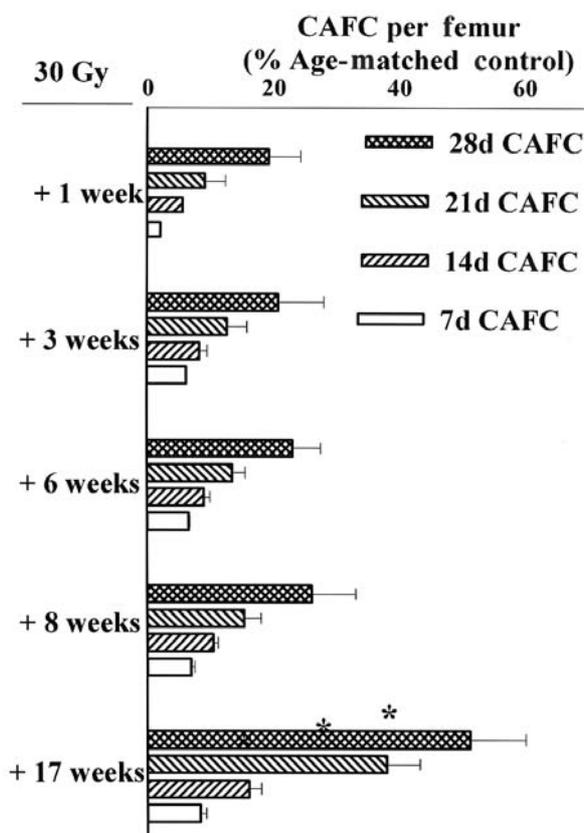


Figure 3. The temporal response of CAFC subpopulations in femoral marrow exposed to an accumulated FxRT dose of 30 Gy. CAFC_{7, 14, 21, 28d} frequency was determined in femoral marrow at intervals from 1-17 weeks after exposure to 30 Gy of FxRT. The data is presented as a percent of non-irradiated, age-matched C57Bl/6 control mice. Each data point was derived from the mean (\pm SEM) CAFC frequency observed in the femurs of 4-6 individual animals. Significant differences between experimental groups amounting to $p \leq 0.05$ are reported in the text.

microscope, CAFC colony formation was assessed on day 7, 14, 21 and 28 of incubation and, accordingly, designated as CAFC_{7-28d}. CAFC frequency was then calculated by a linear regression analysis of the relationship between $-Ln(N_0/N)$ vs. the appropriate cell dilution, where N_0 is the number of wells negative for CAFC for each cell dilution analyzed and N is the total number of cells analyzed. CAFC frequency was expressed as the frequency/ 10^6 cells (18). **LTBMC assay.** LTBMC were established from individual animals by plating the contents of one tibia and one femur in complete medium (Fishers medium supplemented with 10% pretested bovine calf serum, 10% horse serum and 10^{-6} M hydrocortisone-21 hemisuccinate; LTBMC medium) into 25-cm² flasks (15). The cultures were then incubated at 33°C in 5% CO₂ in air and were demi-populated weekly with fresh medium. Hematopoietic cell production [non-adherent cell number], a measure of the integrity of the stromal/stem cell unit in marrow, was determined in the demipopulated culture supernatant using an electronic cell counter.

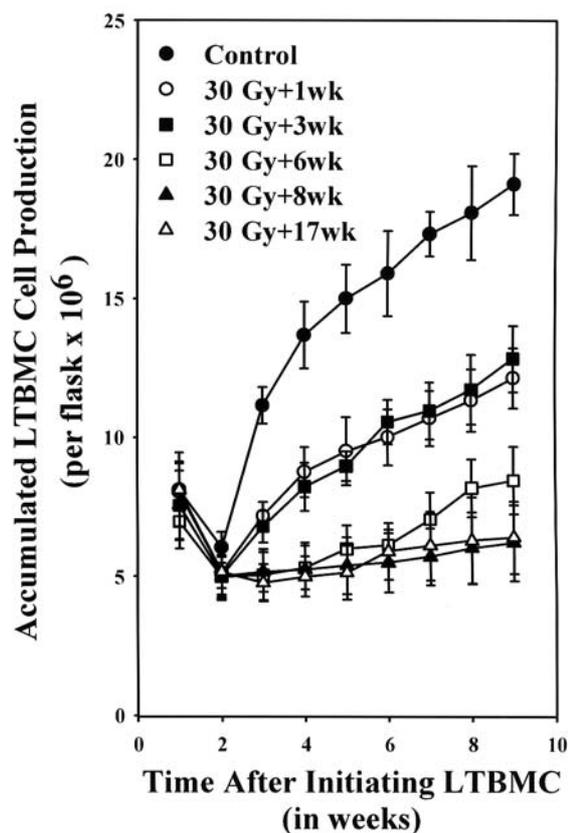


Figure 4. Accumulated cell production over a 9-week interval by LTBMC established from the marrow of animals receiving local exposure to an accumulated FxRT dose of 10 Gy: The effect of time after exposure. Animals received an accumulated dose of 10 Gy of FxRT and LTBMC were established at a time equivalent to 1-17 weeks after exposure. Accumulated cell production by the individual LTBMC was assessed over a 9-week interval and the data presented as the mean (\pm SEM) accumulated cell production by a minimum of 5 LTBMC per experimental group. Significant differences between experimental groups amounting to $p \leq 0.05$ are reported in the text.

Results

The hematopoietic response to 74 Gy of FxRT. As summarized in Table I, each of the 4 CAFC subpopulations was significantly reduced ($p \leq 0.05$) within one week of initiating the FxRT (10 Gy). While the CAFC_{7, 14} and 21 remained at levels of from 1-9% of control throughout the course of the FxRT, the CAFC₂₈ subpopulation leveled out with a nadir of ~14% of control as the accumulating dose of FxRT increased from 10 to 74 Gy. Within 121 days of completing the course of 74 Gy FxRT, both the CAFC₂₁ and CAFC₂₈ subpopulations initiated recovery with restoration of the CAFC₂₈, but not the CAFC₂₁, to normal levels ($p \leq 0.05$) by day 231 after FxRT. At the same time, however, the more mature CAFC₇ and CAFC₁₄ subpopulations remained depressed at levels of between 15-22% of control ($p \leq 0.05$;

Table I). Unlike the CAFC subpopulations, the integrity of corresponding LTBM, assessed by their ability to maintain non-adherent, hematopoietic cell production *in vitro*, remained at comparable levels to age-matched control marrow ($p \leq 0.05$) after the first week of FxRT (Table I). Thereafter, however, the integrity of LTBM established from marrow that continued to be exposed to FxRT was severely compromised, with accumulated cell production in LTBM reduced from 19.2×10^6 (for normal LTBM) to $\sim 5 \times 10^6$ and 1×10^6 cells per flask (for LTBM derived from marrow exposed to 30 and 74 Gy FxRT, respectively). Moreover, there was no evidence of an attempt at restoration of LTBM integrity over a period of from 121 to 231 days following the completion of the FxRT (Table I).

The hematopoietic response to 10 Gy of FxRT. The femoral content of the CAFC₇₋₂₈ subpopulations was established over a 17-week period after receiving 10 Gy of FxRT. As observed in Figure 1, all 4 of the CAFC subpopulations were markedly depressed, reaching levels of from 2% of control for CAFC₇ to 19% of control for CAFC₂₈. This depression lasted for a period in excess of 3 weeks following 10 Gy FxRT. By week 6, however, the CAFC₂₁ and CAFC₂₈ were restored to $\sim 45\%$ and $\sim 60\%$ of control, while the CAFC₇ and the CAFC₁₄, demonstrated only minimal recovery and, as a result, remained depressed. Thereafter, CAFC recovery continued and CAFC₂₁ and ₂₈ approached control levels ($\sim 85-91\%$) by week 17, while CAFC₇ and ₁₄ were restored to $\sim 71-84\%$ of control (Figure 1). Similarly, the hematopoietic activity in LTBM established from irradiated (femoral + tibial) marrow reflected the dampened CAFC subpopulations over the first 3 weeks following 10 Gy of FxRT (Figure 2). Accumulated cell production in LTBM was reduced to 68% and 73% of normal at 1 and 3 weeks following 10 Gy of FxRT, respectively. Consistent with the recovery of the more primitive CAFC subpopulations, the integrity of the LTBM was restored to near normal levels within 6 weeks of 10 Gy FxRT (Figure 2).

The hematopoietic response to 30 Gy of FxRT. After 30 Gy of FxRT, each of the 4 subpopulations of CAFC were reduced to levels similar to that observed after 10 Gy FxRT. However, unlike the response to 10 Gy, CAFC recovery was delayed after 30 Gy FxRT and a significant ($p \leq 0.05$) repopulation of the CAFC₁₄, CAFC₂₁, and CAFC₂₈ (but not the CAFC₇) was only observed between 8 and 17 weeks following FxRT (Figure 3). When compared to CAFC recovery from 10 Gy FxRT, however, the recovery at 17 weeks following 30 Gy FxRT remained significantly ($p \leq 0.05$) dampened. Similar to the response to 10 Gy FxRT, accumulated cell production in LTBM established during the first 3 weeks following 30 Gy FxRT was reduced

to $\sim 65\%$ of normal (Figure 4). Thereafter, unlike the response to 10 Gy FxRT, cell production in LTBM established at 6 to 17 weeks after 30 Gy FxRT was further dampened (44% and 33% of normal for weeks 6 and 8, respectively). Furthermore, by week 17, when restoration of the more primitive CAFC subpopulations was well on its way, accumulated cell production by the LTBM remained suppressed at $\sim 33\%$ of normal.

Discussion

In an earlier report (15), we described the generation of a persisting FxRT-induced lesion in the microenvironmental stroma that appeared to effectively interfere with the normal regulation of hematopoietic differentiation and the recovery of hematopoietic activity in the irradiated marrow. The lesion, assessed by the integrity of LTBM established from marrow that received increasing (accumulating) dose levels (10-74 Gy) of FxRT, was still observed at 121-231 days after the projected completion time (7.4 weeks) for a full course of 74 Gy FxRT. The lesion initially appeared in response to an accumulated threshold dose of 20 Gy and progressively increased in severity with continuing dose accumulations through a complete course of 74 Gy FxRT (16). The studies described herein were carried out to further define the influence of accumulating dose (10 vs. 30 Gy) and subsequent time (0-17 weeks) on the generation of this stromal lesion. Collectively, the data described demonstrate that after a subthreshold dose of 10 Gy FxRT, only a brief transitory interruption of LTBM integrity occurred (LTBM cell production restored to normal by week 6). Conversely, at an accumulated dose above the threshold (30 Gy), the integrity of the LTBM was significantly ($p \leq 0.05$) compromised without evidence of even an abortive attempt at recovery over a 17-week period (Figure 4).

The temporal response of the CAFC subpopulations and the LTBM to an accumulated dose of 10 Gy of FxRT was consistent with a FxRT-induced damage to the blood-forming CAFC subpopulations rather than the microenvironmental stroma. Within 6 weeks of receiving 10 Gy FxRT, cell production by the LTBM had been restored to near control levels (Figure 2), further suggesting that the compromised integrity of the LTBM observed at 1 and 3 weeks was the result of damage sustained by the CAFC subpopulations (Figure 1). In support of this conclusion, the restoration of each of the 4 CAFC subpopulations to near normal values occurred within 17 weeks of receiving 10 Gy of FxRT, while continuing exposure to FxRT maintained the CAFCs in a dampened state (Table I). In contrast, a uniquely different response pattern was observed after exposure to 30 Gy FxRT. First and foremost, as might be expected with additional exposure to FxRT, the damage to the CAFC subpopulations

was sustained well beyond the 6-week interval observed after 10 Gy FxRT. In fact, the initiation of CAFC recovery was first observed between 8 - 17 weeks (*vs.* 3-6 weeks) following 30 Gy (*vs.* 10 Gy) FxRT (Figures 3 *vs.* 1). Secondly, while LTBMC cell production was reduced to similar levels ($\sim 12 \times 10^6$ cells/flask) during the first 3 weeks following both 10 and 30 Gy FxRT ($p > 0.05$), the integrity of LTBMC derived from marrow exposed to 30 Gy FxRT, unlike that for 10 Gy FxRT, remained compromised through week 17 (Figure 4), even though CAFC recovery was initiated (Figure 3). It should be noted that, in our earlier report (15), restoration of control levels of the CAFC subpopulations was limited to the more primitive CAFC₂₈ and CAFC₂₁ subpopulations. As described above, the delayed recovery of the CAFC₇ and ₁₄ was again observed during the present study; this time occurring over a 17-week interval (*vs.* 48 weeks) following 30 (but not 10) Gy FxRT (Figure 3). Furthermore, the delayed recovery of the more mature CAFC subpopulations persisted in excess of a 24-week period following 30 Gy FxRT (data not presented). While it is conceivable that this temporal advantage for the recovery of the more primitive CAFC subpopulations may reflect the dose-sparing effect of the lower α/β ratio of the CAFC₂₈ (5), dose-sparing alone would not explain the FxRT dose-dependent discriminating lag in the recovery of the more mature (*vs.* more primitive) CAFC subpopulations.

A number of factors are known to influence hematopoietic recovery in locally-irradiated marrow. These include (a) the survival of HSC subpopulations (4-7), (b) the mobilization of HSC from non-irradiated marrow (19-21), and (c) a change in the balance between proliferative expansion and differentiation of surviving (or mobilized) HSC (22, 23). Earlier, we proposed that the persistent dampening of CAFC₇ and ₁₄ subpopulations was, in all probability, a result of a stromal dysfunction which limited either the differentiation of the more primitive HSC after recovery, or the clonal expansion of progenitors generated by differentiation (15). The data reported herein also appear consistent with this explanation for the delayed recovery of CAFC₇ and ₁₄. It should be noted, however, that while the factors contributing to recovery may appear to be distinct, and a number of factors may enter into the delayed recovery of the more mature CAFC, the regulation of each of these factors is believed to fall, at least indirectly, within the physiological domain of the marrow microenvironment. Furthermore, the HIM is currently thought to represent a unique interaction between both cellular and molecular elements, collectively creating hematopoietic cell-specific "niches" (24-29). As a result, damage to the hematopoietic "niches" in the microenvironment could, conceivably, influence both HSC survival, the lodging of HSC in the irradiated marrow and, in addition, the relationship between proliferative expansion and differentiation of individual HSC subpopulations during the recovery process itself.

Collectively, the data described above support our original hypothesis that FxRT results in a dynamic expression of hematopoietic radiation damage involving both the blood-forming cells and their regulation by the microenvironmental stroma. Having examined the marrow's response at doses above and below the threshold dose for the generation of the stromal lesion, the observations appear to be consistent with the early development of an acute effect on the blood-forming CAFCs and their subsequent recovery. However, with increasing accumulative dose and subsequent time, the response to FxRT is converted from the acute effect on the blood-forming cells to a persistent phenotypic expression of a dysfunctional microenvironmental stroma which alters the ratio between HSC proliferation and differentiation normally associated with the recovery process. Studies continue in an attempt to define the molecular nature of this stromal lesion resulting from FxRT and the mechanism(s) through which it alters hematopoietic recovery.

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