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Histomorphometric Evidence of Growth Plate Recovery Potential after Fractionated Radiotherapy:

An *In Vivo* Model

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Abstract

This study evaluated the hypothesis that early growth plate radiorecovery is evident by growth rate, histomorphometric and immunohistochemical end points after exposure to clinically relevant fractionated radiation *in vivo*. Twenty-four weanling 5-week-old male Sprague-Dawley rats were randomized into eight groups. In each animal, the right distal femur and proximal tibia were exposed to five daily fractions of 3.5 Gy (17.5 Gy) with the left leg serving as a control. Rats were killed humanely at 7, 8, 9, 10, 11, 14, 15 and 16 days after the first day of radiation exposure. Quantitative end points calculated included individual zonal and overall growth plate heights, area matrix fraction, OTC-labeled growth rate, chondrocyte clone volume and numeric density, and BrdU immunohistochemical labeling for proliferative index. Transient postirradiation reductions occurred early and improved during observation for growth rate, proliferative indices, transitional/hypertrophic zone matrix area fraction, proliferative height, and clonal volume. Reserve and hypertrophic zone height remained increased during the period of observation. The current model, using a more clinically relevant fractionation scheme than used previously, shows early evidence of growth plate recovery and provides a model that can be used to correlate temporal changes in RNA and protein expression during the early period of growth plate recovery.

INTRODUCTION

Radiation treatment of musculoskeletal sarcomas is common in pediatric oncology. Radiotherapy is used as an adjunct to surgical excision for soft tissue sarcomas such as synovial sarcoma and may be used as an alternative to surgery for rhabdomyosarcoma and Ewing's sarcoma. However, in patients with immature skeletons, radiotherapy frequently results in asymmetric limb growth arrest and resultant limb length discrepancy due to physeal damage from local radiation effects when the physis is included in the radiation field (1–3).

The Sprague-Dawley rat has been used to examine the effects of radiation and radioprotectors on the growth plate, because this animal has a relatively short period of rapid growth during which growth plate effects may be evaluated efficiently. Much of the early radiation work with the Sprague-Dawley rat used a single-fraction 17.5-Gy dose of radiation for simplicity and to allow proof of concept in establishing efficacy of radioprotectors (4,5). This model has been

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well characterized by sequential histomorphometry, growth rates, limb discrepancies, and immunohistochemistry (6–9).

However, in clinical practice, fractionation of the doses of therapeutic radiation is the standard of care and is routinely employed to minimize the damaging effects of radiation on normal tissues. Hence any means used to lessen the damaging effects of radiation on the growth plate must have efficacy above and beyond the protective effects of fractionated radiation doses alone. A well-characterized model of fractionated radiation is needed to allow more clinically relevant experimentation. Our laboratory has previously used various fractionated doses, but the emphasis in those experiments was simply on demonstrating efficacy of radioprotectors on limb length discrepancies² (10). The histomorphometry, growth rates and immunohistochemistry of a clinically relevant fractionated model have not been described.

In this experiment, we proposed to establish and characterize effects of clinically relevant fractionated radiation therapy on the proximal tibial growth plate of weanling Sprague-Dawley rats using histomorphometry, growth rates and immunohistochemistry for BrdU incorporation. We hypothesized that this model of fractionation would show early changes of increased area matrix fraction and diminished total growth plate height, zonal height, growth rate and BrdU proliferative index compared to contralateral control growth plates with subsequent early improvement toward control levels over time of observation.

METHODS

Twenty-four weanling 5-week-old (75–100 g) male Sprague-Dawley rats (Taconic Farms, Germantown, NY) were randomized into eight groups of three animals each. Sprague-Dawley rats are an established animal model for examination of skeletal growth effects because they are large enough to permit precise bone measurements while allowing amplification of skeletal growth disturbances (11–13). All animals received a total of 17.5 Gy radiation in five fractions of 3.5 Gy to the right knee only delivered on consecutive days. The biologically equivalent dose of 3.5 Gy in five fractions is calculated to be 9 Gy using the α/β formalism. The eight groups of three animals each were killed humanely at intervals of 7, 8, 9, 10, 11, 14, 15 and 16 days after the first day (day 0) of irradiation. The animal protocols in this study were approved by the Committee for the Humane Use of Animals, Upstate Medical University.

Prior to radiation treatment, the animals were anesthetized using Telazol (30 mg/kg i.p., Fort Dodge Animal Health, Fort Dodge, IA). The animals were weighed and then placed on an acrylic sheet with the right leg extended, secured with tape, and placed under the radiation source (Philips MGC 30; Shelton, CT). Radiation was administered using 300 kV X rays at 10 mA with a 30-cm source-to-skin distance and a dose rate of 2.56 Gy/min (3). The exposure time for each 3.5-Gy fraction was 1.37 min. Using beam collimation and lead shields, only the right knee regions were exposed (distal half of femur and proximal half of tibia) (14). The 17.5-Gy dose administered as a single fraction had consistently produced a significant reduction of growth in our earlier study and in those of others (12–14). Mean body weight on the first day of radiation exposure was 87.9 g (80–96 g).

Oxytetracycline (OTC, 50 mg/kg) was given i.p. 25 h before killing to determine the current growth rate. A single injection of 5-bromo-2-deoxyuridine (BrdU, 25 mg/kg) was given 30 min before killing to label cells actively engaged in DNA synthesis immediately prior to the assay.

²T. Damron, R. Tamurian, J. Spadaro and L. Damron, Combined effects of fractionation and radioprotection in sparing of radiation induced physeal damage. Presented at the Connective Tissue Oncology Society's 5th Annual Scientific Meeting, 1999.

On the day of planned assay (7, 8, 9, 10, 11, 14, 15 and 16 days after the first day of radiation treatment), the animals were killed by via CO₂ inhalation. Based on our earlier work with a single 17.5-Gy fraction, the recovery changes should have largely begun by 14 days after initiation of irradiation, and since our ultimate purpose in establishing this more clinically relevant model of fractionation was to analyze early changes leading to this postirradiation growth plate recovery, we carried the study only through 16 days. The hind limbs were disarticulated at the hip. The proximal tibiae were removed and split in the sagittal plane. One half of each split tibia was placed in P/G/R fixative [2% paraformaldehyde, 2% glutaraldehyde with 0.7% hexamine ruthenium (III) chloride (RHT) in a 0.1 M cacodylate buffer], dehydrated through ascending concentrations of ethanol, and embedded in methylmethacrylate (MMA) resin using a cold-polymerization protocol (15). This half of each tibia was used for stereological estimates of growth plate and individual zonal heights, area matrix fraction (Aa matrix), and clone number/density. The more regular, less undulating proximal tibial growth plate is the standard source for growth plate histomorphometry in the Sprague-Dawley rat model of phy-seal growth. The other half of each tibia was immediately frozen using dry ice and later transferred to a -80°C freezer for RNA analysis (reported elsewhere).

The femurs were fixed in ethanol and used for BrdU immunohistochemistry and to calculate the growth rates of OTC-labeled cells in the distal femoral metaphysis. The disarticulated femora were then preserved in 70% ethanol until they were processed similarly through ascending concentrations of ethanol and embedded at low temperature in methylmethacrylate. Based on our previous experience showing very small length differences at these relatively early times after even higher doses of radiation by radiographic assessment of bone length, the decision was made not to perform radiographs for length measurements.

Growth Plate Histomorphometry

Histomorphometric measurements were taken from tibial tissues sectioned at 5 µm on a Leica RM 2155 rotary microtome and mounted on APES-coated slides. Three 5-µm-thick sections were sampled at uniform random intervals of 80 µm in the sagittal plane and stained for quantitative histomorphometric analysis with a polychrome stain described previously (8). Three tibial specimens for both the irradiated right limb and control left limb per time were processed in this fashion for analysis of the histomorphometric parameters described below. In addition, three femoral specimens for both the irradiated right limb and control left limb per time were processed for immunohistochemical detection of BrdU incorporation. Micrographs were made using a Polaroid Digital Microscope Camera and a Nikon Eclipse E400 microscope at 10× magnification. All images were analyzed with the imaging program Image Pro Plus 4 (Media Cybernetics, Silver Spring, MD).

Quantitative histomorphometry end points calculated included individual and overall growth plate heights, area matrix fraction, OTC-labeled cell growth rate, and chondrocyte clone volume and numeric density. Individual zonal heights were measured for the morphologically defined reserve, proliferative, transitional and hypertrophic zones of the growth plates (Figs. 1, 4). The reserve zone (RZ) was defined as the region from the epiphyseal chondro-osseous junction (eCOJ) to the first flattened chondrocyte at the base of a cell column. The proliferative zone (PZ) is defined as the region of chondrocytes displaying a flattened-disc morphology; it extends from the first flattened cell at the top of a chondrocytic column to the level at which cells assume a more a rounded cellular morphology. This shape change is a morphological hallmark of the transitional zone (TZ) chondrocyte, and the TZ is defined as beginning at the level of first shape change to the presence of typical cylindrical hyper-trophic cells. The hypertrophic zone (HZ) is defined as the region beginning with the consistent cylindrical cellular profile and extending to the metaphyseal chondro-osseous junction (mCOJ) (8). Oxytetracycline binds calcium deposited into the extracellular matrix by terminal hypertrophic

chondrocytes at the metaphyseal chondro-osseous junction (mCOJ), and it can be visualized in tissue section by epifluorescence microscopy using a UV-1a filter set (excitation 365 nm/emission >400 nm, band pass). Subsequent chondrocytic hypertrophy and turnover at the mCOJ cause the fluorescent band of pulse-labeled matrix to migrate into the metaphysis at a rate approximating the rate of elongation by this growth plate. Thus we have estimated daily growth rate by measuring the average vertical distance between the band of OTC fluorescence and the metaphyseal chondro-osseous junction and dividing this distance by the time elapsed between injection and killing, as described previously (14). These measurements were made from three sections per limb, with sections sampled at uniform random intervals of 80 μ m. Tissue cellularity was estimated by determining the matrix area fraction (Aa_{matrix}) of each morphologically defined zone of the growth plate by applying the disector method (16). This extension of the Cavalieri principle is a sensitive estimate of tissue cellularity (17,18). Briefly, an orthogonal grid of points was superimposed on 10 $\times$ micrographs at random angles of rotation, and the number of points intersecting cellular profiles or extracellular matrix was counted and divided by the total number of points distributed over a given field. Thus the counted number of matrix-intersecting points per total points is the estimate of matrix area fraction, Aa_{matrix} .

For the clonal analysis, chondrocytic colonies consisting of more than eight adjacent cellular profiles in serial section were considered a single clonal expansion in the irradiated growth plates. This minimum cell number indicates an expansion that has completed at least three successive mitotic cycles, thus representing a progeny of viable cells descended from a common progenitor cell (19). These colonies were generally observed in serial sections as conical expansions of chondrocytes with epiphyseal to metaphyseal polarity and were observed as early as the seventh day after the initial radiation exposure. Numerical density of clones (Nv_{clone}) was determined using the disector method adapted for vertical uniform random sections of growth cartilage (16). This estimation procedure was previously validated using Cruz-Orives's number counting method (20) from which the disector is mathematically derived. In this analysis, an individual clonal structure is regarded as a single object in a volume of aggregate tissue (19,21). Nv_{clone} was estimated as the number of individual clones counted in a volume of irradiated tissue sampled at serial 80- μ m intervals. The average volume of individual clonal condensations, V_{clone} , was estimated as the sum of the products of clone profile areas determined by point counting in serial sections and the sampling interval thickness for each colony identified (21).

To determine growth plate proliferative activity, we calculated an index of BrdU immunoreactivity in ethanol-fixed distal femoral growth plates as follows. First, antigen retrieval based on the technique of Baroukh *et al.* (22) was performed by treatment with 0.1% trypsin for 30 min at 37°C in a humid chamber followed by further treatment with 2 M HCl for 30 min. Non-specific binding was blocked by incubation in 0.1 M phosphate-buffered saline (PBS, pH 7.0) with 10% normal horse serum and 2% bovine serum albumin for 1 h. Then the slides were incubated overnight at 4°C with a mouse anti-BrdU antibody (1:100, Becton Dickinson, San Jose CA) diluted in blocking buffer. The next day all sections were washed in PBS and incubated with a biotinylated anti-mouse IgG (1:200, Vector Laboratories, Burlingame, CA) diluted in blocking buffer. Horseradish peroxidase-conjugated avidin was then used to detect the secondary antibody (ABC Elite Kit, Vector Laboratories), visualized with DAB as the chromogen (Vector Laboratories), and counterstained with 2% methyl green, and sections were covered with cover slips. From these sections, we calculated an index of BrdU incorporation by determining the proportion of BrdU-labeled nuclear profiles per total cellular profile (9,23). Since the 5- μ m-thick sections used for this assay were less than one cell diameter in thickness, this index was adjusted to account for the probability of observing a nucleated cellular profile within any randomly selected cell profile (23). The correction factor for this experiment was 0.715.

Statistical comparisons were made between the subgroups. ANOVA with Fisher's PLSD was used within limbs to demonstrate temporal differences, and Student's paired *t* test was used to determine the significance of pairwise differences between limbs at each time using StatView 5.0.1 (SAS Institute, Cary, NC). For both tests, differences were considered significant when $P \leq 0.05$. Data presented reflect the mean value determined from three animals and the standard deviations.

RESULTS

Qualitative Effects

The overall effects of this fractionated regimen of irradiation of the growth plate produced very subtle histomorphological changes (Fig. 1). However, compared to the control left proximal tibial growth plate, there was a subtle decrease in cellularity and, conversely, a relative increase in the area fraction of the growth plate comprised of matrix was most evident at 1 week.

By 2 weeks after the first dose of radiation, there was an improvement in both cellularity and growth plate height compared to those same measurements at 1 week. However, chondrocytic clones did not predominate the growth plate even at 2 weeks after initiation of irradiation (Fig. 2).

Quantitative Effects

Oxytetracycline (OTC)-labeled cell growth rates were significantly lower ($P < 0.05$) for the right irradiated tibiae compared to the unirradiated left at 7, 8, 9, 10 and 11 days (Fig. 3). Beginning on day 7 after initiation of irradiation, the growth rate for the right was only 51% of that of the left side (93.6 and 184.1 $\mu\text{m}/\text{day}$). However, by the 14th day, the rate for right side had returned to 77% of that of the left, and the difference between sides was no longer significant ($P = 0.055$). As the right tibial growth rate gradually increased and approached that of the left nonirradiated control tibiae, differences remained insignificant at days 15 (83% of left side growth rate, $P = 0.086$) and 16 (93.3% of left side, $P = 0.56$). In fact, while the left side control growth rate was relatively constant, OTC growth rates increased significantly over time on the right side. The mean OTC growth rates for the right side were significantly lower at 7, 8, 9 and 10 days (each) than at days 14, 15 and 16 (individually) after initiation of the fractionated-dose irradiation ($P < 0.05$). The mean right side growth rate at 11 days was also significantly less than that at 15 and 16 days, and that for day 14 was significantly less than at day 16.

The overall growth plate height failed to show significant differences between sides at any time (Fig. 4). However, the right growth plate height progressively increased over time. At its thinnest on days 7 and 9, the right proximal tibial growth plate was significantly smaller than at its thickest on day 15 ($P < 0.05$).

Individual zonal heights after irradiation did show differences compared to the nonirradiated control side. Reserve zone height was on average greater on the irradiated right side than on the left side at all times, although the difference reached statistical significance only on days 11, 14 and 16 ($P < 0.05$) (Fig. 4). Compared to the left side, the right side mean reserve zone height was 17% to 86% greater. Proliferative zone height on the irradiated side was significantly lower at days 7 and 8 after irradiation compared to the left (75% and 61%, respectively; $P < 0.03$). Thereafter, except at day 9, the right side proliferative zone height remained on average lower than that of the left, but insignificantly so. The transitional zone height remained essentially constant. For the hypertrophic zone height, beginning at day 10 and continuing through day 16, the mean right side was considerably thicker than the left side, reaching statistical significance on day 16 ($P = 0.016$) (Fig. 4).

The matrix area fraction of the total growth plate was greater at 7 days after initiation of irradiation in the right irradiated tibial growth plate than for the control left side (0.68 compared to 0.58), but this difference was not statistically significant ($P = 0.07$). Thereafter, from days 8 through 16, the total area matrix fraction was nearly identical on the right and left sides (not significant) (Fig. 5).

For the most part, while the individual zonal Aa_{matrix} on average tended to be slightly greater on the irradiated side at most times, there were few statistically significant differences for individual zonal Aa_{matrix} within the reserve, proliferative, transitional or hypertrophic zones for any of the times. However, there were exceptions. In the proliferative zone, there was a significant difference ($P = 0.021$) at 15 days, with a greater Aa_{matrix} in the right irradiated side (0.87 compared to 0.82, respectively) compared to the left. In the transitional zone, the Aa_{matrix} was significantly greater on the right side at 7 days (0.79 compared to 0.60, $P = 0.021$), 15 days (0.83 compared to 0.74, $P = 0.027$), and 16 days (0.83 compared to 0.73, $P = 0.049$) after irradiation started. For the hypertrophic zone, the irradiated right side had a significantly greater Aa_{matrix} at 7 days (0.55 compared to 0.45, $P = 0.048$) and 15 days (0.69 compared to 0.62, $P = 0.034$).

In the irradiated right tibial growth plate, average clone volume was at its lowest at the first time and increased progressively throughout the experimental period (Fig. 6). Clone numeric density, by contrast, remained essentially unchanged over the periods evaluated (Fig. 6).

The BrdU indices were transiently less on the irradiated right side compared to the left nonirradiated side (Fig. 7). There were no significant differences between right and left sides at any time for any zone.

DISCUSSION

Radiation therapy for pediatric malignant tumors carries the potential for growth arrest, angular deformity, pathological fractures, and postirradiation sarcomas. Strategies for minimizing growth arrest and angular deformities have included fractionation, hyperfractionation and the use of chemical radioprotectors. Fractionation of radiotherapy is the clinical standard of care, and a clear fractionation effect on bone growth has been demonstrated. However, standard fractionation still results in premature growth arrest in children. Techniques that will provide clinically meaningful reduction in growth arrest beyond that achieved with fractionation alone are clearly needed. Our laboratory has been successful in demonstrating beneficial effects of a number of radioprotectors in an earlier study using three fractions totaling 17.5 Gy (24) or 25 Gy (10). However, these preventative effects have been incomplete. The potential remains for further improvement using selective stimulation of the growth plate through a radiorecovery paradigm, but to select candidates for such a treatment, a better understanding of the process of growth plate recovery after clinically relevant fractionated radiotherapy is needed. In the current study, the histomorphometric, growth rate, clonal analysis and proliferative capacity changes after fractionated irradiation have been characterized. Our findings only partially support our hypothesis. Transient postirradiation reductions in this model of fractionated doses occurred early and improved during observation for oxytetracycline-labeled cell growth rate, BrdU proliferative index, transitional/hypertrophic zone area matrix fraction, proliferative height, and clonal volume. Reserve and hypertrophic zone height remained increased during the period of observation. Neither overall growth plate height nor overall area matrix fraction was significantly changed.

In earlier work with a single 17.5-Gy dose, similar end points were examined at 0.5, 1, 2, 3 and 4 weeks after radiation treatment (25). The earliest qualitative histological changes were loss of normal columnation at 0.5 weeks leading to gross cellular distortion and loss of orderly

columnation by 1 week. Regenerative clones were a prominent feature beginning at 2 weeks. In the current fractionated model, less overall distortion was noted at each time assessed (Figs. 1, 2). However, a similar early pattern was observed in this study. Changes such as decreased overall growth plate and individual zonal heights as well as increased matrix area fraction were most noticeable at 1 week after the first fraction was given. No earlier times comparable to the 0.5-week time in the earlier single-fraction study were analyzed, because animals would have had to be killed prior to completion of the fractionated irradiation. By 2 weeks after the last fraction, these same parameters had improved and were approaching those of the normal growth plate. No later end points were examined in this model because the ultimate purpose was to validate a model for examination of the early recovery effects.

Clonal analysis in this study suggested that, after the current radiation treatment, there were relatively constant numbers of chondrocytic clones present in the irradiated growth plate between 1 and 2 weeks (Figs. 2, 6). However, during this same period, the clonal volume increased. This pattern suggests that the number of cells that contribute to regeneration of the growth plate after irradiation may be determined within the first week after irradiation. The subsequent process that leads to restoration of more normal growth plate morphology than would be expansion of this fixed number of clones.

Growth rate in the current study decreased less (to a mean 51% of the nonirradiated growth plate at 1 week) than in the earlier single-fraction study (to a mean 18% of nonirradiated growth plate at 1 week) (25). The peak reduction in proximal tibial growth plate height at 1 week to 91% of contralateral nonirradiated growth plate in the current study was a smaller reduction in height than the value of 65% relative to controls in the earlier study of non-fractionated radiation at 1 week. Changes in overall growth plate height in the earlier study were accounted for nearly exclusively by changes in the hypertrophic zone height after single-fraction irradiation, but in the current fractionated study this early decrease was primarily due to a reduction in proliferative zone height. Only the later increase in growth plate height to 127% of that of the control side was manifest primarily by an increase in the height of the hypertrophic zone. In both the earlier study and the current one, growth rate began to increase toward normal after the 1-week nadir.

The relatively high mitotic index of the growth plate chondrocytes makes this tissue highly susceptible to radiation-induced cell death and accelerated terminal differentiation, with the net effect being a reduction in the rate of endochondral bone elongation resulting in limb length discrepancy. While the exact lethal dose to chondrocytes remains to be determined, the dose used in series for this study appears to approximate this threshold because clonal chondrocyte expansions are evident as soon as 2 days after the final exposure. Such persistence suggests that this dose given in daily fractions is not uniformly fatal to growth plate chondrocytes and may be a more clinically relevant model than previous work using a single large X-ray exposure. Despite the initial growth depression, which appears to be transient, significant growth was observed in the irradiated limb, and daily growth rates had begun a return toward rates in nonirradiated growth plates. Future studies will be directed toward strategies that would promote a more robust recovery of growth rate. Such protocols, when used in conjunction with selective radioprotection of non-tumor tissues, may reduce such iatrogenic disruption to a level that does not result in crippling limb length discrepancy.

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FIG. 1.

Representative histology of control (panel A) and irradiated proximal tibial growth plates is shown at 7 days (panel B) and 14 days (panel C) after initiation of fractionated irradiation totaling 17.5 Gy. Contours (yellow) identifying the morphologically defined epiphyseal chondro-osseous junction (eCOJ), reserve (RZ), proliferative (PZ), transitional (TZ), and hypertrophic zones (HZ) and metaphyseal chondro-osseous junction. Polychrome stain at original magnification of 10×; Bar is 100 μm (mCOJ).

FIG. 2. Representative histology illustrating the volumetric expansion of individual chondrocytic clones, encircled in yellow, at 7 (panel A), 11 (panel B), and 14 days (panel C) after the initial radiation dose. Poly-chrome-stained clones are shown at an original magnification of 10 $\times$; bar is 100 μ m.

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FIG. 3. Oxytetracycline-labeled growth rate in control and irradiated tibiae. Points are means $\pm$ 1 SD for three measurements per limb. * $P \leq 0.05$ by paired t test. Note that the growth rates that are initially significantly less for the right irradiated side and then reach a level that is statistically indistinguishable from the left nonirradiated side by 14 days after initiation of irradiation.

FIG. 4.

Zonal and total growth plate height. No temporal differences were observed in the control limbs, so measurements from the control limbs were pooled, and the data presented reflect the means $\pm$ SD for 24 animals. For the irradiated limbs, data reflect the means $\pm$ SD for three tibiae per time.

FIG. 5. Total growth plate matrix area fraction in control and irradiated tibial growth plates. Means $\pm$ 1 SD; $n = 3/\text{limb}$; no significant differences were seen at any time.

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FIG. 6.

Proximal tibial growth plate clonal volume and numerical density. V_{clone} = clonal volume (μm^3); Nv_{clone} = numerical clone density (clones/ mm^3). Means ± 1 SD, $n = 3/\text{limb}$.

FIG. 7. Index of proliferative activity by BrdU incorporation in control and irradiated distal femoral growth plates. Mean $\pm$ 1 SD, $n = 3/\text{limb}$; * $P \leq 0.05$ by paired t test.