Sequential Histomorphometric Analysis of the Growth Plate Following Irradiation with and without Radioprotection

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Background: The availability of radioprotectant drugs that selectively protect normal cells but not tumor cells has rekindled interest in the effects of irradiation on the growth plate. The purpose of the present study was to quantitatively examine the sequential histomorphometric effects of irradiation and pretreatment with a free radical scavenger radioprotectant, amifostine, on the growth plate over time.

Methods: Sixty four-week-old male Sprague-Dawley rats were randomized into five groups of twelve animals that were to be killed at 0.5, one, two, three, or four weeks after irradiation. One-half of the animals also received amifostine (100 mg/kg) prior to irradiation. In all animals, the right knee was treated with a single 17.5-Gy dose of radiation. End points were assessed with quantitative histomorphometric analysis of the growth plate, BrdU labeling for evidence of proliferation, evaluation of chondroclast cellularity, and determination of growth rates by means of oxytetracycline labeling.

Results: The mean lengths of the femur, tibia, and hind limb continued to increase at each time-interval following treatment, but by one week the mean limb length was 4% less on the irradiated side than on the control side, and this difference remained significant for four weeks ($p < 0.05$). The proximal tibial growth rate decreased during the first week to 18% of the control level. Nevertheless, growth continued even at the earliest time-periods, began to return toward normal at two weeks, and ultimately returned to at least 80% of normal by four weeks after irradiation. The area fraction of matrix in the hypertrophic zone increased initially and returned to control levels at three and four weeks. The administration of the radioprotectant resulted in significant increases in growth, growth rate, growth plate height, hypertrophic zonal height, and chondroclast profiles compared with the values for limbs in which irradiation had not been preceded by treatment with amifostine.

Conclusions: We found an initially profound but transient direct inhibitory effect of irradiation on growth plate chondrocytes. Recovery of growth plate function after irradiation corresponded temporally with the appearance of newly formed islands of proliferating chondrocytes. Accumulation of matrix led to a transient increase in overall growth plate height, which was most pronounced in the hypertrophic zone. This was due, in part, to the sensitivity of chondroclasts to irradiation. The radioprotectant amifostine reduced these effects on growth rate, growth plate height, matrix accumulation, and limb length.

Clinical Relevance: The transient effects of irradiation on the growth plate are reduced by a clinically utilized radioprotectant drug. Use of radioprotectants may have potential for reducing the damaging effects of irradiation on the growth plate while preserving the desirable effects of irradiation on tumors.

Irradiation of tumors of the extremities in children frequently results in adverse effects on the involved growth plates[1,2]. Recently, there has been renewed interest in the use of selective radioprotectant drugs for the protection of normal tissues at the expense of tumor cells[3-6]. The potential of these drugs to protect against growth-plate injury during therapeutic irradiation has rekindled interest in understanding the response of the growth plate to irradiation.

Previous studies on the qualitative growth-plate changes following irradiation in the Sprague-Dawley rat indicated that there is a temporary inhibition of growth of the treated limb followed by recovery of some growth function[7-12]. However,

TABLE I Experimental Design

Treatment Group	Time After Treatment of Right Limb with 17.5 Gy of Radiation *(wk)*	Number of Animals Receiving No Amifostine	Number of Animals Receiving 100 mg/kg Amifostine
A	0.5	6	6
B	1	6	6
C	2	6	6
D	3	6	6
E	4	6	6
Total	—	30	30

the specific quantitative effects of irradiation on histomorphometric parameters over time have not been reported. Additionally, there has been no analysis of the serial effects of the free radical scavenger radioprotectant drug amifostine, which has been shown to ameliorate the adverse effects of irradiation on the growth plate[3-6]. The purpose of the present study was to quantify the temporal changes in limb bone length, growth rate, growth-plate height, matrix area fraction, and chondroclast and osteoclast activity after irradiation of the growth plates of the knee in Sprague-Dawley rats with and without pretreatment with a free radical scavenger radioprotectant. These quantitative changes also were correlated with the qualitative changes in growth-plate morphology and proliferative potential following irradiation.

Materials and Methods

Sixty four-week-old male Sprague-Dawley rats were randomized into five treatment groups (Table I). In all animals, the right knee (the distal part of the femur and the proximal part of the tibia) was treated with a single 17.5-Gy dose of radiation[3,13]. In all animals, the left hind limb was used as an internal control. One-half of the animals in each group also received amifostine (100 mg/kg) intraperitoneally twenty minutes prior to irradiation[3]. The animals were killed by carbon-dioxide asphyxiation at 0.5, one, two, three, or four weeks after irradiation, with twelve animals killed at each time-period. These studies were approved by the Institutional Use and Care of Animals Committee (IUCAC).

Prior to radiotherapy treatment, the animals were anesthetized by means of an intraperitoneal injection of Telezol (tiletamine hydrochloride and zolazepam; 30 mg/kg) and were then placed on a Plexiglas sheet with the right leg extended, secured with tape, and placed under the radiation source (Philips MGC 30; Shelton, Connecticut)[3,13,14]. Radiation was administered with use of 250-kVp x-rays at 15 mA with a 30-cm source-to-skin distance and a dose rate of 3.0 Gy/min. With use of beam collimation and lead shields, only the right knee region (the distal half of the femur and the proximal half of the tibia) was exposed. The proximal part of the femur and the distal part of the tibia were shielded from radiation[3,13].

Forty-eight hours before they were killed, the animals received an injection of oxytetracycline (50 mg/kg) to allow for bone growth measurements[15]. Oxytetracycline labeling provides a measurement of growth rate over time; in the present study, the growth rate was calculated in the last twenty-four hours before the animals were killed. Thirty minutes before they were killed, the animals received an intraperitoneal injection of the thymidine analog 5-bromo-2'-deoxyuridine (BrdU; Sigma, St. Louis, Missouri), at a dose of 25 mg/kg, as an indicator of proliferative potential. After each animal was killed, both lower extremities were harvested, radiographs of the extremities were made, and the bones were removed. Radiographs of all 120 limbs, made at baseline and following dissection, were scanned and measured with use of NIH Image 1.62 software to determine femoral length, tibial length, and overall limb length (femoral length plus tibial length)[3-6]. Lengthening was calculated with use of the formula $L_s - L_o/L_o$, where L_s is the length of the skeletonized limb and L_o is the length at baseline.

The proximal part of the tibia was split sagittally and was fixed in 2% glutaraldehyde and 2% paraformaldehyde with 0.7% hexamine ruthenium (III) chloride (RHT) in a 0.1-M cacodylate buffer[16]. Ten tibial halves (two per time-point, including one control and one irradiated specimen) were further cut into approximately 1-mm cubes that included the growth plate and were embedded in epon-araldite. The tibiae were then sectioned at a 1-μm thickness for BrdU analysis. In addition, twelve tibial halves per time-period were embedded in methylmethacrylate and were sagittally sectioned with a rotary microtome at a 5-μm thickness for all other histomorphometric and bone growth measurements.

The 5-μm sagittal tibial sections were stained for quantitative histomorphometric analysis with a combination of 2% periodic acid, 1% methylene blue (in 1% borax), 0.15% basic fuchsin (in 10% EtOH), and azure II (with azure II and basic fuchsin mixed in equal parts). Three tibial specimens for each of the four types of limbs (the irradiated right limb and the control left limb in animals treated with radiation only and the irradiated and amifostine-pretreated right limb and the control left limb in animals treated with radiation following amifostine pretreatment) were analyzed in this fashion at each time-period for each of the histomorphometric parameters; thus, twelve limbs per time-period, or a total of sixty limbs, were analyzed in this fashion. Visualization of incorporated

BrdU on the 1-µm epon-araldite sections was accomplished with use of a monoclonal antibody (Becton Dickinson, San Jose, California) and a commercially available kit (Zymed Laboratories, South San Francisco, California)[17]. BrdU could not be identified with use of this same technique on the 5-µm methylmethacrylate sections. Micrographs were made with use of a Polaroid Digital Microscope Camera (Polaroid, Waltham, Massachusetts) and a Nikon Eclipse E400 microscope (Nikon, Melville, New York). All image analysis was accomplished with the Image Pro 4 imaging program (Media Cybernetics, Silver Springs, Maryland).

Overall growth-plate height was measured by selecting a region centered on the long axis of the tibia. Horizontal lines were drawn along the contours of both the epiphysis on the proximal side of the growth plate and the chondro-osseous junction on the distal side of the growth plate. Ten lines, spaced 100 µm apart, were drawn between these contours, centered on the longitudinal axis. The ten height values were then averaged to generate a total growth-plate height for a particular animal. Measurements were made for three animals per treatment group for each time-period observed.

Individual zonal heights were measured for the reserve zone, the proliferative zone, and the hypertrophic zone of the growth plate. The reserve zone was defined as the region from the epiphysis to the first flattened chondrocyte at the base of a cell column. The proliferative zone extended from there to the first chondrocyte in which a shape change was evident. The hypertrophic zone extended from there to the chondro-osseous junction[15]. For each zone, the area fraction of matrix was determined by means of color thresholding with use of Image Pro. The growth rate was calculated on the basis of the measurement of the distance from the fluorescent oxytetracycline band to the chondro-osseous junction by means of epifluorescence microscopy with use of a 400-nm UV-1A dichroic mirror. In order to enhance the demarcation of the chondro-osseous junction in the older animals and those that had received irradiation, color thresholding was employed.

Additional tibial sections were stained with tartrate-resistant acid phosphatase (TRAP) (Leukocyte Acid Phosphatase Kit 387-A; Sigma, St. Louis, Missouri) and were counterstained with use of a 2% methyl green solution for the observation of osteoclasts and chondroclasts. An estimate of the number of osteoclasts and chondroclasts was made within three selected regions within the metaphysis. These regions included (1) the chondro-osseous-junction region, which extended 75 µm below the chondro-osseous junction; (2) region 1^0, which was within the primary spongiosa (the metaphyseal bone closest to the physis that undergoes remodeling changes during growth) and was located 100 µm below the chondro-osseous junction; and (3) region 2^0, which was within the sec-

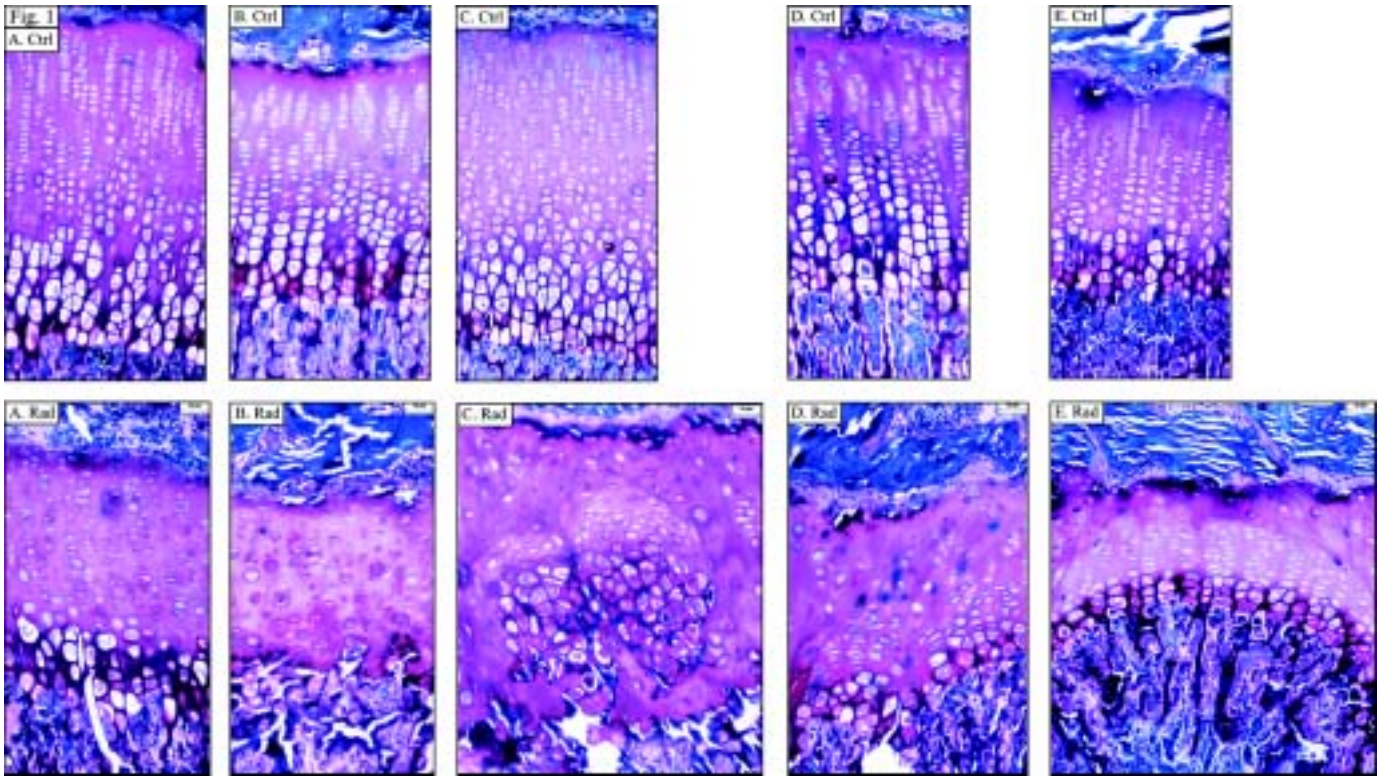

Fig. 1

Proximal tibial growth-plate morphology for both the nonirradiated control (Ctrl) left limbs (above) and the irradiated (Rad) right limbs (below) of the Sprague-Dawley rat at (A) 0.5 week, (B) one week, (C) two weeks, (D) three weeks, and (E) four weeks following a single 17.5-Gy dose of radiation. Staining was performed with use of 2% periodic acid, 1% methylene blue (in 1% borax), 0.15% basic fuchsin (in 10% EtOH), and azure II (with azure II and basic fuchsin mixed in equal parts) (×400).

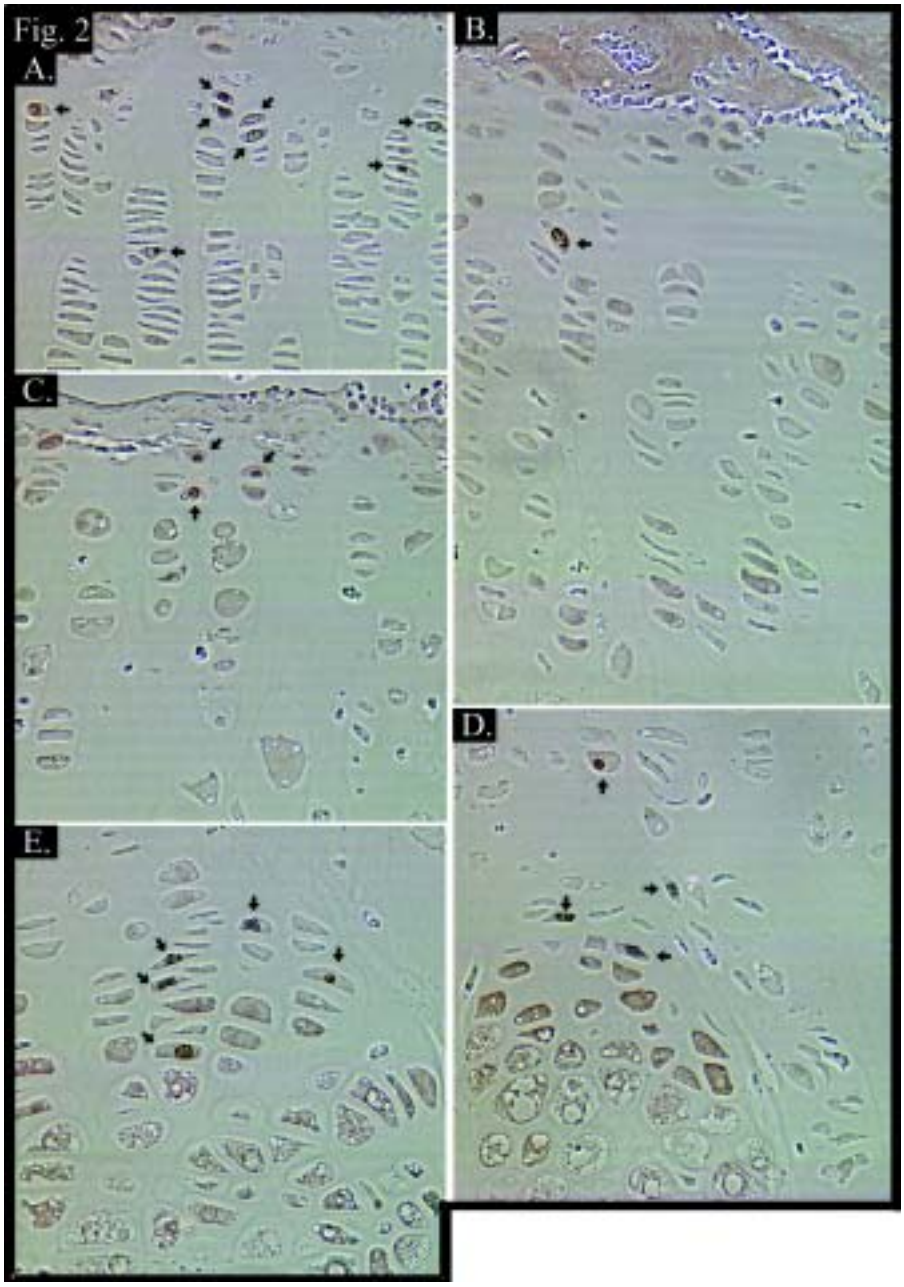

Fig. 2

Immunohistochemical staining of the proximal tibial growth plate of the Sprague-Dawley rat for BrdU labeling of proliferative potential in control (A) and irradiated limbs at 0.5 week (B), one week (C), two weeks (D), and four weeks (E) following irradiation (×400). Note the paucity of labeling (arrows) at 0.5 and one week. Labeling increases through four weeks to nearer-normal levels.

ondary spongiosa (the metaphyseal bone more distant from the physis that undergoes remodeling changes during growth) and was located 800 μm below the chondro-osseous junction. A box of fixed dimensions that followed the contours of the chondro-osseous junction was generated, with the chondro-osseous-junction box being 75 μm × 1.5 mm and the 1^0 and 2^0 boxes being 100 μm × 1.5 mm. Profiles of tartrate-resistant acid phosphatase-positive osteoclasts and chondroclasts were counted in a constant area within the metaphyseal region from the chondro-osseous junction to the secondary spongiosa. Cellular profiles were considered to be osteoclasts or chondroclasts if (1) they stained positively for tartrate-resistant acid phosphatase, (2) they had at least three nuclear profiles, and (3) they apposed either bone (osteoclasts) or cartilage (chondroclasts) with an active resorption front. The ratio of the total number of cellular profiles to the area measured was then calculated[18,19].

A qualitative scale was used to portray the direct and re-

TABLE II Direct and Restorative Qualitative Changes Following Irradiation of the Four-Week-Old Sprague-Dawley Rat

	Magnitude of Change Compared with Controls				
	0.5 Week	1 Week	2 Weeks	3 Weeks	4 Weeks
Direct changes					
Loss of columnation	++	+++	+++	+++	++
Distorted cellular morphology	++	+++	+	0	0
Metaphyseal distortion	0	+	++	0	0
Restorative changes					
Regenerative clones	0	0	+	++	+++
Loss of proliferative BrdU staining	+++	+++	++	++	+

0 = identical to controls, + = mild change, ++ = moderate change, +++ = pronounced change.

storative changes. Scores ranging from 0 (indistinguishable from control) to +++ (pronounced change compared with control) were assigned, with intermediate scores reflecting mild (+) or moderate (++) change. Sections were initially reviewed and assessed for these changes in a random order to minimize bias.

Statistical analysis was performed with StatView software (SAS Institute, Cary, North Carolina) with use of analysis of variance and Fisher's PLSD for the post hoc comparison of growth, growth rates, and objective histomorphometric changes between radiation, control, and amifostine-treated radiation and control groups. The level of significance was $p \leq 0.05$.

Results

Qualitative Effects

Qualitative changes in the tibial growth plate were seen at all time-periods when the irradiated limbs were compared with the contralateral, control (nonirradiated) limbs (Table II and Fig. 1). Beginning at 0.5 week, a loss of normal cell columnation was evident (Fig. 1, *A*). In addition, the cellular morphology within individual zones was distorted, although individual zones were still discernible. The most dramatic differences were seen at one week (Fig. 1, *B*). At that time, the height of the growth plate was notably less than that on the control side and normal columnation was completely absent. Cellular distortion became more pronounced within all zones, with more pyknotic nuclei evident. The zonal architecture became much less obvious even than it was at 0.5 week. Metaphyseal spicules of unresorbed cartilage were first observed at one week.

At two weeks, regenerative clones of chondrocytes first appeared (Fig. 1, *C*). Loss of columnation was still pronounced within and outside of the clones. Cellular morphology within the clones appeared closer to normal, although the hypertrophic cells maintained some distortion. Cellular morphology outside of the clones continued to be highly distorted. The overall height of the irradiated growth plate was as great as or greater than that in the controls, and the height of the hypertrophic zone represented a relatively larger proportion of the overall height of the growth plate than it did in the controls. Metaphyseal spicules were longer and wider than those seen at one week and contained some cellular profiles. At three weeks, the number of regenerative clones had increased but cellular morphology outside of the clones remained distorted (Fig. 1, *D*). Metaphyseal projections were sparse. By four weeks, the number of regenerative clones had increased further and the clones often abutted each other (Fig. 1, *E*). However, even when the clones abutted each other, the cells within them failed to completely regain the normal orderly columnar pattern of organization. The cellular morphology within the clones at both three and four weeks was close to normal and resembled that of proliferating chondrocytes.

Proliferative potential, as assessed with BrdU labeling, was evident throughout the proliferative zone in the normal (control) growth plates (Fig. 2, *A*). However, at 0.5 and one week after irradiation, proliferative potential was markedly diminished (Fig. 2, *B* and *C*). Some of the labeling that was evident at one week occurred within the region that, in a normal growth plate, would be considered the reserve zone. By two weeks, increased BrdU labeling was evident within the proliferative portion of the regenerative clones (Fig. 2, *D*). This labeling increased to nearer-normal levels by four weeks but was still mostly within the proliferative portion of the regenerative clones (Fig. 2, *E*).

Quantitative Effects

In this section, the effects of irradiation alone are described first and the effects of amifostine radioprotectant are described second. However, the figures that are cited in the text display the data for both the radiation effects and the radioprotectant effects simultaneously.

Quantitative Effects of Irradiation Alone

At one week the mean length of the hind limb on the irradiated side was still within 4% of that on the control side, but by two weeks it was 11% shorter (Table III). At four weeks, there was a 15% limb-length discrepancy between the irradiated and control limbs.

The mean lengths of the femur, tibia, and hind limb continued to increase at each time-interval following treatment, but by 0.5 week the increase in total hind-limb length was 27% to 30% less for the irradiated (right) limb than for the control

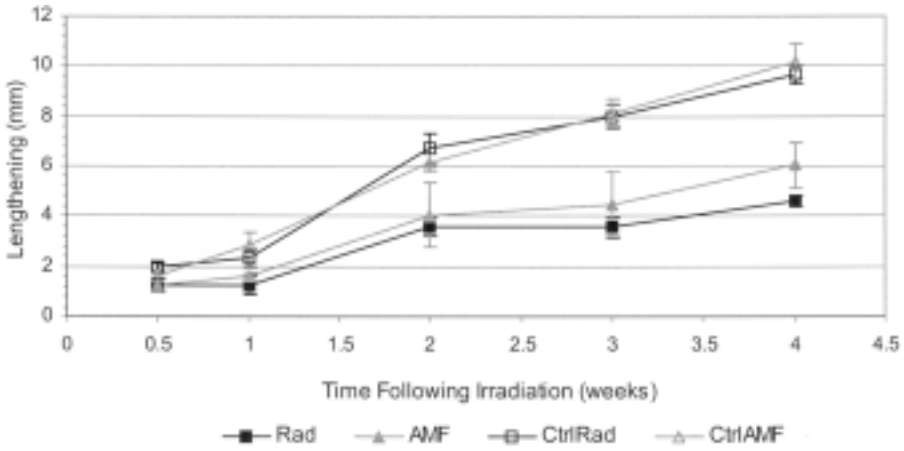

Fig. 3

Growth curves for the hind limb following a single 17.5-Gy dose of radiation. Rad = irradiated (right) limb of animals that did not receive amifostine (100 mg/kg), AMF = irradiated (right) limb of animals that received amifostine, CtrlRad = nonirradiated control (left) limb of animals that did not receive amifostine, and CtrlAMF = nonirradiated control (left) limb of animals that received amifostine.

(left) limb ($p < 0.01$) (Fig. 3). The increase in total hind-limb length was 45% to 48% of control levels at one to two weeks and 57% to 63% of control levels at three to four weeks ($p < 0.05$ for all comparisons). The cumulative increase in tibial length was significantly greater for the nonirradiated control (left) limb than for the irradiated (right) limb beginning at 0.5 week and continuing through four weeks ($p < 0.0001$).

Following irradiation, the proximal tibial growth rate as determined by means of oxytetracycline labeling in the twenty-four hours before the animals were killed decreased dramatically during the first week to 18% of the control level. Nevertheless, some growth continued even at the earliest time-periods, and growth began to return to normal beginning as early as two weeks. Growth ultimately returned to at least 80% of normal by four weeks after irradiation (Fig. 4). The tibial growth rate on the irradiated side was significantly less than that on the nonirradiated (control) side at each time-point ($p \leq 0.001$).

The overall height of the growth plate was reduced most notably (to 65% of the control value) at one week after irradiation and then showed a distinctive increase (to greater than the control level) at two weeks before returning to near normal (to 80% of the control level) by four weeks ($p \leq 0.01$ for all comparisons) (Figs. 5 and 6). The height of the hypertrophic zone on the irradiated side changed in the same direction as the overall height of the growth plate at each time-point. The height of the hypertrophic zone on the irradiated side was significantly different from that on the control side at 0.5, one, and three weeks ($p < 0.05$) and showed a trend toward significance at two weeks ($p = 0.059$). In contrast, the heights of the reserve and proliferative zones on the irradiated side remained close to or insignificantly below those on the control side throughout the period of observation. The height of the hypertrophic zone on the irradiated side was not significantly different from that on the nonirradiated side at four weeks.

The mean number of chondroclast profiles at the chondro-osseous junction in irradiated limbs was significantly lower than that in nonirradiated controls at 0.5 through two weeks before increasing to beyond control levels at three weeks and then returning to control levels by four weeks (Fig. 7, *A*, *B*, and *C*). The mean numbers of osteoclast profiles in both the primary and the secondary spongiosa regions were signifi-

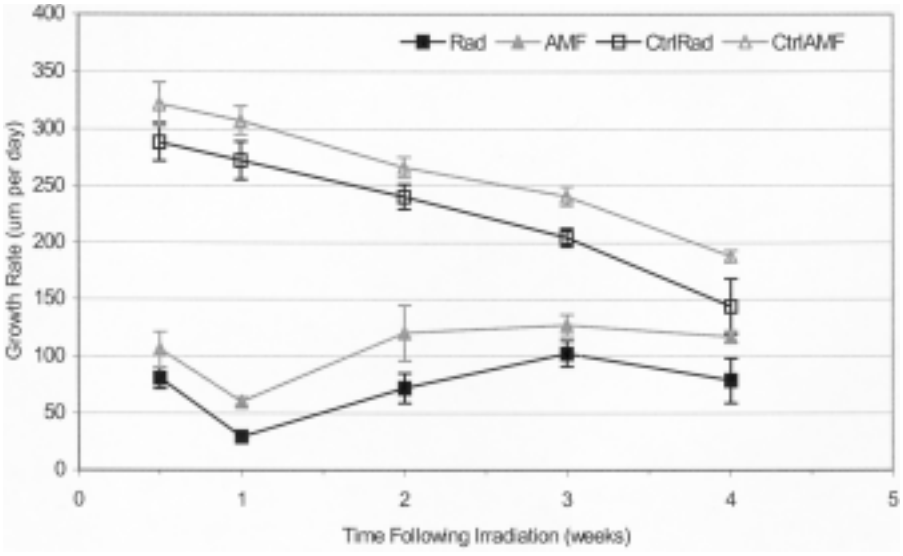

Fig. 4

Tibial oxytetracycline-based proximal tibial growth rates over time following a single 17.5-Gy dose of radiation. Rad = irradiated (right) limb of animals that did not receive amifostine (100 mg/kg), AMF = irradiated (right) limb of animals that received amifostine, CtrlRad = nonirradiated control (left) limb of animals that did not receive amifostine, and CtrlAMF = nonirradiated control (left) limb of animals that received amifostine.

TABLE III Mean Length of Hind Limb Following Irradiation with and without Amifostine Pretreatment

	0.5 Week		1 Week	
	Left Limb (Control)	Right Limb (Irradiated)	Left Limb (Control)	Right Limb (Irradiated)
Radiation-only group*				
Limb length† *(mm)*	51.6 ± 0.9	50.4 ± 0.9	57.8 ± 1.4	55.6 ± 1.3
Side-to-side difference (%)	–2.3%	–3.8%	—	–3.4%
Amifostine-pretreatment group‡				
Limb length† *(mm)*	51.8 ± 0.8	50.8 ± 0.5	59.6 ± 1.0	57.4 ± 1.3
Side-to-side difference (%)	—	–1.9%	—	–3.7%

*The animals in the radiation-only group received a single 17.5-Gy dose of radiation without amifostine pretreatment. †The values are given as the mean and the standard deviation. ‡The animals in the amifostine-pretreatment group received pretreatment with amifostine (100 mg/kg) prior to irradiation with a single 17.5-Gy dose of radiation.

cantly lower than control levels at 0.5 week, reached a nadir at two weeks, increased to greater than control levels at three weeks, and returned to control levels at four weeks.

The area fraction of matrix increased progressively to a peak at two weeks after irradiation, predominately as a result of an increase in the area fraction of matrix in the hypertrophic zone (Fig. 8). These differences in the overall area fraction of matrix reached significance compared with control values only at one week ($p < 0.05$). However, the area fraction of matrix in the hypertrophic zone of the irradiated tibia was significantly greater than that of the nonirradiated tibia from 0.5 through two weeks after irradiation ($p < 0.05$). The area fraction of matrix in the hypertrophic zone returned to control levels at three and four weeks, corresponding with the increasing number of regenerative clones identified during those time-periods and with the return of chondroclasts.

Quantitative Effects of Amifostine Radioprotectant

The mean increase in total limb length was significantly greater in the animals that had been pretreated with amifostine than in the animals that had been treated with irradiation alone at three ($p < 0.01$) and four weeks ($p = 0.01$) (Fig. 3). The mean increase in tibial length was also significantly greater in the pretreated animals at three and four weeks, whereas the mean increase in femoral length was significantly greater in the pretreated animals only at three weeks ($p < 0.05$). Despite these significant improvements in lengthening in the limbs that had been pretreated with radioprotectant compared with those that had been treated with radiation only, the increase in length remained significantly less in the radioprotected limbs than in the control limbs ($p < 0.05$). This resulted in a persistent 13% limb-length discrepancy between the amifostine-pretreated irradiated limbs and the control limbs after four weeks (Table III).

The administration of amifostine prior to irradiation significantly reversed the radiation-induced reduction in the twenty-four-hour tibial growth rate, resulting in a significantly higher growth rate, compared with that in the tibiae treated with irradiation alone, from one to four weeks after irradiation ($p < 0.05$) (Fig. 4). Nevertheless, the growth rate in the amifostine-protected limbs did not approach normal until four weeks after irradiation. Prior to four weeks, the mean twenty-four-hour growth rate for the amifostine-treated limbs remained less than half of that for the controls.

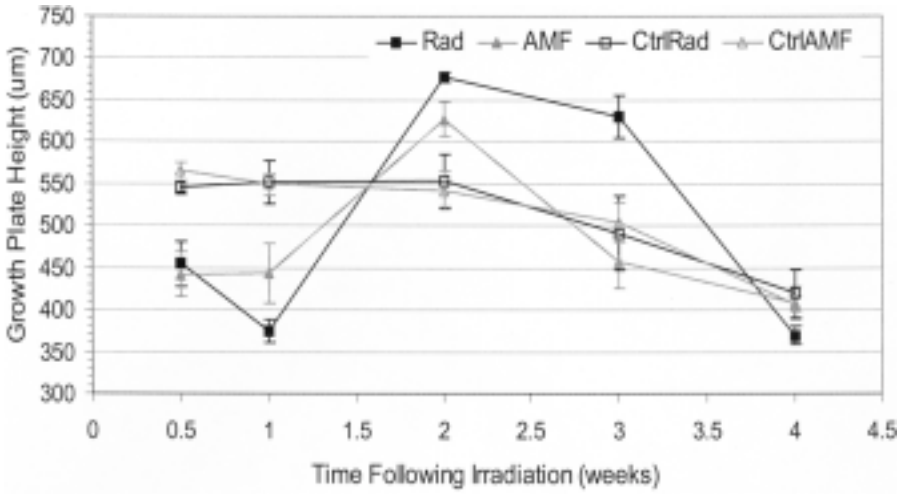

Fig. 5

Overall height of the growth plate following a single 17.5-Gy dose of radiation. Rad = irradiated (right) limb of animals that did not receive amifostine (100 mg/kg), AMF = irradiated (right) limb of animals that received amifostine, CtrlRad = nonirradiated control (left) limb of animals that did not receive amifostine, and CtrlAMF = nonirradiated control (left) limb of animals that received amifostine.

TABLE III (continued)

2 Weeks		3 Weeks		4 Weeks	
Left Limb (Control)	Right Limb (Irradiated)	Left Limb (Control)	Right Limb (Irradiated)	Left Limb (Control)	Right Limb (Irradiated)
62.0 ± 1.0	55.3 ± 1.2	65.3 ± 1.7	56.4 ± 2.0	69.6 ± 1.4	59.2 ± 1.4
—	–10.8%	—	–13.6%	—	–14.9%
62.7 ± 0.6	57.4 ± 1.8	65.7 ± 2.3	58.1 ± 1.9	69.7 ± 1.3	60.9 ± 1.1
—	–8.5%	—	–11.6%	—	–12.6%

The administration of radioprotectant prior to irradiation also significantly reversed the radiation-induced changes in the overall height of the growth plate ($p < 0.05$ at one through four weeks) and the height of the hypertrophic zone ($p < 0.01$ at one and three weeks) (Figs. 5 and 6). The overall height of the growth plate in the limbs that had been pretreated with amifostine was not significantly different ($p > 0.05$) from that in the nonirradiated limbs at three and four weeks after irradiation.

At all time-points, the number of chondroclast profiles in the region of the chondro-osseous junction was significantly higher in limbs that had been pretreated with amifostine than in limbs that were treated with radiation only, irrespective of whether the amifostine-pretreated limbs were irradiated or not (Fig. 7). In contrast, the numbers of osteoclast profiles in both the primary and the secondary spongiosa regions of limbs that had been pretreated with amifostine were indistinguishable from those of limbs that were treated with radiation only.

During the first two weeks, the area fraction of matrix in the hypertrophic zone of limbs that had received radioprotectant prior to irradiation was intermediate between that of control limbs and that of limbs that received irradiation only (Fig. 8). By three and four weeks, this area fraction was indistinguishable among control limbs, irradiated limbs that had been pretreated with radioprotectant, and irradiated limbs that had not been pretreated with radioprotectant.

Discussion

The present report describes and quantifies the damaging effects of a single 17.5-Gy dose of gamma radiation on an active growth plate in the Sprague-Dawley rat model. These findings are in general agreement with previous reports that have supported a direct inhibitory effect of radiation on the growth plate chondrocyte[7-12]. This effect was illustrated by the dramatic reduction in proliferative activity and growth rate that we observed as early as 0.5 week after irradiation and by the marked loss of cellularity and organization that reached its nadir at one week after irradiation. Arguelles et al.[7], in a study of nineteen rabbits, investigated the effects of a single 10-Gy dose of gamma radiation on the basis of subjective histological findings, tritiated thymidine uptake, and a fluorescent marker-based growth rate. In that study, the sensitivity of the rabbit growth-plate chondrocyte to irradiation was demonstrated by nonexistent tritiated thymidine uptake at twenty-four hours and one week after irradiation. The BrdU labeling utilized in the current Sprague-Dawley rat model demonstrated a marked reduction of proliferative potential within proximal tibial physeal chondrocytes at 0.5 and one week following irradiation, but the presence of some labeling even at the earliest time-periods suggests that some proliferative activity was maintained, at least in isolated cells, after the administration of a 17.5-Gy dose of radiation. The presence of BrdU labeling in some areas of the reserve zone at one week implies that at least some reserve-zone cells had proliferative potential during the post-irradiation recovery. The oxytetracycline-based growth rate in the proximal tibial physis in the current rat model also did not decrease to zero but rather was maintained at no less than 18% of the control growth rate at these time-periods, suggesting incomplete inhibition of growth within the radiation field, another novel finding.

The maintenance of growth even at the nadir point following irradiation may have been attributable to any of the components known to contribute to active growth, including proliferation, matrix production, and cellular hypertrophy[15]. The finding of diminished but active proliferation demonstrates that continued proliferation contributes in at least a small way to this elongation, perhaps by way of reserve-cell repopulation of the proliferative zone. In their normal state, reserve-zone growth-plate chondrocytes may be less susceptible to damage from irradiation than actively proliferating cells are. Cellular hypertrophy, the major source of elongation in the rapidly elongating rodent growth plate, appears unlikely to have contributed to this process at the point of maximal damage as overall cellularity appeared to be diminished at that time, at least relative to the area composed of matrix. Matrix production, however, appears likely to have been the most important contributor to continued elongation at the nadir at

one week. This conclusion is supported by the significantly greater area fraction of matrix in the hypertrophic zone in the irradiated limbs than in the control limbs at that time-point. Both the height of the growth plate and the area fraction of matrix then reached their peak at two weeks. This finding corresponded with a time when chondroclasts were also noted to be nearly completely absent, suggesting an inability to resorb matrix. Only after the chondroclasts were noted to have returned did the percent area occupied by matrix and the overall height of the growth plate return to normal. These observations regarding the accumulation of matrix after irradiation are supported by the finding, reported by Jikko et al.[20] , that irradiation does not affect the synthesis of type-II collagen, the major collagen of the growth plate matrix. Furthermore, Wilsman et al.[15] demonstrated that the mean contribution of matrix synthesis to normal growth of the proximal part of the tibia in the four-week-old Long-Evans rat was 32%, comparable in magnitude to the 18% of the control growth rate that we observed at the nadir after irradiation. More sophisticated BrdU-labeling studies will be necessary to determine the major contributor to elongation in this setting[17].

Recovery from this initial growth-plate injury following irradiation has been demonstrated previously in other animal models, albeit at varying time-courses specific to the animal model and radiation dose. Barr and colleagues, in 1943, apparently were the first to describe light microscopic evidence of regeneration as "clusters of viable chondrocytes which are normal in appearance but not in distribution."[8] These clusters were later labeled "clones" by Kember[11,12]. Arguelles et al., in a rabbit model, observed that subjective evidence of growth-plate injury began at seven days after irradiation and became most advanced at fourteen and twenty-one days, with "regen-

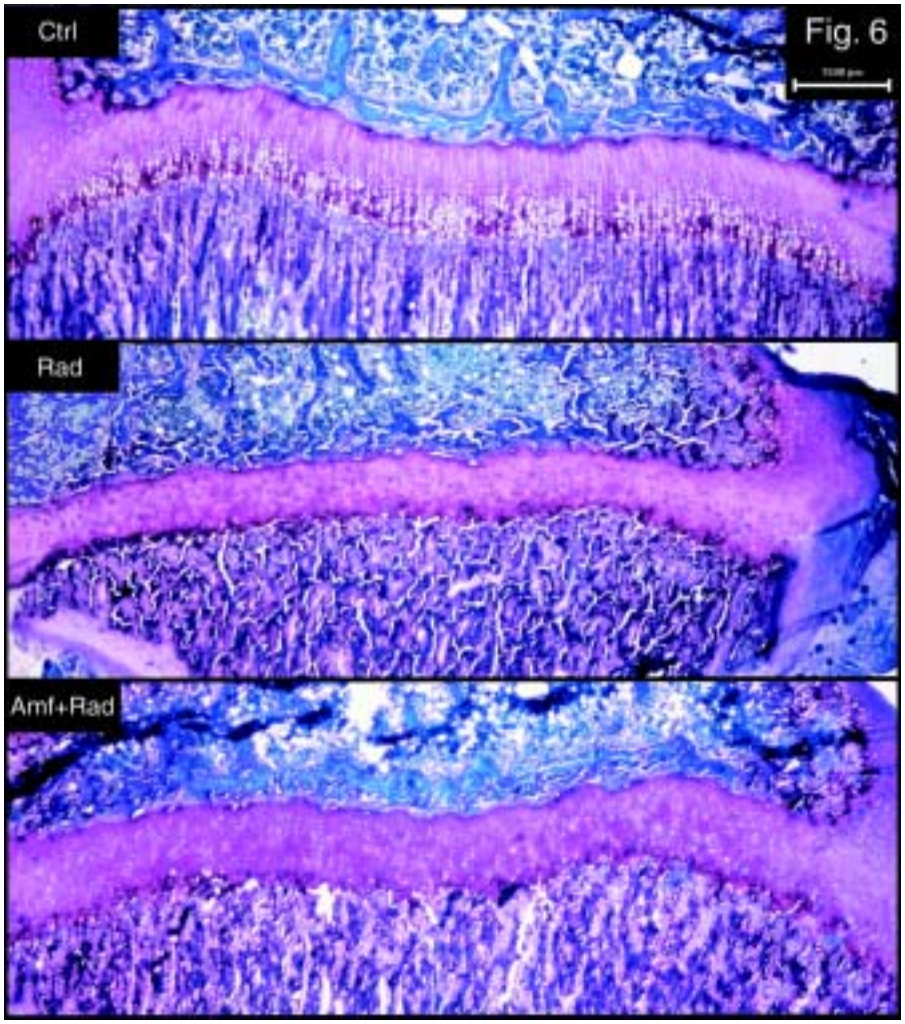

Fig. 6

Histological effects of irradiation and amifostine treatment at one week compared with controls. *Top,* Entire width of control (left) nonirradiated proximal tibial growth plate from an animal that received irradiation of the right limb without amifostine pretreatment. *Middle,* Irradiated right proximal tibial growth plate from an animal that did not receive amifostine pretreatment. *Bottom,* Irradiated right proximal tibial growth plate from an animal that received amifostine pretreatment. Staining was performed with use of 2% periodic acid, 1% methylene blue (in 1% borax), 0.15% basic fuchsin (in 10% EtOH), and azure II (with azure II and basic fuchsin mixed in equal parts) (×400).

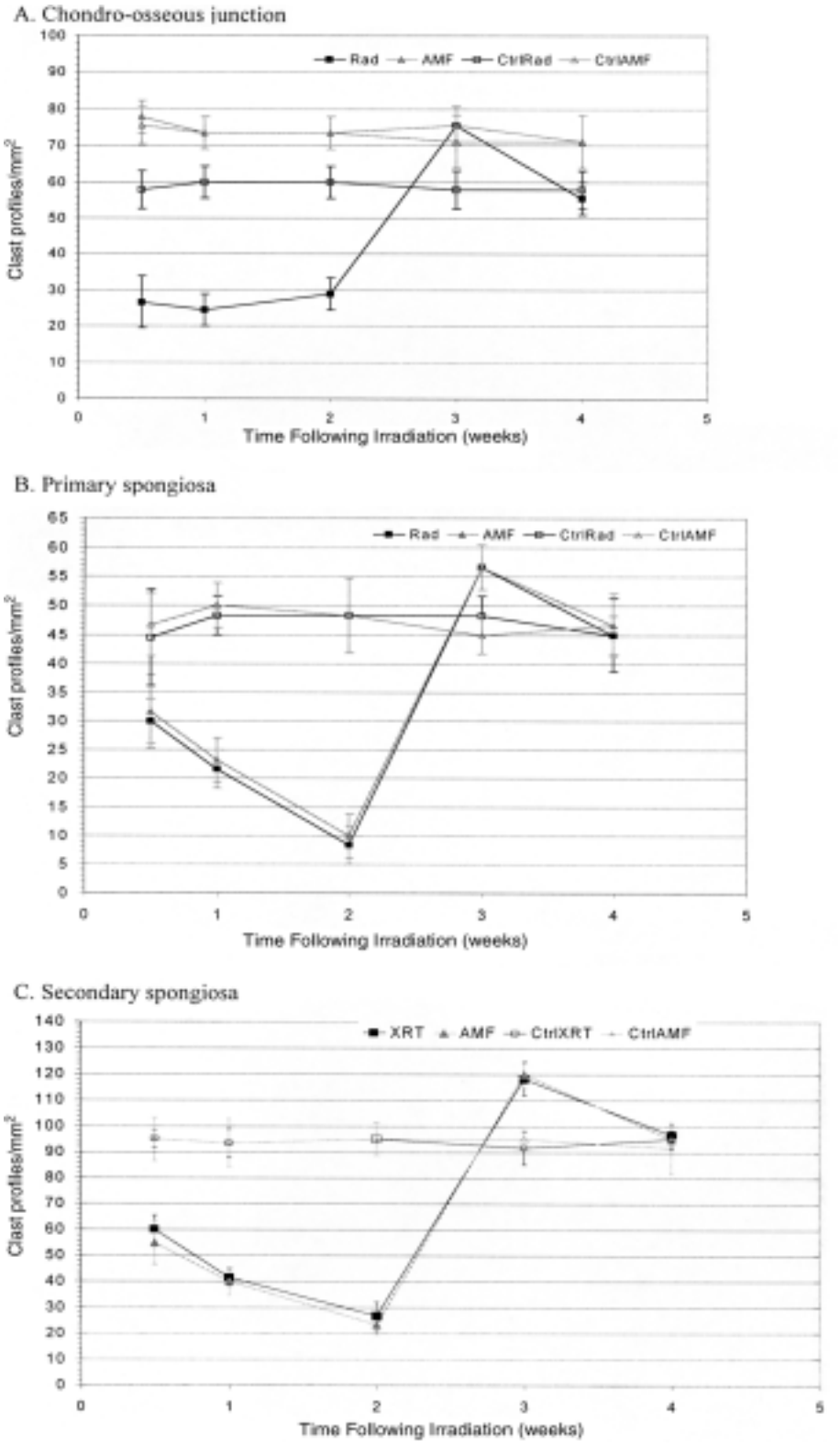

Fig. 7

Illustrations depicting the number of chondroclasts per area within the chondro-osseous junction region (A), the primary spongiosa region (B), and the secondary spongiosa region (C). Rad = irradiated (right) limb of animals that did not receive amifostine (100 mg/kg), AMF = irradiated (right) limb of animals that received amifostine, CtrlRad = nonirradiated control (left) limb of animals that did not receive amifostine, and CtrlAMF = nonirradiated control (left) limb of animals that received amifostine.

eration" beginning at four weeks[7]. In the present study of Sprague-Dawley rats, the effects of irradiation were evident morphologically at 0.5 week and were most obvious at one week after irradiation. Recovery was demonstrated by the return toward a nearer-normal growth rate beginning at two weeks, with the growth rate increasing to approximately 80% of normal by four weeks. Further objective evidence of the return of proliferative activity was demonstrated by the in-

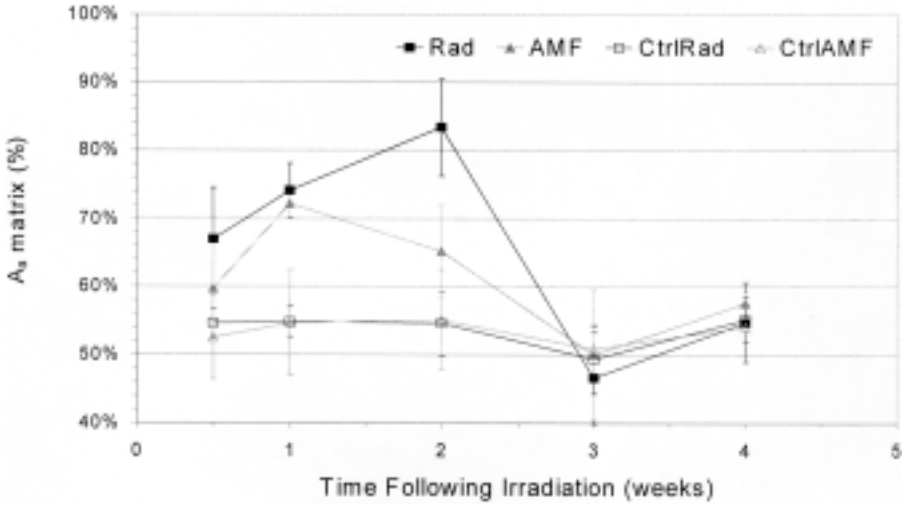

Fig. 8

Area fraction of matrix in the hypertrophic zone following a single 17.5-Gy dose of radiation. Rad = irradiated (right) limb of animals that did not receive amifostine (100 mg/kg), AMF = irradiated (right) limb of animals that received amifostine, CtrlRad = nonirradiated control (left) limb of animals that did not receive amifostine, and CtrlAMF = nonirradiated control (left) limb of animals that received amifostine.

creased BrdU labeling within regenerative clones that began at two weeks after irradiation and continued through four weeks. The present study appears to be the first to provide objective evidence that the functional recovery of the growth plate after irradiation corresponds temporally with the appearance of proliferative clones.

There is evidence in the literature that irradiation may have little effect on matrix production by the growth plate. Jikko et al. showed that, in a mature rabbit chondrocyte culture system in which chondrocytes had established extensive cartilage matrix production, irradiation with up to 10 Gy did not affect the rate of proteoglycan synthesis[20]. Other in vitro studies of growth-plate chondrocytes have shown suppression of mitogenic factors (bFGF, TGF-beta) with retention of collagen expression following irradiation[21]. We also observed indirect evidence supporting reduction in growth-plate matrix resorption. The numbers of profiles of chondroclasts and osteoclasts reached a nadir at two weeks, corresponding with the time at which the height of the growth plate was the greatest. The numbers of profiles of chondroclasts and osteoclasts then increased beyond normal levels at three weeks, corresponding with the return toward normal growth-plate height. Hence, the inhibitory effect of irradiation on chondroclasts and osteoclasts appeared to be temporally related to the changes in the height of the growth plate. One unexpected observation was the difference between chondroclasts and osteoclasts in terms of their response to amifostine. The numbers of chondroclast profiles per area in the amifostine-treated limbs, whether irradiated or not, were greater than those in even the nonirradiated control limbs without amifostine treatment (Fig. 7, *A*). In contrast, amifostine had little effect on osteoclasts (Fig. 7, *B* and *C*). This observation may have implications with regard to the potentially differing origins and differentiation of the two cell types.

The effects of amifostine in ameliorating the damaging effects of irradiation were apparent in the growth measurements at all time-periods examined. When administered prior to irradiation, amifostine resulted in growth-plate changes that generally were intermediate between those observed for nonirradiated and irradiated growth plates. The lack of consistently significant differences in terms of the increase in limb length between the limbs that were treated with amifostine (100 mg/kg) and the limbs that received radiation without radioprotectant at these early time-points is not surprising. The effects of amifostine on the growth plate are dose-dependent[5]. The dose of amifostine (100 mg/kg) that was used in the present experiment is at the low end of the spectrum of previously documented effectiveness in bone[5]. The improvement in limb growth with this low dose of amifostine has been shown to reach significance by six weeks after irradiation[3].

A limitation of the present experiment lies in the relatively late time-points examined following the radiation insult and in the absence of an examination for potential mediators of the response. One of the major direct results of radiation is cell death. Classically, radiotherapy has been thought to result in cell death due to unrepaired DNA strand breaks or reproductive or mitotic cell death[22]. Morphological evidence of this type of cell death typically appears between twenty-four and ninety-six hours after irradiation. Increasingly, apoptosis has been recognized as an important cause of cell death following irradiation. Apoptosis is also referred to as programmed cell death or interphase death, and it includes cell death that occurs before the first post-irradiation mitosis. This latter type of cell death occurs within four to six hours after irradiation[22]. Hence, examination of the growth plate at much earlier time-points is needed in order to better evaluate the mechanism of the effects of radiation and radioprotectant treatment on cell death, particularly that due to apoptosis.

The importance of these findings is the demonstration of quantifiable transient changes in the growth plate following irradiation that are reduced by a clinically utilized radioprotectant drug. Radioprotectants delivered prior to therapeutic irradiation may have potential for reducing the damaging effects on the growth plate while preserving desirable effects on a tumor. Additional study of the effects of radioprotectants on the growth plate is warranted. ■

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