Radiation Effects on Osteoblasts In Vitro

A Potential Role in Osteoradionecrosis

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Objective: To evaluate the factors involved in bone remodeling and wound healing that may be altered by radiation therapy.


Subjects: MC3T3-E1 mouse osteoblasts.

Interventions: Cells were irradiated at 0, 2, 4, or 6 Gy. Specimens were harvested at 1, 7, 14, 28, and 42 days following irradiation for immunohistochemical analysis of transforming growth factor β1 expression and transforming growth factor β1 type I and II receptor expression. Collagen production was measured at 1, 7, 28, 35, and 49 days after irradiation. The effects of dexamethasone on collagen production and cell proliferation were also examined.

Results: Irradiated cells demonstrated decreased cell proliferation and a dose-dependent, sustained reduction in collagen production when compared with control cells. An increase in transforming growth factor β1 type I and II receptor expression was noted in irradiated cells when compared with controls.

Conclusion: Radiation-induced alterations of factors related to bone remodeling and wound healing have a potential role in the pathogenesis of osteoradionecrosis.

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OSTEORADIONECROSIS is a complication of radiation therapy for carcinoma of the head and neck that occurs in 3% to 10% of irradiated patients. Its pathogenesis has been classically described as a progression of irradiation, trauma, and infection. Several theories exist about its etiology. Osteoradionecrosis has been attributed to radiation fibrosis of small blood vessels. An increased incidence of osteoradionecrosis has likewise been associated with dental caries within the field of irradiated bone. The risk of bone necrosis is believed to be related to the amount of radiation, although the injury does not always occur at the area of highest dosage.

Most of the literature on osteoradionecrosis has generally been retrospective. Therapy consists of adequate debridement and stabilization. Hyperbaric oxygen is believed to play a role in treatment through the reversal of tissue hypoxia, although its success has not been universal. The effects of radiation on bone at the molecular level have received little attention. In vitro studies have been primarily limited to survival curves and dose-response relations of marrow cells to study the treatment of myelogenous disorders. This scarcity of data is largely secondary to the difficulty in maintaining the osteoblast phenotype in culture.

Our initial studies involved immunohistochemical analysis of archived specimens of osteoradionecrosis of the mandible. Because of the necrosis and cellular debris present in all specimens tested, significant artifact and background staining resulted, rendering interpretation of the studies difficult at best. It was thought that perhaps this was a result of inadequate sampling of affected bone in the specimens; however, similar difficulties were encountered when sufficient specimens of osteoradionecrotic mandible from more recent cases were sampled. We considered the possibility that perhaps by the time patients with osteoradionecrosis presented for surgical debridement, there was already too much bone loss for any useful conclusions to be formulated from surgical specimens.

This pilot study examined the effects of radiation on the osteoblast phenotype in culture. In vitro studies have been primarily limited to survival curves and dose-response relations of marrow cells to study the treatment of myelogenous disorders. This scarcity of data is largely secondary to the difficulty in maintaining the osteoblast phenotype in culture.

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This pilot study examined the effects of radiation on the MC3T3-E1 osteoblast cell line in an attempt to establish that the clinical phenomenon of osteoradionecrosis was attributable, to some extent, to radiation-induced sublethal damage, resulting in an alteration of the expression of growth factors necessary for normal bone formation and bone healing in the surviving fraction. Alteration in the expression of these growth factors may account for the inability of oth-
MATERIALS AND METHODS

TISSUE CULTURE

MC3T3-E1 mouse osteoblasts were clonally derived from murine calvariae; this cell line maintains osteoblast phenotype despite repeated subcultivation. They are unlike primarily obtained cell populations from calvariae in that the interpretation of the data obtained from these cells is not limited by the presence of other cell populations (fibroblasts and chondroblasts) at various developmental stages. The MC3T3-E1 cell line has been shown to be a viable model for exploring the molecular mechanism of osteoblast proliferation, maturation, and differentiation. Cells were grown to approximately 80% confluence in minimal essential medium supplemented with a combination of 1% penicillin and streptomycin sulfate and 10% fetal bovine serum, and maintained by incubation at 37°C in 5% carbon dioxide. Cells were subcultured every 3 to 4 days using 0.001% pronase using a standard technique for adherent cells.

PHENOTYPE CHARACTERIZATION

Osteoblast phenotype was confirmed by von Kossa staining for the presence of calcium (data not shown) and by alkaline phosphatase assay. For the alkaline phosphatase assay, known quantities of cells were harvested and lysed with a nonionic reagent (Triton X-100; Sigma-Aldrich Inc, St Louis, Mo). Cell lysate was then incubated with disodium p-nitrophenol phosphate. Alkaline phosphatase activity was quantified using spectrophotometric measurement of the resulting free nitrophenol at 410 nm. Alkaline phosphatase activity was measured in cells treated with TGF-β (R&D Systems, Inc, Minneapolis, Minn) 5 ng/mL of media, or bone morphogenetic protein 4 (Genetics Institute, Inc, Cambridge, Mass), 40 ng per 6-well plate. In well-differentiated osteoblasts, alkaline phosphatase production is normally enhanced by the addition of bone morphogenetic protein 4 and suppressed by the addition of TGF-β1.

IRRADIATION METHODS

Cells were irradiated in cooperation with the Department of Radiation Oncology, University of South Florida, Tampa, using a linear accelerator (Mevatron KD; Siemens Medical Systems, Oncology Care Systems Group, Concord, Calif). Because correlation between in vitro and clinical dose range is unknown, preliminary studies were conducted to determine a dose range appropriate for the study of this particular cell line. A dose fraction of 2700 Gy/min was used in earlier studies and appeared to be adequate for the purposes of this experiment. Because the intent was not to examine osteoblast death rates but rather to examine the effects of radiation on the surviving fraction, a dose range of 0 to 6 Gy was used. Preliminary data suggested that this range was sufficient for the examination of sublethal injury and alteration of growth factor expression. Doses tested above 6 Gy demonstrated significant death rates, with degeneration of the cell line. Cells were irradiated at doses of 2, 4, and 6 Gy, and were compared with control cells.

COLLAGEN PRODUCTION

The effects of radiation on collagen production were measured. Cells, 5 x 10^4 per well, were radiolabeled with tritiated proline, 1 µCi/mL (DuPont, Wilmington, Del) in 6-well plates for 2 hours. Cell layers were lysed by freeze thawing and extracted in 0.5% nonionic reagent (Triton X-100). Samples were precipitated with trichloroacetic acid and chilled, and insoluble material was collected by centrifugation. Tritiated proline incorporation into collagen and noncollagen protein was measured by differential sensitivity to bacterial collagenase free of nonspecific protease activity.

A 2-way analysis of variance was used to examine the effect and interaction of radiation dosage and time on the production of collagen.

IMMUNOHISTOCHEMISTRY

Immunohistochemical analysis was performed on slides obtained from paraffinized cell blocks that were prepared from cell suspensions harvested at appropriate intervals. Specimens were stained for TGF-β1 at 1:100 dilution, TGF-β type I receptor (Santa Cruz Laboratories, Santa Cruz, Calif) at 1:100 dilution, and TGF-β type II receptors (T.M.-A.) at 1:50 dilution. Samples were collected for staining at 1, 7, 14, and 28 days following radiation. A pathologist (C.A.M.-C.), blinded to the radiation dose, measured the intensity of staining, which was graded on an ordinal 4-point scale.

The technique was performed as follows. Endogenous peroxidase was quenched with a 3% solution of hydrogen peroxide for 20 minutes at 37°C. Slides were washed twice in deionized water. Antigen retrieval was performed as follows. Slides were placed in citrate buffer (4.5 mL of citric acid, 0.1 mol/L; 21.5 mL of sodium citrate, 0.1 mol/L; and 225 mL of deionized water) in a clear plastic container with a vented top and microwaved on high power 2 times for 5 minutes. Slides were allowed to cool for 10 to 20 minutes, rinsed well in deionized water, and placed in phosphate-buffered saline for 5 minutes. Slides were drained, and blocking serum was applied. Slides were incubated in a humid chamber for 20 minutes at room temperature. After draining slides, the first antibody was applied at 1:100 dilution for 1 hour at room temperature. Slides were rinsed and placed in phosphate-buffered saline for 5 minutes. For detection, an avidin-biotin complex kit (Vectastain ABC Kit, Rabbit IgG, Elite series; Vector Laboratories, Burlingame, Calif) was used. The biotinylated secondary antibody was applied for 20 minutes at room temperature in a humid chamber. Slides were rinsed and placed in phosphate-buffered saline for 5 minutes. The avidin-biotin complex was added, and slides were incubated in a humid chamber for 30 minutes at room temperature. Slides were rinsed and placed in phosphate-buffered saline for 5 minutes. Diamino benzidine was applied, and color was developed until the desired intensity was reached (2-5 minutes). Slides were rinsed in water and counterstained for 30 seconds with Mayer hematoxylin. Slides were rinsed in running water for 10 minutes, dehydrated, cleared, and mounted with resinous mounting medium.

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RESULTS

Normal production of calcium was confirmed in irradiated and control cells by von Kossa staining. Alkaline phosphatase activity, which is increased by the addition of bone morphogenetic protein 4 to the media and is unaffected by treatment with TGF-β1, was also examined. The cells demonstrated a phenotype characteristic of well-differentiated osteoblasts, and maintained this phenotype in the presence of radiation treatment, although the alkaline phosphatase activity was slightly decreased in irradiated cells (Figure 1). These assays confirmed that the change in irradiated cells did not result from an alteration in phenotype.

Osteoblast proliferation was inhibited by radiation treatment. The altered rate of proliferation appeared to be a function of an attenuated growth rate in irradiated cells and not a function of initial radiation-induced cell death, as it remained evident with repeated subcultivation of viable cells. Osteoblast proliferation was decreased in cells treated with dexamethasone, consistent with the known effects of corticosteroids on bone formation. However, this effect appeared to be augmented by the addition of radiation (Figure 2).

Irradiated cells demonstrated a prolonged reduction in collagen production when compared with controls. A distinct difference in collagen production was seen between cells treated at 0 and 2 Gy when compared with those treated at 4 and 6 Gy (Figure 3). Cells irradiated at 4 and 6 Gy demonstrated a statistically significant decrease in collagen production when compared with controls ($P = .03$ and $P = .04$, respectively). This effect appeared to diminish over time. While dexamethasone diminished collagen production in control groups, this effect was less pronounced in the presence of radiation (Figure 4).

The glucocorticoid effects of bone loss may be mediated by a decrease in TGF-β1 type I receptors. As we were able to demonstrate a radiation-induced decrease in collagen production, we explored the possibility that this was due to an alteration of TGF-β1 receptor expression. The effects of radiation on TGF-β1 receptor (type I and II) expression were measured at 1, 7, 14, 28, and 42 days following radiation. The effects were most dramatic at 7 days after therapy (Figure 5). An alteration in levels of TGF-β1 protein expression after radiation was not demonstrated by immunohistochemical methods.

In summary, the effects of radiation on irradiated MC3T3-E1 osteoblasts include decreased proliferation, decreased collagen production, and increased TGF-β type I and II receptors; the additional effects of dexamethasone on irradiated MC3T3-E1 osteoblasts include a further decrease in proliferation and no significant effect on collagen production.

COMMENT

To our knowledge, few studies have looked at the process known as osteoradionecrosis at the cellular or molecular
level. In 1983, Marx\textsuperscript{12} examined 26 cases of osteoradionecrosis of the mandible. Bacterial cultures from deep sections of the necrotic bone were negative for microorganisms in all cases. Only cultures from the bone surface grew microorganisms. He concluded that osteoradionecrosis is not a result of infection but of wound healing, and likened the process to that of a diabetic ulcer of the extremity rather than to osteomyelitis, as previously believed.\textsuperscript{12} He contended, however, that this process was the result of tissue hypoxia and not a result of radiation-induced effects on the osteoblast itself. Rohrer et al\textsuperscript{13} looked at the effects of cobalt 60 irradiation on the mandible of 8 monkeys. Within the field of irradiation, the periosteum exhibited a loss of cellularity, a loss of vascularity, and a loss of osteoid formation. Interestingly, they also noted a marked proliferation of new bone within the medullary area.\textsuperscript{13} Studies\textsuperscript{14} using technetium Tc 99m medronate scintigraphy also showed increased uptake and, therefore, increased metabolic activity in mandibular specimens with osteoradionecrosis, which demonstrated increased sclerosis with large areas of resorption and fibrosis. The researchers do not speculate, however, as to the origin of this intimal fibrosis. The theory of radiation-induced vascular compromise of the mandible is brought into question by the relative infrequency of osteoradionecrosis in irradiated mandibles when compared with normal mandibles.

Bras et al\textsuperscript{15} studied the histology of 17 mandibular specimens with osteoradionecrosis, which demonstrated necrosis with large areas of resorption and fibrosis. The significance of the consistent finding of intimal fibrosis within the inferior alveolar artery was emphasized. This, in conjunction with the failure of revascularization by branches of the facial artery to the periosteum, was believed to be the etiology of the radiation-induced ischemic necrosis, or osteoradionecrosis.\textsuperscript{15} The researchers do not speculate, however, as to the origin of this intimal fibrosis. The theory of radiation-induced vascular compromise of the mandible is brought into question by the relative infrequency of osteoradionecrosis in irradiated vascularized bone grafts.\textsuperscript{16}

Ideally, the radiation dose used for the study of normal tissue in vitro would be similar to the clinical dose in vivo. Using this dose, the radiation exposure to the mandible could be simulated and its effects on the osteoblasts could thus be appropriately correlated. While correlations have been made between in vitro sensitivity of squamous cell carcinoma to radiation and clinical response, no definitive relationship has been established between in vitro radiation dose and in vivo dose.\textsuperscript{17–21} The modifying effects of hypoxia and intercellular contact are believed to contribute to the differences between in vivo and in vitro radiation effects. It has been shown that the discrimination between cell lines is best around 2 Gy. Acutely, at low doses (<2 Gy), cells repair most sublethal DNA damage successfully. The extent of recovery reflects the incidence of repairable lesions rather than recovery capacity. Radiosensitivity at low doses is, therefore, determined by the induction of nonrepairable lesions.\textsuperscript{22} In this study, cells irradiated at 2 Gy demonstrated similar results to controls, as would be expected. Radiation effects were most pronounced at 4 Gy when compared with controls, while doses above 6 Gy demonstrated significant radiation toxicity and death, rendering them inadequate for study.

Stimulatory effects of TGF-\(\beta\) on type I collagen synthesis is important for the structural integrity of bone. Collagen production is essential for the creation of osteoid, necessary for the formation and remodeling of bone. There is some indication that the increased levels of type I collagen are a result of activation of type I collagen genes by TGF-\(\beta\). A relation between levels of the TGF-\(\beta\) receptor and collagen production in bone has been demonstrated.\textsuperscript{11} However, it is likely that the net increase in collagen production as a result of TGF-\(\beta\) activity probably involves multiple transcriptional, posttranscriptional, and posttranslational events.\textsuperscript{3} For example, TGF-\(\beta\) has been shown to increase the synthesis of dinoprostone in the MC3T3-E1 osteoblast cell line. Dinoprostone enhances production of insulin-like growth factor I, which in turn enhances type I collagen formation in bone matrix.\textsuperscript{23}

While there appears to be an alteration in TGF-\(\beta\) receptor expression in irradiated osteoblasts, no distinct relation was apparent that could provide an explanation for the observed decrease in collagen production. The observed decrease in collagen production may be due to an alteration in the overall ratio of type I–type II receptors, which cannot be detected by this technique. Alterations in receptor ratios have been attributed to changes in collagen production in previous studies.\textsuperscript{24} It may also be the result of alteration of TGF-\(\beta\)-independent pathways through changes in other growth factors not examined in this study.

Immunohistochemistry may not be the most sensitive means by which to demonstrate alteration in expression of DNA coding for the proteins relevant to this study. It is possible that in situ hybridization or, more likely, poly-
merase chain reaction analysis may prove to be a more appropriate means to investigate this question. Despite this, differences in the degree of growth factor receptor expression between irradiated cells and controls were evident. This implies that the clinical entity of osteoradionecrosis may to some degree be the result of an alteration in the ability of irradiated bone to remodel and regenerate. This may be due to altered sensitivity to growth factors such as TGF-β, or others, resulting in an impairment of collagen production.

Osteoradionecrosis most commonly occurs clinically months to years after exposure to radiation, a difficult phenomenon to simulate in the laboratory. However, we believe that the data derived from this experiment are applicable to the disease process for several reasons. First, normal cell repopulation in vitro is much more rapid than in vivo. Thus, an in vitro incubation time of 4 to 6 weeks represents a significantly longer fraction of time in the life cycle of an in vivo osteoblast. Second, the process of radiation-induced bone injury is a progressive process that may begin well before the disease becomes clinically apparent. If osteoradionecrosis is indeed a function of alteration in growth factor expression in the irradiated osteoblast, this should be apparent at the cellular level long before one would expect to see the disease clinically. Clinical presentation of disease may be a function of individual treatment tolerance, or the degree to which this alteration of receptor activity occurs in vivo. The degree to which subclinical changes occur in bone subjected to radiation therapy is unknown.

Clinically, osteoradionecrosis involves tissue death and impaired healing of tissues that contribute to normal bone health. It likely involves the complex interaction between radiation effects on the numerous cell types present in bone. For example, in contrast to the effects on osteoblasts noted in this study, radiation has been shown to augment in vitro production of collagen in several fibroblast cell lines.24-26 This was confirmed in an ancillary study in our laboratory using comparable radiation doses (data not shown). The interaction between these cell types in vitro was not examined.

The uniqueness of this particular study is that it demonstrates a discrete alteration in factors involved in bone remodeling and wound healing that is independent of the effects of tissue hypoxia. The significance of this finding requires further study.

**CONCLUSIONS**

Osteoblasts demonstrate changes in vitro that cannot be attributed solely to tissue hypoxemia or impaired vascularity. Irradiated osteoblasts demonstrate diminished rates of proliferation and production of collagen when compared with controls. This effect is enhanced by treatment with dexamethasone. Irradiation results in an increase in TGF-β, type I and II receptor expression. The role of impairment or alteration in factors involved with normal bone remodeling and wound healing resulting from a radiation-induced injury to otherwise normal bone at the cellular level in the development of osteoradionecrosis requires further study.

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