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The role of myofibroblasts in the development of osteoradionecrosis in a newly established rabbit model☆

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ABSTRACT

This study aimed to establish a proper animal model of osteoradionecrosis of jaws (ORNJ) and to observe preliminarily the characteristics of myofibroblasts, the key effector cell of fibrosis, in ORNJ. Rabbit mandibles were irradiated at three different doses based on a human equivalent radiation schedule, and examined by gross manifestation, single-photon emission computed tomography (SPECT), micro−computed tomography, sequential fluorochrome labeling, and histology. Immunohistochemistry staining of α-SMA was applied to detect the existence of myofibroblasts. The exposed necrotic bone, which is the main indication of ORNJ, started to be observed at all rabbits at 9 Gy. With the radiation dose increasing, the microarchitecture of the irradiated mandibles was more destroyed, the metabolism and mineralization of the irradiated mandibles diminished, the osteocytes number decreased, and more mature bones were substituted by fibrosis in the irradiated mandibles. In addition, as the radiation dose increased, the myofibroblast number increased and collected around the separated sequestrum, which indicated that myofibroblasts might relate to the pathogenesis of ORNJ. In summary, a clinically translational ORNJ model was successfully established in our study, and the role of myofibroblasts in the pathogenesis of ORNJ is described for the first time.

1. Introduction

Osteoradionecrosis of jaws (ORNJ) is the most devastating long-term complication after head and neck radiotherapy, which can cause severe pain, ulcer, necrotic bone exposure, pathological fracture, and, in extreme cases, death (Vanderpuye and Goldson, 2000). Although improvements in radiotherapy techniques can decrease the radiation toxicity, the incidence of ORNJ is still as high as 6% (Eisbruch et al., 2010).

However, effective prevention and treatment measures for ORNJ are limited by inadequate understanding of its pathogenesis (Rice et al., 2015). Until recent years, a new understanding of the pathophysiology that showed that the radiation-induced fibrosis (RIF) may be the reason for ORNJ has led to the development of new therapeutic modalities (Madrid et al., 2010; Kahenasa et al., 2012). In some clinical trials, doctors found that using anti−radiation fibrosis drugs to treat ORNJ achieved satisfactory therapeutic effects (Delanian et al., 2011; Robard et al., 2014). However, the precise pathogenesis of ORNJ has not been fully clarified, which poses obstacles to earlier diagnoses and effective treatment.

To elucidate the mechanism of ORNJ, a proper ORNJ animal model should be established for research (Teng and Futran, 2005); however, until now, no universally accepted animal model has been built yet. To adequately mimic the clinical radiotherapy

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regimen, we used a hypofractionated radiation schedule in building the animal model and the dose linear quadratic equation (Tchanque-Fossuo et al., 2011a, b) to quantify the human equivalent radiation dose in rabbits. The left mandibles of rabbits were irradiated at three different doses, and the mandibular molars were extracted 2 months after radiation. Clinical manifestation, single-photon emission computed tomography (SPECT), micro-computed tomography (micro-CT), sequential fluorochrome labeling, and histopathology were used to evaluate the ORNJ animal model. We assumed that ORNJ, like fibrosis in other organs, was caused by fibrogenesis in which myofibroblasts are the key effector cells; hence, immunohistochemistry was carried out to explore the function of myofibroblasts in the development of ORNJ in our study.

2. Material and methods

2.1. Experimental design

All the protocols for animal experiments were conducted in accordance with international standards on animal welfare as well as being approved by the ethics committee of the Fourth Military Medical University. Twenty-four adult New Zealand rabbits weighing 2.0–2.5 kg were selected for the study. The animals were obtained from the experimental animal center of the Fourth Military Medical University. The rabbits were randomly allocated to a control group and three experimental groups ($n = 6$). The left mandibles of the control group were sham irradiated. The left mandibles of experimental groups 1, 2, and 3 received radiation at doses of 7.0 Gy, 8.0 Gy, and 9.0 Gy per fraction, respectively, and 5 fractions (once every 2 days) in total. These doses were equivalent to the total doses of 70 Gy, 90 Gy and 110 Gy in clinical conventional regimens for head and neck cancer patients. This radiation protocol was calculated by biological equivalent dose linear quadratic equation (biological equivalent dose [BED] $= nd\,[1 + d/(\alpha/\beta)]$, where n represents the irradiation times, d the fractionated dose, and α/β approximates 3 in bone tissue) (Tchanque-Fossuo et al., 2011a,b).

2.2. Irradiation and teeth extraction

Under general anesthesia with 3% pentobarbital sodium, the area from the incisor to the last molar of the left mandibles of rabbits in the three experimental groups were irradiated by means of a linear accelerator (6 MVe, 21EX, Varian Inc, California, USA) at the doses and according to the methods mentioned above. The surrounding areas were covered by lead plates. Two months after irradiation, all left mandibular molars of all animals were extracted under aseptic conditions, without primary suture of gingiva.

2.3. Animal sacrifice

Four months after teeth extraction, the animals were humanely euthanized. The mandibles were harvested, photographed, and examined by micro-CT. Then the left mandibular specimens at the molar site were hemi-sected medially to allow non-decalcified–based fluorochrome analysis and decalcified-based histological analysis.

2.4. Micro-CT examination

Mandibles were placed under a CT microscope (Siemens Inveon, 80 kV, 100 μA, a high resolution of 15 μm). Regions of interest (ROI) were selected as follows: from the anterior to the posterior border of the extraction socket; from the most superior aspect of the tooth socket where both medial and lateral cortices were seen (in an axial view) down to the nadir of the inferior border; and separation of cortical bone and trabecular bone (Cohen et al., 2011). The internal program was adopted to reconstruct the three-dimensional images and to analyze the microarchitecture parameters.

2.5. SPECT examination

SPECT was performed the day before the animals were sacrificed. Bolus ^{99m}technetium-methylene diphosphonate (^{99m}Tc-MDP) was injected into each ear vein at a dose of 18.5 MBq/kg. After 4 h, delayed static bone scanning was performed with 128 × 128 acquisition matrices. For semiquantitative analyses, manually drawn rectangular ROIs were established on the left mandibular molar sites, and the uptake ratio of ^{99m}Tc-MDP of the ROIs was calculated (Zhou et al., 2010).

2.6. Fluorochrome labeling and analysis

Sequential different-colored fluorochrome labeling of mineralizing bone was performed to evaluate the bone–mineral apposition. Two fluorochromes were used in this study: calcein (green fluorescence) (0.5% in 2% $NaHCO_3$ solution, 8 mg/kg body weight; Sigma Aldrich, Saint Louis, Missouri, USA) and tetracycline (orange fluorescence) (2.5% in normal saline solution, 50 mg/kg body weight; Sigma Aldrich). Calcein was administered intramuscularly 2 days before animals were sacrificed. Intramuscular injection of tetracycline was performed 14 days before calcein administration. The mandibular samples were put into 80% ethanol for 5 days, then dehydrated and embedded in methyl methacrylate. Sections of 35 μm were cut on a microtome (SP1600, Leica, Germany) and observed under a fluorescence microscope (IX-71, Olympus, Japan) for fluorochrome analysis. The distance between the two labels was measured, and the mineral apposition rate (MAR) was calculated as the distance/14 d (interval days between the two fluorochrome administrations).

2.7. Histological analysis

The other specimens were decalcified with 10% ethylenediaminetetraacetic acid (EDTA; pH 7.4, changed every 2 days until full decalcification was achieved), and then dehydrated and paraffin embedded. Decalcified sections of 4 μm were stained with hematoxylin–eosin (HE) and Masson's trichrome, and observed under a light microscope (XTJ30, Nikon, Japan). Using ×200 magnification, nine high-power field (HPF) images were randomly selected in the HE staining per specimen. The point counting of osteocytes and empty lacunae was performed by three independent reviewers (Tchanque-Fossuo et al., 2011b). Monoclonal primary antibody against α-SMA (1:200, Abcam, Cambridge, Massachusetts, USA), the most commonly used specific molecular marker of myofibroblasts, was used for immunohistochemistry. Stromal spindle cells positive for α-SMA were regarded as myofibroblasts. Five appropriate HPF images (×200 magnification) per slide were selected, and the number of myofibroblasts was counted.

2.8. Statistical analysis

Quantitative data from the experiments are expressed as mean ± SD. Significance was determined by one-way analysis of variance followed by the Holm–Sidak test. Statistical significance was set at $P < 0.05$.