ORIGINAL RESEARCH ARTICLES

A MODEL OF MANDIBULAR IRRADIATION IN THE RABBIT: PRELIMINARY RESULTS

Anne-Gaëlle Bodard [1-2], Soufyane Debbache [1], Stephan Langonnet [1], Frédéric Lafay [2], Bertrand Fleury [2]

[1]Institut de Chirurgie Expérimentale, Centre Régional de Lutte Contre le Cancer, Lyon, France and LabTAU - Inserm U556 [2]Centre Léon Bérard, Lyon , France

Abstract
Radiotherapy is widely used in the treatment of head and neck cancers. Its major adverse effect is osteoradionecrosis, which can occur during the whole life of the patient, involving the vital prognosis. The aim of the study was to develop a model for irradiation of the rabbit mandible in order to have a better knowledge of radiotherapy-induced bone alterations and thus a better prevention and treatment of osteoradionecrosis.
The control group consisted in 7 rabbits and was used to assess anatomical and histological parameters of the rabbit's mandible. A first group of 14 rabbits was weekly irradiated at doses of 5.5 Gy during 5 weeks, at a total dose of 46.8Gy. Sacrifices were done at 1 week, 4 weeks, 12 weeks and 24 weeks. As histological analysis did not reveal statistical differences with the control group, a second group (3 rabbits) was weekly irradiated at 8.0, 8.5 and 9 Gy during 5 weeks. The first histological results seem to show vascular alterations, bone cells decrease and alterations of bone architecture. The role of intra alveolar collagen sponges, PRF®, ultrasounds and stem cells in bone regeneration after radiotherapy will be further studied.

Résumé
La radiothérapie est une modalité thérapeutique utilisée quasi systématiquement dans le traitement des cancers des voies aérodigestives supérieures. Son principal effet secondaire est l'ostéoradionécrose, qui peut survenir tout au long de la vie du patient et compromettre le pronostic vital. Le but de ce travail est de mettre au point un modèle d'irradiation des maxillaires chez le lapin afin de mieux connaître la pathogénie de l'ostéoradionécrose et proposer une prévention et des traitements plus efficaces.
Un groupe contrôle de 7 lapins a permis de connaître l'anatomie et l'histologie de la mandibule de lapin. Un premier groupe de 14 lapins a été irradié à raison d'une séance hebdomadaire de 5.5 Gy pendant 5 semaines, soit un équivalent de dose de 46.8 Gy. Ils ont été sacrifiés à 1, 4, 12 et 24 semaines. L'analyse statistique n'ayant pas montré de différences significatives avec le groupe contrôle, un second groupe de 3 lapins a été irradié à une séance hebdomadaire de 8.0, 8.5 et 98.0 Gy respectivement pendant 5 semaines. Les premiers résultats histologiques montrent une altération vasculaire, la diminution du nombre de cellules osseuses et des modifications de l'architecture osseuse. Le rôle des éponges collagéniques intra alvéolaires, du PRF®, des ultrasons et des cellules souches sera étudié ultérieurement.

Keywords
Animal model
Head and neck
Radiotherapy
Adverse effects

Mots clés
Modèle animal
Tête et cou

Radiothérapie
Effets secondaires

Introduction

Radiotherapy is widely used in head and neck cancer (HNSCC) treatment but its adverse effects concern all orofacial tissues. One of the most important is osteoradionecrosis (ORN). It is caused by hypoxia, hypovascularization and decrease of bone cells (Marx, 1983). It provokes alterations of the endothelium and increases collagen excretion (Gevorgyan, 2008). Decrease of vasculature, cell activity and differentiation, collagen synthesis and growth factors expression are observed (Delanian, 2002) Thus, bone becomes unable to heal after traumatic or iatrogenic exposure. Even if the occurrence of ORN has dramatically decreased over the past 20 years, it remains a major adverse effect which can endanger the prognosis of the patient. Nonetheless, some elements of its pathogenesis remain unknown. Many studies have been made to assess the effects of radiotherapy on bone, but most of them concern femur of rabbits or rats (Johnsson, 1999; Phulpin, 2009). A canadian study has developed a model of irradiation of the orbito-zygomatic complex in growing rabbits (7 weeks) (La Scala, 2005). Radiotherapy was delivered at doses ranging from 25 to 35 Gy. Zhang (2010) studied mandibular bone regeneration after distraction osteogenesis. Rabbits received 5 sessions of 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 Gy. Bone alterations were observed at doses over 8.0 Gy. Distraction osteogenesis was performed 1 month after radiotherapy.

The aim of the study was to develop an animal model of radio-induced mandibular lesion, which will allow a better understanding of the pathogenesis of ORN and lead to propose prevention and earlier treatment of this pathology.

Material and method

New Zealand white female rabbits (weight: 3.5-4 kgs) were used. Acclimatizing was done during 7 days. Rabbits were divided in 3 successive groups. Seven rabbits (G0) were control group. Fourteen rabbits (G1) received 5 fractions (one per week) of 5.5 Gy each and were sacrificed at 1 week (2 rabbits), 4 weeks (4 rabbits), 12 weeks (4 rabbits) and 24 weeks (4 rabbits). The last 3 rabbits (G2) received 5 fractions of 8.0, 8.5 and 9.0 Gy each and were sacrificed at 8 weeks.

A scanner was performed on one rabbit to calculate the dosimetry of radiotherapy [Figure 1]. Before each session of radiotherapy, rabbits were anaesthetized with ketamin / xylazin / glycopyrolate. A silicon sheet was used to increase the thickness of soft tissues and concentrate maximal dose on bone. A Digital Reconstruct Radiograph was performed to validate the positioning of the animal before the beginning of radiotherapy. Radiotherapy was performed on a linear accelerator used for therapeutic irradiation, delivering 6MeV photons. Sedation was maintained thanks to re-injection when necessary. Irradiation was performed on the mandible and the maxilla, avoiding brain and eyes, thanks to lead protections. Alpha-bêta ratio was 2 to reproduce long time side effects on bone. At the end of the session, animals were immediately transferred under infrared lights and monitored until complete recovering. Daily clinical examination was performed. Pain symptoms, food intake and hydratation were noticed. In case of reaching a limit standard point, animal should be sacrificed and withdrawed from the study. Standard limit points are: permanent decubitus, convulsions, severe anemia, fever, anorexia superior to 24 hours, pain symptoms resistant to analgesia procedure.

Animals were sedated and sacrificed by 4ml intravenous pentobarbital.

Bone harvesting was made thanks to a thin

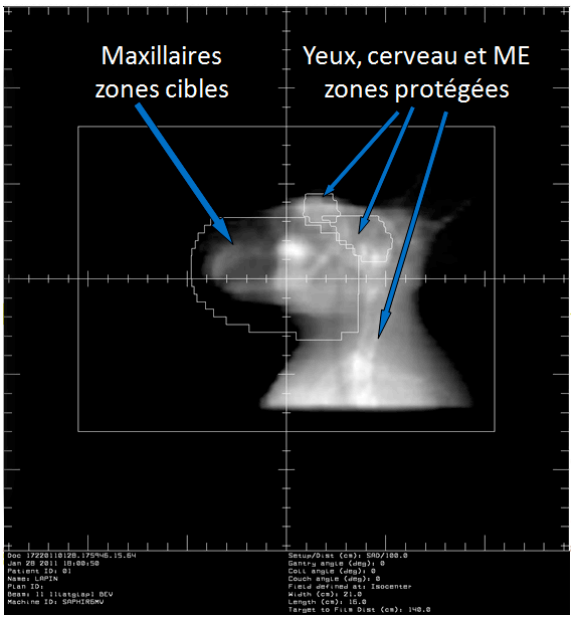

Figure 1: Pre-irradiation CT scan with delineation of critical organs.

surgical saw. Harvest zone was comprised between incisors and premolars. Bone fragments were put into formaldehyde 4%.
Criteria for histological analysis were: number and aspect of bone cells (osteoblasts, osteocytes, and osteoclasts), osteoid tissue, presence of alkaline phosphatase, number and aspect of vessels, presence of collagen I and aspect of bone structure.
For G0 and G1, decalcification was made with trichloracetic acid. For group 2, decalcification was slowly made with EDTA.
Histologic analysis was performed with tartrate resistant acid phosphatase (TRAP), Goldner trichrome and hematoxylin-phloxin-safran.

Results

The protocol respected all ethical principles related to animal experimentation and was examined by an internal ethic committee.
For group 1, radiotherapy was performed according to the protocol, delivering an equivalent of 46.8 Gy. Alpha/beta ratio was 2 in all groups.
Harvested bone fragments were about 2 cm length, 1 cm wide and concern the total height of the mandible. Maxillary bone fragments were harvested in the G0 group. In the G2 group, bone seemed visually less vascularized and less hard.
Clinical examination noticed no weight loss in G0 and G1 animals, food and water intake was correct and there were no signs of pain. In group 1, animal sacrificed at 4 weeks and over presented with depilation in the submental area and color change in the cheek area. For group 2, all animals had a 24 hours interruption of food and water intake. They were controlled every 2 hours. After 24 hours they began to drink and then eat normally. In this group, submental depilation was intense and associated with erythema.
Group 0 allowed the histological analysis of 52 slides. Mandibular bone of rabbits was characterized by thin cortical bone and cancellous bone including a large amount of fat tissue and only few hematopoietic tissues. Bone trabeculations were thick and non parallel, which signs an intense bone remodeling. Few fibroblasts around vessels and nerves were noticed [Figure 2].
In group 1, 120 slices were observed. No signs of inflammation were noticed in rabbits sacrificed at 1 week. There were no signs of fibrosis or pathologic tissue. Vessels and nerves had a normal aspect. Only few osteoclasts

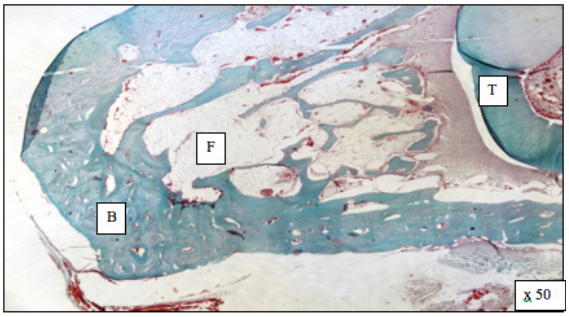

Figure 2: histological aspect of the mandibular bone in the control group (Goldner)
F: fat tissue; B: bone with ostocytes, osteoblasts and clasts ; T: tooth

Discussion

The animal is the New Zealand White female rabbit. Despite important differences between rabbit mandibular bone and human mandibular bone, it remains an interesting model at its size is compatible with repetitive manipulation and irradiation. Furthermore, two studies (La Scala, 2005; Zhang, 2010) allow comparisons. Other studies concern rat femur (Phulpin, 2009) or Beagles. Rabbit seems to be a valuable compromise between reliability of the study, cost and manipulation of the animals.
The choice of the animal was also due to radiotherapy and the will to perform radiotherapy under clinical conditions in a linear accelerator. The aim of the study was to develop an animal model of long term effects of radiotherapy on mandibular bone. Radiotherapy was performed for the group 1 according to clinical doses: 5.5 Gy weekly during 5 weeks, which is used in digestive tumors and corresponds to a dose of 46.8 Gy. Ratio alpha/beta was 2 to assess long time adverse effects (Clark 2006; Hopewell, 2003). To observe long time adverse effects, we needed a species with rapid bone turn over. The rabbit is known to have a bone turn over 3 times faster than humans (Johnsson, 1999). Furthermore, it is easy to manipulate and bring into the linear accelerator for irradiation.
In group 0, 7 rabbits were included, which means 14 samples (mandibular left, mandibular right) divided in 52 slices. Maxillary harvesting was tested but rapidly stopped due to the poor quality of maxillary bone and poor bone volume: aeric cavities are very developed and allowed the harvest of only a few millimeters of bone. Furthermore, histological analysis of maxillary bone showed important differences with mandibular bone and no interest for this

study.
In group 1, 14 rabbits were included, with 2 rabbits sacrificed at 1 week (4 samples) and 4 sacrificed at 4, 12 and 24 weeks (16 samples each). At 1 week post radiotherapy, signs of acute inflammation were expected (Williams, 2003). Only 2 rabbits were sacrificed at this time as the main objective was long term adverse effects and not acute early effects.
To assess reproducibility of the positioning during sessions of radiotherapy, anatomical points were drawn on the wedge placed under the head of the rabbit: nose, eye and ear base. Laser lights guiding the positioning of the rabbits were also drawn on the wedge. The silicon sheet used to concentrate the radiotherapy on bone and limit cutaneous toxicity didn't allow the use of thermoplastic mask for precise repositioning. Thus, a digital reconstruct radiograph was made before each irradiation so the team could check that brain and eyes were not included in the irradiation field. These critical organs were protected thanks to lead.
The results in the first group didn't show statistical difference with control group. Many hypotheses can be made to explain these results. First of all, the lack of anatomical and histological knowledge on rabbit mandibular bone in the literature complicated the analysis. The rabbit is quite a weak animal and tolerance to repetitive anaesthesia was not proven. It has been shown in this study that repetitive anesthesia is not a limitation of the protocol, as all animals underwent 5 sessions. Only addiction was observed, obliging to slightly increase the doses of anesthetics during the last session.
Secondly, the histological process for group 0 and 1 consisted in a rapid decalcification, which probably destroyed some information, especially for tartate resistant acid phosphatase analysis.
Finally, the dose or the fractionation for radiotherapy was probably insufficient. Bone healing seems to be very quick and bone recovery after 5.5 Gy was probably completed after one week. Two choices were possible for the last group (G2): increasing the dose or doing 2 sessions of radiotherapy per week. In the literature Zhang (2010) highlights bone alterations after 8.5 or 9 Gy weekly during 5 weeks in rabbit mandibular bone ad after distraction osteogenesis. The objective of the second group was to validate results of the literature (Zhang, 2010) and thus, only 3 rabbits were irradiated following this protocol. Histological analysis is currently done but clinical acute toxicity was very important and the first histological observations show bone alterations, even without any bone trauma (i.e. tooth extraction, distraction osteogenesis…). Two weekly sessions of irradiation per animal did not seem acceptable, as the veterinary was not sure that the rabbit could bear 2 sessions per week under general anaesthesia. Furthermore, logistic constraints render this protocol difficult to develop.

Perspectives
The aim of the study was to develop an animal model of mandibular bone alteration after radiotherapy. Precise and reproducible knowledge of radio-induced bone alterations and their chronology were seeked. Rabbit osteoblasts will be irradiated to determine the precise dose to administrate and thus precise modalities for irradiation. Secondly, effects of healing materials will be studied in irradiated bone after creation of standardized surgical wounds: collagen sponges, Platelet-Rich fibrin, and their possible enrichment with adipose derived stem cells. Different harvesting sites for ADSCs in rabbits have been described and it seems that abdominal harvested cells have better properties (Chen, 2012). Harvest is made thanks to lipoaspiration. It seems that hypoxia stimulates their differentiation and adhesion (Chung, 2009). This could be interesting in irradiated bone, as hypoxia is one of the characteristics of irradiated tissues.
Two other projects are linked: the role of high frequency ultrasounds (HIFU) in alveolar bone healing, and radioprotection induced by fat injection to prevent hair loss during radiotherapy. Short term clinical applications are homogenization of dental extractions in irradiated bone, and a decrease of incidence of ORN, which, even if it is rare, remains a heavy complication involving the prognosis of the patient. Moreover, maintaining a correct amount of bone should allow a widely use of dental implants which are, in many cases, the only way to have a functional rehabilitation of oral functions.
In the future, the model could be enlarged to bisphosphonate-induced bone alterations or to delayed alveolar healing due to targeted therapies. Both can provoke the occurrence of osteochemonecrosis (ONJ), due to alterations of the vascularization. Until today, many steps of its pathogenesis remain unclear. Fur-

thermore, no treatment has currently shown its efficacy for the treatment of ONJ. The aim would be preventive, especially when removing teeth in patients with bisphosphonates, and curative of ONJ.

Acknowledgements
This work is supported by the IFRO.

References

Marx RE. Osteoradionecrosis: a new concept of its pathophysiology. J Oral Maxillofac Surg. 1983 May;41(5):283-8

Gevorgyan A, Sukhu B, Alman BA, Bristow RG, Pang CY, Forrest CR. Radiation effects and radioprotection in MC3T3-E1 mouse calvarial osteoblastic cells. Plast Reconstr Surg 2008 ;122 :1025-1035

Delanian S, Lefaix JL. Radionécrose de l'os mature : connaissance physiopathologique récente motrice d'une thérapeutique médicale innovante. Cancer/Radiother 2002 ;6 :1-9.

Johnsson AA, Saxaii T, Jacobsson M, Granström G, Turesson I. A hsitomorphometric study of bone reactions to titanium implants in irradiated bone and the effect of hyperbaric oxygen treatment. Int J Oral Maxillofac Implants Sep-Oct;14(5):699-706.

Phulpin B, Gangloff P, Tran N, Bravetti P, Merlin JL, Dolivet G. Rehabilitation of irradiated head and neck tissues by autologous fat transplantation. Plast Reconstr Surg. 2009 Apr;123(4):1187-97.

La Scala GC, O'Donovan DA, Yeung I, Darko J, Addison PD, Neligan PC, Pang CY, Forrest CR. Radiation induced craniofacial bone growth inhibition: efficacy of cytoprotection following a fractionated dose regimen. Plast Reconstr Surg 2005; Jun,115(7):1973-85.

ZhangWB, Zheng LW, Chua D, Cheung LK. Bone regeneration after radiotherapy in an animal modelJ Oral maxillofac Surg 2010; 68:2802-09.

Clark CL, Strider J, Hall C, Fregusson HW, Armstrong KL, Runner RR, Baum DA. Distraction osteogenesis in irradiated rabbit mandibles with adjunctive hyperbaric oxygen therapy. J Oral Maxillofac Surg 2006 Apr;64(4):589-93.

Hopewell JW. Radiation-therapy effects on bone density. Med pediatr Oncol 2003;41:208-211.

Williams J, Chen Y, Rubin P, Finkelstein J, Okurieff P. The biological basis of a comprehensive grading system for the adverse effects of cancer treatment. Semin Radiat Oncol 2003 Jul;13(3):182-8.

Chen L, Peng EJ, Zeng XY, Zhuang QY, Ye ZQ. Comparison of the proliferation, viability and differentiation capacity of adipose-derived stem cells from different anatomic sites. Cells Tissues Organs 2012;196(1):13-22.

Chung HM, Won CH, Sung JH. Responses of adipose-derived stem cells during hypoxia: enhanced skin-regenerative potential. Expert Opin Biol Ther. 2009 Dec;9(12):1499-508.