

THE EFFECTS OF EPIDERMAL GROWTH FACTOR (EGF) AND BASIC FIBROBLAST GROWTH FACTOR (bFGF) ON IRRADIATED RAT PAROTID GLANDS

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INTRODUCTION

Radiotherapy is the most commonly used method of treatment for head-and-neck cancer, during which the salivary glands are often included within the radiation field [1]. The most common side effect of this treatment is the development of oral dryness (xerostomia), which is due to a change in composition and a reduction in the secretion of saliva from the major and minor salivary glands [1,2]. As a consequence, patients experience impaired ability to swallow, chew, and talk, loss of taste acuity, increased incidence of dental caries, and oral ulcerations.

According to Cooper [3], the serous acinar cells are the target cells of radiation injury in the salivary glands, and cell death soon after irradiation cannot be attributed to mitosis since these cells are not normally growing and dividing; therefore, death of serous acinar cells is attributed to apoptosis.

Currently, no satisfactory treatment is available for patients with salivary gland damage. Some treatments for post-radiation xerostomia include topical fluorides to retard caries, saliva substitutes such as water and artificial saliva, and sialogogues to improve oral function and comfort [4,5].

In this study a new strategy is proposed for correcting radiation-induced salivary gland damage. This strategy consists of the administration of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF or FGF2) at physiological concentrations before and after irradiation in order to repair radiation-induced damage in salivary gland cells. As a preliminary examination of the efficacy of this approach we have characterized the effects of EGF and bFGF on the apoptotic response of irradiated salivary gland cells. Also, we have developed a controlled-release delivery system to effectively administer the growth factor to the gland.

MATERIALS AND METHODS

Rat parotid glands were cut into pieces of at least 1 mm² with curved scissors. The rat parotid gland pieces were maintained in RPMI 1640 1X culture media (Mediatech, Herndon, VA) without phenol red, supplemented with 300mg/L L-Glutamine, penicillin

(1000 U/ml), and streptomycin (1 mg/ml), and were kept in a 95% O₂ 5% CO₂ atmosphere at 37 °C. Parotid gland pieces were incubated four hours prior irradiation and 24 hr post irradiation in 12-well plates containing media supplemented with EGF (100ng/ml), bFGF (100 ng/ml), or 10% fetal bovine serum (FBS). A linear accelerator (6 MeV) was used to irradiate the tissues at 15 Gy. Immediately after irradiation the culture media was replaced by fresh media. The gland fragments were incubated for 24 hr and then they were fixed for future paraffin embedding.

Apoptosis of parotid acinar cells from paraffin-embedded tissue samples was determined by the TUNEL assay which is based on the binding of terminal deoxynucleotidyl transferase (TdT) to exposed 3'-OH ends of DNA fragments generated in response to apoptotic signals. An insoluble colored substrate at the site of DNA fragmentation is generated, which allows visualization of apoptotic cells microscopically. Tiled field mapping images were taken from each TUNEL sample to count the apoptotic nuclei. A morphometric microscope and a stereology software were used to count all the nuclei in samples stained with Hematoxylin and Eosin.

bFGF-loaded microspheres were prepared by the water-oil-water (W/O/W) emulsion technique. The polymer used was poly(lactide-co-glycolide) (PLGA) ratio of 50/50 (M_w 75,400 Da) from Birmingham Polymers. In addition to bFGF, five more additives were encapsulated in the following concentrations: 15% bovine serum albumin (BSA), 3% Mg(OH)₂, 0.01% EDTA, 0.6% sucrose, 0.0025% heparin, and 0.0025% bFGF. All additives were dissolved in 1 ml PBS, pH 7.4, and emulsified in methylene chloride containing 1% (w/w) PLGA. This emulsion was added to 0.1% polyvinyl alcohol (PVA) solution at room temperature under continuous stirring at 800 rpm for 24 hours.

To characterize the microspheres, five tests were performed: encapsulation efficiency, protein release kinetics, SEM analysis, size distribution, and bioactivity of bFGF. To determine the encapsulation efficiency, 10 mg of dried microspheres was dissolved in 1 ml methylene chloride. 10 ml PBS was added and the mixture was vortexed for 2 min. The solution was filtered and the protein concentration was determined using the Bradford reagent (Sigma, St.

Louis, MO). For release kinetics determination, 20 mg of dried microspheres was suspended in 0.5 ml PBS containing 0.01% sodium azide (w/v). The suspension was incubated at 37°C under continuous orbital rotation. At predetermined intervals, the media was removed with a pipette after centrifugation and replaced with an equal volume of fresh media. Protein concentration was determined using the Bradford reagent. Surface morphology of the microspheres was examined a SEM after carbon coating, and size distribution was determined with a Beckman Particle Characterization Coulter. To test bFGF bioactivity, we examine if encapsulated and released growth factor induced cell proliferation using the MTT assay (R&D Systems, Minneapolis, MN) which measures the ability of cells to convert the tetrazolium compound MTT by the action of succinate dehydrogenase to water-insoluble formazan crystals.

RESULTS AND DISCUSSION

Figure 1 shows the results of the TUNEL assay. Irradiated samples treated with EGF yielded the same results as the irradiated samples incubated in unsupplemented media (negative control). On the other hand, irradiated samples treated with bFGF yielded a lower apoptotic index than the negative control. After running a two-way analysis of variance (ANOVA) and the Tukey test, it was shown that the difference between irradiated bFGF samples and the negative control was statistically significant ($p < 0.05$). Administration of bFGF prior to and immediately after 15-Gy irradiation partially protected the parotid gland and prevented 44% of the increment in the apoptotic index by irradiation.

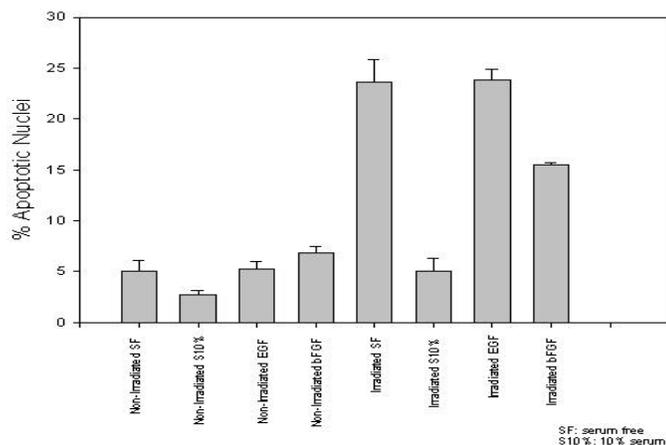


Figure 1. TUNEL assay

As can be seen in Figure 2 (A), microspheres appeared spherical in shape. The encapsulation efficiency was calculated to be 68.61 % \pm 4.26% (mean \pm standard error of the mean, SEM). The majority of the particles were approximately 2 μ m in diameter, and a few microspheres were between 10 and 50 μ m in diameter.

The surface morphology of the microspheres at day 0 was smooth, as shown in Figure 2 (A). As time progressed, the microspheres degraded and the surfaces became rough, Figure 2 (B). In addition, the smaller microspheres appeared to meld with larger ones as they degraded. By day 28 the microspheres degraded to an extent that all spherical morphology was lost, Figure 2 (C).

The release kinetics of the 1:6000 bFGF/BSA microspheres showed two phases. The first phase was an initial burst release followed by a lower steady-state release. The burst release rate was $2.25 \pm 0.12 \mu$ g/ml (mean \pm SEM).

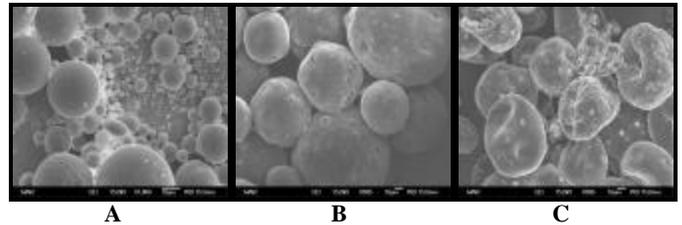


Figure 2. SEM of degrading microspheres

Dermal fibroblast cells responded to bioactive bFGF by proliferating. The cells exposed to exogenous bFGF or supernatant from bFGF microspheres showed an increased rate of proliferation compared to the negative control ($p < 0.05$). In addition, the bFGF from the microsphere supernatant was more potent than exogenously added bFGF, which could be explained by the encapsulation of BSA and heparin along with bFGF.

CONCLUSIONS

Administration of bFGF prior to an immediately after irradiation partially protected the rat parotid gland. The radioprotective effectiveness of the growth factor could be enhanced by encapsulation of bFGF within 50/50 PLGA microspheres. bFGF encapsulated with protein carriers as BSA and heparin would remain stable for a longer period of time and would not adhere to glass and plastic surfaces so avidly. Further studies need to be conducted to compare the radioprotective effects of encapsulated and released bFGF to the exogenously added growth factor.

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