Radiotherapy and Oncology 124 (2017) 448–454

Contents lists available at ScienceDirect

Radiotherapy and Oncology

journal homepage: www.thegreenjournal.com

Radiosensitivity and stem cells

Role of glial-cell-derived neurotrophic factor in salivary gland stem cell response to irradiation

Xiaohong Peng [a], Kärt Varendi [b], Martti Maimets [a,c], Jaan-Olle Andressoo [b,d], Rob P. Coppes [a,*]

[a] Departments of Cell Biology and Radiation Oncology, University Medical Centrum Groningen, University of Groningen, The Netherlands; [b] Institute of Biotechnology, University of Helsinki, Finland; [c] BRIC-Biotech Research and Innovation Centre, Copenhagen, Denmark; [d] Institute of Biosciences and Medical Technology – BioMediTech, University of Tampere, Finland

ARTICLE INFO

Article history:
Received 1 May 2017
Received in revised form 6 July 2017
Accepted 6 July 2017
Available online 4 August 2017

Keywords:
Salivary glands
GDNF
Stem cells
Spheres
Radiation-induced xerostomia

ABSTRACT

Background and purpose: Recently, stem cell therapy has been proposed to allow regeneration of radiation damaged salivary glands. It has been suggested that glial-cell-derived neurotrophic factor (GDNF) promotes survival of mice salivary gland stem cells (mSGSCs). The purpose of this study was to investigate the role of GDNF in the modulation of mSGSC response to irradiation and subsequent salivary gland regeneration.

Methods: Salivary gland sphere derived cells of Gdnf hypermorphic ($Gdnf^{wt/hyper}$) and wild type mice ($Gdnf^{wt/wt}$) were irradiated (IR) with γ-rays at 0, 1, 2, 4 and 8 Gy. mSGSC survival and stemness were assessed by calculating surviving fraction measured as post-IR sphere forming potential and population doublings. Flow cytometry was used to determine the $CD24^{hi}/CD29^{hi}$ stem cell (SC) population. QPCR and immunofluorescence was used to detect GDNF expression.

Results: The IR survival responses of mSGSCs were similar albeit resulted in larger spheres and an increased cell number in the $Gdnf^{wt/hyper}$ compared to $Gdnf^{wt/wt}$ group. Indeed, mSGSC of $Gdnf^{wt/hyper}$ mice showed high sphere forming efficiency upon replating. Interestingly, GDNF expression co-localized with receptor tyrosine kinase (RET) and was upregulated after IR *in vitro* and *in vivo*, but normalized *in vivo* after mSGSC transplantation.

Conclusion: GDNF does not protect mSGSCs against irradiation but seems to promote mSGSCs proliferation through the GDNF-RET signaling pathway. Post-transplantation stimulation of GDNF/RET pathway may enhance the regenerative potential of mSGSCs.

Dry mouth syndrome or xerostomia is a common radiation-induced side effect resulting from damage to salivary glands (SGs) which severely affects cancer patients' quality of life [1–3] Xerostomia is a multi-faceted syndrome which manifests in oral dryness and infections, dental caries, and difficulties with food mastication [1]. Currently, no cure is available therefore the main focus is optimizing regenerative potential of the gland post-irradiation. This can be achieved by reducing the damage caused to the glands by limiting the irradiation dose given [4] or move the submandibular glands out of the irradiation field before radiation therapy [5,6], or pharmacologic treatments such as amifostine [7,8] or pilocarpine [9,10]. If these methods are unsuccessful, only the generally supportive measurements like water drinking, artificial saliva and special diets remain [1]. Recently, stem cell therapy has been suggested as an optional treatment for xerostomia [11–14]. Several signaling pathways involved in stem cell maintenance, such as WNT and NOTCH [13,15–18], have been suggested to be involved in mouse salivary gland stem cell (mSGSC) self-renewal, proliferation, and radiation response [19]. However, knowledge on the molecular cues underlying the maintenance of mSGSCs after therapeutic irradiation is scarce. Recently, it was suggested that GDNF may play an important role in survival of mSGSCs and therefore could be used as a tool for SG function restoration after irradiation-induced damage [2,12]. GDNF is a member of GDNF family ligands, which belongs to the transforming growth factor-β (TGF-β) superfamily [20]. GDNF promotes the survival of dopamine neurons, acts as a morphogen in renal branching morphogenesis and regulates the differentiation of spermatogonia [21,22].

To investigate the role of GDNF in mSGSC response to irradiation and subsequent salivary gland regeneration, we modulated GDNF expression. To this end, we used hypermorphic (gain of function) Gdnf ($Gdnf^{wt/hyper}$) mice, which generated basically by replacing 3′UTR of GDNF with a 3′UTR which is less responsive to

* Corresponding author at: Department of Cell Biology, University Medical Centrum Groningen, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands.
E-mail address: r.p.coppes@umcg.nl (R.P. Coppe.

http://dx.doi.org/10.1016/j.radonc.2017.07.008
0167-8140/

negative regulation by microRNAs, so that exhibit elevated levels of GDNF expression from *Gdnf* native locus, enabling analysis of endogenous GDNF function *in vivo* [23] or recombinant GDNF was added *in vitro*. We used our recently developed 3D salivary gland stem cell culture system [11,13] to show the survival of mSGSCs with upregulated GDNF expression in response to irradiation. We demonstrated that endogenous GDNF does not act as a radioprotector, but does stimulate proliferation and regeneration following irradiation, potentially through increasing mSGSC self-renewal ability. Expression of GDNF and its receptor RET is upregulated in mSGSCs and in irradiated tissue, but is reduced after mSGSC transplantation. Post-transplantation stimulation of GDNF/RET pathway may enhance the regenerative potential of mSGSCs and therefore could represent a promising treatment for radiation-induced hyposalivation and consequential xerostomia.

Methods and Materials

Detailed descriptions of all materials and methods are provided in Supplementary Materials and Methods.

Mice

8 to 12-week-old female C57BL/6 mice were purchased from Harlan, The Netherlands. 8 to 12-week-old female $Gdnf^{wt/hyper}$ mice (129Ola/ICR/C57bl6), $Gdnf^{wt/wt}$ mice (129Ola/ICR/C57bl6) [23], were bred in the Helsinki University, Finland. The mice were maintained under conventional conditions. All experiments were approved by the Ethics Committee on animal testing of the University of Groningen.

Isolation of salivary gland cells

Salivary glands (SGs) were dissected. SG cells were isolated and cultured to form spheres as described previously [24–26] (Suppl Materials and Methods).

Self-renewal assay

Dispersed salivary gland cells were cultured in basement membrane extract (Matrigel) in enriched medium (EM) [24] and passaging every 7 days (Suppl Material and Methods).

Luciferase assay

GFL-GFRa1/RET-luc reporter gene system (Strata1LUC cell line) was used for Luciferase assay [27] (Suppl Material and Methods).

Flow cytometry

Primary salispheres were harvested after 3 days of culture [24]. Cells were incubated with a combination of cell-surface marker antibodies and flow cytometry was performed on XDP Flow Cytometer machine. Data were analyzed with FlowJo software (Ashland, OR) (Suppl Material and Methods).

Irradiation treatment (in vitro)

Single mSGSCs were γ-irradiated at 0 Gy, 1 Gy, 2 Gy, 4 Gy, 8 Gy and sphere forming efficiency (SFE), stem cell surviving fraction (SSF) and population doublings were determined as described earlier [3] (Suppl Material and Methods).

In vivo irradiation and transplantation assay

The irradiation and transplantation assay performed here was described earlier [13]. In brief, Female C57BL/6 mice salivary glands were X-ray irradiated at 15 Gy. One month later, 10,000 passage 7 single-cells of donner cells were suspended in α-MEM (Gibco) and injected intraglandularly into both SMGs of irradiated mice. 3 months later, irradiated mice were sacrificed and SGs were embedded in paraffin.

Immunostaining

Salivary glands were fixed with 4% formaldehyde and embedded into paraffin. 5 μm paraffin sections were dewaxed and immunostaining was performed (Suppl Material and Methods).

Image analysis

Immunofluorescence images of tissue sections and organoid sections were acquired with Leica SP8 confocal microscope. TissueFAXS high-throughput fluorescence microscope was used to analyze the GDNF expression of the whole tissue section after IR.

Protein extraction and Western blot analysis

Cell pellets were harvested at certain time points after irradiation and were lysed in RIPA buffer containing protease inhibitor (80 μl in 1 ml RIPA), followed by 5 s sonicate. Protein concentration and Western blot were performed using routine methods in the field (Suppl Material and Methods).

Cell cycle analysis

Mouse cells treated with or without GDNF were harvested 3 days after 4 Gy irradiation and processed to single cells from Matrigel as described above. Cell cycle analysis was performed using flow cytometry (Suppl Material and Methods).

qRT-PCR

Cells were collected at the end of each passage. Total RNA was extracted using the RNA Miniprep kit (Agilent Technologies), following the manufacturer's instructions (Suppl Material and Methods).

Data analysis

All calculations were performed using GraphPad Prism software. Two-way ANOVA tests were applied to the long-term passaging sphere formation efficiency. T-tests were applied to analysis of GDNF expression in WB or TissueFAXS. Numbers (n) for tested groups are stated in the figure legends.

Results

To investigate the role of endogenous GDNF on mSGSC survival after irradiation, we used salivary glands obtained from $Gdnf^{wt/hyper}$ mice that have an increased, but spatially unchanged, GDNF expression [23]. Using our mSGSC expansion method [24], $Gdnf^{wt/hyper}$ and $Gdnf^{wt/wt}$ mice mSGSCs were cultured as spheres and re-seeded as single cells in Matrigel with enriched medium (EM) (Fig. 1A). After passaging, sphere-derived single cells were irradiated (IR) with γ-rays (Fig. 1A), after which sphere formation efficiency (SFE) (Fig. 1B), surviving fraction (Fig. 1C) and cell