Regulation of DNA Repair Following Radiation-Induced Salivary Gland Dysfunction

By:
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Abstract:

Ionizing radiation is one of the most commonly used cancer treatments. Radiation can be extremely affective at killing malignant tissue; however, the treatment comes with a wide range of debilitating side effects. A large portion of the side effects due to radiation treatment are a direct result of the radiosensitivity of nearby tissues. The salivary gland is incredibly sensitive to ionizing radiation, and damage to this tissue may result in a drastic decrease in the quality of life of the patient. One way to decrease the side effects of radiation damage to the salivary gland is to activate DNA repair following radiation treatment. Changes in DNA damage of irradiated mice with and without IGF1 (insulin-like growth factor 1) were quantitatively measured over time with a comet assay. DNA repair response following radiation treatment was measured by amounts of γ-H2AX, phospho-RPA, and Sirtuin 1 present in mice at time points corresponding to those used in the comet assay. In mice, we have found that injection of IGF1 prior to radiation treatment results in the same amount of initial DNA damage; however, over time DNA damage quantitatively decreases at time points corresponding to the visual activation of DNA repair.

Statement of Purpose:

Each year an estimated 40,000 new cases of xerostomia with a concomitant loss of salivary gland function result from radiation treatment of head and neck cancer (NIDCR website). Radiation is a primary or secondary therapeutic modality in most head and neck radiation cases and results in chronic salivary gland dysfunction in most patients. The ensuing salivary gland hypofunction results in significant morbidity, diminishes the effectiveness of anti-
cancer therapies and decreases the quality of life for these patients. Using a mouse model, we have shown that temporary suppression of apoptotic pathology in salivary glands has significant benefits to glandular function (Limesand, KH et al 2009 PLoS One).

**Statement of Relevance:**
This project is highly relevant to cancer treatment and the expansion of the field of salivary gland research. By uncovering the role of IGF1 in DNA repair and preservation of salivary gland function, the goal of helping to ease radiation side effects become more of a reality.

**Introduction:**

Head and neck cancer affects over 1.5 million people a year in the United States and is generally treated with ionizing radiation (USCS, 2009). Radiation treatment directed at this region of the body usually results in a variety of debilitating side effects (Grundmann, Mitchell, & Limesand, in press). One direct problem is loss of saliva production due to the extreme radiosensitivity of the salivary gland (Avila, Grundmann, Burd, & Limesand, 2008). The salivary gland’s low resistance against radiation is apparent in the large amounts of DNA damage and cell death visible in irradiated tissue.

In order to sustain the function of salivary cells, it is essential to activate DNA repair in damaged tissue. The DNA damage and cell death pathways are largely regulated by p53 which receives DNA damage stimuli (Berns, 2006). IGF1 has been found to inhibit the apoptotic response of p53 and is hypothesized to simultaneously activate the cell cycle arrest and DNA repair pathways in irradiated mice (Limesand, Barzen, Quissell, & Anderson, 2003). The implications of IGF1’s ability to activate DNA repair in irradiated tissue may have a great impact
on the future of head and neck cancer treatment by alleviating the symptoms associated with radiation treatment.

**Methodology:**

Mice.

All experiments were conducted on four to five week old FVB female mice. P53 knockout mice were provided by Dr. Carla van den Berg (University of Texas, Austin, TX, USA). Mice were derived from a Balb/c background and backcrossed with FVBs for eleven generations. Genotyping of p53 knockdown mice was conducted as previously described (1,2) using PCR primers from integrated DNA technology (IDT, Coralville, IA, USA). Mice were maintained and treated in accordance with protocols approved by the University of Arizona Institutional Animal Care and Use Committee.

Treatment.

Mice were treated with ionizing radiation and IGF1, simultaneously or separately. Irradiated mice were anesthetized with avertin (0.4-0.6mg/kg) and irradiated directly on the head and neck region (\(^{60}\)Co therapeutic irradiator, Theratron-80, Atomic Energy of Canada Ltd, Ottawa, Canada) as previously described (3). IGF1 treated mice received 5μg of human recombinant IGF1 (GroPrep, Adelaide, Australia) intravenously as previously described (4). If treated with IGF1 in combination with radiation, IGF1 was administered immediately prior to radiation treatment.

Western blotting.
Parotid glands were homogenized in RIPA buffer(1) with SIGMAFAST protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA) and 5mM sodium orthovanadate. 100 mg/ml of PMSF was added and samples were boiled for 10 min and sonicated until homogenous. Protein concentrations were determined using Coomassie Plus Protein Assay Reagent (Thermo Fisher Scientific). 100 mg of each sample was loaded on polyacrylamide gels ranging in percentage from 5 to 15. Gels were transferred to a 0.45um Immobilon-P membrane and 0.20um Immobilon-P SQ (Millepore, Bedford, MA, USA), and probed with one of the following antibodies: anti-ERK (Promega, Madison, WI, USA), antiphosphorylated p53 (Ser15), phospho histone H2A.X, SirT2 (Cell Signaling Technologies, CST, Beverly, MA, USA), phospho-RPA (S4/S8) (Bethey Lab Incorporation, Montgomery, TX, USA), Sirt1 (H-300) (Santa Cruz Biotechnology Incorporated, Santa Cruz, CA, USA). Secondary antibodies, goat anti-mouse and goat anti-rabbit, were conjugated with HRP (Bio-Rad, Hercules, CA, USA) and ECL substrate (Thermo Fisher Scientific) was used for detection as instructed by the manufacturer. Membranes were stripped with Restore western blot stripping buffer (Thermo Fisher Scientific), reblocked and reprobed as previously described (1).

Neutral Comet Assay:
Parotid glands were placed in 2ml dispersion media containing 30ml Hank’s Modified Solution, 30mg Collagenase (Roche Diagnostic Corporation, Indianapolis, IN, USA), and 30mg Hyalluronidase (Sigma-Aldrich, St Louis, MO, USA), at pH 7.4. Glands were minced and passed through a 21 gauge syringe. Samples were incubated at 37°C for 10 minutes and centrifuged. Cells from mouse parotid glands or C5 rat parotid cells were resuspended in 1 x PBS (phosphate-buffered saline). Samples were diluted to approximately 20 million cells per ml and resuspended in 1% low melting agarose (Thermo Fischer Scientific) and cast onto microscope slides
(Mercedes Medical, Sarasota, FL, USA). The remainder of the procedure was performed with minimum light exposure. The slides were placed in 100ml of lysis buffer for 3 minutes (150mM NaCL, 1mM tris, 18mM SDS, 4mM EDTA, at pH 7.4) followed by a 10 minute deionized water rinse. Slides were transferred to an electrophoresis chamber in 1.5L of electrophoresis buffer (230mM tris, 180mM boric acid, and 0.2mM EDTA). Electrophoresis was run for 25 minutes at 25 constant V. 2.5 ug of propidium iodide (MP Biomedicals, Solon, OH, USA) was added to each slide. Images were taken with a Leica DM5500 (Leica Microsystems, Wetzlar, Germany) and 4 megapixel Pursuit camera (Diagnostic Instruments, Inc, Sterling Heights, MI, USA). A minimum of 20 comets were analyzed per gland using TriTek CometScore tail moment data.

**Literature Review:**

Head and neck cancer is classified as any malignancy arising in the nasal cavity, sinuses, lips, mouth, throat, or salivary gland (Head and Neck Cancer, 2005). Head and neck cancer comprises approximately 5% of all cancer types in the U.S. and is most prevalent in people over fifty years of age (Head and Neck Cancer). Radiation therapy is a common treatment for this type of cancer; however, it comes with a variety of adverse secondary side effects (Grundmann, in press). Side effects include xerostomia (decreased salivation), oral mucositis (ulceration of mucous membrane), and a heightened risk of periodontal disease which have a significant impact on the quality of life of head and neck cancer patients (Avila, 2008).

Ionizing radiation works by directing high energy photons at the tumor site (Gudkov, 2003). The radiation creates free radicals in the tissue which cause DNA damage through single and double strand breaks (Gudkov). Inadvertently, healthy tissues, such as the salivary gland, receive the same dose of radiation due to its close proximity to the tumor. Salivary glands are composed of three unique glands including the parotid, submandibular, and
sublingual glands. For the purpose of this study, the focus will rely heavily on results from the parotid gland due to its heightened radiosensitivity and role in production of stimulated saliva (Limesand, Said, & Anderson, 2009). The salivary glands are located in the upper and lower jaw region (Figure 1) and are highly likely to receive a dose of radiation if any portion of the head or neck is radiated. Radiation is administered in doses measured in Gy, with usual clinical exposure to the salivary gland staying below 2Gy/day (Grundmann, in press).

Figure 1 (U.S. National Library of Medicine, 2009)

Radiosensitivity describes the degree of vulnerability certain tissues have following exposure to ionizing radiation. Tissues with a high proliferation rate and low differentiation, such as the small intestines and bone marrow, tend to be most susceptible to the effects of radiation (Gudkov, & Komarova, 2003). The radiosensitivity of the salivary gland is in many ways an anomaly due to its low rate of proliferation, making research in this area of great importance. Acinar cells, which are responsible for saliva production and transport in the salivary gland are extremely sensitive to radiation and are excellent indicators of damage to the gland. Following large doses of radiation a 50% reduction in saliva production by acinar cells has been witnessed (Limesand, Schwertfeger, & Anderson, 2006). Adequate saliva is essential to a healthy lifestyle due to its role in food digestion, speech, and protection of the oral mucosa (Limesand, 2003).
order to diminish these harmful side effects and make the recovery of head and neck cancer patients more successful, the pathways of cell damage and death are being explored.

Healthy cell function is based on a critical harmony between cell survival, growth, and death (Limesand, 2006). When the DNA of a cell is damaged, in this case by radiation, a variety of damage detecting sensors are alerted. Once the cell receives these damage sensing stimuli, it has the ability to activate one of three pathways (Figure 2). The first option is for the cell to begin to repair its damaged DNA, the second path requires the cell to enter a state of cell cycle arrest, and the third pathway leads to apoptosis, also known as programmed cell death, with all pathways involving some form of transcription. The path taken usually depends on the severity of the DNA damage.

Figure 2 (Sancar, 2004)

A cell that receives a minimal amount of DNA damage will usually enter the path of DNA repair. Here, a cell will begin to transcribe new DNA to replace broken strands prior to continuing its path of growth and proliferation. It is hypothesized that if a cell enters the path of
DNA repair that it fully fixes all damage prior to proliferating. If damage is not sufficiently repaired, the cell has the capability to undergo apoptosis. If apoptosis is defective the cell has a heightened capacity to become cancerous upon division.

Cell cycle arrest occurs when a cell cycle checkpoint is triggered by DNA damage sensors. At this point, the cell will stop all cycle progress until the cell is repaired. Unfortunately if the cell is unable to repair itself, the state of cell cycle arrest will become irreversible. The cell will remain locked at a cell cycle checkpoint and will be unable to repair its DNA damage, grow, or divide (Gudkov, 2003).

Apoptosis, the final possibility following DNA damage, will occur if the cell is too damaged to repair itself and does not enter cell cycle arrest. It is anticipated that cells with substantial DNA damage will die rather than proliferate due to the possibility of becoming cancerous. However, the excessive death of normal tissue would have negative effects on function and therefore, it is hoped that through further understanding of the pathways of DNA repair and apoptosis a way to increase a cell’s ability to successfully repair itself following DNA damage will be found.

There are two key players in the selection and execution of these three pathways, p53 and Akt. The p53 protein has long been known to play a role in apoptosis. p53 has been shown to direct cell death based on both DNA damage as well as cancer detection. When p53 receives sufficient stimuli from DNA damage, it will likely lead the cell down an apoptotic pathway. It has been shown that if p53 is not present, as seen in p53 null mice, apoptosis will not occur; illuminating the direct link between p53 and cell death (Berns, 2006).

Akt, also known as kinase B, is a known component of cell survival and has been shown to inhibit p53 by suppressing apoptosis in a variety of cell systems. Akt inhibits p53 by
activating MDM2 (murine double minute clone 2) which negatively regulates p53 (Limesand, 2006). By binding to p53, MDM2 causes its degradation which prevents apoptosis of the cell (Figure 3). With the knowledge that Akt can negatively influence p53 to prevent cell death, the need to activate Akt in the salivary gland prior to radiation treatment may be the key to preventing both cell death and activating DNA repair.

Figure 3 (Grundmann, in press.)

Akt has been shown to be stimulated by a variety of growth factor through the P13K pathway which leads to cell survival (Limesand, 2003). IGF1 (insulin like growth factor 1), is the most potent activator of Akt in salivary acinar cells and has been linked to the maintenance of adequate levels of saliva production (Limesand, 2003). IGF1 has been shown to suppress apoptosis in C5 cells (rat parotid gland cells) following radiation through the activation of Akt which inactivates a variety of proapoptotic substrates (Limesand, 2003). Myr-Akt mice, which have been developed to express constitutively active Akt, showed the same anti-apoptotic
response as C5 cells with treatment of IGF1. This showed that IGF1 does successfully activate Akt in a manner that prevents apoptosis and contributes to the maintenance of salivary function following radiation.

Since IGF1 has been shown to prevent radiation induced apoptosis in vivo and prevent loss of salivary function, the next point of interest is to see how IGF1 affects DNA damage over time. If IGF1 has the ability to decrease DNA damage following radiation treatment of the salivary gland, then we are one step closer to understanding the mechanisms of DNA damage repair as well as the possibility of applying palliative care prior to radiation treatment of head and neck cancer patients.

**Results:**

FVB mice were irradiated at 5Gy of radiation with some being injected with IGF1 prior. Mice were dissected at time points corresponding to those shown in Figure 4. The fold tail moment of each time point shows an increase in visible DNA damage as early as 15 minutes post treatment. Damage levels decrease over time with IGF1 treated mice returning to untreated levels by 3 hours.
Figure 4. DNA damage response in vivo comet assay

Figure 5, shows the same data exhibited in Figure 4, however this presentation of the data allows for the examination of the rate of DNA repair. Both treatment groups have relatively congruent rates of repair until 1 hour post treatment, at which point the IGF1 + 5Gy treatment group undergoes an elevated rate of repair as indicated by the decreased slope of the line.

Figure 5. DNA damage response in vivo comet assay 2
\(\gamma\)-H2AX is a DNA damage marker. It appears at increased levels at sites of DNA damage. Figure 6 displays a western blot of protein samples from FVB mice that were dissected at the corresponding time points following irradiation. DNA damage indicated by the presence of \(\gamma\)-H2AX is seen from 5 minutes until 8 hours at which point it begins to decrease.

Figure 6. 5Gy \(\gamma\)-H2AX, 5min – 8hr

Somewhat different results were seen in 5Gy + IGF treated mice. Radiation alone mice, showed a substantial amount of \(\gamma\)-H2AX presence, but it 5Gy + IGF samples the presence of the protein in question is minimal and inconsistent beyond 30 minutes post treatment.

Figure 7. 5Gy + IGF \(\gamma\)-H2AX, 5min – 8hr

Several other DNA repair proteins were examined in this study. P-RPA is a necessary component in active DNA repair. In 5Gy samples, the presence of P-RPA is consistent with untreated levels, but it is clearly elevated in sample treated with IGF prior to irradiation.
Sirt-1, another protein necessary in DNA repair is shown via western blot in Figure 9. It is clearly visible that sirt-1 expression is drastically reduced in mice treated with radiation alone. When mice are treated with a combination of radiation and IGF, they maintain levels of sirt-1 comparable to untreated mice. This pattern is sustained through four hours post treatment.

Analysis:

Based on the presented data, it is clear that IGF1 mediates a different repair response in irradiated mice than those not treated with IGF1. As can be seen by the comet analysis, DNA damage decreases more quickly in IGF1 treated mice. At approximately two hours the rate of repair seen in IGF1 treated mice is much faster. This possibly shows the existence of an alternative pathway responsible for this increased rate of repair. Increased repair is essential for
head and neck cancer patients, because it will allow for preservation of salivary function post treatment. It appears that the same amount of initial damage occurs in both treatment groups, indicating the IGF1 does not have protective properties. The increased rate of repair however does show that an alternative repair pathway is being activated and utilized.

γ-H2AX corroborates the comet data, by indicating that there is less damage in IGF1 treated mice. It remains elevated in radiation alone treatment groups until eight hours, indicating that a significant amount of damage remains in the cells. The presence of γ-H2AX wanes by one hour in mice pretreated with IGF1. This solidifies the comet data by showing that there is a decrease in damage in this treatment group. These findings led us to further examine repair pathways to determine which proteins IGF1 was utilizing to activate this elevated repair.

P-RPA and sirt1 are necessary DNA repair proteins. In IGF1 treated mice, the amount of p-RPA increased from untreated and radiation alone samples. This indicates an upregulation of this repair protein by IGF1. Interestingly, sirt1 is also affected by IGF1, but in a different way. Instead of increasing levels of sirt1, the presence of IGF1 preserves its elevation levels. Without IGF1 prior to radiation treatment, sirt1 levels plummet. These correspondingly different responses to repair proteins by IGF1 indicate that IGF1 is enhancing repair by multiple mechanisms.

Further studies on this topic are currently being performed to investigate the effects of IGF1 on p53, which mediates multiple repair factors. This examination will allow us to identify where in the DNA repair pathway IGF1 makes its initial impact. Sirt1 is also being further examined to explore how its deacetylation properties play a role in this mechanism.
References:


