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Identifying cell and molecular stress after radiation in a three-dimensional (3-D) model of oral mucositis

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ABSTRACT

Mucositis is a debilitating adverse effect of chemotherapy and radiation treatment. It is important to develop a simple and reliable in vitro model, which can routinely be used to screen new drugs for prevention and treatment of mucositis. Furthermore, identifying cell and molecular stresses especially in the initiation phase of mucositis in this model will help towards this end. We evaluated a three-dimensional (3-D) human oral cell culture that consisted of oral keratinocytes and fibroblasts as a model of oral mucositis. The 3-D cell culture model was irradiated with 12 or 2 Gy. Six hours after the irradiation we evaluated microscopic sections of the cell culture for evidence of morphologic changes including apoptosis. We used microarrays to compare the expression of several genes from the irradiated tissue with identical genes from tissue that was not irradiated. We found that irradiation with 12 Gy induced significant histopathologic effects including cellular apoptosis. Irradiation significantly affected the expression of several genes of the NF-kB pathway and several inflammatory cytokines, such as IL-1B, 1L-8, NF-kB1, and FOS compared to tissue that was not irradiated. We identified significant upregulation of several genes that belong to damage-associated molecular patterns (DAMPs) such as HMB1, S100A13, SA10014, and SA10016 in the 3-D tissues that received 12 Gy but not in tissues that received 2 Gy. In conclusion, this model quantifies radiation damage and this is an important first step towards the development 3-D tissue as a screening tool.

1. Introduction

Despite the benefits of chemotherapy and radiation therapy for cancer patients, mucositis remains a debilitating adverse effect. Patients with mucositis may suffer from ulcers in both their mouths and intestines. In mucositis, the mucosal surfaces of the mouths and intestines of patients are morphologically and physiologically changed following radiation and chemotherapy. Mucositis is a direct cause of suffering, distress, and severe debilitation in 40–100% of patients undergoing cancer treatment, and occasionally even leads to death [1]. Mucositis develops in five stages: The first phase, the initiation phase, occurs immediately after direct mucosal tissue damage due to the generation of reactive oxygen species. The second phase, primary damage response (signaling), is characterized by upregulation and message generation. In the second phase NF-kB, tumor necrosis factor (TNF), and other cytokines, such as interleukin-6 (IL-6) are upregulated. The third phase is the amplification phase. During the third phase, signal amplification, the proinflammatory cytokines increase production of cytokines, resulting in ulceration (fourth phase), during which the epithelium breaks and is colonized by bacteria. It is this fourth stage where mucositis becomes clinically evident. The last phase of mucositis is the healing phase, which is accompanied by restored epithelium integrity; this occurs after the cessation of radiation therapy [1]. The initiation phase is considered as one of the most critical stages, "a gatekeeper" in the development of mucositis. By delaying or stopping the initiation phase the irradiation injury may be prevented or minimized [2].

When cells experience mechanical, or chemical, stress, they produce molecular signals alerting their neighboring cells of imminent danger. Collectively, the signals that alert the cells to respond to damage are called damage-associated molecular patterns (DAMPs) [3]. Cells that experience chemotherapy or radiation stress may result in release of endogenous chemotherapy and or radiation induced damage-associated patterns (CRAMPs) which may play an important role in initiating toxicity [2]. Recognizing DAMPs or CRAMPs in the initial phase of mucositis will help us

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understand the initiation phase of mucositis and formulate prophylactic and therapeutic interventions.

Currently, there are only a few effective regimens for the prophylaxis or treatment of oral mucositis. Part of the difficulty of finding new and effective therapies for the treatment and prevention of mucositis is the lack of both clear molecular stress signatures after radiation, and a suitable research model for oral mucositis. Monolayer cultures of oral keratinocytes have been used in the study of mucositis but two-dimensional (2-D) cell cultures are not accurate representations of reality because cells live in three dimensions. Efforts to develop oral mucositis models have been reported using biopsies [4] and in-vitro reconstructed skin [5–7]. The transcriptional response of primary skin fibroblasts and keratinocytes (cultured separately in 2D cell cultures) to ionizing radiation has been evaluated by microarray analysis [8].

We wished to evaluate a three-dimensional (3-D) human cell culture of oral epithelia as a model of oral mucositis. The 3-D tissue, which consisted of both keratinocytes and fibroblasts, was irradiated in such a way as to emulate the effect of irradiation on a patient's oral mucosa. Six hours after the irradiation we checked the morphology of the 3-D human cell culture oral mucosal tissue using hematoxylin and eosin (H&E) staining, evaluated apoptosis using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, and used microarrays to compare the expression of several genes from the irradiated tissue with identical genes from tissue that was not irradiated. We studied the effect of irradiation on apoptosis, and on signaling pathways such as NF-kB, TNF, and IL-6and examined the DAMPs after irradiation. We found that irradiation with 2 Gy does not significantly alter histopathology and cell apoptosis. However, irradiation with 12 Gy induced significant histopathologic effects, apoptosis, and affected the expression of several genes of the NF-kB pathway, and several inflammatory cytokines.

2. Materials and methods

2.1. 3-D tissues

The tissue model described here is an organotypic culture of normal human oral keratinocytes grown on the top of fibroblasts, and cultured in serum-free medium to form a 3-D differentiated tissue histologically similar to buccal mucosa. The 3-D tissue EpiOral was purchased from MatTek Corporation (Ashland, MA). The cell culture inserts consisted of human fibroblasts on the bottom and human oral keratinocytes on top of the fibroblasts. After several days of submerged culture, the culture inserts containing the developing tissues were elevated to the air–liquid interface to induce stratification and differentiation. Growth medium was placed beneath the culture inserts, and thus the tissue was nourished by medium permeating through the microporous membrane on the bottom of the cell culture inserts.

2.2. Irradiation

The 3-D oral mucosal tissue was irradiated at the City of Hope facilities. The tissues were exposed to gamma radiation at doses of 0, 2, and 12 Gy. Subsequent to irradiation, the tissues were incubated for 6 h at 37 °C and 10% CO_2. Next, the tissues were harvested, and some were placed in 10% formalin for histopathologic studies; others were used for the extraction of total RNA.

2.3. Histology

Tissues that were placed in 10% formalin were used for staining with hematoxylin and eosin. The samples were then processed to facilitate visual examination of radiation damage.

2.4. TUNEL assay

In order to visualize the apoptotic cells, we performed a TUNEL assay. The TUNEL assay is a fluorimetric assay designed to detect apoptotic cells within a cell population. It is a non-radioactive method that provides accurate detection of apoptotic cells by measuring DNA fragmentation in the nuclei. Nuclear DNA fragmentation is the hallmark of apoptosis in cells. The In situ Cell Death Detection Kit (Roche, Philadelphia, PA) was used for the TUNEL assay. Briefly, the tissue was treated with Proteinase K and rinsed twice. The area around the tissue was wiped, and an equilibrium buffer that contained the labeled nucleotide mix and TdT enzyme was added; the mixture was left to incubate at 37 °C for 1 h in a humidified chamber, and the staining was observed using a Nikon Eclipse fluorescence microscope.

2.5. Gene expression

Total RNA was extracted using RNeasy Plus Mini Kit (Qiagen, Germantown, MD). DNA microarray analysis was performed using Human Whole Genome OneArray™ (Phalanx Biotech, Palo Alto, CA). RNA quality and integrity were determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), and absorbance at A260/A280. Only high quality RNA with a RIN of >7.0, and an A260/280 absorbance ratio of >1.8, was used for further experimentation. RNA was converted to double-stranded cDNA and amplified using in vitro transcription that included amino-allyl UTP; the RNA product was subsequently conjugated with Cy5™ NHS ester (GEH Lifesciences). Fragmented RNA was hybridized at 42 °C overnight using the HybBag mixing system with 1× OneArray Hybridization Buffer (Phalanx Biotech), and 0.01 mg/ml sheared salmon sperm DNA (Promega, Madison, WI, USA), at a concentration of 0.025 mg/ml labeled target. After hybridization, the arrays were washed according to the OneArray protocol.

Raw intensity signals from each microarray were captured using a Molecular Dynamics™ Axon 4100A scanner, measured using GenePixPro™ Software, and stored in GPR format. The data from all microarrays in each experimental set was then passed to Rosetta Resolver (Rosetta Biosoftware) for analysis. Testing was performed in triplicate by combining the technical replicates and performing statistical analyses using Rosetta Resolver's proprietary modeling techniques. The data were then imported into an Excel (Microsoft) database, with the corresponding gene names.

3. Results and discussion

Radiation damage in the epithelium was studied in vitro using 3-D cultures of human oral mucosal keratinocytes and fibroblasts. To determine the effect of radiation on the quality of the epithelium in the 3-D oral mucosal tissue, H&E staining was performed. Non-irradiated samples showed a well-differentiated, multilayered epithelium that resembled the natural mucosal epithelium. The epithelium of the non-irradiated 3-D tissue was also well organized into a basal monolayer with cylindrical cells (stratum basalis), and a regular suprabasal spinous layer (stratum spinosum). On the top of the 3-D tissue, there were flattened keratinocytes forming the stratum corneum (Fig. 1A). The stratum corneum is usually lacking in squamous epithelium lining mucosal surfaces, but is always a constituent of the squamous epithelium covering the skin.

Irradiation with 2 Gy did not cause significant morphological changes to 3-D tissue (Fig. 1B). Irradiation with 12 Gy, however, caused significant changes in the morphology of the 3-D tissue. In the non-irradiated tissue, focal epithelial hyperplasia was evident, and the stratum corneum formed by flattened cells was intact; however, the 12 Gy-irradiated tissue lost its coherence, and

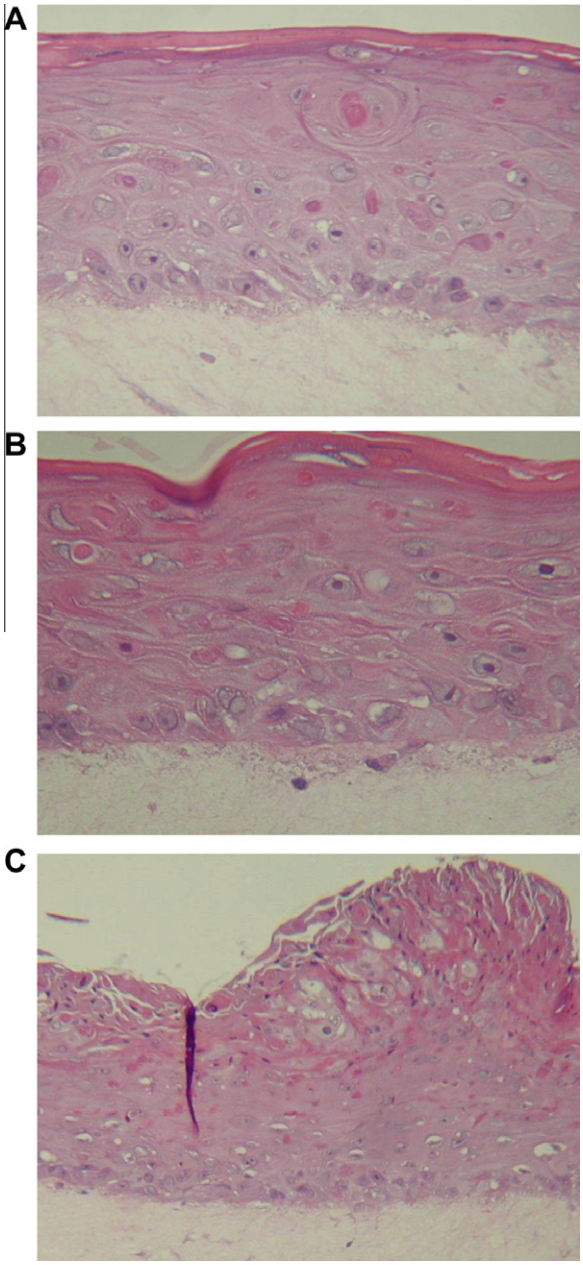

Fig. 1. Hematoxylin and eosin staining of 3-D tissue before irradiation. The epithelium is shown in pink. It consists of human keratinocytes. The epithelium is divided into the stratum corneum (the flat horizontal cells), the stratum spinosum (the spindle-shaped cell layer), and the stratum basalis (basal layer), which is at the bottom of the keratinous epithelium (A). A 3-D cell culture of human oral mucosa 6 h after irradiation with 2 Gy. The stratum corneum, spindle layer, and stratum basalis are clearly differentiated. There is no significant difference in the appearance of the sample irradiated with 2 Gy and the one that was not irradiated (control) (B). 3-D human oral mucosal tissue after 12 Gy irradiation. The keratinous epithelium shows an atypical proliferation, especially at the top, and an increase in the numbers of pyknotic cells, especially in the proliferative stratum basalis (C).

parts of it disappeared altogether, to be replaced by swollen vacuolated cells exhibiting loss of polarity (Fig. 1C). Furthermore, the 3-D tissue exposed to 12 Gy was marked by the appearance of apoptotic cells. Donetti et al. observed that irradiation had consequences for the integrity of human oral mucosa [4].

3.1. Apoptosis

The TUNEL assay stains fragmented DNA in the nuclei of apoptotic cells. It is a non-radioactive method that can accurately detect apoptosis. The apoptotic areas are colored bright red. In our experiments, the TUNEL assay showed that non-irradiated 3-D tissue had minimal apoptotic cells (Fig. 2A). Also, the 3-D tissue treated with 2 Gy resembled the non-irradiated tissue in terms of the number of apoptopic cells (Fig. 2B). In contrast, the 3-D tissue irradiated with 12 Gy showed large areas populated with apoptotic cells (Fig. 2C).

3.2. NF-kB pathway

Radiation-induced upregulation of early key genes may cause a domino effect that result in the activation of other genes with consequent tissue damage [9,10]. In this study, 19 genes of the NF-kB pathway were significantly affected, upregulated or downregulated after exposure to 12 Gy radiation (Table 1). Most prominently, IL-8, IL-1B, NF-kB1, and NF-kB1A were significantly affected ($P < 0.05$) in 12 Gy-irradiated tissue, whereas the same genes in the tissue treated with 2 Gy were not significantly

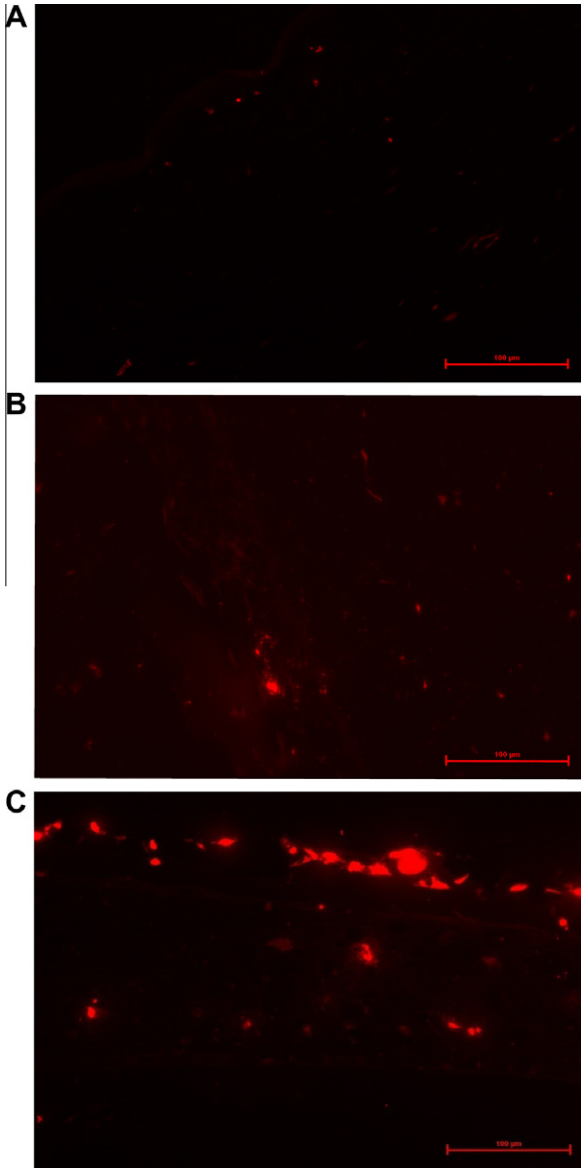

Fig. 2. TUNEL assay results to determine the degree of apoptosis in 3-D human oral tissue culture. The apoptotic cells are stained bright red. Non-irradiated 3-D tissue (A). 2 Gy irradiated 3-D tissue (B). 12 Gy irradiated 3-D tissue showing a significantly greater degree of bright red staining (C).

Table 1
NF-kB pathway, inflammatory response and cytokine genes upregulated or downregulated in the 3-D oral human tissue culture after 12 and 2 Gy irradiation.

Gene symbol	NF-kb pathway, inflammatory response and cytokine genes Times upregulated or downregulated compared with the untreated control		Gene_ID
	Tissue exposed to 12 Gy	Tissue exposed to 2 Gy	
IL-1B	0.54*	1.27	3553
IL-8	1.81*	1.29	3576
MYD88	0.68*	0.87	4615
TLR1	1.53*	0.71	7096
NF-kB1	0.74*	1.76	4790
NF-kBIA	1.66*	1.11	4792
RELA	1.62*	1.10	NA
STAT1	1.53*	1.66	6772
MYD88	0.68*	0.87	4615
SLC20A1	0.50*	1.27	6574
TNFSF10	0.78*	0.69	8743
HMOX1	0.64*	0.76	3162
RHOA	1.50*	1.31	387
CASP1	1.50*	0.95	834
ICAM1	1.25*	0.74	3383
EGR1	2.57*	2.23	1958
FOS	1.96*	2.11	2353
JUN	1.85*	1.16	3725
TNFAIP3	1.83*	0.90	7128
CCL5	2.46*	2.34	6352
CCL8	0.66*	0.85*	6355
CCL11	0.54	1.48	6356
CXCR4	0.89	0.51*	7852
CEBPB	1.56	2.13*	1051
ABCF1	0.67*	1.00	23

* $P < 0.05$.

affected. Furthermore, there were significantly higher levels of early response genes, such as JUN and FOS, in the 12 Gy-irradiated tissue, but not in the 2 Gy tissue. We also observed upregulation of TNFAIP3, TNF alpha-induced protein, and MAPK3, dual specificity mitogen activated protein kinase 3, as well the CXXC5-type zinc finger protein 5.

3.3. Inflammatory response

Several inflammatory response elements and cytokines were affected by 12 Gy irradiation but not by 2 Gy irradiation. Small chemokines were affected by 12 Gy irradiation, including CCL11 (also known as eotaxin), which selectively recruits eosinophils; CCL5, which recruits eosinophils and basophils to areas of inflammation; and CCL8, which is monocyte chemotactic and activates many different immune cells, such as mast cells, eosinophils, and basophils [11,12]. Imadome et al. found upregulation of stress response elements such as CCL3 when tumors were irradiated [13]. ABCF1, ATP-binding cassette sub-family F member 1 was significantly downregulated in the 12 Gy group (Table 1). The proteins encoded by this group are transport proteins, but ABCF1 in particular is regulated by TNF-alpha, which is involved in inflammatory and autoimmune reactions [14,15].We found a significant downregulation of IL-1B 6 h after irradiation; other researchers have observed a significant upregulation of the same gene at days 12 and 15 following irradiation but not earlier than 12 days [16,17]. IL-8 another important inflammatory cytokine is significantly upregulated 6 h after 12 Gy irradiation (Table 1). Its primary function is to recruit neutrophils to phagocytose the antigen [18]. IL-8 has also been reported to be upregulated after irradiation [19]. After 12 Gy irradiation the ATF4 transcript was significantly induced in our experiments. Genes in the ATF family are stress inducible and the ATF3 gene was significantly upregulated in normal keratinocytes 4 or 8 h after a high dose of radiation [20].

3.4. DAMPs

Trauma and pathogens cause tissue and cell damage but the tissue has ways to deal with such damage. When tissues are injured, they release molecules that communicate the injury to the rest of the tissue. Damage associated tissue patterns, DAMPs, are initiated after trauma or tissue damage, and are recognized at the cell level. The term DAMPs also includes pathogen-associated molecular patterns (PAMPs) and alarmins which are molecules that signal tissue and cell damage. Specifically, alarmins are endogenous molecules that signal tissue and cell damage [3]. A subset of alarmins are CRAMPs which are endogenous molecules released after chemotherapy and radiation damage [2]. All of these molecules (a) are rapidly released following non-programmed death of cells, (b) can be released from live cells of the immune system, (c) promote the adaptive immune response because they recruit and activate receptor-expressing cells of the immune system such as dendritic cells, and (d) promote reconstruction of the damaged tissue [3,21]. The list of DAMPs includes high mobility group box 1 (HMGB1), S100, heat shock proteins, interleukin-1alpha, defensins and annexins [3,21,22]. In this experiment, we identified several putative DAMPs, including S100A13, SA100A14, and S100A16, which are calcium-binding proteins associated with inflammation (Table 2). The most characteristic DAMPs molecule is HMGB1. This is a protein in the nucleus of the cell that binds to nucleosomes. When released from necrotic cells, it induces inflammation, dendritic cell maturation, and T-cell activation [18]. HMGB1 plays a central role in inflammatory responses [23]. The gene LGALS8 was significantly upregulated 6 h after irradiation with 12 Gy. It encodes galectin-8 protein which provides co-stimulatory and proliferative signals to T lymphocytes. Galectins are a family of mammalian carbohydrate binding proteins which interact with a wide range of cell surface glycoproteins to regulate the immune cell function [24]. In this experiment, HMGB1 was significantly upregulated (1.6 times) by both radiation treatments. In the microarray, 738 genes were upregulated in the group exposed to 12 Gy irradiation, and 465 were upregulated in the group exposed to 2 Gy irradiation ($P < 0.05$). Of these genes, 155 were common to both groups. A total of 233 genes were downregulated in the 12 Gy group, and 258 genes in the 2 Gy group ($P < 0.05$); 22 of these genes were common to both groups.

In summary, we observed a significant effect of 12 Gy irradiation on certain genes, the majority of which were not affected by 2 Gy irradiation. Significant histological changes were also caused by

Table 2
Putative DAMPs genes upregulated or downregulated in the 3-D oral human tissue culture after 12 and 2 Gy irradiation.

Gene symbol	Putative DAMPs genes Times upregulated or downregulated from tissue expo0073ed to the untreated control		Gene_ID
	Tissue exposed to 12 Gy	Tissue exposed to 2 Gy	
HMGB1	1.61*	1.22*	3146
S100A13	1.73*	1.78*	57402
S100A14	1.80*	1.37	6284
S100A16	1.43*	1.72*	140576
HSPA1A	0.50*	0.98	NA
HSPA4	2.53*	1.74	3308
EDN1	1.69*	0.95	1906
LGALS8	3.56*	2.82	3964
ANXA4	2.08*	1.20	307
PANX1	1.578*	1.41	24145
ANXA5	1.438*	1.06	308
TLR1	1.53*	1.40*	7096
IL-1R2	2.03*	1.78*	7850

* $P < 0.05$.

12 Gy irradiation in the 3-D human cell culture oral mucosal model. Furthermore, apoptosis was profound in 12 Gy-irradiated tissue, but no significant apoptosis or histological changes were observed in 2 Gy tissues. These experiments indicate that the of the 3-D human cell culture of oral mucosal model quantifies radiation damage and this is an important first step towards the development of the 3-D tissue as an in vitro screening tool for mucositis drugs.

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