Inhibition of caspases protects mice from radiation induced oral mucositis and abolishes the cleavage of RNA binding protein HuR

Sudha Talwar¹, Reniqua House¹, Santhanalakshmi Sundaramurthy¹, Sundaravadivel Balasubramanian², Hong Yu¹, and Viswanathan Palanisamy¹

¹Department of Craniofacial Biology and Center for Oral Health Research, College of Dental Medicine, Medical University of South Carolina, Charleston, SC 29425, USA
²Department of Cardiology, College of Medicine, Medical University of South Carolina, Charleston, SC 29425, USA

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Address correspondence to: Viswanathan Palanisamy, Department of Craniofacial Biology, Medical University of South Carolina, Charleston, SC 29425, USA; Email: visu@musc.edu; Telephone: 843-792-5701. Fax: 843-792-6696.

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Background: In oral mucositis, cleavage product-1 of RNA-binding protein HuR regulates cell death.

Results: Activation of caspases initiates HuR cleavage modification and stabilizes BAX, thereby promoting apoptosis in oral mucositis.

Conclusion: Inhibition of caspases reduced the severity of oral mucositis through HuR and the expression of BAX.

Significance: This is the first study implicating the RNA-binding protein HuR in oral mucositis.

Abstract

The oral mucosal epithelium is typically insulted during chemotherapy and ionizing radiation (IR) therapy and disposed to mucositis, which creates painful inflammation and ulceration in the oral cavity. Oral mucositis alters gene expression patterns, inhibits cellular growth, and initiates cell death in the oral epithelial compartments. Such alterations are governed by several different factors, including transcription factors, RNA-binding proteins (RBPs), and microRNAs. IR-induced post-transcriptional regulation of RBPs exists but is poorly studied in clinically relevant settings. We herein report that the RBP Human antigen-R (HuR) undergoes cleavage modification by caspase-3 following IR-induced oral mucositis, and subsequently promotes the expression of the pro-apoptotic factor Bcl-2-associated X protein (BAX), as well as cell death. Further analyses revealed that the HuR cleavage product-1 (HuR-CP1) directly associates and stabilizes the BAX mRNA, and concurrently activates the apoptotic pathway. On the other hand, a non-cleavable isoform of HuR promotes the clonogenic capacity of primary oral keratinocytes and decreases the effect of IR-induced cell death. Additionally, specific inhibition of caspase-3 by a compound, NSC321205, increases the clonogenic capacity of primary oral keratinocytes and causes increased basal layer cellularity, thickened mucosa, and elevated epithelial cell growth in the tongues of mice with oral mucositis. This protective effect of NSC321205 is mediated by a decrease in caspase-3 activity and the consequent inhibition of HuR cleavage, which reduces the expression of BAX in mice with IR-induced oral mucositis. Thus, we have identified a new molecular mechanism of HuR in the regulation of mRNA turnover and apoptosis in oral mucositis, and our data suggest that blocking the cleavage of HuR enhances cellular growth in the oral epithelial compartment.

Oral mucositis is a side effect of cancer treatment and frequently occurs in patients with head and neck squamous cell carcinoma (HNSCC) who have been treated with chemo- and radiation therapy directed at the oral cavity (1). It is characterized by the complete breakdown of the epidermis and mucosal epithelia, and ulcerative
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lesions that result in the restriction of oral intake and, most importantly, provide sites for secondary infection and microbial entry portals (2-4). Mucositis limits the ability of patients to tolerate optimal anti-cancer treatment modalities, thereby compromising cancer therapy outcomes and patient survival (2). Several drugs are known to reduce the severity of mucositis (2), but drugs such as palifermin or intravenous injection of human recombinant keratinocyte growth factor (KGF) are commonly used to reduce the severity of mucositis (5). Moreover, indirect damage to normal oral epithelial tissues during cancer radiotherapy is considered significant and still remains a major issue (6). Therefore, the healthcare cost associated with mucositis is substantially high (7). Hence, accurate diagnosis and therapeutic interventions for oral mucositis after the initiation of cancer treatment is important.

Apoptosis is considered an important constituent of chemoradiation-induced mucosal injury (2), but the magnitude of apoptosis in oral mucositis has not been well-defined. Both chemotherapy and radiotherapy damage the mucosal lining and cause apoptosis (2). Ionizing radiation (IR) activates several transduction pathways that activate transcription factors such as p53 and nuclear factor kappa B (NF-κB) (8). Interestingly, NF-κB activation leads to activation of the expression of both pro-apoptotic and anti-apoptotic genes that are significant factors in determining the fate of normal tissues following radiation or chemotherapy (8). All of these sequential events induce apoptosis by the activation of the pro-apoptotic factor Bcl-2-associated X protein (BAX) (9), resulting in mucositis. On the other hand, radiotherapy is capable of causing apoptosis in submucosal fibroblasts, which precedes epithelial injury (10). The activation of apoptosis in the mucosal epithelium appears to be linked with caspase-3 activity in fibroblasts (11). Both caspase-3 and BAX are known to be critical mediators of apoptosis in mammalian cells (12,13). BAX is predominantly present in the cytosol of normal cells, but it translocate to the mitochondria following DNA damage and triggers caspase activation, resulting in apoptosis (14,15). Thus, activation of BAX in association with caspase activity is a serious consequence leading to IR-induced apoptosis.

Mammalian Hu Antigen R (HuR) is an essential and ubiquitous member of the embryonic lethal abnormal vision/Hu family of RNA-binding proteins (RBPs) and plays a central role in the regulation of cell growth and cancer progression (16). In contrast, HuR promotes cells to undergo differentiation and senescence, thereby exiting the proliferative cell pool (17). In particular, HuR promotes the stability of mRNAs encoding pro-survival proteins in a variety of cells (18). HuR regulates both pro- and anti-apoptotic functions by controlling the stability of target mRNAs (19-21). Under various stress conditions, including ionizing radiation, HuR undergoes post-translational modification and regulates mRNA turnover (22). These post-translational modifications include cleavage of HuR by the activation of caspase-3, and these HuR cleavage products increase the apoptotic response and associate with the AU-rich regions of the mRNA (23,24). While the functions of HuR have been extensively studied for cell proliferation, few of the apoptosis-related functions have been identified. Recent report revealed that HuR shifts its role from pro-survival to the cell death pathway during the cell response to severe stress (24). Although the balance between shifting HuR functions from survival to cell death is known, its physiological significance under diseased condition is remains elusive. We thus hypothesized that ionizing radiation (IR) induces the post-translational modification of HuR and in turn controls the expression of AU-rich elements (AREs) containing pro-apoptotic mRNAs during the development of oral mucositis. Here we report that HuR undergoes cleavage, and the cleavage product-1 of HuR (HuR-CP1) promotes the stability of the pro-apoptotic BAX mRNA, both in vitro and in vivo. A recently characterized small molecular compound NSC321205, known to be an allosteric inhibitor of caspases (25), inhibits caspase-3 activation after IR, thereby repressing the cleavage of HuR and reducing the rate of apoptosis both in vitro and in vivo. These observations compelled us to examine whether IR-induced oral mucositis has any effect on the cleavage of HuR and whether the subsequent inhibition of caspase-3 activity could alleviate oral mucositis.
Results

IR-induced activation of caspase-3 promotes cleavage of HuR in normal oral keratinocyte cells compared with oral cancer cells. Cancer regimens, such as chemotherapy and radiotherapy, alter protein expression patterns, and influence gene expression in mammalian cells. For instance, HuR controls the stability of target mRNAs that encode proteins which promote both growth and apoptosis, and thus is influenced by both ionizing radiation (22) and chemotherapeutic treatments (26). Previously, we have shown that surgically removed human tongue tissues obtained after chemo- or radio-therapy showed significant cleavage of HuR in adjacent to normal tissues compared with cancer tissues (23). In addition, oral cancer cells subjected to chronic hypoxic stress exhibit activation of caspase-3, which promotes the cleavage of HuR (23). To further establish how HuR cleavage occurs in normal and cancer cells, we asked whether IR can induce apoptosis and HuR cleavage in normal primary human oral keratinocytes (HOKs) and UM74B oral cancer cells. First, we treated both cell lines with 16 Gy IR and studied the cleavage of HuR in a time-dependent manner. As shown in Figure 1A, cleavage of HuR in normal HOKs began after 15 min of IR treatment, and considerably cleaved at 2 h. In contrast, oral cancer cells required a minimum of 12 h to trigger HuR cleavage (Fig. 1A, right panel), whereas 12 h after IR, normal cells had completely undergone cell death (data not shown) and were unable to produce sufficient amounts of protein for detection. These observations suggest that HuR is cleaved in normal cells more readily than in cancer cells. Second, HuR was localized in the nucleus of HOK cells under normal conditions, whereas within 2 h after IR treatment, it was exported to the cytoplasm (Fig. 1B). In contrast, lethal stress was required to induce the export of HuR from the nucleus to the cytoplasm in oral cancer cells (23). Thus, nuclear export and cleavage of HuR is cell-specific and highly dependent on physiological stress conditions. Third, caspase-3 is known to induce the cleavage of HuR (23,27); therefore, we investigated whether the cleavage of HuR in HOK cells after radiation is dependent on caspase-3 activity. We have observed an increased activity of caspase-3 during the cleavage of HuR (Fig. 1C) and activity of caspase-3 and HuR cleavage were both abolished by the addition of the caspase inhibitor benzoylcarboxyl-Val-Ala-Asp (zVAD), (Fig. 1D). This observation demonstrates that HuR cleavage is dependent on the activity of caspase-3 under IR. Interestingly, increased expression of pro-apoptotic factor BAX is observed during active cleavage of caspase-3 and HuR (Fig. 1C and right panel depicts the quantitative informations of both HuR cleavage product-1 and BAX). Inhibition of caspase-3 with zVAD also reduces the expression of BAX under IR (Fig. 1D). These observations suggest that IR promotes cleavage of both caspase-3 and HuR and concurrently increases the expression of BAX. Finally, to confirm whether IR-treated cells promote apoptosis, we quantified the percentage of annexin V/propidium iodide-positive cells using flow cytometry. IR treatment significantly increased the number of annexin V-positive cells compared with untreated control cells (Fig. 1E). Taken together, our data indicate that IR induces the nuclear export and cleavage of HuR in association with activation of caspase-3 and expression of BAX in HOK cells.

IR-induced HuR influences steady-state levels of BAX mRNA. To investigate whether IR-induced changes directly influence the targeting of ARE-containing mRNAs by HuR, we first analyzed known HuR target transcripts that are altered during IR (22) using reverse transcription quantitative polymerase chain reaction (RT-qPCR) and examined alterations in their abundance in HOK cells. We observed increased expression of mRNAs encoding murine double minute protein-2 (MDM2; \( p < 0.05 \)), Bcl-2-like protein 11 (BCL2L11), and BAX (\( p < 0.01 \)), and no change in Bcl-associated athanogene 5 (BAG5) and Bcl-associated death promoter (BAD) after radiation (Fig. 2A, right panel), compared with untreated HOK cells. To confirm that the amounts of ARE mRNAs are directly controlled by HuR under IR induction; we analyzed their expression levels after knockdown of HuR using RT-qPCR. As shown in Figure 2B, under IR, the quantity of BAX is significantly (\( p < 0.05 \)) reduced in HuR-knockdown cells compared with control siRNA-transfected cells. We did not see significant changes in the levels of other mRNA species.
Thus, following IR, either HuR or its cleavage product controls the expression of BAX mRNA. Next, to investigate whether HuR influences the steady-state levels of target mRNAs, we examined the half-lives of BAX and BAG5 mRNA after IR. The mRNA quantities of BAX and BAG5 in untreated and IR-treated HOK cells were measured by RT-qPCR at 0, 1, 2, and 4 hr time points after actinomycin D (ActD) treatment and the half-life of the mRNA was calculated. The half-life of the BAX mRNA was 2.2 h in untreated HOK cells, and increased to more than 4 h in IR-treated HOK cells (Fig. 2C). However, we did not see a dramatic change in the stability of BAG5 mRNA (from 2.75 h to 3.2 h) after IR treatment (Fig. 2D). These data indicate that the steady-state level of BAX mRNA is increased following IR-induced HuR cleavage.

Next, to examine if IR alters the association of HuR with target mRNAs, ribonucleoprotein (RNP) immunoprecipitation (IP) was carried out with an anti-HuR antibody, followed by RT-qPCR analysis to detect HuR targets BAX, MDM2, BCL2L11 and BAG5 mRNAs. BAX is one of the known targets of HuR during IR treatment (22) and BAG5 is an anti-apoptotic protein (28) containing AU-rich consensus sequences in the 3' UTR of its mRNA (29). In agreement with the relative expression levels of BAX and BAG5, we observed an approximately 2-fold enrichment of HuR-bound BAX (Fig. 2E) in IR-treated cells compared with both unbound IgG beads and untreated cells. Surprisingly, MDM2, BCL2L11 and BAG5 did not exhibit significant association with HuR in either treated or untreated cells (Fig. 2E). Thus, IR induces HuR to preferentially associate with BAX and acts as a factor for its stability. Collectively, these data support our hypothesis that IR induces HuR cleavage and concurrently overexpress BAX mRNA in HOK cells.

**HuR-CP1 directly associates with and increases the stability of BAX mRNA.** First, to examine whether the overexpression of HuR isoforms does play a role in mRNA binding and stability; we transfected HOK cells with GFP, GFP-HuR-FL, GFP-HuR-CP1, and GFP-HuR-D226A (in which the cleavage amino acid aspartate 226 was mutated to alanine) and studied their cleavage patterns under irradiation. IR induces cleavage of GFP-HuR-FL and failed to induce cleavage of GFP-HuR-D226A compare to control cells (Fig. 3A). We did not see cleavage of cellular HuR partly due to strong expression of exogenous proteins masking the effect of radiation to the endogenous proteins. To determine if the cleavable and non-cleavable isoforms of HuR associated with BAX and BAG5 mRNAs, we expressed GFP, GFP-HuR-FL, GFP-HuR-CP1, and GFP-HuR-D226A in oral keratinocytes (Fig. 3A) and performed RNP IP using an anti-GFP antibody followed by RT-qPCR analysis. After IR treatment, HuR-CP1 is associated significantly with BAX (p < 0.01) in comparison with HuR-D226A (p < 0.05) and GFP (Fig. 3B). This suggests that HuR-CP1 is more selectively associated with BAX, and possibly promotes cell death after IR treatment. No significant changes were observed in the association of BAG5 with GFP, GFP-HuR-FL and GFP-HuR-D226A; however, it exhibited little association with HuR-CP1. These data suggest that HuR-CP1 may exhibit greater RNA-binding capacity than its native full-length form. Next, to validate the changes we observed in the association of BAX and BAG5 mRNAs with HuR isoforms, we estimated the steady-state levels of BAX and BAG5 in cells transfected with GFP, GFP-HuR-CP1, or GFP-D226A. Overexpression of HuR-CP1 resulted in an increase in the t½ of BAX mRNA (Fig. 3C) compared with GFP and GFP-D226A mRNA. However, we did not observe substantial changes in BAG5 mRNA stability with HuR isoforms (Fig. 3C). Together, these findings demonstrate that IR-induced HuR associates with BAX, leading to increased mRNA stability. To further understand whether the rate of apoptosis is influenced by HuR isoforms, we measured apoptosis in HOK cells after IR. An increased rate of apoptosis was observed in GFP-HuR-CP1 cells (34.2%) compared with HuR-FL and HuR-D226A (12.3% and 10.6%, respectively (Fig. 3D). These data suggest that the overexpression of HuR-FL and HuR-D226A blocks apoptosis following IR treatment. The impact of the expression of HuR isoforms in primary oral keratinocytes cells is quite remarkable. Previously, we have shown that cell survival was increased during hypoxia upon treating oral cancer UM74B cells with HuR-D226A (23). In this study, using the same model, we irradiated HOK cells and
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studied colony size and colony-forming efficiency. HuR-D226A-expressing cells significantly enhanced the colony size of HOKs compared with GFP and GFP-HuR-CP1 cells, and this increment is maintained after irradiation (Fig. 3E). Along with the increase in colony size, HuR-D226A increased the colony-forming efficiency of control and irradiated HOK cells (Fig. 3E, right panel). These findings indicate that HuR-D226A increases survival rate and blocks cell death after irradiation in HOK cells. This effect may be due to the decreased expression of BAX under irradiation due to the decreased steady-state level of BAX, as shown above. Collectively, our data suggest that HuR-CP1 promotes apoptosis, decreases cell survival, and promotes the stability of BAX after IR treatment.

Allosteric inhibition of caspase-3 protects primary oral keratinocytes from cell death after IR. A recent high-throughput screening (HTS) study identified four different compounds (NSC321205, NSC277584, NSC321206, and NSC310547) that exhibit allosteric inhibition of caspases such as caspase-3, -7, and -9 (25). Based on kinetic, biochemical, and structural analyses, the authors concluded that all of the four compounds bind to the catalytic site of caspase-3, -7 and -9, and inhibit cytochrome c-mediated caspase activation. Hence, we wanted to test whether these compounds were able to block cellular apoptosis through the inhibition of caspase-3 and block the cleavage of HuR in primary oral keratinocytes after IR. The intrinsic apoptotic pathway was triggered by IR in HOK cells, and treatment with 10 µM concentration of compound NSC321205 (named Comp-A) substantially reduced the activation of caspase-3 following IR (Fig. 4A). Notably, inhibition of caspase-3 activity by Comp-A after irradiation has not only abolished HuR cleavage but also reduced the expression of BAX in HOK cells (Fig. 4A, right panel depicts the quantity of HuR-CP1 and BAX proteins). The percentage of annexin-V and propidium iodide (PI) measured using flow cytometry confirmed the inhibition of IR-induced apoptosis by Comp-A in HOK cells (Fig. 4B). These data suggest that Comp-A inhibits the activation of caspase-3 and subsequently reduces HuR cleavage and BAX expression after IR. We next tested whether Comp-A can modulate the distribution of HuR between the nucleus and cytoplasm of HOK cells after IR. The ability of HuR to alter the post-transcriptional mRNA stability mechanism is strictly dependent on its cytoplasmic distribution (30). Although HuR localization has no role in caspase-3 inhibition, we wanted to test whether caspase-3 inhibition plays any role in the nuclear export of HuR. Before irradiation, HuR was localized to the nucleus whereas within 2 h of IR treatment, HOK cells exported HuR to the cytoplasm (Fig. 4C). When added to the culture medium during irradiation, Comp-A was able to block the nuclear export of HuR (Fig. 4C, bottom panels). This observation is very interesting because we believe that complete inhibition of caspases may have a role in cell survival, which might influence the distribution of HuR. To confirm that Comp-A was able to block the IR-induced export of HuR in these cells, we treated the cells with a known caspase inhibitor zVAD and observed HuR translocation by immunofluorescence microscopy. Under untreated conditions, HuR was predominantly localized in the nucleus, whereas after treatment with IR, it was exported to the cytoplasm (Fig. 4D). But, zVAD treated cells; HuR was retained in the nucleus compare to irradiated cells (Fig. 4C, bottom panels). These data suggest that inhibiting the activity of caspases might influence the distribution of HuR between nucleus and cytoplasm. Considering the remarkable effect of Comp-A in HOK cells, we next asked if blocking HuR cleavage could influence the expression and steady-state level of BAX mRNA. We observed increased BAX mRNA expression and no significant change in Comp-A-treated HOK cells following IR when compared with untreated cells (Fig. 4E). To confirm the changes in BAX mRNA levels, we examined the half-life \( t_\frac{1}{2} \) of the BAX mRNA after IR. In untreated and Comp-A-incubated HOK cells, the \( t_\frac{1}{2} \) of BAX mRNA was 2.6 h and 2.72 h, respectively, whereas in IR-treated HOK cells, the \( t_\frac{1}{2} \) increased to more than 4 h (Fig. 4F). The mRNA levels of the control, BAG5, did not significantly change with IR treatment (Fig. 4F). These data indicate that IR increases the \( t_\frac{1}{2} \) of BAX mRNA, but Comp-A decreases the stability of BAX through the mechanism of caspase-3 inhibition and repression of HuR cleavage. Furthermore, a colony assay showed increased clonogenic efficiency of Comp-
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A-treated HOK cells after radiation compared with control radiated cells (Fig. 4G). Collectively, our data suggest that HuR cleavage-associated BAX expression is dependent on caspase-3 activity following the IR-induced apoptosis in HOK cells.

HuR undergoes cleavage modifications in experimental oral mucositis animal model. Oral mucositis closely follows the paradigm of an acute mucosal damage phase, characterized by inflammation, epithelial cell apoptosis, and ulcerative lesions, followed by a self-healing phase with the restoration of the mucosal epithelium and barrier function (2). To determine if HuR plays a role in oral mucositis in vivo, we developed an IR-induced oral mucositis animal model, as described previously (31). Briefly, oral mucositis was induced in mice using 5 fractions of 8 Gy or single dose of 22.5 Gy head-only irradiation. After 7 days, IR induced ulceration in tongue tissue (Fig. 5A) and the percentage of ulceration was significantly higher (50–60%) in IR-treated mice than in control animals (Fig. 5A, right panel). This observation clearly demonstrates that IR induces ulceration in tongue tissues and that this ulceration is similar to that seen in clinical human oral mucositis, as described previously (31). Hematoxylin and eosin (H&E) staining revealed that little or no inflammation was observed in control tissues compared to the very strong inflammation, ulceration, and disintegrated epithelium that was observed in IR-treated tissues (Fig. 5B). As shown in Figure 5B, radiation caused extensive tongue mucosal injury as evidenced by reduced mucosal basal epithelial layer thickness compared to control. Immunohistochemistry analysis revealed the presence of nuclear HuR in the epithelial and stromal regions of both control and IR-treated animals, but enlarged nuclei, disintegrated epithelium, and cytoplasmic HuR were additionally observed in the IR-treated animal group (Fig. 5C, bottom panel). This observation clearly indicates that HuR is exported from the nucleus in IR-treated cells and could be involved in post-transcriptional regulation. Previously, it has been shown that ultraviolet light (32) and other stresses (30) induce HuR translocation from nucleus to cytoplasm. Herein, we show that in vivo, HuR is exported to the cytoplasm in IR-induced oral mucositis. To determine if HuR translocation is associated with cell death during radiation, we stained the tissues with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and caspase-3 and performed immunofluorescence analysis. Activation of caspase-3 and TUNEL was observed in IR-treated mouse tissues, compared with control tissues (Fig. 5D). Next, to determine whether IR promotes the cleavage of HuR in vivo, we tested the cleavage of HuR in oral mucositis tongue tissues. Western blotting analysis of tongue tissue sections revealed significant HuR cleavage in IR-treated animals (Fig. 5E) compared with control animals. Interestingly, almost 50% of the full-length HuR was cleaved in IR-treated animals (Fig. 5E, right panel). This observation clearly demonstrates that IR induces HuR cleavage in the experimental oral mucositis animal tongue tissues and agrees with our in vitro observation. Thus, our data suggest that irradiation-induced mucosal damage of the oral epithelium results in the export of HuR to the cytoplasm, concurrently triggering HuR cleavage and cell death. Finally, to test whether the cleavage of HuR is dependent on the activity of caspase-3 and influences BAX, we analyzed HuR the expression of HuR, caspase-3, and BAX in the tongue tissue extracts by western blotting. As evidenced in vitro (Fig. 1C), increased BAX expression is observed in IR-treated animals compared with control animals. This observation suggests that IR promotes cleavage of both caspase-3 and HuR and subsequently increases the expression of BAX in oral mucositis tissues.

Comp-A protects oral mucosa from IR-induced epithelial cell death in mice. As Comp-A potentially inhibits caspase-3 in primary HOK cells, we next wanted to test the protective effect of Comp-A against IR-induced oral mucositis in animals. First, mice were pretreated with 3 times daily injections of Comp-A (10 to 30 mg/kg) before irradiation and then the tongue tissue was harvested after 7 days and examined for the extent of mucosal damage. As shown in Figure 6A, radiation caused extensive tongue ulceration (approximately 60%), but Comp-A treatment protected the mice from mucosal ulceration.
Likewise, western blotting analysis showed that the administration of Comp-A abolished caspase-3 activation, subsequently protected against HuR cleavage, and reduced BAX expression compared with untreated tongue tissues (Fig. 6B and Fig. 6C: graphical representation of BAX expression in irradiated and Comp-A+ irradiated mice). These observations suggest that Comp-A blocks the cleavage of caspase-3 and HuR in vivo and controls the expression of BAX. Morphometric analysis of H&E-stained tongue sections were used to confirm the protective effect of Comp-A in oral mucositis in mice. While radiation reduced the mucosal basal layer epithelial thickness in tongue compared with control, Comp-A treatment significantly increased basal layer epithelial thickness in tongue mucosa (Fig. 6D). A similar protective effect of Comp-A was also seen in cheek mucosa in the oral cavity of irradiated mice (data not shown). Next, immunohistochemistry analysis of HuR before and after IR in the presence and/or absence of Comp-A revealed increased cellularity and epithelial expression of HuR in control and Comp-A treated mice compared with IR-treated mice (Fig. 6E). This observation clearly indicates that HuR plays a critical role in epithelial regeneration in its native form compared with the cleaved form. To further assess the anti-apoptotic effects of Comp-A during oral mucosinal injury, we tested the expression of caspase-3 and performed the TUNEL assay in oral tongue tissues. Consistent with our in vitro results, we did not observe robust apoptotic activity differences between control and Comp-A treated mice in the visible accumulation of caspase-3 in basal epithelial layers after radiation compared with IR-induced mucositis tissues (Fig. 6F).

**Discussion**

Collectively, these results demonstrate that the caspase inhibitor Comp-A might be a potent epithelial regenerative molecule and is an effective therapeutic agent in reducing irradiation-induced oral mucosal injury in mice. We document that HuR is cleaved in an oral mucositis mouse model and promote the expression of BAX to facilitate cell death. Interestingly, allosteric inhibition of caspase-3 by Comp-A prevents HuR cleavage and reduces BAX expression. As HuR cleavage is an important molecular event during radiotherapy, it could play a key role in post-transcriptional gene regulation and promote oral mucositis.

Detailed biochemical and molecular studies on the mechanisms of HuR modulation in cancer cells have emerged in recent years. However, exactly how HuR activation is regulated in various primary cells remains largely unexplored. This is particularly important as both cancer cells and normal cells may modify their apoptotic threshold in order to function in, and adapt to, different physiological conditions. Typically, HuR is activated by various physiological insults and itself undergoes post-translational modification, thereby altering gene expression patterns (16). For example, HuR plays a key role in modulating apoptosis by controlling the stability of mRNAs that encode proteins involved in survival or in the cell death pathway (33). We and others have previously demonstrated that in cells responding to lethal stress, HuR is exported to the cytoplasm and is cleaved in a caspase-dependent manner, yielding two cleavage products (HuR-CP1 and HuR-CP2) (23,27). Recently, HuR is known to shift its physiological function from survival to cell death by caspase-mediated apoptosis in response to lethal stress by targeting mRNAs that encode apoptotic proteins such as caspase-9 (24). Although, the cleavage products of HuR are known, the physiological significance of the cleavage products are moderately established and shown to be regulating muscle development (34). Herein, our data strengthen the model that HuR is exported to the cytoplasm after IR (Fig. 1B) and generates HuR-CPs to subsequently promote cell death in oral mucositis. Furthermore, HuR-CPs triggers apoptosis in the absence of lethal stress, in which no engagement of apoptosis or HuR cleavage occurs (27). However, a non-cleavable mutant of HuR (HuR-D226A) failed to rescue apoptosis in cells depleted of cellular HuR, and acted as a dominant negative factor (23,27). The difference between the effect of non-cleavable and cleaved HuR on the cell death pathway is quite remarkable. We observed that HuR-CP1 promotes apoptosis, whereas non-cleavable HuR blocks apoptosis (Fig. 3E). Under chronic hypoxic conditions, HuR undergoes cleavage via caspase-3.
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in oral cancer cells of epithelial origin, which are obtained from the base of the tongue tissue (23). Here, our results show that the process of HuR cleavage is different in normal oral keratinocytes, where it undergoes cleavage in a rapid manner compared to oral cancer cells (Fig. 1A). This effect may be due to the rapid constitutive activation of apoptosis in oral keratinocytes, whereas oral cancer cells are uncoupled from rapid apoptotic pathways under stress stimuli. The full repertoire of HuR expression and its altered mRNAs is unknown in HOK cells, but BAX mRNA, a known target of HuR, is overexpressed, stabilized, and associated with HuR after radiation.

HuR was known to be a central player in regulating gene expression patterns and cell survival in response to IR via the novel mechanism of binding mRNA targets through the checkpoint kinase Chk2, a kinase that phosphorylates HuR (22). The phosphorylation of HuR is one of the critical molecular functions that dictate the association and dissociation of the mRNA-protein complex. In human HCT116 colorectal carcinoma cells, IR activated Chk2, triggered the dissociation of the HuR:mRNA complex, and altered the expression of its target mRNAs (22). On the other hand, in Chk2-null (CHK2-/-) HCT116 cells, IR did not cause the dissociation of the HuR:mRNA complex. Thus, IR can influence the HuR-mediated mRNA turnover pathway through the Chk2 signaling pathway. Contrary to this observation, our results herein demonstrated that IR activated HuR cleavage in normal HOK cells, triggered the association of HuR-CP1:mRNA complex, and altered the expression of BAX mRNA. On the other hand, our data supports the previous model that BAX mRNA showed less association between full-length and non-cleavable HuR. As Chk2 expression is cell-specific, the differential effect of mRNA stability by HuR may be dependent on the cell type. Our cells did not express Chk2 proteins either in the presence or absence of IR (data not shown). Hence, IR-induced alterations in the Chk2 pathway of normal cells call for further investigations. However, our model indicates that normal HOK cells exhibit intrinsic apoptotic signals that could play a key role in HuR cleavage during stress conditions. As evidenced in Figure 1, cancer cells require 12 h to cleave HuR, whereas normal cells undergo HuR cleavage within 15 minutes, clearly demonstrating that export and cleavage are solely dependent on cell type. Since, apoptosis is indispensable for the promotion of HuR as a pro-apoptotic factor, HuR-CP1 acts as cell death modulator and not a bona fide pro-survival factor (24).

Using HuR-CPs and non-cleavable proteins as modulators of the cell death pathway in a clinically relevant oral mucositis model is potentially risky due to the following points. First, HuR-CP1 is potentially known to exacerbate cell death; hence, introducing this protein into the oral mucositis model will not be beneficial. Second, using a non-cleavable HuR-D226A mutant in a mucositis model presents the potential risk of promoting cancer cell growth, because HuR-D226A is known to enhance cell survival and block cell death (23). Thus, we avoided using the overexpression of HuR isoforms in the mucositis model, and instead decided to block cell death and study HuR function in mucosal damage. We have extended our in vitro observations and have shown that radiation stimulates epithelial injury in the oral mucosa of the mice via augmentation of caspase-3, thereby amplifying the cleavage of HuR and BAX. The inhibition of caspase-3 by Comp-A promotes physiological regeneration in radiation-induced oral mucositis and restores the damaged oral mucosa. Our work establishes oral mucositis as a model for HuR-dependent apoptosis, providing a powerful tool for identifying novel regulators of IR-induced, HuR-dependent apoptosis, which is clinically relevant.

Oral mucositis closely follows the paradigm of an acute mucosal damage phase characterized by inflammation, epithelial cell apoptosis, and ulcerative lesions, followed by a self-healing phase with restoration of mucosal epithelium and barrier function (3). We thus assessed the effect of Comp-A in reducing epithelial cell apoptosis in both HOK cells and oral mucositis in irradiated mice (Fig. 4 and 6). Our observation revealed that Comp-A specifically inhibits active caspase-3 in irradiated HOK cells and is effective in the injury phase of oral mucositis in mice by increasing the basal layer of the epithelial cell density in tongue. In addition, Comp-A treatment is also effective in
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reducing ulcerative lesions in mice receiving head-only radiation. Therefore, the use of Comp-A appears to be effective during the injury phase of experimental oral mucositis in mice. The integrity of the oral epithelium is highly dependent on self-renewing stem cells, which are highly vulnerable to chemo- or radiotherapy (2). Recent studies illustrate that rapamycin, an inhibitor of mammalian target of rapamycin (mTOR), increases the clonogenic capacity of primary human oral keratinocytes and their resident self-renewing cells by preventing stem cell senescence in an oral mucositis model (35). This protective effect of rapamycin is mediated by an increase in the expression of mitochondrial superoxide dismutase (MnSOD), and by the consequent inhibition of ROS formation and oxidative stress that ultimately prevents the loss of basal epithelial stem cells upon radiation therapy. Moreover, irradiation increases the mRNA levels of the MnSOD transcript in normal cells through the 3'-UTR, which contains canonical ARE sequences (36). Conversely, the MnSOD mRNA is also known to be a target of HuR, and is expressed at very low levels during senescence (37). Also, reduced HuR expression emphasized the senescent phenotype, whereas overexpression of HuR restored the phenotype (37). Collectively, these studies highlight a critical role for HuR during the process of replicative senescence; in particular, cell senescence is a key factor in oral mucositis development (35). We speculate that HuR could alter the expression of MnSOD through association or dissociation with its 3'-UTR following radiation, but this and other possibilities regarding MnSOD mRNA warrant further investigation.

It is very important to prevent oral mucositis by blocking apoptosis and dampened keratinocyte proliferation during radiotherapy. Several drugs have been shown to be effective against oral mucositis and initiate keratinocyte proliferation. Palifermin is a mitogen that promotes keratinocyte proliferation (38), but the proliferative and anti-apoptotic effect of Smad7 was recently shown to be more effective than palifermin (39). Moreover, the overexpression of Smad7 in oral epithelial tissues blocks apoptosis through the activation of Rac1, and mediates keratinocyte migration and wound closure in oral mucositis. Hence, blocking epithelial basal layer apoptosis after IR is critical in the regeneration of epithelial tissues. To block apoptosis in the oral epithelial basal layer, we established a caspase inhibitor Comp-A, known to be a reversible inhibitor that binds to the dimerization interface of caspases (25). Comp-A, which has been shown to inhibit apoptosis induced by multiple stimuli in several cell types, also inhibits caspase-1-mediated interleukin generation in macrophages (25). Our study suggests that Comp-A increases the clonogenic capacity of primary human oral keratinocytes after radiation. Considering the remarkable effects of Comp-A in vitro, it was effective at limiting the loss of the basal epithelial layer in mice, thereby enhancing tissue regeneration and preventing the appearance of ulcers. Hence, establishing the therapeutic potential of Comp-A in vivo is a promising approach to alleviate oral mucositis.

Materials and Methods

Cell Lines, Constructs, and Transfection Experiments. Normal HOKs (ScienCell) were grown in keratinocyte serum-free medium supplemented with bovine pituitary extract and epithelial growth factor (EGF; Gibco, BRL). GFP plasmid vectors containing GFP, HuR-FL, HuR-D226A, and HuR-CP1 (a kind gift from I. Gallouzi, McGill University) were used as described (27). Plasmid transfections were performed using Lipofectamine (Invitrogen). For HuR knockdown analysis, cells were transfected with siRNAs (20 nM) in keratinocyte serum-free medium. Subsequently, either the control siRNA (20 nM; GTTCAATTGTCTACAGCTA) or siRNAs targeting HuR (20 nM; mixture of the siRNA oligos AATCTTAAGTTTCGTAAGTTA, TTCGTAAGTTATTTCCCTTAA, and AGTGCAAAGGGTTTGGCTTT) (40) were used for the experiments. siRNA transfections were carried out using HiPerFect (Qiagen).

Experimental animals. Eight-week-old female C57 Black/6 (JAX laboratories) mice were used for the study. All aspects of animal research were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Medical University of South Carolina.
Animal irradiation. The head and neck area was irradiated by placing each animal in a specially built Lucite Jig (Precision X-ray, New Haven, CT) to avoid anesthetics, as described (41). The jig was fitted with a Lucite cone surrounding the head to prevent head movement during radiation. Fractionated doses of irradiation at (8 Gy/day for 5 days) were delivered by a JL Shepherd Model 143 137 Cesium irradiator. An associated adapter was used to expose each animal. The irradiator produces an unshielded exposure rate of 2.35Gy/min. After irradiation, animals were removed from the jig, housed in a climate- and light-controlled environment, and allowed free access to food and water.

Demonstration of mucositis. Oral mucositis was demonstrated as previously described (42). Briefly, excised tongues were stained in a solution of 1% toluidine blue in 10% acetic acid, rinsed with 1% acetic acid, and then rinsed with the tap water. Ulceration in the tongue is visible as a blue color after staining. Macroscopic images were recorded using NIH image J software and the stained areas were measured in pixels. Increased toluidine blue staining and a change in epithelial thickness were used as markers of oral mucositis (31).

Apoptosis Assays. The Annexin V-EGFP cell apoptosis assay was performed according to the manufacturer’s protocol (Biovision). Fluorescence was measured using a FACSCalibur Flow Cytometer (Becton-Dickinson, San Jose, CA).

Immunohistochemistry, Immunofluorescence and Western Blot Analysis. Three-micron tongue tissue sections were stained with H&E and examined microscopically. Immunohistochemical analyses for TUNEL, caspase-3, and HuR were performed as described previously (23). Briefly, deparaffinized sections were processed for TUNEL staining using the ApopTag TUNEL kit (Chemicon S7110) following the manufacturer’s instructions. The sections were further labeled with anti-mouse HuR (Santa Cruz) or anti-caspase-3 (Cell Signaling) primary antibodies overnight at 4°C, secondary antibodies (Anti-mouse Alexa Fluor-647 or anti-rabbit Alexa Fluor-555, both from Invitrogen) for 1.5 h at RT and mounted in DAPI mounting medium (Prolong; Invitrogen #36935). The sections were imaged using a 1X81 confocal microscope (Olympus) and processed using Photoshop. For immunofluorescence, HOK cells were grown on cover slips overnight and treated as previously reported (23) using the following antibody dilutions: Anti-HuR, 1:300 and anti-β-Actin, 1:100 (Santa Cruz). For western blotting, protein extracts (40–60μg) were resolved by SDS-PAGE and transferred onto PVDF membranes. Immune complexes were visualized using the ECL system (Pierce). Blots were stripped and re-probed with anti-β-actin antibody (Sigma).

RNA Extraction and qPCR. Total RNA was prepared from tongue tissues and HOK cells using the RNeasy Mini kit (Qiagen). qPCR for all mRNA targets was performed using an Applied Biosystems StepOne Plus system with the TaqMan® One-Step RT-PCR kit (Applied Biosystems). Primer sequences (5'-3') used in this study is shown in Table 1.

HuR and GFP RNP IP analysis. HuR RNP IP was performed as previously described (43). Briefly, cell lysates were prepared from exponentially growing HOK cells that had been treated with IR or left untreated as a control. Equal amounts of protein were used (100–300 μg). HuR monoclonal antibody 3A2 (Santa Cruz), GFP antibody (Clontech), or isotype control IgG (Sigma) were pre-coated onto Protein A/G Sepharose beads (PAS). Lysates were pre-absorbed with IgG (30 μg) and then removed by the addition of PAS beads. Individual pull-downs were performed at 4°C for 1–2 h to minimize the potential re-assortment of mRNAs. For RNA analysis, the beads were incubated with 1 ml of NT2 buffer containing 20 U of RNase-free DNase I (15 min, 30°C), washed twice with 1 ml of NT2 buffer, and further incubated in 1 ml of NT2 buffer containing 0.1% SDS and 0.5 mg/mL proteinase K (15 min, 55°C) to digest the proteins bound to the beads. RNA was extracted using phenol and chloroform and precipitated in the presence of glycogen. For the analysis of individual mRNAs, the RNA isolated from the immunoprecipitate was subjected to reverse transcription (RT) using random hexamers and SuperScriptII reverse transcriptase (Invitrogen). Amplification and quantification of the PCR products were performed using the Applied Biosystems StepOne Plus system (Applied Biosystems) and Power
SYBR Green PCR Master Mix (Applied Biosystems). Input GAPDH mRNA was used as a loading control.

Statistics. The data are expressed as the mean ± standard deviation. Two-sample t tests with equal variances were used to assess differences between the means. Results with P-values less than 0.05 and 0.01 were considered significant.

References

1. Vissink, A., Jansma, J., Spijkervet, F. K., Burlage, F. R., and Coppes, R. P. (2003) Oral sequelae of head and neck radiotherapy. Critical reviews in oral biology and medicine : an official publication of the American Association of Oral Biologists 14, 199-212
2. Sonis, S. T. (2004) The pathobiology of mucositis. Nat Rev Cancer 4, 277-284
3. Sonis, S. T. (2007) Pathobiology of oral mucositis: novel insights and opportunities. J Support Oncol 5, 3-11
4. Sonis, S. T. (2010) New thoughts on the initiation of mucositis. Oral diseases 16, 597-600
5. Sonis, S. T. (2010) Efficacy of palifermin (keratinocyte growth factor-1) in the amelioration of oral mucositis. Core evidence 4, 199-205
6. Citrin, D., Cotrim, A. P., Hyodo, F., Baum, B. J., Krishna, M. C., and Mitchell, J. B. (2010) Radioprotectors and mitigators of radiation-induced normal tissue injury. The oncologist 15, 360-371
7. Nonzee, N. J., Dandade, N. A., Patel, U., Markossian, T., Agulnik, M., Argriris, A., Patel, J. D., Kern, R. C., Munshi, H. G., Calhoun, E. A., and Bennett, C. L. (2008) Evaluating the supportive care costs of severe radiochemotherapy-induced mucositis and pharyngitis: results from a Northwestern University Costs of Cancer Program pilot study with head and neck and nonsmall cell lung cancer patients who received care at a county hospital, a Veterans Administration hospital, or a comprehensive cancer care center. Cancer 113, 1446-1452
8. Sonis, S. T. (2002) The biologic role for nuclear factor-kappaB in disease and its potential involvement in mucosal injury associated with anti-neoplastic therapy. Crit Rev Oral Biol Med 13, 380-389
9. Kitada, S., Krajewski, S., Miyashita, T., Krajewska, M., and Reed, J. C. (1996) Gamma-radiation induces upregulation of Bax protein and apoptosis in radiosensitive cells in vivo. Oncogene 12, 187-192
10. Wu, X., Chen, P., Sonis, S. T., Lingen, M. W., Berger, A., and Toback, F. G. (2012) A novel Peptide to treat oral mucositis blocks endothelial and epithelial cell apoptosis. International journal of radiation oncology, biology, physics 83, e409-415
11. Manakova, S., Puttonen, K. A., Raasmaja, A., and Mannisto, P. T. (2003) Ara-C induces apoptosis in monkey fibroblast cells. Toxicol In Vitro 17, 367-373
12. Tait, S. W., and Green, D. R. (2010) Mitochondria and cell death: outer membrane permeabilization and beyond. Nature reviews. Molecular cell biology 11, 621-632
13. Youle, R. J., and Strasser, A. (2008) The BCL-2 protein family: opposing activities that mediate cell death. Nature reviews. Molecular cell biology 9, 47-59
14. Brunelle, J. K., and Letai, A. (2009) Control of mitochondrial apoptosis by the Bcl-2 family. Journal of cell science 122, 437-441
15. Dewson, G., and Kluck, R. M. (2009) Mechanisms by which Bak and Bax permeabilise mitochondria during apoptosis. Journal of cell science 122, 2801-2808
16. Abdelmohsen, K., and Gorospe, M. (2010) Posttranscriptional regulation of cancer traits by HuR. Wiley interdisciplinary reviews. RNA 1, 214-229
17. Yi, J., Chang, N., Liu, X., Guo, G., Xue, L., Tong, T., Gorospe, M., and Wang, W. (2010) Reduced nuclear export of HuR mRNA by HuR is linked to the loss of HuR in replicative senescence. *Nucleic Acids Res* **38**, 1547-1558

18. Abdelmohsen, K., Pullmann, R., Jr., Lal, A., Kim, H. H., Galban, S., Yang, X., Blethrow, J. D., Walker, M., Shubert, J., Gillespie, D. A., Furneaux, H., and Gorospe, M. (2007) Phosphorylation of HuR by Chk2 regulates SIRT1 expression. *Mol Cell* **25**, 543-557

19. Kawai, T., Lal, A., Yang, X., Galban, S., Mazan-Mamczarz, K., and Gorospe, M. (2006) Translational control of cytochrome c by RNA-binding proteins TIA-1 and HuR. *Mol Cell Biol* **26**, 3295-3307

20. Lal, A., Kawai, T., Yang, X., Mazan-Mamczarz, K., and Gorospe, M. (2005) Antiapoptotic function of RNA-binding protein HuR effected through prothymosin alpha. *Embo J* **24**, 1852-1862

21. Mazan-Mamczarz, K., Hagner, P. R., Corl, S., Srikantan, S., Wood, W. H., Becker, K. G., Gorospe, M., Keene, J. D., Levenson, A. S., and Gartenhaus, R. B. (2008) Post-transcriptional gene regulation by HuR promotes a more tumorigenic phenotype. *Oncogene* **27**, 6151-6163

22. Masuda, K., Abdelmohsen, K., Kim, M. M., Srikantan, S., Lee, E. K., Tominaga, K., Selimyan, R., Martindale, J. L., Yang, X., Lehrmann, E., Zhang, Y., Becker, K. G., Wang, J. Y., Kim, H. H., and Gorospe, M. (2011) Global dissociation of HuR-mRNA complexes promotes cell survival after ionizing radiation. *Embo J* **30**, 1040-1053

23. Talwar, S., Jin, J., Carroll, B., Liu, A., Gillespie, B. M., and Palanisamy, V. (2011) Caspase-mediated cleavage of RNA-binding protein HuR regulates c-Myc expression after hypoxic stress. *The Journal of biological chemistry* **16**, 32333-32343

24. von Roretz, C., Lian, X. J., Macri, A. M., Punjani, N., Clair, E., Drouin, O., Dormoy-Raclet, V., Ma, J. F., and Gallouzi, I. E. (2013) Apoptotic-induced cleavage shifts HuR from being a promoter of survival to an activator of caspase-mediated apoptosis. *Cell Death Differ* **20**, 154-168

25. Feldman, T., Kabaleeswaran, V., Jiang, S. B., Antczak, C., Djaballah, H., Wu, H., and Jiang, X. (2012) A class of allosteric caspase inhibitors identified by high-throughput screening. *Mol Cell* **47**, 585-595

26. Costantino, C. L., Witkiewicz, A. K., Kuwano, Y., Cozzitorto, J. A., Kennedy, E. P., Dasgupta, A., Keen, J. C., Yeo, C. J., Gorospe, M., and Brody, J. R. (2009) The role of HuR in gemcitabine efficacy in pancreatic cancer: HuR Up-regulates the expression of the gemcitabine metabolizing enzyme deoxyxycytidine kinase. *Cancer Res* **69**, 4567-4572

27. Mazroui, R., Di Marco, S., Clair, E., von Roretz, C., Tenenbaum, S. A., Keene, J. D., Saleh, M., and Gallouzi, I. E. (2008) Caspase-mediated cleavage of HuR in the cytoplasm contributes to pp32/PHAP-I regulation of apoptosis. *J Cell Biol* **180**, 113-127

28. Takayama, S., Sato, T., Krajewski, S., Kochel, K., Irie, S., Millan, J. A., and Reed, J. C. (1995) Cloning and functional analysis of BAG-1: a novel Bcl-2-binding protein with anti-cell death activity. *Cell* **80**, 279-284

29. Lebedeva, S., Jens, M., Theil, K., Schwanhausser, B., Selbach, M., Landthaler, M., and Rajewsky, N. (2011) Transcriptome-wide Analysis of Regulatory Interactions of the RNA-Binding Protein HuR. *Mol Cell* **43**, 340-352

30. Doller, A., Pfeilschifter, J., and Eberhardt, W. (2008) Signalling pathways regulating nucleocytoplastic shuttling of the mRNA-binding protein HuR. *Cell Signal* **20**, 2165-2173

31. Zheng, C., Cotrim, A. P., Sunshine, A. N., Sugito, T., Liu, L., Sowers, A., Mitchell, J. B., and Baum, B. J. (2009) Prevention of radiation-induced oral mucositis after adenoviral vector-mediated transfer of the keratinocyte growth factor cDNA to mouse submandibular glands. *Clin Cancer Res* **15**, 4641-4648

32. Fernau, N. S., Fugmann, D., Leyendecker, M., Reimann, K., Grether-Beck, S., Galban, S., Ale-Agha, N., Krutmann, J., and Klotz, L. O. (2010) Role of HuR and p38MAPK in ultraviolet B-induced post-transcriptional regulation of COX-2 expression in the human keratinocyte cell line HaCaT. *J Biol Chem* **285**, 3896-3904
33. Kuwano, Y., G. I.-E., & Gorospe M (2010) Role of the RNA-Binding Protein HuR in Apoptosis and Apoptosome Function. Apoptosome, eds Ceconi F & D’Amelio M (Springer Netherlands), pp 203-220. Kuwano, Yuki, Galloulzi, I.-E., and Gorospe, M. (2010) Role of the RNA-Binding Protein HuR in Apoptosis and Apoptosome Function. in Apoptosome (Ceconi, F., and D’Amelio, M. eds.), Springer Netherlands. pp 203-220

34. Beauchamp, P., Nassif, C., Hillock, S., van der Giessen, K., von Roretz, C., Jasmin, B. J., and Gallouzi, I. E. (2010) The cleavage of HuR interferes with its transportin-2-mediated nuclear import and promotes muscle fiber formation. Cell Death Differ

35. Iglesias-Bartolome, R., Patel, V., Cotrim, A., Leelahavanichkul, K., Molinolo, A. A., Mitchell, J. B., and Gutkind, J. S. (2012) mTOR inhibition prevents epithelial stem cell senescence and protects from radiation-induced mucositis. Cell stem cell 11, 401-414

36. Chaudhuri, L., Nicholson, A. M., Kalen, A. L., and Goswami, P. C. (2012) Preferential selection of MnSOD transcripts in proliferating normal and cancer cells. Oncogene 31, 1207-1216

37. Wang, W., Yang, X., Cristofalo, V. J., Holbrook, N. J., and Gorospe, M. (2001) Loss of HuR is linked to reduced expression of proliferative genes during replicative senescence. Mol Cell Biol 21, 5889-5898

38. Henke, M., Alfonsi, M., Foa, P., Giralt, J., Bardet, E., Cerezo, L., Salzwimmer, M., Lizambri, R., Emmerson, L., Chen, M. G., and Berger, D. (2011) Palifermin decreases severe oral mucositis of patients undergoing postoperative radiochemotherapy for head and neck cancer: a randomized, placebo-controlled trial. J Clin Oncol 29, 2815-2820

39. Han, G., Bian, L., Li, F., Cotrim, A., Wang, D., Lu, J., Deng, Y., Bird, G., Sowers, A., Mitchell, J. B., Gutkind, J. S., Zhao, R., Raben, D., ten Dijke, P., Refaeli, Y., Zhang, Q., and Wang, X. J. (2013) Preventive and therapeutic effects of Smad7 on radiation-induced oral mucositis. Nature medicine 19, 421-428

40. Kuwano, Y., Kim, H. H., Abdelmohsen, K., Pullmann, R., Jr., Martindale, J. L., Yang, X., and Gorospe, M. (2008) MKP-1 mRNA stabilization and translational control by RNA-binding proteins HuR and NF90. Mol Cell Biol 28, 4562-4575

41. Sonis, S. T. (2011) Oral mucositis. Anti-cancer drugs 22, 607-612

42. Chen, P., Lingen, M., Sonis, S. T., Walsh-Reitz, M. M., and Toback, F. G. (2011) Role of AMP-18 in oral mucositis. Oral oncology 47, 831-839

43. Lopez de Silanes, I., Zhan, M., Lal, A., Yang, X., and Gorospe, M. (2004) Identification of a target RNA motif for RNA-binding protein HuR. Proc Natl Acad Sci USA 101, 2987-2992

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Footnotes

The abbreviations used are: H&E, hematoxylin and eosin; HuR-CP1, Hu Antigen R- Cleavage product-1; HuR-D226A, non-cleavable isoform of HuR; RBP, RNA-binding protein; ARE, AU-rich elements; 3′ UTR, untranslated region; HNSCC, head and neck squamous cell carcinoma; IR, ionizing radiation.

Author contributions: S.T., S.S., S.B., and V.P. designed the study; S.T., S.S., R.H., and H.Y. performed the research; S.T and V.P analyzed the data; and V.P. wrote the paper.
**Figure legends**

**Figure 1. IR-induced activation of caspase-3 promotes HuR cleavage and increases the level of BAX in human oral keratinocytes.** (A) Cleavage of HuR in normal cells compared with cancer cells. Total protein was isolated from human oral keratinocyte cells and oral cancer UM74B cells at the indicated time points after irradiation with a dose of 16 Gy to identify HuR cleavage (appearance of a 24-kDa product as indicated) using western blot analysis. β-actin was used as a loading control. (B) HuR is exported to the cytoplasm in HOK cells after IR. Immunofluorescence detection of HuR in HOK cells either left untreated, or after treatment with 16 Gy IR. Distribution of cytoplasmic HuR (merged panel) is observed after IR. Blue: DAPI nuclear staining; Red: β-actin to detect cytoplasm; Green: HuR. Scale bar denotes 20 µm. (C) Cleavage of Caspase-3 and HuR after IR. HOK cells were irradiated with 16 Gy, followed by western blotting for HuR, active caspase-3, and BAX performed. Right panel depicts the quantitative values of Western blots of HuR-CP1 and BAX. β-actin serves as a loading control. (D) Inhibition of activation of caspase-3 abolishes the cleavage of HuR. Cells were either treated or untreated with IR and IR + zVAD followed by Western blotting for HuR, performed as described above and probed with antibodies to active caspases-3 and BAX. β-Actin serves as a loading control. (E) HOK cells were irradiated with 16 Gy radiation, and after 2 h the cells were analyzed by staining with annexin V-FITC and propidium iodide by flow cytometry. The percentage of apoptotic cells (left boxes) upon IR treatment was determined. The values were normalized to control untreated cells. The graph represents the number of apoptotic cells after treatment as described in the left boxes. Values are the means ± S.E. (error bars) from three independent experiments. (**, P < 0.01, n = 3).

**Figure 2. Altered expression of ARE mRNAs after IR.** (A) The relative quantity of ARE mRNAs measured by RT-qPCR in control versus IR-treated after 2 h; GAPDH serves as a loading control. (B) The relative amounts of ARE mRNAs expressed in HOK cells transfected with HuR siRNA or control siRNA after IR were measured by RT-qPCR. The values are normalized using a factor calculated from GAPDH gene expression. (C and D) The decay rates of the BAX and BAG5 mRNAs as indicated were assessed in HOK cells by RT-qPCR after IR followed by transcription inhibition with actinomycin D. (E) HuR associates with BAX mRNA after IR. HOK cells were subjected to 16 Gy IR and after 2 h the lysates were subjected to RNP IP followed by RT-qPCR analysis to measure the relative quantities of BAX, BCL2L11, MDM2 and BAG5 mRNAs in HuR IP compared with control IgG IP. GAPDH serves as a loading control. Error bars denote the S.E. of three sets of experiments. *, p < 0.05; **, p < 0.01, n = 3.

**Figure 3. HuR-CP1 associates with and increases the stability of BAX mRNA, and initiates apoptosis in comparison with non-cleavable mutant HuR-D226A.** (A) HOK cells were transfected with the indicated plasmid vectors; IR treatment was applied and after 2 h the cell extracts were analyzed by western blotting using antibodies against HuR and β-actin. All cells were normalized based on GFP counts using flow cytometry for transfection efficiency. (B) Forty-eight hours after transfection with a control plasmid (GFP) or plasmids over-expressing HuR-FL, HuR-D226A and HuR-CP1, HOK cells were treated with IR and subjected to RNP IP using an anti-GFP antibody. The extracted RNA was subjected to RT-qPCR analysis to measure the relative quantities of BAX and BAG5 mRNA. GAPDH served as a loading control from the input lysates. (C-D) The decay rates of BAX and BAG5 mRNAs in HOK cells transfected with the respective GFP-tagged HuR isoforms was assessed by RT-qPCR after treatment with IR followed by the transcription inhibitor actinomycin-D. (E) HOK cells were transfected with the indicated plasmids and treated with 16 Gy of IR. After 2 h, the cells were stained with annexin V–FITC and PI and analyzed by flow cytometry. The percentage of apoptotic cells after IR treatment was determined (left boxes), and the bar graph represents the number of apoptotic cells after treatment (right panel). (F) Representative culture dishes from clonogenic assays of cells transfected with indicated HuR isoforms after IR. Right panel depicts the colony forming efficiency from clonogenic assays of HOK
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cells. Data are presented as the means ± SD from three independent experiments.*, p < 0.05; ** p < 0.01 n = 3.

Figure 4. Caspase-3 inhibition by Comp-A protects HOK cells from apoptosis and reduces the cleavage of HuR and expression of BAX. (A) Comp-A diminished IR-induced apoptosis. HOK cells were irradiated with a dose of 16 Gy and either DMSO or 100 nM Comp-A was added 6 h prior to the IR to the culture medium. Total protein was isolated from HOK cells to determine HuR cleavage, caspase-3 activity, and BAX expression using western blot analysis. β-actin was used as a loading control. Right panel illustrates the quantitative Western blot values of HuR-CP1 and BAX. (B) Annexin V/PI staining and flow cytometry of IR-treated HOK cells. Cells were irradiated with IR in the presence or absence of Comp-A (100 nM). Cells were collected 2 h after IR, stained with annexin V–FITC and PI, and analyzed by FACS. Data are presented as the means ± SD from three independent experiments.*, p < 0.05. (C-D) Comp-A and zVAD blocks the nuclear export of HuR. Immunofluorescence detection of HuR in HOK cells either untreated or treated with IR in the presence or absence of Comp-A (100 nM). Distribution of cytoplasmic HuR (merged panel) is observed after IR. Blue: DAPI nuclear staining; Red: β-actin to detect cytoplasm; Green: HuR. The scale bar denotes 20 µm. (E) Comp-A reduces the expression of BAX mRNA after IR. The graph represents relative quantity of BAX mRNA measured by RT-qPCR in control versus IR-treated after 2 h in the presence and absence of Comp-A (100 nM) and GAPDH serves as a loading control. (F) BAX mRNA is unstable after treating with Comp-A. The decay rates of BAX and BAG5 mRNAs in HOK cells treated with IR in the absence or presence of Comp-A was assessed by RT-qPCR after treatment with IR followed by the transcription inhibitor actinomycin-D. (G). Representative culture dishes from clonogenic assays of HOK cells incubated with and without Comp-A. Right panel depicts the colony forming efficiency from clonogenic assays of HOK cells. ** p < 0.01, *** p < 0.001.

Figure 5. IR induces inflammation and apoptosis, and triggers cleavage of HuR in oral mucositis tongue tissues in vivo. (A) Toluidine blue staining of tongue tissues (n = 6) after fractionated dose (8 Gy/5 days) irradiation at day 8. Arrows indicate toluidine staining of ulcers on the dorsal surface of the base of the tongue. The right panel depicts the quantitative difference in the ulcerated area between control and IR-treated mouse tissues. These results are representative of duplicate experiments. (B) Light micrographs of control and IR-treated mouse tongue tissues. Tissue sections from untreated and IR-treated animals were subjected to staining with H&E. Scale bar denotes 100 µm. (C) Tissue sections from representative oral mucositis ulcers were subjected to immunohistochemistry using a primary monoclonal antibody to HuR followed by a peroxidase-conjugated goat anti-mouse secondary antibody. The scale bar denotes 50 µm in the left-hand image and 20 µm for the insert. (D) Immunofluorescence detection of HuR, TUNEL, and caspase-3 in mouse tongue tissues either untreated or treated with IR. Distribution of cytoplasmic caspase-3 and HuR (merged panel) is observed after IR. Blue: DAPI nuclear staining; White: HuR; Green: TUNEL to visualize apoptosis; Red: caspase-3 to detect apoptosis. Scale bar denotes 20 µm. (E) Total protein from control and IR-treated tongue tissues was used for western blot analysis. The blots were probed for HuR and GAPDH (used as the loading control). HuR-FL (36 kDa); HuR-CP1 (24 kDa). The right panel depicts the quantitative values of protein expression for full-length HuR and HuR-CP1. (F) Total protein was isolated from oral mucositis tongue tissue to identify HuR cleavage, activation of caspase-3, and expression of BAX using western blot analysis. β-actin was used as a loading control.

Figure 6. Comp-A prevents apoptosis after radiation in normal oral epithelia in vivo. (A) Representative images of toluidine blue staining of tongue tissues (n = 6) after fractionated dose (8 Gy/5 days) irradiation at day 8 after they received vehicle (radiated mouse) or Comp-A (radiation + 30mg/kg body weight Comp-A). The right panel depicts the quantitative difference in the ulcerated area between IR and Comp-A+IR-treated mouse tissues. These results are representative of duplicate experiments. (B) Comp-A blocks HuR cleavage and reduced BAX via inhibition of caspase-3 activity. Total protein from IR-treated and Comp-A (30mg/kg body weight)+IR-treated tongue tissues were used for western blot
analysis. The blots were probed for HuR, caspase-3, BAX, and GAPDH (used as the loading control). (C) The graph depicts the quantitative values of BAX protein expression (panel -B) in IR-treated and Comp-A+IR-treated mouse tongue tissues. (D) Light micrographs of control, IR-treated and IR+Comp-A treated mouse tongue tissues. Tissue sections from the control, IR-treated and IR+Comp-A-treated animals were subjected to staining with H&E. The scale bar denotes 100 μm. (E) Tissue sections from representative oral mucositis tongue sections were subjected to immunohistochemistry using a primary monoclonal antibody to HuR followed by a peroxidase-conjugated goat anti-mouse secondary antibody. The scale bar denotes 50 μm. (F). Immunofluorescence detection of HuR, TUNEL, and caspase-3 in mouse tongue tissues either untreated or treated with IR in the presence and absence of Comp-A (30mg/kg body weight). Blue: DAPI nuclear staining; White: HuR; Green: TUNEL to visualize apoptosis; Red: caspase-3 to detect apoptosis. Scale bar denotes 20 μm. The location of the basal layer is indicated by a white line.
Table 1. Primers used in this study.

| S.N. | Primer name   | Sequence 5'-3'                     | Tm in °C |
|------|---------------|------------------------------------|----------|
| 1    | BAX sense     | TCTACTTTGCCAGCAAACCTGGTGC          | 60       |
|      | BAX antisense | TGTCAGGCTATGGTTCCTG                | 60.3     |
| 2    | BAG5 sense    | CATGTCATGGGATGTTCTTTT              | 60.2     |
|      | BAG5 antisense| ACACCTGGACCCACATGTTCTCAA           | 60.2     |
| 3    | GAPDH sense   | GGTGGTCTCTCTGACTTTCAACA            | 66.6     |
|      | GAPDH antisense| GGTGCTGAGCAAATTCTGTTGT            | 65.9     |
| 4    | HuR sense     | ACGCAAGAATGTCAGTTACAGGC            | 59.9     |
|      | HuR antisense | TGTCTGGCCCTGGCTATTTCAACA           | 60.1     |
| 5    | β-Actin sense | CGTCTCCCTCCCTCATCG                 | 54.8     |
|      | β-Actin antisense| CGTCTATGGTACGCAGC                  | 51.7     |
| 6    | GFP sense     | CACATGAAGGACGACGACG                | 64.2     |
|      | GFP antisense | GGTGCTGCCTGGCTCTT                | 62.9     |
| 7    | MDM2 sense    | GGAGCAGGCAATGTCAGCAC              | 60.2     |
|      | MDM2 antisense| ATGGCTTTGGTCTAACCAGGGTCT          | 60.1     |
| 8    | BCL2L11 sense | AGGTCTGAGTGTCAGCACAGGTA            | 59.5     |
|      | BCL2L11 antisense| TCCTGCTTTGCTGCTGTCTGTA            | 59.3     |
Figure 1

A

| Normal Oral Keratinocytes | Oral Cancer UM74B cells |
|---------------------------|-------------------------|
| 0                         | 0                       |
| 0.25                      | 2                       |
| 0.5                       | 4                       |
| 1                         | 8                       |
| 2                         | 12                      |

- Time (hrs)

HuR - FL (35kD)
HuR - CP1 (24kD)
β-Actin (42kD)

B

Nucleus | Cytoplasm | HuR | Merged

Untreated Cells

IR-treated Cells

C

| 0 | 0.25 | 0.5 | 1 | 2 |
|---|------|-----|---|---|

- Time (hrs)

HuR-FL (35kD)
HuR-CP1 (24kD)
Caspase-3 (32kD)
Cleaved Caspase-3 (17kD)
Bax (23kD)
β-Actin (42kD)

D

No IR | IR | IR + Z-VAD

FL-HuR
HuR-CP1
Caspase-3
Cleaved Caspase-3
Bax
β-Actin

E

Control | Irradiation

Annexin V FITC vs. Annexin V APC

Percent Apoptosis

0% | 10%

Control | Radiation

% Apoptotic Rate

0% | 10%
Figure 4

A

B

C

D

E

F

G
Inhibition of caspases protects mice from radiation induced oral mucositis and abolishes the cleavage of RNA binding protein HuR
Sudha Talwar, Reniqua House, Santhanalakshmi Sundaramurthy, Sundaravadivel Balasubramanian, Hong Yu and Viswanathan Palanisamy

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