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A Hyaluronic Acid-Based Compound Inhibits Fibroblast Senescence Induced by Oxidative Stress In Vitro and Prevents Oral Mucositis In Vivo

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Virtually all patients receiving radio- and chemotherapy for cancer develop oral mucositis, a severe and highly debilitating condition. The onset of mucositis is thought to involve the production of reactive oxygen species (ROS) in the submucosa. Here we investigated a possible protective effect of a commercial formulation of hyaluronic acid (HA) enriched with amino acids (Mucosamin®) against the damage induced by oxidative stress both in vitro and in vivo. Transient exposure of normal human oral fibroblasts to hydrogen peroxide (H_2O_2) led to irreversible senescence, as demonstrated by sustained increase in the levels of $p16^{INK4A}$ and SA-βGal. Conditioned media from senescent fibroblasts induced detrimental effects on keratinocytes, as shown by reduced metabolic activity and migration capability. Pre-treatment with Mucosamin® prevented H_2O_2-induced, but not TGF-β-induced, fibroblast senescence with a concomitant reduction of fibroblast-induced loss of keratinocyte vitality and functional activity. Finally, data from a case-series of patients undergoing radio/chemotherapy strongly suggested that prophylactic use of the hyaluronic acid-based compound in the form of a spray may be effective in preventing the onset of oral mucositis.

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Virtually all patients who receive radiation therapy with or without concomitant chemotherapy develop ulcerative mucositis in the mouth (Trotti et al., 2003). Oral mucositis is among the most common tissue toxicities associated with radiation therapy used for the treatment of cancers of the head and neck. It results in the development of diffuse ulcerative lesions of the mucosa of the mouth and oropharynx, with consequent pain of such severity as to require opioid-level analgesics (Elting et al., 2008). Patients consistently report that it is the most bothersome side effect of the total treatment that they experience (Sonis, 2011). In addition, oral mucositis significantly increases the likelihood of unplanned breaks or delays in treatment, reduction in chemotherapy dose, use of feeding tube placement or total parenteral nutrition use, the need for an intravenous line, opioid use and hospitalization (Sonis et al., 2004; Nonzee et al., 2008). Effective treatments for this highly debilitating condition would improve dramatically the quality of life of patients and also, would limit the use of health resources.

It has been calculated that the incremental cost of oral mucositis in patients population with head and neck cancer is more than $17,000 (Nonzee et al., 2008). Despite a major clinical and economic impact, the recommended treatment for mucositis is largely palliative and singularly few agents, such as palifermin and benzydamide, have been approved to date. Palifermin is expensive and its use is limited to patients with hematological malignancies undergoing stem cell transplants (Raber-Durlacher et al., 2013); benzydamine has been approved for use in Europe, but its efficacy seems to be limited to patients receiving radiation-only regimens (Sonis, 2011). None of the drugs used for the treatment of oral mucositis are specific to the mechanism of action of the disorder and surprisingly, no single preventive measure has been approved

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to date. Some level of effectiveness in preventing the development or severity of oral mucositis has been shown with a local application of PTA (polymyxin E, tobramycine, and amphotericin B), granulocyte macrophage-colony-stimulating factor/granulocyte colony-stimulating factor (GM-CSF/G-CSF), oral cooling and the systemic administration of amifostine together with GM-CSF/G-CSF (Stokman et al., 2006), although this has not been confirmed in a recent systematic review (Raber-Durlacher et al., 2013). To date, therefore, no single intervention completely prevents or reverts oral mucositis. New therapeutic or preventive mechanism-based strategies are urgently required.

In 2001, the US Food and Drug Administration approved a gel based on sodium hyaluronate (SH) as a Class 1 medical device for use in the management of pain relief and which also proved to be useful in patients with oral mucositis. More recently, we and others have demonstrated that Aminogam®, a compound containing a pool of synthetic amino acids (L-proline, L-leucine, L-lysine, and glycine) combined with SH, accelerated healing (Favia et al., 2008) and helped manage pain in patients with oral mucositis (Colella et al., 2010). The mechanisms by which hyaluronic acid (HA) promotes the healing in oral mucositis, however, are poorly understood. Studies on Polyvinylpyrrolidone-SH preparations in the form of bioadherent gel (Gelclair®) suggest that the compound acts merely as a physical barrier between the oral environment and oral mucosa, thus reducing pain and possibly, promoting healing (Buchsel, 2008). Since the amino acid-enriched HA compound (Aminogam®) is not a bioadherent gel, the mechanisms underlying its efficacy in wound healing and oral mucositis are more likely to involve biomolecular and physiological changes in keratinocytes and mesenchymal cells (Mariggiò et al., 2009; Colella et al., 2012). Surprisingly, despite the relatively low cost of commercial preparations, the effectiveness of HA-based compounds in preventing, rather than treating, oral mucositis has not been investigated to date.

The pathogenesis of oral mucositis is currently the subject of intense debate. Because the disease peaks near the completion of treatment, approximately 7–9 weeks after initiation of radiotherapy, and usually resolves between 2 and 10 weeks after the end of treatment (Pauloski et al., 2011), it is reasonable to speculate that slowly appearing but long lasting changes take place in the oral mucosa, in addition to the acute toxicity associated with apoptosis of keratinocytes (Talwar et al., 2014). Further, mucosal alteration causing impaired oral intake often persists long into the first year after cancer treatment when the impact of oral mucositis would be expected to have dissipated (Pauloski et al., 2011). It is now thought, however, that chemoradiation-induced mucosal injury is actually the result of a complex series of biological and cellular events that take place predominantly in the submucosa, with the epithelium being the final target tissue (Sonis, 2009). Today, mucositis is recognized as an epithelial and subepithelial injury and is thought to develop in a five-stage model: (1) initiation, (2) up-regulation with generation of messengers, (3) signaling and amplification, (4) ulceration with inflammation, and (5) healing (Sonis, 2004). At a molecular level, both chemotherapy and radiation-induced mucositis are thought to be associated with the production of reactive oxygen species (ROS) at early pre-clinical stages and consequent activation of oxidative stress pathways (Criswell et al., 2003; Yoshino et al., 2013). The changes taking place in the mesenchyme would ultimately culminate in cell death of the keratinocytes in the basal epithelium, but the mechanisms through which oxidative stress can induce alteration of the oral mucosa still remain to be elucidated. Understanding and targeting the patho-physiological processes that lead to mucositis is crucial in order to develop effective preventive and/or therapeutic strategies.

We have shown recently that senescent fibroblasts regulate major aspects of keratinocyte behaviour, including adhesion and invasion (Hassona et al., 2013, 2014), through paracrine mechanisms. In the present study, we demonstrate that a commercial compound based on HA attenuates the paracrine effects of fibroblast senescence on keratinocyte behaviour and show that it may have clinical therapeutic benefit.

Materials and Methods

Cell strains, culture conditions, and treatments

Details of the cell strains and culture conditions have been published previously (Lim et al., 2011; Cirillo et al., 2012). Normal human oral fibroblasts (NHOF) were cultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS) and 2 mM L-glutamine and grown in a humidified atmosphere of 5% CO_2/95% air; cells were examined at passages <12. Keratinocytes were cultured in DMEM-F12 supplemented with 10% (v/v) FBS and 0.5 μg/ml hydrocortisone; cells were grown under the same standard conditions (5% CO_2, 37°C). Serum-free keratinocyte growth medium (KGM) was used in certain experiments. Ethics Committee approval was obtained for oral primary cultures (E5133, Bristol, UK). The mesenchymal origin of the fibroblast strains was confirmed by positive and negative labelling with vimentin and pan-cytokeratin/keratin 14 antibodies, respectively. By contrast, the origin of the keratinocyte lines was confirmed by positive and negative labelling with pan-cytokeratin/keratin 14 antibodies and vimentin, respectively.

The compound tested was Mucosamin® (Errekappa Euroterapici Spa, Milan, Italy), a commercial adjuvant gel composed of 1.33% (w/v) HA with molecular mass of 600–800 kDa, not cross-linked, in combination with a pool of synthetic amino acids, namely L-proline (0.75% w/v), L-leucine (0.15% w/v), L-lysine (0.1% w/v), and glycine (1% w/v).

Collection of conditioned medium

Cells were grown in 75 cm flasks until they were 70–90% confluent, washed with serum free media (×3) and PBS (×3) and then incubated in serum free media for a further 48 h. The conditioned media (CM) were centrifuged at 2000 RPM for 5 min to remove dead cells. The viable attached cells were trypsinised and counted; the CM were normalised for 0.5×10^6 fibroblasts and 1×10^6 keratinocytes. CM was stored at −20°C.

Induction of oxidative stress, senescence, and detection of 8-hydroxy-2-deoxyguanosine

1×10^4 NHOF were seeded into 60 mm culture dishes and grown under standard conditions. At 60% confluence, the fibroblasts were treated with 600 μM H_2O_2 for 2 h each day for 5 days followed by a 5-day period of recovery in fresh culture media (recorded as time 0). Untreated cells were used as positive controls. Senescence was also induced with 4 ng/ml TGB-β1 for 4 h every day for 8 days, and end of treatment was recorded as time 0.

The presence of oxidative DNA damage was confirmed by immuno-detection of 8-hydroxy-2-deoxyguanosine (8-oxo-dG). This was undertaken using the anti-oxo-dG antibody (Clone 2E2; CAT Number 4354-MC-050; Trevigen, USA) according to the supplier's protocol. Positive cells were counted in 20 microscope fields and the ratio of positive cells to the total number of cells counted was calculated. A minimum of 100 cells was counted for each time-point.

Detection of cellular senescence

Cell senescence was examined by measuring senescence-associated β-galactosidase (SA-ßGal) activity (Bio Vision, USA). Fibroblasts were grown overnight in 12 well culture plates, the culture media decanted and then, the cells were washed in 1 ml PBS followed by fixation in the fixative solution of the commercial kit for 15 min at room temperature. The cells were washed again in PBS (×2) and incubated overnight in 500 μl staining solution (470 μl staining solution, 5 μl staining supplement, 1 mg/ml X-gal in DMSO) in a dark environment. Senescent cells with positive dark green staining were counted in 20 microscope fields and the ratio of positive cells to the total number of counted cells was calculated. A minimum of 100 cells was counted for each time-point. The induction of senescence was confirmed by $p16^{INK4A}$ and α-SMA expression, as demonstrated by Western blot analysis.

Detection of metabolic activity using MTT

Assessment of the activity of living cells, based on mitochondrial function, was determined by the ability of cells to convert soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into the insoluble purple formazan reaction product, as detailed by us previously (Cirillo et al., 2007b). Briefly, cells were plated on 12-well dishes and after 24 h were subjected to the experimental treatments. During the last 4 h of incubation time, the media were replaced by MTT solution (10% (v/v) in DMEM (without phenol red), 1 ml for each well. The MTT solution was then aspirated and formazan was dissolved by addition of 1 ml 0.1 N HCl. The absorbance of the supernatants was read at 570 nm wavelength and normalized on cell number. The percentage of cell viability (MTT conversion into purple formazan compared to control values) indicates the rates of mitochondrial respiration or activity of mitochondrial dehydrogenases.

Western blotting

Protein extraction and Western blotting were undertaken using standard protocols (Cirillo et al., 2010). $p16^{INK4A}$ was detected using anti-human $p16^{INK4A}$ (1:100, BD Pharmingen, UK) and α-tubulin identified using anti-human α-tubulin (1:10000, Sigma, UK). Sheep anti-mouse (1:1000, Sigma, UK) was used as secondary antibody. Selected proteins were detected using Amersham ECL™ Western Blotting Detection Reagent (Amersham Biosciences, UK) and exposed to radiograph film (Kodak, UK).

Migration assays

Wound healing. Cells were seeded and grown to confluence in KGM and DMEM (KAD) medium (Cirillo et al., 2007a) in six-well dishes. Scratches were made with a sterile blue 1 ml pipette tip perpendicular to the bottom of the dish to facilitate the identification of three sites for each well at which migration could be determined. Cells were rinsed and the wounded area was examined microscopically to ensure that cellular debris was removed. The wells then received fresh KAD medium with or without test compounds. The culture was photographed at each line/scratch intersection at time 0 and again after 6, 12, 24 and 36 h in at least two independent experiments. Images were processed and measurements performed using the FIJI open source package (Schindelin et al., 2012).

AGKOS assay. Keratinocytes were processed for the assay as reported previously (Zia et al., 2000). In the chemokinesis AGKOS assay, 1×10^4 cells per 10 μl were loaded into 3-mm well in an agarose gel. After an overnight incubation to allow the cells to settle and to form a megacolony, the cultures were fed with KGM containing 25% (v/v) CM from fibroblasts exposed to several treatments (H_2O_2, Mucosamin®, or TGF-β, alone or in combination) versus no treatment (control) and then incubated for 7 days in a humid CO_2 incubator with daily changes of medium. The migration of keratinocytes was stopped by fixing the cells in 0.25% glutaraldehyde followed by staining with Wright's stain. To measure the effects of the treatments on the random migration distance (RMD) (i.e., the distance outward from the original 3-mm well to the leading edge), the image of the megacolony was magnified by projecting it onto a screen and the blueprint obtained. A transparent grid corresponding to the original size of the colony was overlaid to visualize the starting point, and the migration distance was measured. To standardize measurements, three segments were drawn through the center of each megacolony at 60° intervals, with a total of six points intercepting the original megacolony (A1–A6) and the larger colony after migration (B1–B6). The RMD was computed in μm using the following formula:

$$RMD = (B1B4–A1A4) + (B2B5–A2A5) + (B3B6–A3A6)/6$$

(Chernyavsky et al., 2004).

Patients and treatments

After obtaining appropriate informed consent, five patients undergoing radiotherapy, chemotherapy, or both, were enrolled in the present study. Each patient received Mucosamin® three times per day starting 4 days before every cycle. The study received Ethics Committee (Naples, Italy) approval in 2010.

Statistics

Data were analyzed using one-way analysis of variance (ANOVA) with Tukey's multiple comparison test being used as a post test. Data were expressed as a mean ± SD (error bars) of three independent experiments, unless otherwise stated. $P < 0.05$ was considered statistically significant.

Results

Fibroblasts exposed to transient oxidative stress reduce cell viability and migration of oral keratinocytes in a paracrine manner

Since the molecular mechanisms underlying oral mucositis involve the production of ROS, we wanted to investigate the effects of oxidative stress in the epithelial-mesenchymal cross-talk.

NHOF were transiently treated with H_2O_2 for 5 days, as described in the methods. Oxidative DNA damage, as revealed by the expression of 8-oxo-dG, was increased in H_2O_2-treated fibroblasts relative to untreated controls at the end of the observation period (Fig. 1a–j). Flow cytometry measurements confirmed that the levels of ROS were considerably higher ($P < 0.005$) in fibroblasts exposed to H_2O_2 compared to controls (Supplementary Figure 1).

To investigate whether fibroblasts exposed to oxidative stress could affect the function of oral keratinocytes, the metabolic activity and migratory capacity of normal oral keratinocytes incubated with conditioned media from H_2O_2-treated oral fibroblasts were assessed. Cell viability as determined in the MTT assay was significantly reduced in keratinocytes incubated with CM from H_2O_2-treated fibroblasts, but not in controls (Fig. 2A). In a wound healing assay, keratinocytes treated with CM derived from H_2O_2-treated fibroblasts displayed a significantly ($P < 0.01$) reduced ability to close the epithelial wound by ~45% when compared to controls (Fig. 2B,C). The RMD of the keratinocytes was also impaired by ~35% in the presence of CM from fibroblasts incubated with H_2O_2, as assessed by the AGKOS assay (Fig. 2D). Consistently, the use of antioxidants prevented in most part the detrimental effects of fibroblast CM on oral keratinocytes (Supplementary Figure 2). These data demonstrate that oxidative stress is involved in the paracrine crosstalk between fibroblasts and keratinocytes. More specifically, fibroblasts undergoing oxidative damage reduce the cell viability and migration of normal keratinocytes.

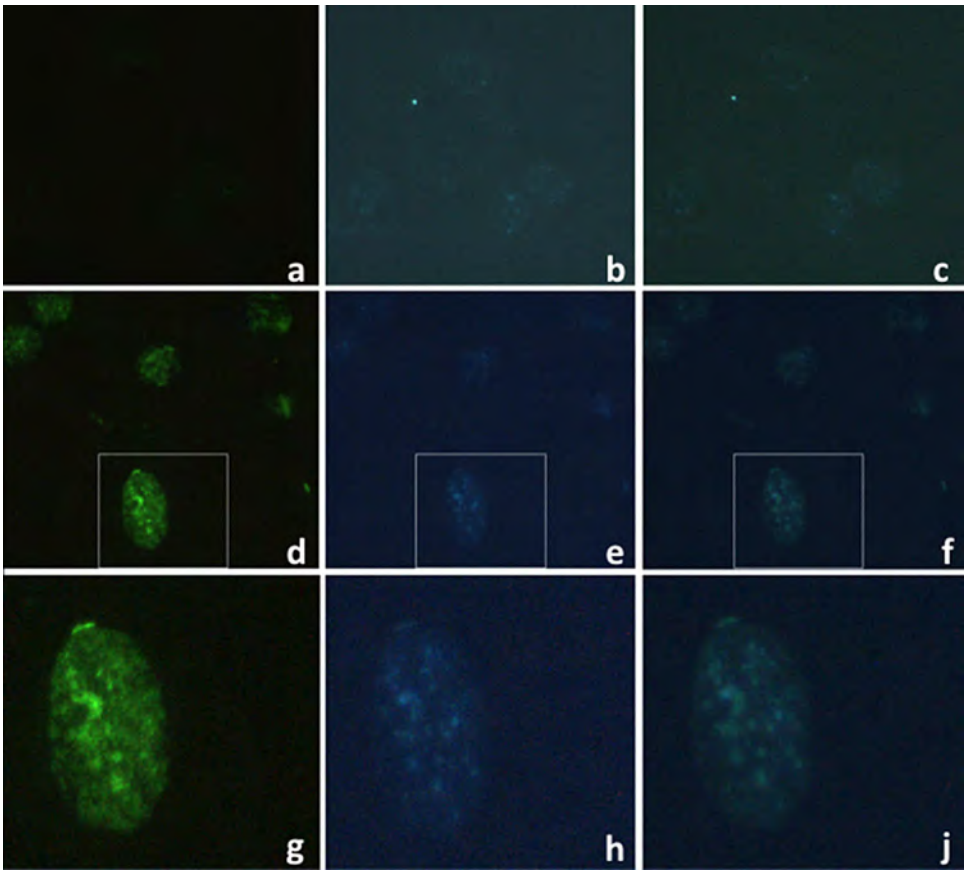

Fig. 1. Staining with antibody against 8-oxo-dG (a,d), Hoechst (b,e), and merged (c,f) of normal oral fibroblasts (a–c) or oral fibroblasts treated with H_2O_2 (d–f) at the end of recovery phase. Nuclei of H_2O_2-treated fibroblasts are also reported at higher magnification (g–j).

Role of Mucosamin® in the development of senescence induced by oxidative stress in normal oral fibroblasts

To gain a better understanding of the mechanisms underlying the paracrine effects of H_2O_2-treated fibroblasts on keratinocytes, we investigated the morphological and molecular consequences of oxidative stress on normal oral fibroblasts. Fibroblasts were monitored on various time points after the recovery period. The cells were attached to the plate throughout the observation period but failed to reach confluence and displayed major morphological changes (Fig. 3). Specifically, the cells lost the classical spindle shape and assumed an enlarged, flat cellular appearance. These alterations were typical of senescent cells; consistently, H_2O_2-treated fibroblasts but not normal untreated fibroblasts stained positively with SA-βGal (Fig. 3A,B). Induction of fibroblast senescence by oxidative stress was confirmed by a time-dependent increase of $p16^{INK4A}$ expression in NHOF treated with H_2O_2, but not controls, as revealed by Western blotting (Fig. 3C). Approximately 10–15% ($P < 0.05$) of the fibroblasts were senescent as early as 4 days after recovery and the number of senescent fibroblasts increased with time to reach over 40% (Fig. 3D). Interestingly, senescence progressed in the absence of exogenous stimulation, as H_2O_2 was not added to the fresh media during the recovery time. Thus, transient oxidative stress induced irreversible cellular senescence in otherwise normal oral fibroblasts.

Having found a strong link between oxidative stress and senescence, we wanted to investigate the effects of a test HA-based compound on the alterations caused by H_2O_2. Oral fibroblasts were pre-treated with Mucosamin® (1% v/v) 30 min before adding H_2O_2 to induce senescence, as per protocol. At the end of the observation time, fibroblasts were assessed for senescence and oxidative DNA damage (Fig. 4). In the presence of Mucosamin®, fibroblasts proliferated with a duplication time of up to 36 h, as opposed to controls exposed to H_2O_2 only where the proliferation rate was reduced dramatically (Fig. 4A). Similarly, the percentage of SA-βGal positive cells was significantly lower in cells pre-treated with Mucosamin® (Fig. 4B). The ability of the test compound to mitigate cellular senescence induced by oxidative stress was confirmed by a lack of expression of $p16^{INK4A}$ in test cells compared with controls (Fig. 4C). Taken together, the results demonstrate that pre-treatment of fibroblasts with Mucosamin® has a protective effect against senescence induced by oxidative stress.

Use of Mucosamin® in fibroblasts treated with H_2O_2, but not with TGF-β, prevents the detrimental effects of CM in keratinocytes

When CM from H_2O_2-treated fibroblasts pre-incubated with Mucosamin® were used to treat keratinocytes, the epithelial cells demonstrated comparable levels of viability as CM from untreated fibroblasts (Fig. 5A,B).

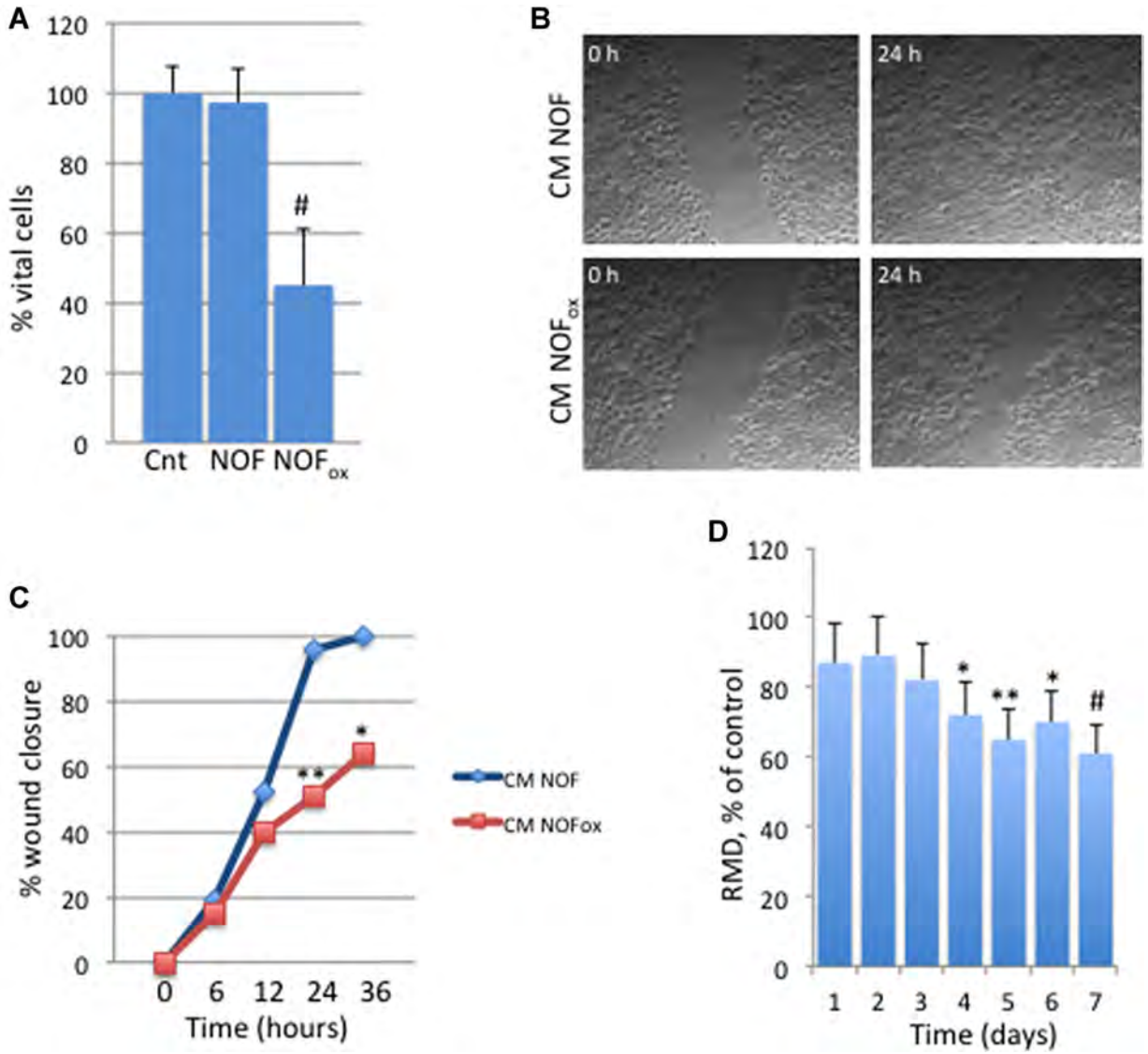

Fig. 2. Keratinocytes were incubated with serum free medium, with conditioned medium (CM) from untreated oral fibroblasts or from fibroblast treated with H_2O_2. Epithelial metabolic activity was assessed using the MTT assay and the average absorbance of controls was referred to as 100% cell viability (A). Keratinocyte monolayers were scratched and wound closure was followed at time 0, 6, 12, 24, and 36 h (time 0 and 24 h is shown in the pictures) in the presence of CM from normal or H_2O_2-treated fibroblasts (B,C). Random migration was assessed with the AGKOS chemokinesis assay and monitored for 7 days (D). Statistical significance refers to test compared to controls at each time point. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{\#}P < 0.005$.

To understand whether the protective effects of Mucosamin® were specific to oxidative stress, we studied the effects of CM from fibroblasts exposed to TGF-β, a well-known inducer of fibroblast activation and senescence (Minagawa et al., 2011). Western blotting (Fig. 5C) confirmed that TGF-β induced a progressive increase in the expression of markers of activation (α-SMA) and senescence ($p16^{INK4A}$). Surprisingly, the level of senescence seen in controls (TGF-β only) did not change significantly in TGF-β-treated fibroblasts pre-incubated with Mucosamin® (Supplementary Figure 3). Keratinocytes incubated with CM from TGF-β-incubated fibroblasts displayed a marked reduction in viability and migratory capability (Fig. 5A,B), similar to the effects of CM from H_2O_2-treated fibroblasts. When CM from TGF-β-treated fibroblasts pre-incubated with Mucosamin® were used, there was no evidence of a protective effect (Fig. 5A,B).

Taken together, the data demonstrate that the HA-based compound Mucosamin® prevented H_2O_2-induced, but not TGF-β-induced, fibroblast senescence. This, in turn, resulted in a protective effect against the secondary detrimental effects of senescent fibroblasts on keratinocytes.

Pretreatment with Mucosamin® prevents severe mucositis in patients undergoing radiochemotherapy: case series

The data obtained so far demonstrate that a HA-based compound not only prevents the onset of senescence induced

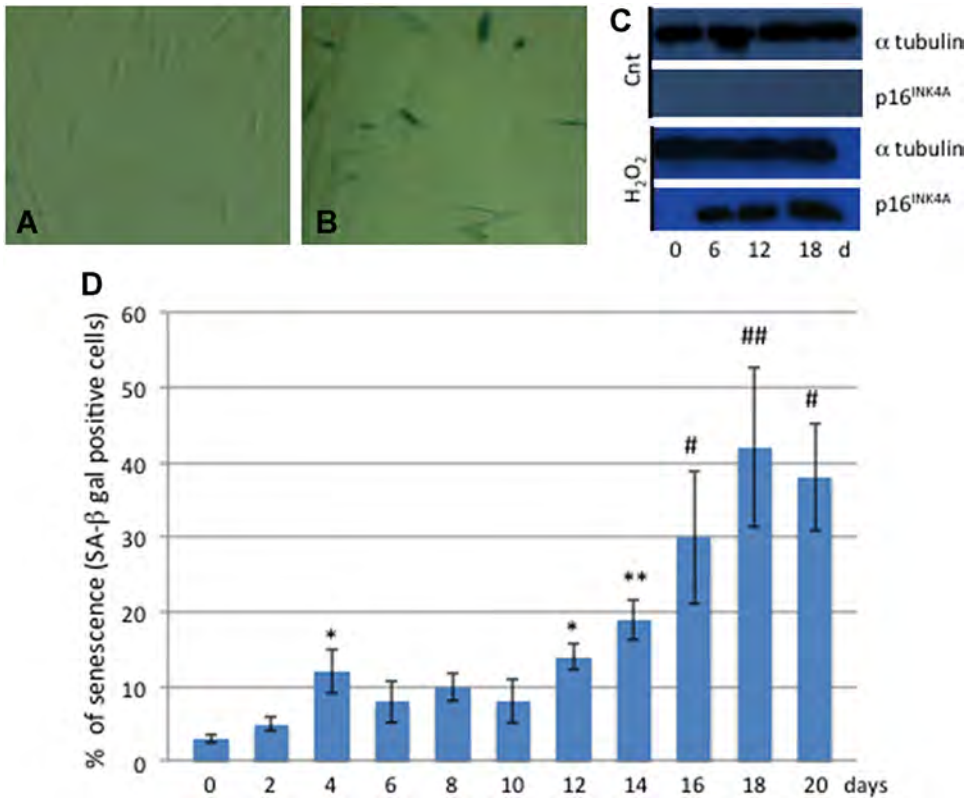

Fig. 3. SA β-gal staining of normal (A) and H_2O_2-treated (B) oral fibroblasts at day 18. Senescence was investigated both in normal and H_2O_2-treated fibroblasts by Western blotting of $p16^{INK4A}$ at 6, 12, and 18 days after recovery (C). Staining of SA β-gal was quantified in H_2O_2-treated cells at different time points throughout the observation period and matched against untreated controls at the same time points to undertake statistical analysis (D). Statistical significance refers to H_2O_2-treated cells compared to control (time 0). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{\#}P < 0.005$; $^{\#\#}P < 0.001$.

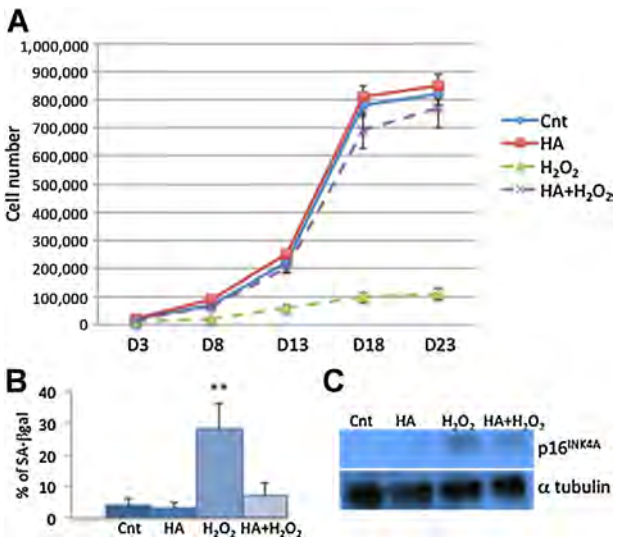

Fig. 4. Normal oral fibroblasts were grown in 2D cell culture and treated with H_2O_2 and Mucosamin® (hyaluronic acid plus amino acids, HA), alone or in combination, or left untreated (Cnt). Cells were seeded in triplicate and counted at day 3, 8, 13, 18, and 23 (A). Senescence was investigated by SA β-gal staining at day 18 (B) and by Western blotting against $p16^{INK4A}$ (C). $^{**}P < 0.01$.

by H_2O_2 in oral fibroblasts but, also, can protect keratinocytes from the detrimental effects of senescent fibroblasts. To translate these results into practice, we investigated whether Mucosamin® spray was effective when used prophylactically to prevent oral mucositis in patients undergoing ratio- and/or chemotherapy (Table 1).

Case 1. This 59-year-old female underwent radical mastectomy plus chemotherapy (doxorubicin i.v. 40 mg/m^2 given every 28 days for 6 cycles) in 2003 with the development of grade 3 mucositis after the second cycle. She had recurrence of the primary cancer in 2011. Chemotherapy (doxorubicin i.v. 40 mg/m^2 given every 28 days for 6 cycles) was initiated, together with the HA-based test compound Mucosamin® three times a day starting from 4 days before every cycle. No appearance of mucositis has been registered to date.

Case 2. Radiotherapy (35 cycles) plus chemotherapy (cisplatin 4 cycles, 1 cycle every 21 days) was started in this 63-year-old male with squamous cell carcinoma (SCC) of the tongue. Mucosamin® spray was used three times/day starting from 4 days before every cycle. After 22 cycles of radiotherapy and 3 cycles of chemotherapy, mild oral mucositis (grade 1, VAS 35) appeared transiently and healed completely after 72 h. Extraoral manifestations included dull erythema (RTOG/EORTC 1). No further episodes of mucositis have been seen to date.

Case 3. The same treatment as in case 2 was used for a 51-year-old male with SCC of the tongue base. The treatment was stopped after 35 cycles of radiotherapy and 4 cycles of chemotherapy due to renal failure. To date, no signs of oral

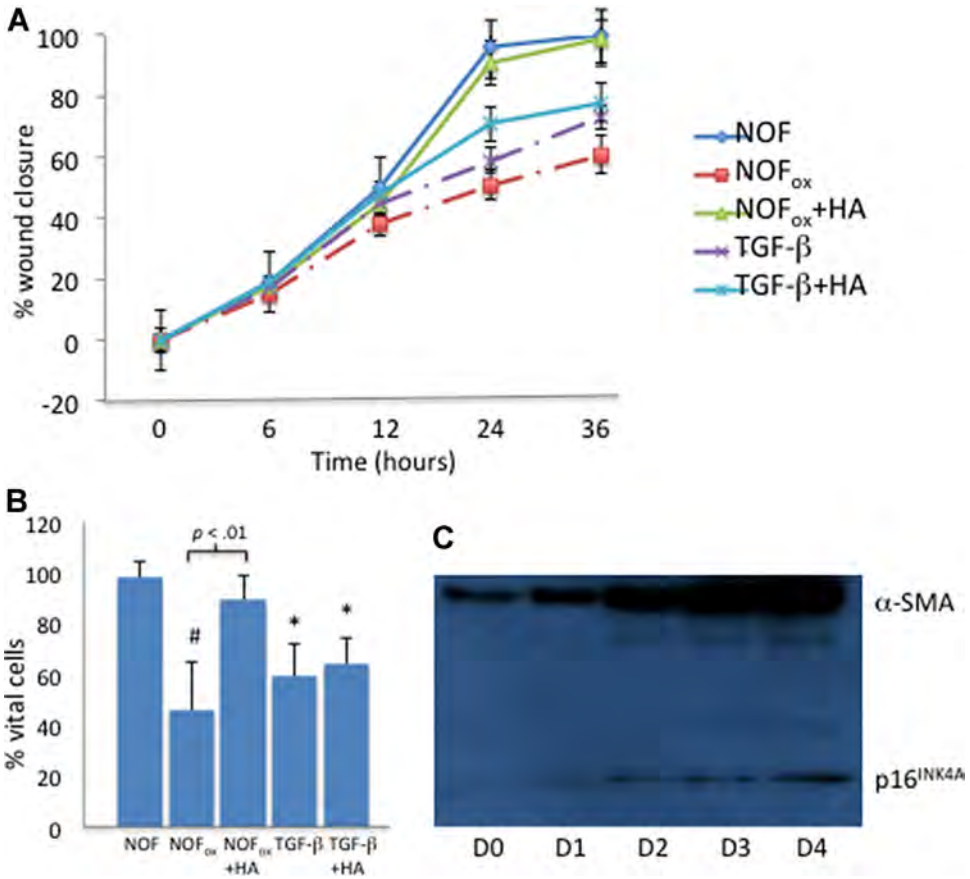

Fig. 5. Keratinocytes were exposed to conditioned media (CM) from untreated normal oral fibroblasts (NOF) or from those treated with H_2O_2 or TGF-β, with or without Mucosamin® (hyaluronic acid plus amino acids, HA). In some experiments, 95% confluent monolayers were scratched and wound closure was followed at time 0, 6, 12, 24, and 36 h (A). The viability of unwounded confluent keratinocytes was measured using the MTT assay (B). The ability of TGF-β to induce both activation and senescence was shown by Western blotting against α-SMA and p16^{INK4A}, respectively, from day 0 (D0) to day 4 (D4). The immunoreactivity was first probed with anti- α-SMA and anti-p16^{INK4A} IgG separately, in two independent experiments. Then, the filter shown in the figure (C) was incubated with both antibodies and overexposed to visualize both markers in the same blot (see Supplemental Figure 4). $^{*}P < 0.05$; $^{\#}P < 0.005$.

mucositis have presented. Extraoral manifestations included dull erythema (RTOG/EORTC 1).

Case 4. This 61-year-old male underwent post-operative radiotherapy (36 cycles) for insufficient surgical resection (excision margins) of an SCC of the buccal mucosa. Mucosamin® spray three times daily starting from 4 days before every cycle was used and no appearance of oral mucositis was registered. Interestingly, the patient developed severe radiodermitis with bright erythema and desquamation (RTOG/EORTC 3).

Case 5. Post-operative radiotherapy (36 cycles) was started in an 80-year-old male patient after recurrence of SCC of the buccal mucosa. Mucosamin® was used as per protocol and no signs of mucositis have been seen throughout the treatment. Appearance of bright erythema (RTOG/EORTC 2) was recorded.

Discussion

In the present study, we demonstrate that the detrimental effects of oxidative stress in human fibroblasts on keratinocytes can be prevented by Mucosamin® in vitro. We then translated the results into clinical practice to provide the first evidence that prophylactic use of a HA-based compound may prevent

TABLE 1. Details of five patients that have completed chemo/radiotherapy for cancer

Pt	Sex/age	Tumor	Previous treatment	Previous mucositis	Treatment	Mucositis/VAS	RTOG/EORTC
1	F/59	Breast	CH (doxorubicin)	Grade 3 (WHO)	CH (doxorubicin)	0/0	N/A
2	M/63	Tongue	NO	NO	RT (35 cycles) + CH (CIS)	1/35[a]	1
3	M/51	Tongue	NO	NO	RT (35 cycles) + CH (CIS)	0/0	1
4	M/61	Cheek	NO	NO	RT (36 cycles)	0/0	3
5	M/80	Cheek	NO	NO	RT (36 cycles)	0/0	2

CH, chemotherapy; RT, radiotherapy; CIS, cisplatin; VAS, visual analogue scale; RTOG/EORTC, acute radiation scoring criteria for skin.
[a]After 22 cycles of radiotherapy (RT) and 3 cycles of chemotherapy (CH), oral mucositis (grade 1 WHO, VAS 35) was registered. Complete healing after 72 h, no further mucositis.

the onset of oral mucositis in patients undergoing radio-chemotherapy for cancer.

It is well established that the mechanisms initiating chemoradiation-induced mucosal injury, including oral mucositis, are linked to the production of ROS (Sonis, 2009; Zhao and Robbins, 2009). To investigate the paracrine effects of oxidative stress in the stroma, we used H_2O_2 to induce oxidative damage in human oral fibroblasts in vitro. Stromal fibroblasts in response to H_2O_2 developed oxidative DNA damage and generated ROS which, in turn, initiated cellular senescence. This phenotype was irreversible and progressed regardless of the presence of exogenous H_2O_2. Strikingly, senescence was massively reduced by pre-incubating the fibroblasts with a HA-containing product diluted in culture medium. Binding of high molecular mass HA to free radicals catalyzes the breakdown of HA into smaller fragments and thus inactivates ROS (Mendoza et al., 2007). Thus, it is possible that Mucosamin® prevented the propagation of oxidative stress by sequestrating ROS. Studies show that UVB irradiation leads to a deficiency or inactivation in antioxidant enzymes in corneal epithelial cells that is prevented by HA (Li et al., 2013), and a most recent pilot study (Lockington et al., 2014) has demonstrated that the HA-rich amniotic membrane has free radical scavenging antioxidant properties. The possibility cannot be ruled out that amino acids, rather than HA, play a role in the beneficial effects of Mucosamin®. Glutamine, an amino acid not present in the test compound used in our study, has been found to be effective in the treatment of oral mucositis (Chattopadhyay et al., 2014). Although further research is needed to confirm our hypothesis, the current data suggest that Mucosamin® may prevent mucosal injury initiated by ROS by inactivating the free radicals that are produced by chemoradiation.

Evidence shows that oral mucositis may persist for months after termination of radiation therapy or chemotherapy, a timeframe in which the epithelium would be expected to fully regenerate. We speculated that if senescence was induced in stromal fibroblasts in response to oxidative stress, this phenotype could be responsible for a prolonged induction of detrimental effects in the epithelial compartment. It is indeed established that senescent fibroblasts develop a secretory phenotype that can control epithelial behavior in a paracrine fashion (Parrinello et al., 2005; Laberge et al., 2012). Our data support this view as we demonstrated that irreversibly senescent fibroblasts were able to alter function, metabolic activity and migration of keratinocytes via paracrine mechanisms. Once again, pre-treatment of CM with HA-based compound almost abolished these effects on keratinocytes. It is also interesting to note that Mucosamin® prevented fibroblast senescence induced by oxidative stress (H_2O_2), but not by other pathways (e.g., TGF-β). This finding further reinforces the notion that the effectiveness of the HA-based compound tested in the present study depends, at least in part, on its antioxidant properties.

Since our data demonstrated that Mucosamin® effectively prevented the oxidative damage in vitro, we wondered if it was possible to use the biological properties of this preparation for the clinical benefit of patients undergoing chemoradiation. Among the five cases presented here, only one patient developed mild and rapidly healing oral mucositis. Case 4 was particularly striking in that the patient developed severe radiodermitis extraorally in the irradiated area, but not mucositis in the corresponding intraoral area. Considering that 85–100% of patients undergoing radio- and chemotherapy develop oral mucositis, our pilot clinical data strongly suggest that prophylactic use of Mucosamin® is effective in the prevention of this most debilitating condition.

A large number of diverse interventions have been tested for mucositis, many of which are available over the counter or for off-label use or marketed as devices. Surprisingly, only one agent to date has been approved by the US Food and Drug Administration as a drug for mucositis, albeit in a relatively restricted population (Lalla et al., 2014). Most recent guidelines from the Multinational Association of Supportive Care in Cancer and International Society of Oral Oncology (MASCC/ISOO) suggest that certain preventive meausre, such as oral cryotherapy, palifermin, low-level laser therapy, benzydamine mouthwash, and oral care protocols may be effective in preventing oral mucositis, but only for certain treatment settings (Lalla et al., 2014). Our data were obtained from a small cohort of patients undergoing chemotherapy (n = 1), head and neck radiation therapy (n = 2), or both (n = 2). A major limitation of the clinical study is the limited number of patients enrolled and the absence of controls. Appropriate clinical trials will be needed in order to confirm the data and to provide more robust evidence for efficacy of Mucosamin® in preventing oral mucosal injury. A current multicenter study on a larger scale suggests this to be the case (G. Colella, personal communication). Notwithstanding the limitations of the clinical data, in the authors' opinion the results presented here are important and suited the purpose of the manuscript, which was conceived as a case study of translational research. Specifically: (1) the experimental question was triggered by a common clinical scenario; (2) hypothesis was formulated and tested in vitro; and (3) the results were eventually applied to a clinical setting for patient benefit.

In conclusion, in the present study we used a combined clinical-molecular approach to show that prevention of senescence in oral fibroblasts undergoing oxidative stress in vitro may be applied clinically to oral mucositis in patients undergoing chemoradiation. Specifically, we provide the first evidence for the efficacy of the HA-based compound Mucosamin® as a preventive measure for this most debilitating condition. Further clinical research is ongoing to substantiate our promising data.

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Literature Cited

Buchsel PC. 2008. Polyvinylpyrrolidone-sodium hyaluronate gel (Gelclair): A bioadherent oral gel for the treatment of oral mucositis and other painful oral lesions. Expert Opin Drug Metab Toxicol 4:1449–1454.

Chattopadhyay S, Saha A, Azam M, Mukherjee A, Sur PK. 2014. Role of oral glutamine in alleviation and prevention of radiation-induced oral mucositis: A prospective randomized study. South Asian J Cancer 3:8–12.

Chernyavsky AI, Arredondo J, Marubio LM, Grando SA. 2004. Differential regulation of keratinocyte chemokinesis and chemotaxis through distinct nicotinic receptor subtypes. J Cell Sci 117:5665–5679.

Cirillo N, Gombos F, Lanza A. 2007a. Changes in desmoglein 1 expression and subcellular localization in cultured keratinocytes subjected to antidesmoglein 1 pemphigus autoimmunity. J Cell Physiol 210:411–416.

Cirillo N, Hassona Y, Pignatelli M, Gasparoto TH, Morgan DJ, Prime SS. 2012. Characterization of a novel oral glucocorticoid system and its possible role in disease. J Dent Res 91:97–103.

Cirillo N, Lanza M, Femiano F, Gaeta GM, De Rosa A, Gombos F, Lanza A. 2007b. If pemphigus vulgaris IgG are the cause of acantholysis, new IgG-independent mechanisms are the concause. J Cell Physiol 212:563–567.

Cirillo N, Lanza A, Prime SS. 2010. Induction of hyper-adhesion attenuates autoimmune-induced keratinocyte cell-cell detachment and processing of adhesion molecules via mechanisms that involve PKC. Exp Cell Res 316:580–592.

Colella G, Cannavale R, Vicidomini A, Rinaldi G, Compilato D, Campisi G. 2010. Efficacy of a spray compound containing a pool of collagen precursor synthetic aminoacids (l-proline, l-leucine, l-lysine and glycine) combined with sodium hyaluronate to manage chemo/radiotherapy-induced oral mucositis: Preliminary data of an open trial. Int J Immunopathol Pharmacol 23:143–151.

Colella G, Vicidomini A, Soro V, Lanza A, Cirillo N. 2012. Molecular insights into the effects of sodium hyaluronate preparations in keratinocytes. Clin Exp Dermatol 37:516–520.

Criswell T, Leskov K, Miyamoto S, Luo G, Boothman DA. 2003. Transcription factors activated in mammalian cells after clinically relevant doses of ionizing radiation. Oncogene 22:5813–5827.

Elting LS, Keefe DM, Sonis ST, Garden AS, Spijkervet FK, Barasch A, Tishler RB, Canty TP, Kudrimoti MK, Vera-Llonch M. Burden of Illness Head and Neck Writing Committee. 2008. Patient-reported measurements of oral mucositis in head and neck cancer patients treated with radiotherapy with or without chemotherapy: Demonstration of increased frequency, severity, resistance to palliation, and impact on quality of life. Cancer 113:2704–2713.

Favia G, Mariggio MA, Maiorano F, Cassano A, Capodiferro S, Ribatti D. 2008. Accelerated wound healing of oral soft tissues and angiogenic effect induced by a pool of aminoacids combined to sodium hyaluronate (AMINOGAM). J Biol Regul Homeost Agents 22: 109–116.

Hassona Y, Cirillo N, Heesom K, Parkinson EK, Prime SS. 2014. Senescent cancer associated fibroblasts secrete active MMP-2 that promotes keratinocyte dis-cohesion and invasion. Br J Cancer 111:1230–1237.

Hassona Y, Cirillo N, Lim KP, Herman A, Mellone M, Thomas GJ, Pitiyage GN, Parkinson EK, Prime SS. 2013. Progression of genotype-specific oral cancer leads to senescence of cancer-associated fibroblasts and is mediated by oxidative stress and TGF-β. Carcinogenesis 34:1286–1295.

Laberge RM, Awad P, Campisi J, Desprez PY. 2012. Epithelial-mesenchymal transition induced by senescent fibroblasts. Cancer Microenviron 5:39–44.

Lalla RV, Bowen J, Barasch A, Elting L, Epstein J, Keefe DM, McGuire DB, Migliorati C, Nicolatou-Galitis O, Peterson DE, Raber-Durlacher JE, Sonis ST. Mucositis Guidelines Leadership Group of the Multinational Association of Supportive Care in Cancer and International Society of Oral Oncology (MASCC/ISOO). 2014. MASCC/ISOO clinical practice guidelines for the management of mucositis secondary to cancer therapy. Cancer 120:1453–1461.

Li JM, Chou HC, Wang SH, Wu CL, Chen YW, Lin ST, Chen YH, Chan HL. 2013. Hyaluronic acid-dependent protection against UVB-damaged human corneal cells. Environ Mol Mutagen 54:429–449.

Lim KP, Cirillo N, Hassona Y, Wei W, Thurlow JK, Cheong SC, Pitiyage G, Parkinson EK, Prime SS. 2011. Fibroblast gene expression profile reflects the stage of tumour progression in oral squamous cell carcinoma. J Pathol 223:459–469.

Lockington D, Agarwal P, Young D, Caslake M, Ramaesh K. 2014. Antioxidant properties of amniotic membrane: Novel observations from a pilot study. Can J Ophthalmol 49: 426–430.

Mariggiò MA, Cassano A, Vinella A, Vincenti A, Fumarulo R, Lo Muzio, Maiorano L, Ribatti E, Favia D. 2009. Enhancement of fibroblast proliferation, collagen biosynthesis and production of growth factors as a result of combining sodium hyaluronate and aminoacids. Int J Immunopathol Pharmacol 22:485–492.

Mendoza G, Alvarez AI, Pulido MM, Molina AJ, Merino G, Real R, Fernandes P, Prieto JG. 2007. Inhibitory effects of different antioxidants on hyaluronan depolymerization. Carbohydr Res 342:96–102.

Minagawa S, Araya J, Numata T, Nojiri S, Hara H, Yumino Y, Kawaishi M, Odaka M, Morikawa T, Nishimura S.L, Nakayama K, Kuwano K. 2011. Accelerated epithelial cell senescence in IPF and the inhibitory role of SIRT6 in TGF-β-induced senescence of human bronchial epithelial cells Am J Physiol Lung Cell Mol Physiol 300:L391–L401.

Nonzee NJ, Dandade NA, Patel U, Markossian T, Agulnik M, Argiris A, Patel JD, Kern RC, Munshi HG, Calhoun EA, Bennett CL. 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.

Parrinello S, Coppe JP, Krtolica A, Campisi J. 2005. Stromal-epithelial interactions in aging and cancer: Senescent fibroblasts alter epithelial cell differentiation. J Cell Sci 118: 485–496.

Pauloski BR, Rademaker AW, Logemann JA, Lundy D, Bernstein M, McBreen C, Santa D, Campanelli A, Kelchner L, Klaben B, Discekici-Harris M. 2011. Relation of mucous membrane alterations to oral intake during the first year after treatment for head and neck cancer. Head Neck 33:774–779.

Raber-Durlacher JE, von Bültzingslöwen I, Logan RM, Bowen J, Al-Azri AR, Everaus H, Gerber E, Gomez JG, Pettersson BG, Soga Y, Spijkervet FK, Tissing WJ, Epstein JB, Elad S, Lalla RV. Mucositis Study Group of the Multinational Association of Supportive Care in Cancer/International Society of Oral Oncology (MASCC/ISOO). 2013. Systematic review of cytokines and growth factors for the management of oral mucositis in cancer patients. Support Care Cancer 21:343–355.

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A. 2012. Fiji: An open-source platform for biological-image analysis. Nat Methods 9:676–682.

Sonis ST. 2009. Mucositis: The impact, biology and therapeutic opportunities of oral mucositis. Oral Oncol 45:1015–1020.

Sonis ST. 2011. Oral mucositis. Anticancer Drugs 22:607–612.

Sonis ST, Elting LS, Keefe D, Peterson DE, Schubert M, Hauer-Jensen M, Bekele BN, Raber-Durlacher J, Donnelly JP, Rubenstein EB. Mucositis Study Section of the Multinational Association for Supportive Care in Cancer; International Society for Oral Oncology. 2004. Perspectives on cancer therapy-induced mucosal injury: Pathogenesis, measurement, epidemiology, and consequences for patients. Cancer 100:1995–2025.

Stokman MA, Spijkervet FK, Boezen HM, Schouten JP, Roodenburg JL, de Vries EG. 2006. Preventive intervention possibilities in radiotherapy- and chemotherapy-induced oral mucositis: Results of meta-analyses. J Dent Res 85:690–700.

Talwar S, House R, Sundaramurthy S, Balasubramanian S, Yu H, Palanisamy V. 2014. Inhibition of caspases protects mice from radiation-induced oral mucositis and abolishes the cleavage of RNA-binding protein HuR. J Biol Chem 289:3487–3500.

Trotti A, Bellm LA, Epstein JB, Frame D, Fuchs HJ, Gwede CK, Komaroff E, Nalysnyk L, Zilberberg MD. 2003. Mucositis incidence, severity and associated outcomes in patients with head and neck cancer receiving radiotherapy with or without chemotherapy: A systematic literature review. Radiother Oncol 66:253–262.

Yoshino F, Yoshida A, Nakajima A, Wada-Takahashi S, Takahashi SS, Lee MC. 2013. Alteration of the redox state with reactive oxygen species for 5-fluorouracil-induced oral mucositis in hamsters. PLoS ONE 8:e82834.

Zhao W, Robbins ME. 2009. Inflammation and chronic oxidative stress in radiationinduced late normal tissue injury: Therapeutic implications. Curr Med Chem 16:130–143.

Zia S, Ndoye A, Lee T.X, Webber R.J, Grando S.A, 2000. Receptor-mediated inhibition of keratinocyte migration by nicotine involves modulations of calcium influx and intracellular concentration J Pharmacol Exp Ther 293:973–981.

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