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ORIGINAL ARTICLE

Synergistic effect of 15-lipoxygenase 2 and radiation in killing head-and-neck cancer

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We previously demonstrated that 15-LOX-2 is significantly reduced in head and neck carcinoma and restoration of 15-LOX-2 expression results in tumor inhibition in HNC. The aim of this study is to evaluate 15-LOX-2 as a candidate for targeted radiotherapy. Molecular subcloning was performed to create a radiation-inducible 15-LOX-2 expression vector in which the full-length 15-LOX-2 cDNA was inserted downstream the recombinant *Egr-1* promoter. The radiation-induced downregulations of 15-LOX-2 protein (twofold up) and its main metabolite 15S-HETE (threefold up) were observed in HNC cells transfected with the 15-LOX-2 expression vector after 4 Gy of radiation. Radiation-induced upregulation of 15-LOX-2 resulted in significant induction of apoptosis in HNC cells. Furthermore, survival colony formation was significantly reduced by 4 Gy in the HNC cells containing the 15-LOX-2 expression vector compared with the controls. Radiation-induced upregulation of 15-LOX-2 results in significant induction of apoptosis and enhances killing effect of radiotherapy in HNC. In addition, exogenous addition of 15S-HETE at high concentrations ($\geqslant 10\,\mu M$) but not at low concentrations induced upregulation of its endogenous ligand PPARγ. In conclusion, synergistic effect between radiation and 15-LOX-2 was observed in killing HNC. 15-LOX-2 may be a potential target in radiation-targeted therapy of HNC. The 15-LOX-2 inhibition may not be PPARγ dependent.
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Keywords: head-and-neck cancer; 15-lipoxygenase 2; radiotherapy; *Egr-1* promotor; 15S-HETE

Introduction

Radiotherapy is an important modality for treating head-and-neck cancer (HNC). Multi-institutional prospective studies have demonstrated that high-dose irradiation (for example, altered fractioned or accelerated radiation) improves local control of HNC. However, high doses are associated with a significant increase in toxicity, which negatively impacts patients' quality of life and treatment compliance, although severity of certain toxicity such as xerostomia may be reduced with the recent use of improved radiation delivery technologies such as 3DCRT or IMRT.[1,2] There is an urgent need to develop novel therapeutic approaches that enhance radiation effect, but at the same time, toxic effects of radiation on adjacent normal structures such as the parotid glands, spinal cord, mandible, orbits, optical chiasm and brain were minimized. Combination of radiotherapy and molecular-targeted therapy based on an understanding of the molecular changes that underlie the development of cancer is one of the promising strategies.[3]

The discovery of radiation-inducible genes led to the new concept and development of radiation-targeted gene therapy. The previous study[4] discovered that the upregulation of the early growth response 1 (*Egr-1*) is induced in mammalian cells. Subsequent studies have demonstrated that human *Egr-1* promoter is one of the promising promoters in cancer gene therapy approaches, controlling extopic expression of therapeutic genes with the application of X-rays to target cells in several kinds of carcinoma.[5–8] Ionizing radiation can regulate the spatial distribution and maintain temporal control of specified gene transcription through radical oxygen intermediate formation. The serum response or CArG box (CC(A + T-rich)$_6$GG) elements in the 5′ promoter of the gene are the sequences necessary and sufficient for radioinduction.[9] Recently several molecules are explored as potential candidates for targeted radiotherapy.[10–12] However, optimal targets have not been identified in combination with targeted radiotherapy.

15-lipoxygenase 2 (15-LOX-2) is 76-kD enzyme and consists of 662 amino acids and two domains (www.ncbi.nlm.nih.gov). The domain one called Plat_LOX domain contains 110 amino acids located in residual number 2 through 111. The domain two called lipoxygenase domain contains 518 amino acids located in

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residual number 137 through 654. 15-LOX-2 oxygenates carbon 15 in arachidonic acid (AA) and is one of the important lipid peroxidizing enzymes that have been linked to carcinogenesis including HNC.[13–15] Unlike 15-lipoxygenase 1 (15-LOX-1), 15-LOX-2 has limited tissue distribution and significant substrate preference.[13] 15-LOX-2 is mainly expressed in epithelia from prostate,[16] esophagus,[17] head and neck,[18] breast,[19] bladder,[19] lung[19] and skin.[13,19] 15-LOX-2 preferentially converts AA to 15-S-hydroxyeicosatetraenoic acid (15S-HETE) and metabolizes linoleic acid poorly. 15-LOX-2 has been shown to be a tumor suppressor. Recently, 15-LOX-2 is proposed to be a tumor suppressor in epithelia-derived carcinoma including HNC. First, 15-LOX-2 was significantly reduced in carcinomas of prostate,[16] esophagus,[17] breast,[19] bladder,[19] lung,[19] skin,[19] and head and neck.[18] Second, the reduced expression of 15-LOX-2 was inversely correlated with the degree of tumor differentiation and pathologic grade (Gleason scores) in prostate cancer specimens.[20] Third, results from us[18] and others[16,17] showed that restoration of 15-LOX-2 resulted in inhibition of cell proliferation. Moreover, 15-LOX-2 was found to be a negative cell-cycle regulator in normal epithelial cells[21] and may be associated with the ability to induce cell senescence.[22] All together, 15-LOX-2 plays an anticarcinogenic role in many epithelia-derived carcinomas including HNC.

In this study, we generated a radiation-inducible 15-LOX-2 expression vector in which a full-length 15-LOX-2 cDNA was inserted downstream the *Egr-1* recombinant promoter. We then explored whether radiation-induced upregulation of 15-LOX-2 resulted in apoptosis and the apoptosis is through peroxisome proliferators-activated receptor γ (PPARγ)-dependent mechanism. Finally, we investigated whether radiation-induced upregulation of 15-LOX-2 enhances the effect of radiation in killing HNC.

Materials and methods

Cells and reagents

Head-and-neck cancer cell lines FaDu and UMSCC-1 were cultured at 37 °C in an atmosphere of 5% CO_2 and 95% air in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (GIBCO-Invitrogen, Carlsbad, CA). FaDu were derived from a primary hypopharyngeal carcinoma (ATCC, Manassas, VA). UMSCC-1 provided by Dr Thomas Carey from the University of Michigan (An Arbor, MI) was derived from a primary squamous cell carcinoma of retromolar trigone. Rabbit polyclonal antibody against human 15-LOX-2 was purchased from Oxford Biomedical (Rochester, MI). Primary antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Abcam Inc. (Cambridge, MA). Monoclonal antibodies against PPARγ and caspase 3 were purchased from Santa Crutz (Santa Cruz Biotechnology, CA). 15S-HETE and AA were purchased from Cayman Chemical Inc. (An Arbor, MI).

Detection of luciferase activity driven by recombinant EGR-1 *promoter*

The cells were transfected with the plasmid pΔ7egr-EGFP-GFL or pRSV-EGFP-GFL containing luciferase gene (provided by Dr Rodemann, University of Tuebingen, Germany), as we previously described.[18] Twenty-four hours after transfection, the cells were exposed to a single dose of radiation (2, 4, 6 or 8 Gy, respectively). Forty-eight hours after irradiation, cells were scraped off in 1 × lysis buffer (Promega, Madison, WI). Activity of luciferase in the cell lysates was measured using a luminometer (Autolumat LB953, Berthold Technologies, Oak Ridge, TN). Transfection efficiency was determined by co-transfection of plasmid pCMV-βgal into the cells. Values of luciferase activity were normalized with β-gal activity using the β-gal kit (Invitrogen, Carlsbad, CA).

Cell irradiation

The cells were irradiated using a 6 MV photon beam from a Siemens linear accelerator available in our department. A water-equivalent plate of 1 cm thick was placed on the bottom of the dish to ensure the full backscatter condition. Another water-equivalent plate of 1 cm thick was placed on the top of the flask to serve as built-up material for the 6 MV beam. The attached cells were in the bottom of culture dish and were at a water-equivalent depth of 5 mm, as they were covered by 5 mm-depth culture media. Therefore, the attached cells were at the depth of dose maximum for the 6-MV (1.5 cm). Two p100 dishes or four 35 mm dishes were placed for radiation treatment each time using a 20×20 cm radiation field. *In vivo* diode measurement for radiation dose was performed and the measured doses agreed with the desired doses within 2%.

Generations of 15-LOX-2 expression vector pEgr-LOX2-GFP and control vector pEgr-GFP

A recombinant human *Egr-1* promoter pΔ7egr fragment was obtained through PCR using the above mentioned plasmid pΔ7egr-EGFP-GFL as a template and primers below. The recombinant *Egr-1* fragment was then inserted into the pIRES-hrGFP backbone to produce a control vector pEgr-GFP. The full-length 15-LOX-2 cDNA (provided by Dr Tang[21]) was subsequently inserted into the pEgr-GFP to produce a radiation-inducible 15-LOX-2 expression construct pEgr-LOX2-GFP, in which 15-LOX-2 expression was driven under the recombinant *Egr-1* promoter (Figure 2a). Orientations and sequences of the recombinant *Egr-1* promoter and 15-LOX-2 in the resultant 15-LOX-2 expression vector were verified by restriction digestions and sequencing analyses. Sequences of primers for the recombinant *Egr-1* promoter were: sense 5′-AGCATGCATAGTGCAGGTGCCA GAACATT-3′ (containing *Nsi*I site) and antisense 5′-ATATACCCGGGAACAGTACCGGAATGCCAAG-3′ (containing *Sma*I site).

Immunohistochemistry of 15-LOX-2

At different time point 6, 12 and 24 h after transfection with 15-LOX-2 expression vector or control vector,

cultured cells were harvested through scraping, spun down to remove the medium, held with 10% buffered formalin, and mixed with 40 µl preheated histogel (Richard-Allan Scientific, Kalamazoo, MI). The pellet was then extracted with a spatula, wrapped in filter paper, placed in a cassette, fixed in formalin overnight and embedded into a paraffin block. For immunostaining, the sections (4-µm thickness) were incubated with the 15-LOX-2 antibody (1:100). Immunohistochemistry was performed using the ABC Elite kit (Vector Laboratories, Burlingame, CA) as previously described.[18] The immunostain were then developed with DAB (Sigma, St Louis, MO) solution for 5 min, and were then counterstained in Modified Mayer's hematoxylin. For controls, corresponding sections were incubated with a rabbit IgG at the same concentration. 15-LOX-2 immunostaining was assessed semiquantitatively from 0 to 3+ as previously described:[22] 0, no staining or equivocal staining in $<5\%$; 1+, weak to moderate in $\leqslant 20\%$ of cells; 2+, weak to strong staining in 21–50% of cells; 3+ weak to strong staining in $>50\%$ of cells. At least 200 cells from four random areas in each time point were counted under high magnification.

Western blot analysis

Western blot analysis was performed as previously described.[18] Briefly, the cells were harvested through scraping. Equal aliquots of total cell lysates of each sample were separated in 10% SDS–polyacrylamide gel electrophoresis. The blots were probed with the primary antibody (1:1000) for 3 h at room temperature. GAPDH was measured as an internal control. Horseradish peroxidase conjugated affinity purified goat anti-rabbit secondary antibody (1:5000, Santa Cruz Biotech, Santa Cruz, CA) was then added at room temperature for 1 h. Antigen–antibody complexes were visualized using the ECL system (Santa Cruz Biotech). The integrated density value of 15-LOX-2 band was quantitatively analyzed using Alpha Imager 2000 Documentation & Analysis System (Alpha Innotech Corporation, San Leandro, CA).

Enzyme immunoassay of 15S-HETE

Enzyme immunoassay of 15S-HETE was performed according to the manufacturer's protocol using Enzyme Immunoassay kit (Assay Designs Inc., Ann Arbor, MI) as previously described.[18] Briefly, the cells were first transfected with either 15-LOX-2 expression vector or control for either 6 or 18 h and then received a single dose of 4 Gy. The cells were then harvested 30 min after radiation. The levels of 15S-HETE were measured according to the manufacturer's protocol. The cross-reactivities for a number of other related eicosanoid compounds were $\leqslant 1\%$.

Immunoreactivity of annexin V and caspase 3. The cells were allowed to grow on a Lab-Tek II Chamber Slide for 24 h and were then transfected either with the 15-LOX-2 expression vector or control vector. Twenty-four hours after the transfection, the cells were irradiated with 4 Gy or received no radiation as control. The cells were then treated with AA (50 µM) for 15 h. The cells were then fixed with 4% paraformaldehyde at 4 °C for 20 min and were permeabilized with 1% Triton X-100 in phosphate-buffered saline for 10 min. For annexin V staining, cells were immunostained using the Annexin V-Cy3 apoptosis kit (Biovision, Mountain View, CA). The cells were observed under a fluorescence microscope using a rhodamine filter. For caspase 3 immunostaining, the cells were blocked in 10% normal goat serum followed by incubation with caspase-3 monoclonal antibody (1:50) for 1 h at room temperature. After phosphate-buffered saline washing $\times 3$, cells were incubated with secondary antibody goat anti-mouse IgG-Alexa-594 (1:1000, Molecular Probe, Eugene, OR) for 30 min at room temperature. Two hundreds of green fluorescent protein-positive cells (cells containing either 15-LOX-2 expression vector or control vector) from four different areas in each slide were counted and relative percentages of annexin V- and caspase 3-positive cells were calculated after normalization to the control cells.

Survival colony fraction study

The cells from a stock culture were prepared into a single cell suspension by trypsinization. In unirradiated control dish, 100 cells were inoculated into 35 mm Petri dish and allowed to grow for 10 days until colonies formed. The plating efficiency was termed as the percentage of cells seeded that grew into colonies. Meanwhile, 9×10^4 cells per 35 mm Petri dish were seeded and allowed to grow for 24 h. The cells were then transfected either with the radiation-inducible 15-LOX-2 expression vector or control vector, respectively. The transfected cells were then irradiated with a dose of 4 Gy and cultured for 21 days. Numbers of colony were counted. A colony is defined as 50 cells or more congregated together. Survival colony fraction was then determined as the ratio of colonies produced to cells plated, with a correction necessary for plating efficiency: Surviving fraction = colonies counted/(cells seeded $\times$ (plating efficiency/100)).

Results

Radiation induced activation of the recombinant Egr-1 *promoter in the HNC cells*

The cells were transfected with the luciferase reporter vector containing the recombinant *Egr-1* promoter and were then irradiated with the single dose (2, 4, 6 or 8 Gy) 24 h after the transfection. The luciferase activities under different doses were analyzed. A dose of 4, 6 or 8 Gy induced significant increase of luciferase activity. A dose of 4 Gy induced maximal promoter activation. The luciferase activity measured after 4 Gy was significantly increased to $192.5 \pm 12.6\%$ compared with the control. A dose of 2 Gy did not induce significant increase in luciferase activity. A dose of 6 Gy induced increase of luciferase activity, but was relatively lower than that by 4 or 8 Gy. A dose of 8 Gy induced significant increase of luciferase activity, which was similar to that achieved by a dose of 4 Gy. Representative data are shown in Figure 1.

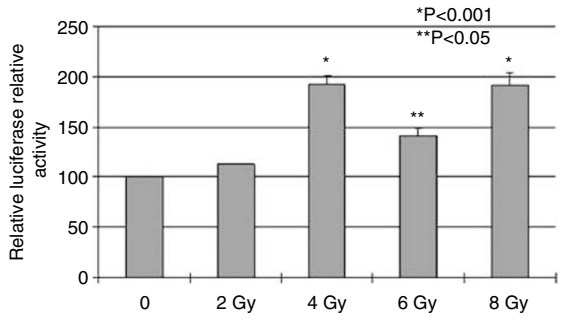

Figure 1 Radiation-induced activation of Δ7egr promoter in HNC cells transfected with pΔ7egr-EGFP-GFL vector containing luciferase gene. Baseline activity of luciferase was calculated as 100% in the control cells that were transfected with the Δ7egr-EGFP-GFL, but received no radiation treatment. The experiment was performed in triplicates and repeated three times with similar results. The results were expressed as mean ± s.e. The vector pGFL (a parental plasmid of pΔ7egr-EGFP-GFL) was used in each experiment and did not show meaningful luciferase activity. The vector pRSV-EGFP-GFL containing RSV gene was used as a control promoter, which showed no significant difference under different radiation doses (data not shown).

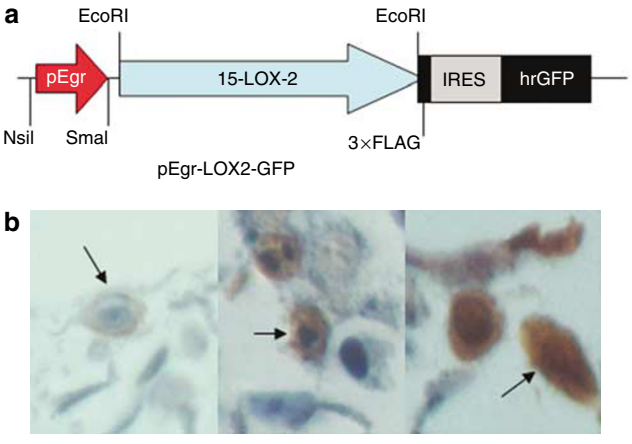

Figure 2 Diagram of the radiation-induced 15-LOX-2 expression vector pEgr-LOX2-GFP (**a**) and representative immunohistochemistry of 15-LOX-2 in head-and-neck cancer cells (**b**). (**a**) 15-LOX-2 gene is driven by the human recombinant Egr-1 gene promoter (pEgr). The translation of humanized recombinant GFP (hrGFP) is separately initiated from an internal ribosomal entry site. Expressed 15-LOX-2 protein is therefore not fused with hrGFP; (**b**) 15-LOX-2 was identified in both nuclei and cytoplasm. Arrowheads show the 15-LOX-2 immunostaining (dark brown DAB as chromogen). Original magnifications, × 400.

Given 4 Gy inducing maximal upregulation of the recombinant *Egr-1* promoter, we chose 4 Gy for subsequent experiments in this study.

Immunohistochemistry of 15-LOX-2 in HNC

After the successful generation of the radiation-inducible 15-LOX-2 expression vector pEgr-LOX2-GFP (Figure 2a), we then determined the basal distribution

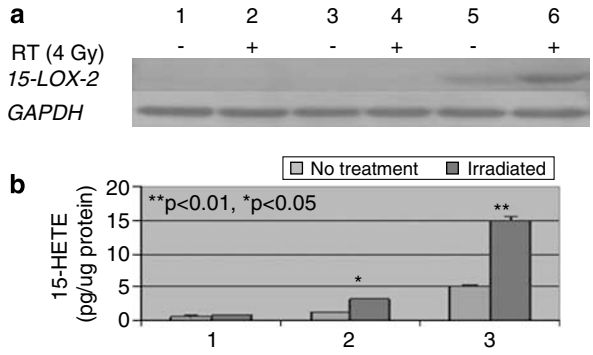

Figure 3 Up-production of 15-LOX-2 and its main metabolite 15S-HETE by 4 Gy of radiation in head-and-neck cancer cells transfected with pEgr-LOX2-GFP vector. (**a**) Western blot analysis of 15-LOX-2 and GAPDH, GAPDH was used as an internal control; lanes 1 and 2, parental carcinoma cells without vector transfection; lanes 3 and 4, transfected with control vector; lanes 5 and 6, transfected with 15-LOX-2 expression vector followed by 4 Gy. Radiation was given 24 h after the transfection and the cells were collected for the Western blot 30 min after radiation. The figure represented one of three experiments with similar results. 'RT (4 Gy)' mean '4 Gy of radiation,' '+' means 'received radiation, '−' means 'received no radiation'; (**b**) 15S-HETE production. (1) Transfected with control vector that received either 4 Gy or no radiation; (2) received 4 Gy or no radiation 6 h after transfection with the 15-LOX-2 expression vector; (3) received 4 Gy or no radiation 18 h after transfection with the 15-LOX-2 expression vector. The experiment was performed in triplicates and was repeated three times. The results were obtained from three experiments and expressed as mean ± s.d.

of 15-LOX-2 in the HNC cells transfected with the pEgr-LOX2-GFP. The immunostaining of 15-LOX-2 was found in the HNC cells at each time point (6, 12 or 24 h) after the transfection. Percentages of the cells containing the 15-LOX-2 immunostaining and the intensity of the immunostaining in each positive cell increased in time-dependent manner. We observed mild (+1), mild-to-moderate (weak +2), moderate-to-strong (strong +2) 15-LOX-2 immunostaining at 6, 12 and 24 h, respectively, after the transfection. The immunostaining of 15-LOX-2 was identified in both nucleus and cytoplasm. Almost no immunostaining of 15-LOX-2 was found in the control cells. Representative data are shown in Figure 2b.

Upregulation of 15-LOX-2 and 15S-HETE in HNC

We measured radiation-induced upregulation of 15-LOX-2 in the HNC cells transfected with the 15-LOX-2 expression vector compared with the control using Western blot analysis. 15-LOX-2 was almost undetectable in the parental cells and cells transfected with the control vector. The radiation did not induce any elevation of endogenous 15-LOX-2 in the cells. Expression of 15-LOX-2 was detected in the cells transfected with the 15-LOX-2 expression vector. Significant upregulation (approximately twofold) of 15-LOX-2 was induced by radiation in the cells transfected with the 15-LOX-2 expression vector. Representative figure is shown in Figure 3a.

Furthermore, we measured radiation-induced up-production of 15S-HETE (the main metabolite of 15-LOX-2) in the above transfected cells using enzyme immunoassay. Without radiation, the production of 15S-HETE was 1.1 ± 0.03 and 5.1 ± 0.21 pg μg^{-1} of protein at 6 and 18 h after the transfection. With radiation, the 15S-HETE product increased to 3.1 ± 0.06 and 15.01 ± 0.30 pg μg^{-1} of protein at 6 and 18 h after the transfection. This was approximately threefold increase at 18-h point. Representative data are shown in Figure 3b.

Apoptosis induction by radiation-induced upregulation of 15-LOX-2 in HNC cells

Many lines of evidence suggest that apoptosis induction in tumor cells has an important role in the efficacy of radiation therapy and chemotherapy.[23,24] Here, we investigated whether radiation-induced upregulation of 15-LOX-2 may induce apoptosis in the HNC cells. Two apoptotic markers (annexin V and caspase 3) were chosen for this study. Significant increases of apoptotic cells containing annexin V and caspase 3 were observed in the cells transfected with the 15-LOX-2 expression vector. Four Gy of radiation resulted in significant increase of apoptotic cells containing annexin V and caspase 3 compared with the control cells without radiation. Addition of AA led to significant increase of apoptotic cells. Representative data are shown in Figure 4.

Downregulation of PPARγ by exogenous 15S-HETE at high concentrations, but not at low concentrations

Results from the previous studies were controversial about whether or not 15-LOX-2 inhibits tumor growth through the effect of its main metabolite 15S-HETE on PPARγ signaling pathway. Here, we investigated whether exogenous 15S-HETE at various concentrations has any effect on the expression of PPARγ in the HNC cells using Western blot analysis. Our results showed significant downregulation of PPARγ and phosphorated PPARγ at higher concentrations ($\geqslant 10\,\mu M$), but not at low concentrations (0.1, 0.25, 0.5, 1 and 5 μM) of 15S-HETE. A representative figure is shown in Figure 5.

Reduction of survival colony fraction

On the basis of our previous report that restoration of 15-LOX-2 expression results in inhibition of cell proliferation in carcinoma cells of head and neck,[18] we hypothesize that upregulation of 15-LOX-2 may enhance the effect of radiation in killing HNC. To achieve this, we transfected the HNC cell lines (FaDu and UMSCC1) with the

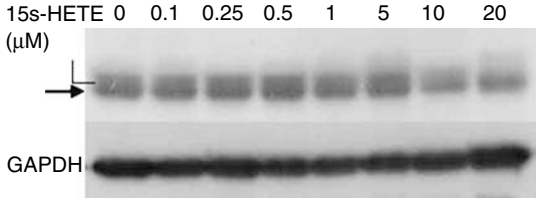

Figure 5 Representative Western blot of PPARγ in head-and-neck cancer (HNC) cells. 15S-HETE at various concentrations (0.1, 0.25, 0.5, 1, 5, 10 or 20 μM) was added to the FaDu HNC cells for 16 h prior to the cell collected for Western blot analysis. The same blot was stripped and probed with GAPDH antibody to verify the equal loading of protein. '⌊' phosphorylated PPARγ. '→' PPARγ. Three experiments were performed with similar results.

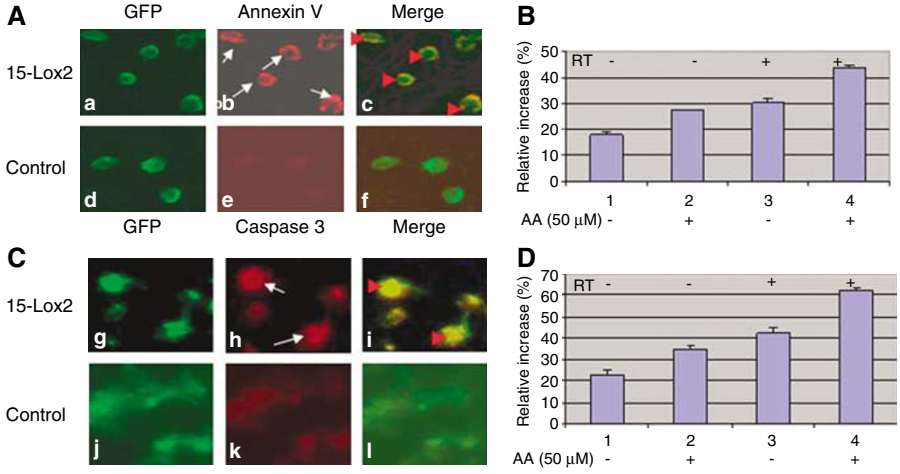

Figure 4 Representative immunoreactivity of annexin V and caspase 3 (apoptotic markers) in head-and-neck cancer cells. Results were expressed in mean ± s.d. and were summarized from three experiments. (**A**) Representative figures of immunoreactive annexin V. (**a–c**) Transfected with the 15-LOX-2 expression vector; (**d–f**) transfected with the control vector, 200X; (**B**) relative increase of annexin V-positive cells after normalization to the control cells in the transfected cells; (**C**) representative figures of immunoreactive caspase 3. (**g–i**) Transfected with 15-LOX-2 expression vector; (**j–l**) transfected with the control vector, 200X; (**D**) relative increase of caspase 3-positive cells after normalization to the control cells in the transfected cells. '→' shows the immunoreactive annexin V or caspase 3. '◀' shows the cells containing both GFP and immunoreactive annexin V or caspase 3. GFP-green, annexin V or caspase 3-red. AA: arachidonic acid. Nuclei were stained with 4,6-diamidino-2-phenylindole (data not shown). RT: radiation of 4 Gy; '−' no addition of AA or RT; '+' addition of AA or RT. *P*-value <0.01 between lane 1 vs lane 2, between lane 1 vs lane 3, between lane 2 vs lane 4 and between lane 3 and lane 4, respectively.

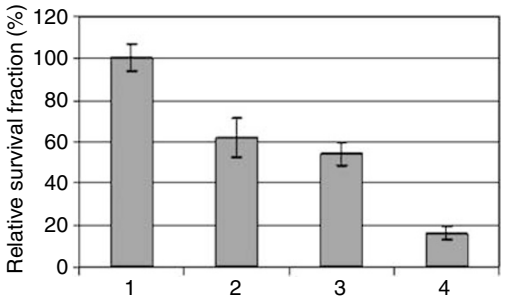

Figure 6 Survival colony assay in head-and-neck cancer FaDu cells. (1) Transfected with 15-LOX-2 nonempty vector pEgr-GFP (control) only; (2) transfected with the 15-LOX-2 expression vector pEgr-LOX2-GFP only; (3) transfected with control vector and irradiated with 4 Gy; (4) transfected with 15-LOX-2 expression vector and irradiated with 4 Gy. A colony was defined as 50 cells or more congregated together. Colony formations were observed for 21 days. The figure showed percentage reduction of survival colonies compared with the cells transfected with the control vector (lane 1) as 100%. Arachidonic acid (50 μM) was added to each experiment. The experiment was performed in triplicates and was repeated three times. The results were calculated from the three experiments and expressed as mean ± s.d. *P*-value <0.01 between lane 1 vs lane 2, between lane 2 vs lane 4 or between lane 3 vs lane 4.

radiation-inducible 15-LOX-2 expression vector, irradiated the transfected cells with 4 Gy and then measured survival colony fractions of the transfected cells compared with the control. Similar results were obtained from these two cell lines. Prior to radiation, we found that the survival colony fraction was reduced to approximately 60% in the cells transfected with the 15-LOX-2 expression vector compared with the control. This indicates that the basal expression of 15-LOX-2 after the transfection resulted in approximately 40% reduction of survival colony fraction. A dose of 4 Gy resulted in approximately 45% reduction of survival colony fraction. The radiation exposure to the cells transfected with the 15-LOX-2 expression vector caused a significant reduction of survival colony fraction from approximately 60 to 15%. This indicates that both radiation and induced upregulation of 15-LOX-2 contributed to approximately 75% reduction of survival colony fraction. Representative results are shown in Figure 6.

Discussion

The *Egr-1* promoter is a well-known radiation inducible vector and has been commonly used in radiation-induced gene therapy approaches in cancer treatment. Recently Meyer and coworkers investigated the mechanisms of radiation induction of the *Egr-1* promoter by the stepwise removal of known regulatory elements from the 5′ upstream region.[10] They mapped the region responsible for radioinduction and created a recombinant promoter fragment pΔ7egr (370 bp, nucleotide −474 to +12, but lacking −259 to −126, containing only the five SRE/Ets binding sites of the wild-type promoter). This recombinant promoter was found to be even more efficiently induced by radiation compared with wild-type *Egr-1* promoter and other recombinant promoters.[10] A single dose of 4 Gy maximally induced luciferase activity downstream the pΔ7egr up to approximately 140% in the malignant glioma cell line U87. Similar data were previously reported.[25,26] Using synthetic promoter constructs consisting merely of repetitive SRE consensus sequences, Marples *et al*.[25] described an upregulation of recombinant promoters by a factor of 1.5–2.5 after radiation exposure in the same glioma cell line. Park *et al*.[26] reported a 2.4-fold increase in relative luciferase activity following exposure to 20 Gy in the human esophageal adenocarcinoma cell line Seg-1 transfected with the wild-type *Egr-1* promoter. Our study demonstrated that a dose of 4 Gy induced an upregulation of 15-LOX-2 by twofold and up-production of its main metabolite 15S-HETE by threefold after the transfection (Figure 3). Results from our subsequent studies suggested that 15-LOX-2 may be a candidate target for targeted radiotherapy of HNC.

The mechanisms by which 15-LOX-2 functions as a tumor suppressor remain unclear. Previous studies primarily centered on its main metabolite 15S-HETE in prostate cancer cells, leading to many interesting discoveries. For example, 15S-HETE and other AA-derived eicosanoids act as endogenous ligands of PPARγ, but at relatively high concentrations.[27,28] Activation of this nuclear receptor PPARγ[29] and use of PPARγ agonists[30,31] is shown to cause cancer cells to undergo a differentiation response and reverse their malignant phenotype. Also, exogenous 15S-HETE was found to activate PPARγ using luciferase reporter assays and inhibit cell proliferation.[32] Exogenous 15S-HETE at a high concentration (30 μM) was shown to affect the phosphorylation of PPARγ through the mitogen-activated protein kinase pathway, thereby inhibiting the activation of PPARγ as a nuclear receptor.[33] Tang *et al*.[21] reported that 15S-HETE at non-physiologically high concentrations (⩾25 μM) induces apoptosis in prostate cancer cells, but in the presence of exogenous AA, the 15-LOX-2 stable clones showed a significant increase in apoptosis.[22] Most recently, Subbarayan *et al*.[34] found that a relatively low concentration of 15S-HETE (1 or 5 μM) was sufficient for PPARγ activation in the presence, but not in the absence of 15-LOX-2 expression, in epithelial cells and cancer cells of prostate. We showed significant induction of apoptosis by restored expression of 15-LOX-2 (in the presence of exogenous AA) in the HNC cells transfected with the 15-LOX-2 expression vector (Figure 4). However, we did not observe any effect on expression of PPARγ or phosphorated PPARγ by 15S-HETE at low concentrations (0.1, 0.25, 0.5 and 1 μM; Figure 5). Induction of apoptosis was not observed with the above low concentrations of exogenous 15S-HETE (data not shown). All together, the above suggests that the exogenous addition of 15-HETE fails to recapitulate the tumor suppressing at low concentrations suggesting that lipoxygenase activity may not be required in the suppression of cultured tumor

cells, however, uptake and subcellular levels of 15S-HETE were not determined in those studies. Therefore, we cannot exclude the possibility that under stimulated conditions, AA may be mobilized resulting in increased 15S-HETE production in the cancer cells, especially in the nucleus, in the presence of 15-LOX-2, which may lead to nuclear receptor PPARγ-dependent growth inhibition. Another possibility is that the direct protein–protein interactions between 15-LOX-2 and PPARγ may also affect the regulation of 15-LOX-2 expression. This is supported by a recent study[35] that the 15-LOX-2 protein can interact through an LXXLL (L, Leucine; X, any amino acid) motif with PPARs and thus act as a co-activator in squamous epithelial cells.

Similar to the previous studies in prostate cancer,[21,22] a significant portion of 15-LOX-2 protein is identified in the nucleus of the HNC cells (Figure 2b). How is 15-LOX-2 imported to the nucleus? Which form, cytosolic, nuclear or both, is responsible for its tumor suppressing function? These questions remain to be answered. Understanding nuclear targeting of 15-LOX-2 might conceptually provide an explanation to the tumor suppressing function of 15-LOX-2. The previous study[22] claimed that one 15-LOX-2 splice variant (15-LOX2sv-b) inhibits tumor growth of prostate cancer cells, but has reduced nuclear targeting and/or no AA metabolizing activity. It is noted that this variant contains a deletion of two exons. These authors believed that changes in protein folding or conformation might somehow mask the responsible nuclear targeting and preclude this variant from interacting with importins required for nuclear transport, and thus prevent its import into nucleus. It is clear that many questions remain. Determination of active form (cytosolic, nuclear or both) of 15-LOX-2 for its tumor suppressing function would be very useful to optimize 15-LOX-2-targeted radiotherapy in future.

Results from our study demonstrated synergistic effect of 15-LOX-2 and radiation in killing HNC, which may represent a novel therapeutic approach that enable restoration of 15-LOX-2 in tumor cells to sensitize the effect of radiation on cell killing. However, it is noted that the *in vitro* cell irradiation used for this study is different from our conventional clinical radiotherapy schedule in which multiple daily fractionated doses (often 1.8–2 Gy per fraction) are often used mainly to reduce chronic toxicity of normal tissue. In addition, cellular environment *in vitro* is significantly different from *in vivo* tumor environment. In this study, we did not observe any significant increase of luciferase activity after a single dose of 2 Gy (Figure 1). The reason for this observation is unknown. In addition, we did not observe a dose-dependent activation of the recombinant *Egr-1* promoter in cultured cells (Figure 1). A dose of 6 or 8 Gy did not yield higher luciferase activity than that observed after a dose of 4 Gy, which is consistent with the previous observation in glioma cell line.[10] The reason for this observation is unknown. This may be due to significant cell death occurring after high doses of radiation, as luciferase activities were measured 24–48 h after the radiation. Therefore, synergistic effect of 15-LOX-2 and radiation observed in this study need to be investigated *in vivo*. An optimal *in vivo* fractionated radiation schedule to activate the recombinant *Egr-1* promoter remains to be identified.

In conclusion, we successfully generated the radiation-inducible 15-LOX-2 expression vector and observed a synergistic killing effect between radiation and radiation-induced upregulation of 15-LOX-2 in HNC. The 15-LOX-2 inhibition may not be PPARγ dependent. Results from this study suggest that 15-LOX-2 may be a candidate target for targeted radiotherapy of HNC.

Conflict of interest

None.

Abbreviations

15-LOX-2, 15-lipoxygenase 2; 15S-HETE, 15S- hydroxyeicosatetraenoic acid; AA, arachidonic acid; Egr-1, early growth response 1 gene; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HNC, head-and-neck cancer; PPARγ, peroxisome proliferators-activated receptor γ.

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