Reoxygenation Reverses Hypoxia-related Radioresistance in Head and Neck Cancer Cell Lines

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Abstract. Background/Aim: Head and neck cancer (HNC) is characterized by epidermal growth factor receptor (EGFR) overexpression and radiotherapy (RT) resistance. Cancer cells are able to survive and proliferate in hypoxic conditions. Hypoxia can be transiently interrupted by phases of reoxygenation. This work aimed to analyze the reoxygenation effect on proliferation in response to radiation in HNC cells. Materials and Methods: HNC cell lines CAL33 and CAL166 were subjected to an 8-Gy radiation dose in hypoxia and/or after reoxygenation. Cell proliferation and molecular factors involved in response to treatments were studied. Results: Cytotoxicity test confirmed radioresistance in hypoxia and highlighted that reoxygenation before RT restores sensitivity in both cell lines. Our results showed a similar proliferation inhibition effect and EGFR modulation but a different cell death mechanism in the two cell lines after treatment. Conclusion: Reoxygenation before RT rescued radiosensitivity in HNC cells.

More than 90% of head and neck cancers (HNCs) present squamous cell histology (head and neck squamous cell carcinoma, HNSCC) and originate in the lip/oral cavity, nasopharynx, oropharynx, hypopharynx and larynx. Epidermal growth factor receptor (EGFR) is highly expressed in more than 95% of HNSCCs and correlates with poor prognosis and resistance to radiation (RT) (1). Microenvironment plays a crucial role during carcinogenesis and solid tumors undergo a reduction in oxygen supply (hypoxia) due to tumor inefficient vasculature and high metabolic requirements. The majority of cancer hypoxia adaptations are orchestrated by the hypoxia-inducible-factor-1α (HIF-1α) (2). However, hypoxia is not a stable phenomenon but subjected to intermittent period of reoxygenation, a mechanism known as “cyclic hypoxia” which contributes to tumor development (3, 4). During the reoxygenation phases, oxidative stress can be generated by production of reactive oxygen species (ROS), which mainly occurs after the interaction of RT with intracellular water. Alternatively, the presence of oxygen can stabilize the highly reactive hydroxyl radicals causing DNA damage and reducing tumor cells’ repairing ability (5).

In our previous work, we demonstrated that the HNC cell line CAL166 was significantly more resistant if irradiated in hypoxia than in normoxia (6). We now extended our study using two different HNC cell lines and investigated radiation sensitivity in hypoxic cells reoxygenated before RT. The role of key biological factors implicated in survival and apoptosis has been evaluated, in hypoxia and after reoxygenation, to analyze the molecular mechanisms underlying response to RT.

Materials and Methods

Cell culture and experimental conditions. CAL166 and CAL33 HNC cell lines, kindly provided by the Centre Antoine Lacassagne (CAL, Nice, France), were used. Both cell lines were maintained as in our previous works (6, 7). Hypoxic conditions were ensured by incubating cells in a "Bug-Box" anaerobic workstation (Ruskinn Technology Ltd, Bridgend, USA) with a 1% oxygen mix. On the basis of preliminary results, we selected 8 h of hypoxia and 24 h of reoxygenation (re-incubating cells in normoxia at 21% oxygen and 5% CO2). The RT dose selected to obtain a 50% growth inhibition (ID50) in both cell lines was 8 Gy, administered using a Varian accelerator equipment. Detailed experimental schedule is reported in Figure 1.

Cell toxicity measurement and Western blot analysis. For proliferation studies, cells were seeded in a 96-well plate at the concentration of 3,000 cells/well and MTT test (8) was performed at
the end of all treatments as described in Figure 1. Results were expressed as % surviving fraction (SF%), thus normalizing the average value of treated cells by the average value of control (CT, untreated) cells. Protein analysis was performed on cells’ lysates collected at the scheduled time reported in Figure 1 (pellet) by standard western blot (6, 7) using primary and secondary antibodies previously described (6).

Statistical analyses. All experiments were conducted at different times in triplicate. In the figures, each value is represented as mean±standard deviation (SD). Differences between mean values were evaluated by one-way ANOVA with Newman-Keuls test, according to data distribution, using GraphPad Software (San Diego, CA, USA). Student’s t-test was used to compare differences between IC50 average values. p-Values were considered statistically significant as indicated in the figures; p<0.05 (*), p<0.01 (**) and p<0.001 (***)

Results

Cell survival analysis. Both cell lines showed a SF% decrease when treated in normoxia with RT8Gy (Figure 2) up to 43.4% in CAL33 and 34.6% in CAL166. Hypoxia induced radioresistance, leading survival to 59.4% in CAL33 (p<0.01) and 56.6% in CAL166 (p<0.05). Radiosensitivity rescued reoxygenating cells for 24 h after 8 h of hypoxia and before RT8Gy, leading survival to 38.6% in both CAL33 (p<0.001) and CAL166 (p<0.001).

Analysis of molecular factors involved in response to radiation in hypoxia and/or reoxygenation. In order to understand the molecular factors involved in treatment response, we evaluated key proteins activated by stress (HIF-1α) or involved in cell-cycle regulation and proliferation (EGFR, p21, p27), as well as in DNA damage repair (excision repair cross-complementation group 1, ERCC1).

HIF-1α (Figure 3A) was up-regulated when both cell lines were grown in hypoxia (p<0.01 for CAL33; p<0.05 for CAL166). Reoxygenation after hypoxia decreased HIF-1α expression (p<0.05 for CAL33; p<0.05 CAL166). RT8Gy given alone did not induce any significant change. Instead, RT8Gy after 8 h of hypoxia showed a down-regulation in both cell lines (p<0.05 for CAL33; p<0.05 for CAL166). When cell lines were irradiated after hypoxia, we observed a HIF-1α expression increase (p<0.001 for CAL33; p<0.01 for CAL166). The effect of irradiation after reoxygenation was seen only in CAL166 cells, where HIF-1α decreased (p<0.01).

Since EGFR expression is strictly correlated to radioresistance (9), we examined the ratio between the phosphorylated (pEGFR) and total EGFR (pEGFR/EGFR) (Figure 3B). Both cell lines showed a significant up-regulation of pEGFR/EGFR ratio when cells were treated with RT alone (p<0.001 for CAL33; p<0.01 for CAL166). A down-regulation was induced by reoxygenation (p<0.05 for CAL33). When cell lines were reoxygenated and then irradiated, we observed pEGFR/EGFR increase with respect to reoxygenation and hypoxia (p<0.05 for CAL33; p<0.01 for CAL166). The pEGFR/EGFR ratio was significantly decreased when cells were irradiated after hypoxia (p<0.001 for CAL33; p<0.01 for CAL 166) but increased again when cells were reoxygenated before RT (p<0.001 for CAL33; p<0.01 for CAL166).

In CAL33, a significant decrease of p21 (Figure 3C) was seen in cells cultured in hypoxia (p<0.05). In CAL166, there was a significant decrease of p21 when cells were treated...
with hypoxia plus reoxygenation and radiation vs. all the other conditions ($p<0.05$ and $p<0.01$). In both cell lines, p27 (Figure 3D) showed a higher expression when cells were reoxygenated after hypoxia compared to control ($p<0.01$ for CAL33; $p<0.001$ for CAL166) and hypoxia alone ($p<0.01$ for CAL33; $p<0.001$ for CAL166). Instead, p27 decreased when the cells were irradiated after reoxygenation ($p<0.01$ for CAL33; $p<0.001$ for CAL166).

Only in CAL166, ERCC1 was significantly increased when cells were irradiated after hypoxia vs. both hypoxia and radiation alone ($p<0.001$) but decreased when cells were reoxygenated before radiation ($p<0.001$) (data not shown).

Figure 3. Analysis of HIF-1α (A), pEGFR/EGFR ratio (B), p21 (C), p27 (D), caspase 3 (E) and caspase 9 cleaved/caspase 9 (F) modulation after treatments in CAL166 and CAL33 cells by Western blot. Experimental conditions are detailed in Figure 1.
Apoptosis study. Caspase 3 (Figure 3E) expression was downregulated when CAL33 were treated with RT both in normoxia ($p<0.01$) and in hypoxia ($p<0.05$ and $p<0.01$). In CAL166, caspase 3 increased after RT in hypoxia vs. hypoxia and RT alone ($p<0.05$ and $p<0.01$). When cells are reoxygenated before RT we observed an up-regulation vs. hypoxia and radiation alone ($p<0.01$) and vs. reoxygenation ($p<0.01$). The ratio caspase 9 cleaved/active form/caspase 9 (Figure 3F), only in CAL166, was reduced when cells were cultured in hypoxia ($p<0.05$), increased when irradiating cells in normoxia ($p<0.01$) but not when cells were irradiated in hypoxia with or without reoxygenation ($p<0.01$).

Discussion

Our previous study confirmed that cells irradiated in hypoxia become radioresistant (6). Herein, we demonstrated that reoxygenation before RT increased cell death in CAL33 and CAL166 HNC cell lines, restoring radiosensitivity. The molecular mechanisms inducing radioresistance and pathways involved to rescue sensibility after reoxygenation seem to be different in the two cell lines, possibly due to their distinct histology.

The main actor in hypoxia response is HIF-1α, which plays an important role in cancer cell proliferation and survival and in regulating the expression of several genes involved in metabolism, metastasis and transition to epithelial-mesenchymal phenotype (10). In our experimental model, HIF-1α was up-regulated when CAL33 and CAL166 cells were grown in hypoxia and down-regulated by RT. This might reflect the in vivo situation where the amount of oxygen present in the microenvironment is increased by RT. We observed a different HIF-1α regulation in the two cell lines irradiated after reoxygenation. In CAL166, the down-regulation might be explained by their basal radiosensitivity and/or different histology compared to CAL33, showing a higher expression of HIF-1α. Conversely, HIF-1α expression was up-regulated when cells were irradiated in hypoxia, becoming radioresistant. Similarly, in resistant lung cancer cells, Kim et al. (11) observed a low expression of HIF-1α and demonstrated that radiation induces de novo HIF-1α protein expression. It has also been demonstrated that reoxygenation and radiation stabilize HIF-1α through ROS production (12) and that RT induces HIF-1α protein accumulation in the reoxygenated regions (13).

EGFR overexpression is generally thought to contribute to solid tumors development and amplification of EGFR contributing to the radioresistance by regulating DNA-double-stranded break repair and activating the MAPK and PI3K pathways (14). Unexpectedly, we showed an overexpression of the pEGFR/EGFR ratio when cell lines were irradiated after 8 h of hypoxia followed by 24 h of reoxygenation and finally RT 8 Gy (HYP8hREOX24hRT8Gy), condition in which we observed a high level of cell death. This apparent discrepancy might be due to a different time schedule of the analysis: pEGFR/EGFR ratio was determined 24 h after treatment, while proliferation was analyzed three days after RT, when cell death itself is more appreciated.

In order to explain the observed cell proliferation decrease, we investigated p27 and p21 proteins, negative regulators of the cyclin-dependent kinases involved in cell division. Green et al. (15) suggested that p21 and p27 are not necessary for cell-cycle arrest induced by hypoxia. In our model, in both cell lines, we did not observed a significant regulation of p27 nor p21 when RT followed reoxygenation, suggesting that, in our model, they are not key regulators in cellular response to treatment. Nonetheless, in CAL166, the significant decrease in p21 in HYP8hREOX24hRT8Gy, which correlates with high of pEGFR/EGFR ratio, suggested that this pathway could be activated to contrast the effect of the high level of EGFR. Moreover, we hypothesized that p21 reduced expression might also correlate with HIF-1α decrease; Goda et al. (16), indeed, demonstrated that HIF-1α expression is essential for p21 induction and arrest into the S phase of the cell cycle.

Since caspase 9 deregulation is known to confer radioresistance and influence cisplatin-induced apoptosis (17), we investigated if cell death could be obtained through a caspase-dependent mechanism. In particular, in CAL166, caspase 9 was induced by RT, suggesting that cells irradiated in normoxia died through apoptosis. After reoxygenation (HYP8hREOX24hRT8Gy), we observed a caspase 3 activation: we hypothesized that this overexpression might contrast the proliferative effect due to EGFR activation.

Finally, we observed an interesting ERCC1 modulation in CAL166 cells when irradiated in hypoxia; probably the low concentration of oxygen did not “fix” the DNA damage induced by ROS and allowed DNA to be repaired, contrasting the apoptotic effect induced by caspase 3. Conversely when cells were reoxygenated before RT, the presence of oxygen impaired cellular DNA repair ability and tumor cells died.

Taken together, our results showed a similar response to treatments in terms of antiproliferative effect and EGFR modulation. Nevertheless, different mechanisms and death/apoptotic pathways seem to be activated in CAL33 and CAL166 cells, possibly due to their distinct tissue of origin.

In conclusion, we demonstrated that reoxygenation before RT rescued radiosensitivity in HNC cell lines. Furthermore, radiosensitivity may be strictly correlated to a combination of molecular mechanisms specific for distinct tumor histology.

Conflicts of Interest

The Authors declare that there is no conflict of interest. This work was partially financed by LILT (Lega Italiana per la Lotta contro i Tumori).
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