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Do altered activities of superoxide dismutases and the level of NF-κB modulate the effects of gamma radiation in HeLaS3 cells?

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Abstract: Most experimental models, including cell culture studies, have demonstrated that over-expression of manganese superoxide dismutase (MnSOD) in cells bearing a carcinoma phenotype has anti-proliferative and tumour suppression characteristics. In contrast, when cervical carcinoma biopsies express MnSOD, there is a poor prognosis and resistance to radiation therapy. The results herein indicate that human cervical adenocarcinoma (HeLaS3) cells have increased MnSOD activity (up to 50 % of the total SOD activity) due to low expression of its repressor p53 and a high level of oxidative stress arising from the cell culture conditions. High MnSOD activity may be related to HeLaS3 cell radioresistance, illustrated by a high IC_{50} of 3.4 Gy and by a relatively high level of cell viability after gamma irradiation. In contrast to MnSOD activity, cytosolic CuZnSOD activity decreased after ionising radiation. The catalase (Cat) activity was unchanged. IR also increased the nitric oxide synthase (NOS) activity. Such conditions lead to increased concentrations of the superoxide radical, hydrogen peroxide and $NO^{\cdot}$, which together may be responsible for the decreased expression of NF-κB and unaltered Cat activity. Therefore, the disturbed redox balance within HeLaS3 cells may be responsible for the cytotoxicity observed at higher irradiation doses. It could be concluded that inhibition of the CuZnSOD activity may be an important target for the selective killing of radioresistant cancer cells.

Keywords: gamma irradiation, antioxidant enzymes, NF-κB, p53, HeLaS3 cells.

INTRODUCTION

Carcinoma of the uterine cervix is one of the most frequent malignancies in the human population with 500,000 newly diagnosed cases annually.[1,2] It is usually treated by surgery in combination with subsequent ionising radiation (IR) therapy.[3–5] Cervical carcinomas are characterised by the fact that approximately

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half of the biopsies express manganese superoxide dismutase (MnSOD) and there is significant association between MnSOD expression, poor prognosis and resistance to IR therapy.[6] A cervical carcinoma cell culture model (HeLaS3 cells) constitutively possesses enhanced MnSOD activity, probably due to the inserted human papilloma virus E6 protein causing ubiquitination and degradation of p53.[7] In p53-deficient cervical carcinoma cells, resistance to irradiation correlates with an increased expression of MnSOD and this enzyme is negatively regulated at the transcriptional level by p53.[8] Adaptation of these cells grown in culture may lead to up-regulation of the antioxidant defence enzymes.[9] Thus, induced over-expression of MnSOD in HeLaS3 cells could further influence their response to a free radical challenge.[10]

Elevated MnSOD activity represents a survival factor which is required for the maintenance of mitochondrial integrity in cells exposed to adverse conditions.[11,12] It seems that there is an established molecular basis for the correlation of MnSOD expression in cervical carcinoma and resistance to IR therapy. As MnSOD is predominantly anti-apoptotic,[13,14] MnSOD over-expression in tumours could offer a survival advantage to tumour cells, leading to their radioresistance due to their low apoptotic potential.[15] High MnSOD activity leads to an increased amount of hydrogen peroxide (H_2O_2) which could *via* NF-κB activation and transactivation of the MnSOD gene build up a positive feed-forward loop.[16]

NF-κB is considered to be a primary oxidative stress-responsive transcriptiton factor which plays a critical role in balancing pro- and anti-apoptotic activities.[17,18] NF-κB is sensitive to concentrations of ROS and can be activated by H_2O_2 in a cell type-specific manner.[19] Increased activity of NF-κB may promote the survival of mutated cells.[20] In contrast, other studies have indicated that over-expression of MnSOD is closely associated with tumour regression *in vivo* and loss of malignant phenotype *in vitro*.[21–23] The current prevailing theory of the tumour-suppressing ability of MnSOD is that an imbalance in the redox state of the cell leads to an inhibition of cell proliferation. One explanation for these growth-suppressive effects of MnSOD could be the resulting imbalance of the anti-oxidant enzymes, which favours H_2O_2 accumulation.[24] Possible mechanisms for the suggested tumour-suppressive consequences of MnSOD over-expression could be the modulation of specific oncogenes,[25] up-regulation of protease inhibitors[26] and inhibition of the transcription factors AP-1 and NF-κB.[27]

In the present study, alterations in the expression of NF-κB and p53 and alterations in the activity of the major anti-oxidant enzymes MnSOD, CuZnSOD and catalase (Cat), and inducible nitric oxide ($NO^{\cdot}$) synthase activity (iNOS) in HeLaS3 cells exposed to IR were explored. The obtained data offer practical information concerning the therapeutic effects of IR when biopsies of human uterine cervical carcinoma express MnSOD.

EXPERIMENTAL

Cell culture. The human uterine cervical adenocarcinoma cell line HeLaS3 was obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained in 95 % Ham's F12 supplemented with 5 % heat-inactivated foetal calf serum, 100 IU ml^{-1} penicillin and streptomycin and 2 mM L-glutamine (all obtained from Sigma–Aldrich, Taufkirchen, Germany). The cells were grown as a monolayer in a humidified atmosphere of 95 % air and 5 % CO_2 at 37 ºC. Asynchronous cultures were chosen for all the experiments because techniques for the synchronisation of mammalian cell lines often lead to perturbation of cell cycle checkpoint mechanisms.[28]

Cell irradiation. IR (2, 5 and 10 Gy of gamma-rays) was delivered during the exponential phase of cell growth using a ^{60}Co source at a fixed dose rate of 20 Gy h^{-1}.

Cell viability. Cell growth and viability were determined using the Trypan Blue exclusion assay.

Sample preparation and Western blot analyses. Trypsinised and washed cells were lysed using a buffer containing 10 mM Tris–HCl pH 7.4, 0.32 M sucrose, 5 mM $MgCl_2$, 1 % Triton X-100 and a protease inhibitor cocktail. The protein concentration in the cell lysates was determined by the Lowry method.[29] Aliquots of lysates were mixed with denaturing buffer according to Laemmli,[30] boiled (100 °C, 2 min), separated by SDS–polyacrylamide gel electrophoresis (60 μg of cell protein per gel lane) and transferred to nitrocellulose membranes. The membranes were blocked using 10 mM Tris buffer pH 7.4 supplemented with 150 mM NaCl, 1 % BSA and 0.1 % Tween-20. The membranes were separately incubated with rabbit anti-actin antibody (CSA-400), anti-mouse p53 antibody (KAM-CC002), both from Stressgen Biotechnologies, Victoria, BC, Canada, and rabbit anti-NF-κB antibody (NF-κB p65, C-20; SC-372, Santa Cruz Biotechnology, CA, USA). A secondary goat anti-rabbit IgG HRP conjugate (SAB-300) and an anti-mouse IgG HRP conjugate (SAB-100) (Stressgen Biotechnologies, Victoria, BC, Canada) were used for detection. The quantification of the specific antigen bands was performed using an UltroScan XL scanning laser densitometer and computer image processing. The level of detected proteins by Western blotting was expressed in arbitrary units (AU mg^{-1} of protein).

Determination of enzyme activities. SOD activity in cell lysates was determined according to McCord and Fridovich as the percentage inhibition of superoxide ($O_2^{\cdot-}$) formation induced by the xanthine–xanthine oxidase system.[31,32] The results are expressed as units mg^{-1} of total cellular protein. Cat activity was determined according to Claiborne.[33] One unit of Cat activity is defined as the amount of enzyme that degrades 1 μmol of H_2O_2 min^{-1} mg^{-1} of protein.

Citrulline assay. The L-citrulline assay was used to measure the inducible NOS activity by a colorimetric assay of deproteinised samples, as previously described by Boyde *et al*.[34] In parallel, L-citrulline was used as a standard and taken through the full assay procedure. All assay reagents were purchased from Sigma–Aldrich.

Statistical analysis. The values are presented as mean ± standard deviation (SD) calculated from four individual experiments. Statistical significance was evaluated using the one-way analysis of variance and the Tukey post-hoc test; $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISSCUSION

To investigate the effect of IR on cell viability and cell growth, HeLaS3 cells were irradiated with 2, 5 and 10 Gy, returned to normal culture conditions and then analysed 24, 48 and 72 h after irradiation. Cell growth inhibition, expressed as the cell viability index, V_i, was significantly reduced after IR treatment. This reduction was both time- and dose-dependent (Fig. 1B). The greatest effect of IR was observed after a dose of 10 Gy, which inhibited cell proliferation by 80 % (at 72 h after irradiation, $p < 0.001$). The calculations indicated that the V_i was 18.1±3.0,

whilst the IR dose that decreased cell growth from 100 to 50 % (IC_{50}) was 3.4±0.2 Gy. This relatively high IC_{50} classifies HeLaS3 as a radioresistant cell line. This was confirmed with the high cell viability, which decreased to only 60 % after 10 Gy at 72 h post irradiation (Fig. 1A). The results indicated that IR-mediated HeLaS3 tumour suppression occurred *via* a non-cytotoxic mechanism.

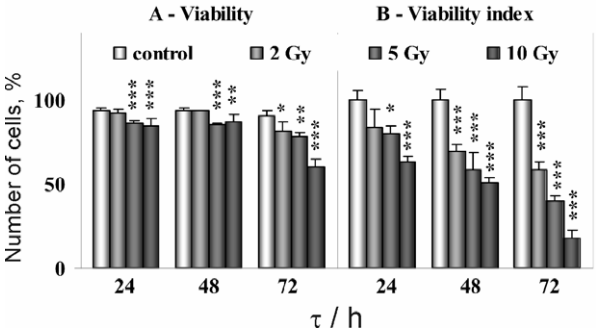

Fig. 1. The time- and the dose-dependence of cell viability (panel A) and cell viability index (panel B) of irradiated HeLaS3 cells. The results are presented as mean ±*SD*, $n = 4$. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, refer to differences between irradiated samples *vs*. the respective control.

In order to investigate the molecular basis of the anti-proliferative action of IR, the activity of the three anti-oxidant enzymes MnSOD, CuZnSOD and Cat, as well as of inducible NOS, was determined. It is known that selective elevation of MnSOD activity increases the resistance of both non-malignant and malignant cells to oxidants and oxidant-generating events such as IR[35–37] but may, under unbalanced conditions culminate in increased sensitivity. The high constitutive MnSOD expression in HeLaS3 cells, potentiated with mild oxidative stress under the cell culture conditions,[9] may be related to their observed radioresistance, illustrated by their high IC_{50} and V_i. However, IR caused a further increase in the MnSOD activity (which is in agreement with observations of other authors)[38] simultaneously with a decrease in the CuZnSOD activity (Fig. 2). Cytosolic CuZnSOD activity has generally not been found to be elevated in tumours. Furthermore, its expression is not dependent on the generation of reactive oxygen species (ROS).[39] This is in accordance with reports indicating that conditions generating O_2^-, such as IR, could induce the synthesis of MnSOD but not of CuZnSOD.[40] The present observations concerning SOD activities were similar to those previously found in HTE epithelial cells and MCF-7 human breast adenocarcinoma cells.[41] As the Cat activity remained unchanged (Table I), such a situation led to unbalanced conditions with a high pro-oxidative potential of endogenously formed H_2O_2. The high H_2O_2 concentration could also have been the reason for the inhibition of CuZnSOD activity, but not of MnSOD activity. Decreased CuZnSOD activity can contribute to decreased cell proliferation, as fibroblasts derived from CuZnSOD knockout animals proliferate more slowly (25 % less) than control cells, emphasising the importance of CuZnSOD activity in cell growth and survival.[42] Inhibition of CuZnSOD activity may cause accumulation of endogenous O_2^- and lead to free radical-mediated mitochondrial membrane damage, release of mitochondrial

cytochrome *c* and increase apoptosis of cancer cells.[43] The present results also indicate that targeting CuZnSOD activity may be a promising approach to the selective killing of cancer cells.[43] Untoxified H_2O_2 may be related to cell cycle arrest in the S phase *via* Cip1 (p21) and cyclin D3 inhibition.[44] IR induces a late S phase arrest in HeLaS3 cells. This latter observation was noted in a previous study[45] as well as in an independent study.[46]

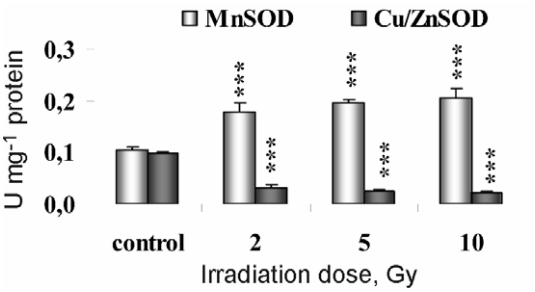

Fig. 2. IR-induced changes in MnSOD and CuZnSOD activity in irradiated HeLaS3 cells (analysed 72 h after irradiation). The results are presented as mean ±*SD*, $n = 4$. ***$p < 0.001$ refers to differences between irradiated samples *vs*. the respective control.

TABLE I. Antioxidant enzyme activity and expression level of transcription factors in irradiated HeLaS3 cells (analysed 72 h after irradiation). The results are presented as mean ±*SD*, $n = 4$. **$p < 0.01$ and ***$p < 0.001$ refer to differences between irradiated samples *vs*. the respective control

	Catalase U mg^{-1}	L-Citrulline % of control	NF-κB AU mg^{-1}	p53 AU mg^{-1}
Control	12.2 ± 1.0	100.0 ± 1.7	100.0 ± 2.9	100.0 ± 0.7
2 Gy	12.8 ± 1.8	107.5 ± 2.5	89.8 ± 4.6	99.5 ± 1.2
5 Gy	13.3 ± 2.2	112.9 ± 2.6**	82.1 ± 6.9**	94.5 ± 6.4
10 Gy	13.9 ± 1.6	115.2 ± 0.8***	75.1 ± 4.7***	94.1 ± 3.7

Key signalling networks which regulate the response to IR also involve the redox-sensitive transcriptional factors p53 and NF-κB. In the current HeLaS3 cell study, in contrast to some other cell lines,[47] IR did not induce expression of p53.[8] However, it led to a decreased expression of NF-κB, by approximately 25 % (Table I). It is well established that NF-κB may protect against a variety of apoptotic signals. In addition, its decreased expression has been shown to enhance the sensitivity of tumours to apoptosis induced by IR. Furthermore, there is correlation between increased $NO^{\cdot}$ production after IR, NF-κB inhibition by $NO^{\cdot}$ and induction of apoptosis in several cell lines. These findings could explain the IR-mediated down-regulation of NF-κB observed in this study. Increased activity of iNOS, measured by the citrulline assay (Table I), could be one of the reasons for the decreased NF-κB expression. Another reason may be a mechanism involving over-expression of MnSOD. Such a signalling pathway linking MnSOD over-expression with down-regulation of NF-κB has been described in MCF-7 cells.[41]

CONCLUSIONS

In conclusion, IR-induced cellular perturbations in HeLaS3 cells involve down-regulation of NF-κB (p65) expression, increased activity of mitochondrial

MnSOD and unchanged Cat activity. Such a situation leads to an increase in the endogenous level of H_2O_2, which may be related to cell cycle arrest in the S phase *via* Cip1 (p21) and cyclin D3 inhibition.[39] The present results also indicated that cytosolic CuZnSOD activity decreased upon IR treatment, which could have been a consequence of a high endogenous concentration of H_2O_2, leading to decreased cell proliferation. Therefore, it is proposed that targeting CuZnSOD with an inhibitory adjuvant drug may be a promising approach to the selective killing of cancer cells.

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ИЗВОД

ДА ЛИ ИЗМЕЊЕНЕ АКТИВНОСТИ СУПЕРОКСИДНИХ ДИСМУТАЗА И НИВОА NF-κB У HeLaS3 ЋЕЛИЈАМА МОДУЛИШУ ЕФЕКТЕ ГАМА ЗРАЧЕЊА?

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Највећи број експерименталних модела, који укључују и студије на ћелијама у култури, показује да повећана експресија манган-супероксид-дисмутазе (MnSOD), у ћелијама које имају малигни фенотип, има антипролиферативне и тумор-супресорске карактеристике. За разлику од тога када су биопсије карцинома цервикса утеруса MnSOD позитивне, постоји лоша прогноза и резистенција на терапију зрачењем. Наши резултати показују да ћелије хуманог аденокарцинома цервикса утеруса (HeLaS3) имају повећану MnSOD активност (до 50 % од укупне SOD активности) због ниске експресије њеног репресора p53 и високог нивоа оксидативног стреса, који проистиче из услова у ћелијској култури. Висока активност MnSOD може се повезати са њиховом радиорезистенцијом, која се огледа у високом IC_{50} од 3,4 Gy и релативно високој вијабилности након озрачивања. За разлику од MnSOD активности, активност цитосолне CuZnSOD се смањује након озрачивања. Активност каталазе се не мења. Јонизујуће зрачење такође повећава активност азот-моноксид-синтазе. Такви услови воде повећању концентрације супероксидног радикала, водоник пероксида и азот моноксида, који заједно могу бити одговорни за смањену експресију NF-κB и неизмењену активност каталазе. Овако поремећена редокс равнотежа у HeLaS3 ћелијама може бити одговорна за цитотоксичност запажену при већим дозама зрачења. Стога се може закључити да инхибиција активности CuZnSOD ензима представља значајан фактор у селективној цитотоксичности радиорезистентних ћелија канцера.

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