

The role of nitric oxide radicals in removal of hyper-radiosensitivity by priming irradiation

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In this study, a mechanism in which low-dose hyper-radiosensitivity (HRS) is permanently removed, induced by low-dose-rate (LDR) (0.2–0.3 Gy/h for 1 h) but not by high-dose-rate priming (0.3 Gy at 40 Gy/h) was investigated. One HRS-negative cell line (NH1K 3025) and two HRS-positive cell lines (T-47D, T98G) were used. The effects of different pretreatments on HRS were investigated using the colony assay. Cell-based ELISA was used to measure nitric oxide synthase (NOS) levels, and microarray analysis to compare gene expression in primed and unprimed cells. The data show how permanent removal of HRS, previously found to be induced by LDR priming irradiation, can also be induced by addition of nitric oxide (NO)-donor DEANO combined with either high-dose-rate priming or exposure to prolonged cycling hypoxia followed by reoxygenation, a treatment not involving radiation. The removal of HRS appears not to involve DNA damage induced during priming irradiation as it was also induced by LDR irradiation of cell-conditioned medium without cells present. The permanent removal of HRS in LDR-primed cells was reversed by treatment with inducible nitric oxide synthase (iNOS) inhibitor 1400W. Furthermore, 1400W could also induce HRS in an HRS-negative cell line. The data suggest that LDR irradiation for 1 h, but not 15 min, activates iNOS, and also that sustained iNOS activation is necessary for the permanent removal of HRS by LDR priming. The data indicate that nitric oxide production is involved in the regulatory processes determining cellular responses to low-dose-rate irradiation.

Keywords: nitric oxide; inducible nitric oxide synthase; hyper-radiosensitivity; low dose-rate

INTRODUCTION

Low-dose hyper-radiosensitivity (HRS) is characterized by a high sensitivity per unit dose for radiation doses below ~0.5 Gy [1, 2]. HRS is, in all probability, the default response of cells to small doses of both high- and low-LET (linear energy transfer) ionizing radiations [3]. For doses of low-LET radiation in the dose-range ~0.5–1 Gy, a more radioresistant response per unit dose is observed. This is called ‘increased radioresistance’ (IRR). HRS is associated with a failure to induce the early (active 0–2 h post-

irradiation) and transient (lasting ~12 h) G₂-phase checkpoint, which arrests cells irradiated in G₂ before entering mitosis, allowing for repair of DNA damage [4–6]. The extent of the HRS-response varies between different cell lines, being more prominent in malignant cells than in cells of normal tissues [7].

Generally, radiation exposure at low dose-rate (LDR) results in higher cell survival than radiation exposure at high dose-rate (HDR). This sparing effect at low compared to HDR has been attributed to repair of sublethal damage during the protracted LDR irradiation, leading to fewer DNA

double-strand breaks [8]. However, differences in response to high- and low-dose-rate irradiation are also reported regarding p53 function and in induction of the adaptive response, in which a small priming dose (0.01–0.5 Gy at dose rates from 0.01–1.0 Gy/min) induces resistance to a subsequent challenge dose (0.5–2.0 Gy) [9, 10].

Exposing HRS-proficient cells to a HDR priming dose of 0.2–0.3 Gy transiently removes the HRS-response to subsequent HDR challenge irradiation [11, 12]. However, previous studies from our laboratory [11, 13, 14] have shown that by reducing the dose-rate for priming irradiation to 0.3 Gy/h (LDR) and giving 0.3 Gy (i.e. 1 h irradiation) results in permanently removed HRS in T-47D breast cancer cells upon later challenge irradiation. The effect of LDR priming irradiation differed not only in duration of the absence of HRS from that of HDR priming irradiation. In addition, HRS was removed in unirradiated cells by medium transferred from LDR-primed but not from HDR-primed cells, and by LDR-primed cell-conditioned medium (i.e. medium given a 1 h priming irradiation after cell conditioning without cells present during irradiation) [11, 14].

Nitric oxide (NO) has been found to be involved in phenomena related to the removal of HRS by LDR priming irradiation or medium transfer from LDR-primed cells. Both cytotoxic bystander effects [15–17], adaptive response [18] and bystander adaptive response [19–21] have been associated with inducible nitric oxide synthase (iNOS)-generated NO.

The aim of the present study was to investigate the mechanism by which LDR irradiation induces effects that are not seen after HDR irradiation. We demonstrate that the effect of LDR irradiation can be mimicked by a chemical nitric oxide (NO)-donor in combination with HDR irradiation, as well as with reoxygenation after chronic hypoxia. The sustained mechanism induced by LDR priming appears to depend on iNOS activity and the permanent removal of HRS by LDR priming was reversed by iNOS inhibitor 1400W.

MATERIALS AND METHODS

Cell culture

Cells of two human HRS-positive cell lines (T-47D breast cancer, T98G glioblastoma) and one human HRS-negative cell line (NHK 3025 cervix cancer) were used. For T-47D and T98G cells the culture conditions were as described previously [11, 13, 14] using RPMI1640 (Roswell Park Memorial Institute) medium (JRH Biosciences, Kansas, USA) supplemented with 10% foetal calf serum (Gibco, Paisley, UK), 2 mM L-glutamine (SIGMA, St Louis, MO, USA), 200 units 11^{-1} insulin (SIGMA), and 1% C. The human cervical carcinoma *in situ* cells NHK 3025 [22, 23] were grown in Minimum Essential Medium Eagle (MEM) (SIGMA) with 15% foetal calf serum, 2 mM L-glutamine (SIGMA) and 2% 2mM L-glutamine (SIGMA).

The cells were kept in exponential growth by reculturing of stock cultures two times a week. The cells tested negative for the presence of mycoplasma.

NO-donor Diethylamine NONOate sodium salt hydrate (DEANO) and iNOS inhibitor 1400W were purchased from SIGMA (D184, SIGMA, St Louis, MO, USA).

Irradiation procedures

The cells were irradiated as described previously [14] with an HDR of ~32 Gy/h used for all challenge irradiations. Because of [^{60}Co]-decay, the LDR used for priming irradiation in the present experiments was ~0.22 Gy/h, compared with 0.3 Gy/h in our previous studies. The total irradiation time for LDR priming was 1 h, so the total dose in all LDR priming irradiations was ~0.22 Gy. In addition, LDR priming irradiation with shorter duration (15 min) was tested (Fig. 1C).

Assessment of anti-phospho-histone H3 (ser28) staining

The method of assessment of anti-phospho-histone H3 staining was adapted from those of Juan *et al.* [24] and Xu *et al.* [25], as described previously [13].

Transfer of medium irradiated without cells present

The medium transfer experiments were performed as described previously [13, 14]. The recipient cells were exposed to the transferred medium for 24 h before being plated for colony formation in fresh medium for 16–20 h before challenge irradiation.

Hypoxic culture

The cells were cultured with two reseeds a week in an IN VIVO₂ 400 (Ruskin, Bridgend, UK) glovebox hypoxia workstation operated to contain 4% O₂ and 5% CO₂ in the gas phase. The cells grown under these conditions were exposed to cycling hypoxia with pericellular oxygen levels decreasing from 4% to below 0.1% between reseeds [26–28].

Statistical analysis

All clonogenic experiments were repeated at least three times using five flasks for each dose and 10 for controls. Within each experiment, the arithmetic means were calculated, weighing the errors. Radiation survival curve data were fit using the linear quadratic (LQ) or the induced repair (IR) model.

The LQ-model is described by the equation:

$$S = \exp(-\alpha d - \beta d^2), \quad (\text{equation 1})$$

where S is the surviving fraction, d the dose and α and β the parameters describing the linear and quadratic components, respectively, of the intrinsic radiosensitivity.

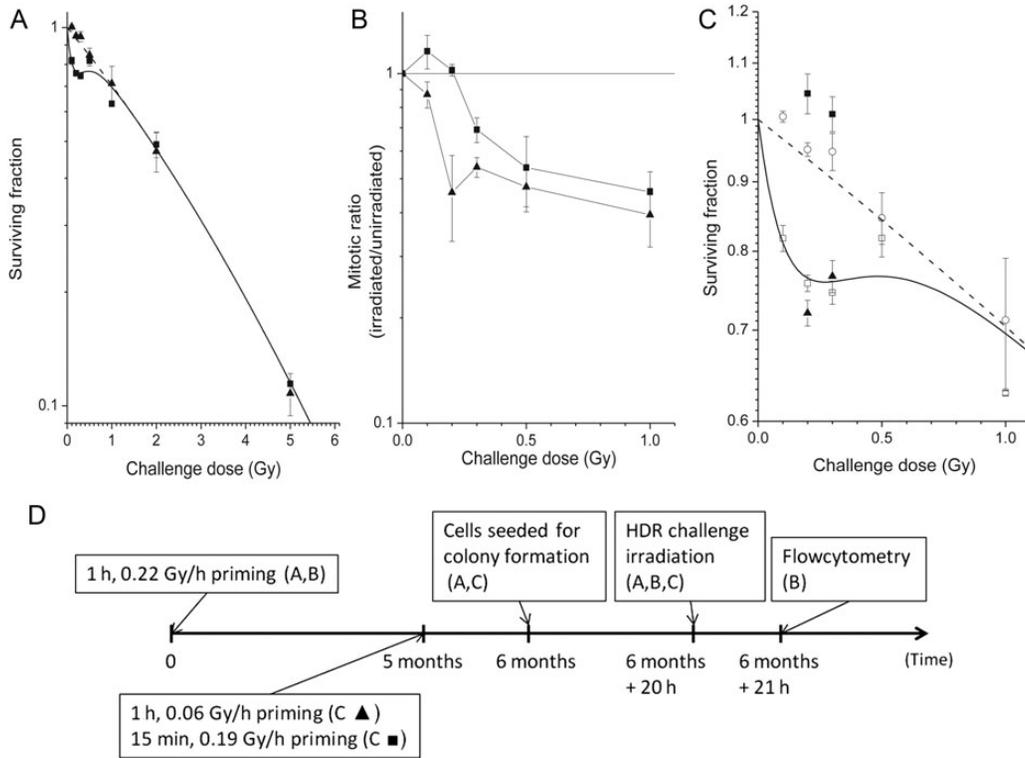


Fig. 1. (A) The surviving fraction, measured by the colony assay, as a function of HDR challenge dose (~32 Gy/h) for unprimed T98G cells (closed squares), and T98G cells primed with LDR irradiation (0.22 Gy/h) for 1 h six months before the HDR challenge dose (closed triangles). The curves represent model-fits to the data from unprimed cells by the IR-model (solid lines) and the LQ-model (dashed lines), respectively. The parameters from the fit to data from unprimed cells by the IR-model are presented in Table 1. (B) The ratio (irradiated/unirradiated) of mitotic cells (staining positive for phosphorylated histone H3) as a function of radiation dose given 1 h before cell harvest (symbols represent cells as described under (A)). The early G₂-check point is activated by doses below the HRS-threshold in LDR-primed cells. (C) The surviving fraction after 0.2 or 0.3 Gy HDR challenge doses to T98G cells. The cells were first exposed to priming doses of 0.06 Gy/h for 1 h = 0.06 Gy (closed squares) or 0.19 Gy/h for 15 min = 0.0475 Gy (closed triangles). Data from T98G controls (open squares) and T98G cells primed with 0.22 Gy/h for 1 h (open circles) are shown as references. $P < 0.01$ for data points (closed squares) compared to T98G controls. (D) Timeline showing the experimental schedules.

In the IR-model α is replaced by:

$$\alpha = \alpha_r \left(1 + \left(\frac{\alpha_s}{\alpha_r} - 1 \right) e^{-\frac{d}{d_c}} \right), \quad (\text{equation 2})$$

where d is dose, α_r is the value of α extrapolated from the high-dose LQ response (equation 2), and α_s is the actual value of α derived from the initial part of the curve (i.e. at very low doses). d_c is the dose where the change from α_s to α_r is 63% complete.

Two-tailed Student's t -test was used to compare the surviving fractions in response to HDR challenge irradiation of pretreated cells compared to controls or LDR-irradiated cells.

Cell-based ELISA

Total iNOS levels in whole cells were measured by an ELISA-based assay using fluorogenic substrates according

to the manufacturer's protocol (KCB9502, R&D systems, Minneapolis, MN, USA). Cells were grown overnight in microplates and fixed and permeabilized by 4% formaldehyde. The cells were then incubated simultaneously with two primary antibodies (iNOS and GAPDH for normalization) and thereafter with two secondary antibodies (horseradish-peroxidase and alkaline phosphatase). The fluorescence was measured using a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA) and the fluorescence of iNOS was normalized to that of GAPDH for each well after background subtraction.

Gene expression analysis

The cells harvested for microarray analysis were either HDR-irradiated, LDR-irradiated or unirradiated T-47D cells. The HDR-irradiated cells were harvested 24 h after a dose of 0.3 Gy at 35 Gy/h, a time when HRS had returned. The

Table 1. Parameters of the fit by the IR-model to the data points from unprimed cells in Fig. 1A and 3C and D. Data on T-47D cells are from [13]

Cell	$\alpha_r \pm \text{SEM} (\text{Gy}^{-1})$	$\alpha_s \pm \text{SEM} (\text{Gy}^{-1})$	$d_c \pm \text{SEM} (\text{Gy})$	$\beta \pm \text{SEM} (\text{Gy}^{-2})$
T98G	0.33 ± 0.03	3.39 ± 1.11	0.18 ± 0.05	0.020 ± 0.006
T-47D	0.19 ± 0.01	1.84 ± 0.34	0.31 ± 0.04	0.018 ± 0.001
NHIK 3025	0.30 ± 0.05			0.031 ± 0.010
NHIK 3025 with 1400W	0.38 ± 0.04	1.02 ± 0.28	0.50 ± 0.24	0.017 ± 0.008

HRS-deficient LDR-irradiated cells were harvested two months after irradiation. Total RNA was purified from cell pellets using a Qiagen RNAeasy minikit (Qiagen 74104, Qiagen, Germany). Two-colour Microarray-Based Gene Expression Analysis (Quick Amp Labelling) was performed using a 44 K human Whole Genome Oligo microarray kit from Agilent Technologies. Agilent Feature Extraction Software (version 9.1.3.1) was used to quantify images scanned on the Agilent Technologies Scanner G2505B US22502537.

Bioinformatic analysis was performed using the Bioconductor package LIMMA (Linear Models for Microarray Data) (www.bioconductor.org), including global loess normalization [29]. Differentially expressed genes between LDR-primed T-47D and unprimed T-47D cells (with HDR-primed T-47D cells as reference) were identified using a linear model with a modified *t*-test comparing the two groups [30]. The genes were ranked using B-statistics, which is the log-odds of whether the gene is differentially expressed or not. The *P*-values were corrected for multiple testing using Benjamini and Hochberg false discovery [31]. Finally, the log₂ fold change was calculated for each gene. The same procedure was used when comparing LDR-primed with HDR-primed T-47D cells (where unprimed T-47D was used as the reference). Both raw and normalized data from the eleven microarrays used in this study are available from Gene Expression Omnibus (GEO) under the accession number GSE41483.

RESULTS

Low-dose-rate priming irradiation of T98G glioblastoma cells

The sustained effect of LDR irradiation (0.3 Gy/h for 1 h) on HRS, previously found in T-47D cells [11], was also observed in another HRS-proficient cell line, T98G glioblastoma cells primed with 0.22 Gy/h for 1 h (Fig. 1A). The data in Fig. 1A and B are from 6 months after LDR priming, but as for T-47D cells, many time-points have been tested with the same result. The parameters from the fit by the IR-model to the data from the unprimed cells are given in Table 1.

Figure 1B shows that the mitotic index 1 h after challenge irradiation, as measured by histone H3-phosphorylation, is different for LDR-primed as compared to unprimed T98G cells. As reported previously for T-47D cells, there was a trend for a decreasing mitotic ratio with increasing challenge radiation dose for LDR-primed T98G cells for the doses as low as 0.1 and 0.2 Gy, in contrast to the wild-type T98G cells where the lowest doses did not decrease the mitotic fraction of cells (Fig. 1B).

The data from Fig. 1A in the challenge dose-range up to 1 Gy is replotted in Fig. 1C (open symbols). In Fig. 1C data are also included the response to two challenge doses in the HRS-range (0.2 and 0.3 Gy) for T98G cells that had previously (1 month before) been given priming irradiation of 0.06 Gy/h for 1 h and 0.19 Gy/h (because of decay not 0.22 Gy/h) for 15 min. The data indicate that the duration of the priming irradiation is important: on the one hand the lowest dose-rate of 0.06 Gy/h for 1 h had the same priming effect as 0.22 Gy/h for 1 h and the response was significantly different from that of unprimed cells ($P < 0.002$ for both challenge doses of 0.2 and 0.3 Gy). On the other hand, 15 min priming irradiation with 0.19 Gy/h did not affect the response to the subsequent challenge doses ($P < 0.002$ for both challenge doses of 0.2 and 0.3 Gy compared with cells primed with 0.22 Gy/h for 1 h). Thus, since the total priming dose was about the same in the two cases, it seems as if the duration of the irradiation is the important factor and that 15 min was too short for the effect to be induced, while 1 h was sufficient.

NO in combination with HDR radiation or reoxygenation after long-term cycling hypoxia mimicked LDR radiation

We hypothesized that NO might be involved in the LDR irradiation-induced response. To test this we first investigated the effect of adding the chemical NO-donor diethylamine nitric oxide (DEANO) (present for 24 h). DEANO was found not to affect the response to the HDR challenge dose in the HRS-range (< 0.5 Gy) in T98G cells. This was tested for DEANO-concentrations up to 1 mM (data not shown). However, as shown in Fig. 2A a DEANO-concentration of just 0.1 mM during HDR priming irradiation with 0.3 Gy was sufficient to mimic the effect of LDR priming irradiation and remove the hypersensitive response for at least two

weeks ($P < 0.004$ for both challenge doses of 0.2 and 0.3 Gy). This effect of LDR irradiation could last up to several years and could remain even after freezing and thawing. In some cases HRS returned within the first week following pretreatment, but never later [13, 14]. Therefore, we considered that if we did not observe HRS two weeks after pretreatment, then it was very likely to be a sustained effect.

In order to test whether the removal of HRS by LDR priming irradiation could be mediated through ROS (reactive oxygen species), another ROS-inducing treatment, namely reoxygenation after hypoxia, was tested (Fig. 2B). T98G cells were cultured for six weeks with 4% oxygen in the gas phase, which exposes the cells to cycling hypoxia, i.e. the oxygen level falls to about 0.1% as the cells proliferate and then there is a sudden increase to 4% oxygen each time the cells are recultured [26]. These cells lost HRS, but within two weeks after the cells were transferred to a CO₂-incubator with air, the HRS-response was regained. However, when DEANO was administered 2 h before the cells were brought from the hypoxia workstation to the normal incubator, wild-type HRS was not regained ($P < 0.004$ for both doses comparing reoxygenation after long-term hypoxia with and without DEANO). It was also tested whether reoxygenation after hypoxia could re-introduce HRS in LDR-primed cells. However, the LDR-primed cells did not regain HRS after culture for two weeks with 4% oxygen in the gas phase (two weeks of cycling hypoxia is enough to induce the effect [26]) followed by three weeks in ambient air ($P < 0.002$ for both challenge doses of 0.2 and 0.3 Gy compared with unprimed T98G cells).

iNOS activity is involved when LDR irradiation induces the HRS-negative phenotype

In order to test if iNOS activity was involved in the mechanism by which LDR irradiation removed HRS, iNOS inhibitor 1400W was added to the cell medium 1 h before LDR priming irradiation of the cells, and removed again 1 h after the irradiation had terminated. For both T98G and T-47D cells, LDR irradiation did not remove HRS when iNOS activity was inhibited (Fig. 3) ($P < 0.00004$ for both challenge doses of 0.2 and 0.3 Gy, comparing LDR priming of T-47D cells with and without 1400W. For T98G cells only the difference between surviving fractions at 0.3 Gy challenge dose was significant ($P = 0.01$)) when comparing LDR priming with and without 1400 W).

iNOS levels in LDR-primed cells

ELISA measurements showed no significant differences in iNOS levels in LDR-primed cells compared with unprimed cells (Fig. 4), indicating that iNOS is activated rather than upregulated in LDR-primed cells. Adding iNOS inhibitor 1400W did not reduce the levels of iNOS protein, for T-47D cells there seemed instead to be a slightly higher amount of iNOS protein ($P = 0.01$).

iNOS activity is involved in maintaining the HRS-negative phenotype

LDR-primed cells (T98G and T-47D) were exposed to medium with 10 μ M iNOS inhibitor 1400W on three consecutive days and were challenge-irradiated 24 h and 3 (T98G) or 5 (T-47D) weeks (with 2-weekly reseeds) after the last treatment (Fig. 5A and B). At all time-points the cells that had been given three days treatment with 1400W had regained wild-type HRS ($P < 0.005$ for both challenge doses of 0.2 and 0.3 Gy compared with LDR-primed cells). Exposure to 1400W for only 4 h did not have this effect ($P > 0.17$ for both challenge doses of 0.2 and 0.3 Gy compared with LDR-primed cells).

Unprimed NHIK 3025 human cervical cancer cells do not express HRS as shown in Fig. 6A. However, treatment with 10 μ M 1400W added on three consecutive days induced the HRS-response even in these cells ($P = 0.003$ for 0.2 and $P = 0.02$ for 0.3 Gy) (Fig. 6A and B). The parameters from the fit by the LQ-model to the data from the untreated cells and by the IR-model to the cells treated with 1400W are given in Table 1. The presence of HRS in NHIK3025 treated with 1400W is corroborated by Fig. 6C showing the mitotic index 1 h after challenge irradiation, as measured by histone H3-phosphorylation. As seen for the LDR-primed T-47D and T98G cell lines (which had lost HRS), there was a trend for decreasing mitotic ratio with increasing challenge radiation dose for NHIK 3025 cells for doses as low as 0.2 and 0.3 Gy. However, the NHIK 3025 cells treated with 1400W showed a response similar to the unprimed (HRS-positive) T98G and T-47D cells in which the lowest doses up to 0.5 Gy did not decrease the mitotic fraction of cells.

iNOS was not differentially expressed in LDR-irradiated (i.e. primed) cells

Microarray analyses were performed comparing mRNA levels in (i) unprimed control T-47D cells, (ii) T-47D cells harvested two months after LDR-priming irradiation (0.3 Gy at 0.3 Gy/h), and (iii) T-47D cells harvested 24 h after HDR-priming irradiation (0.3 Gy at 40 Gy/h), a time when HRS had been regained in these cells. The LIMMA analysis resulted in 2045 differentially expressed genes (Agilent Probe IDs where $B > 0$) in LDR-primed vs HDR-primed cells, and 5401 differentially expressed genes in LDR-primed cells vs unprimed controls. Combining these results yielded 128 upregulated and 42 downregulated unique Gene Names. DAVID annotation was used to infer which pathways were significantly up- or downregulated assuming a minimum of five genes must be present in the same pathway. The MAPK (mitogen-activated protein kinase) signaling pathway was found to contain the highest number of genes [11] among the upregulated genes (endocytosis with six genes, and general pathways in cancer with seven genes being the only others), while no pathways passed the

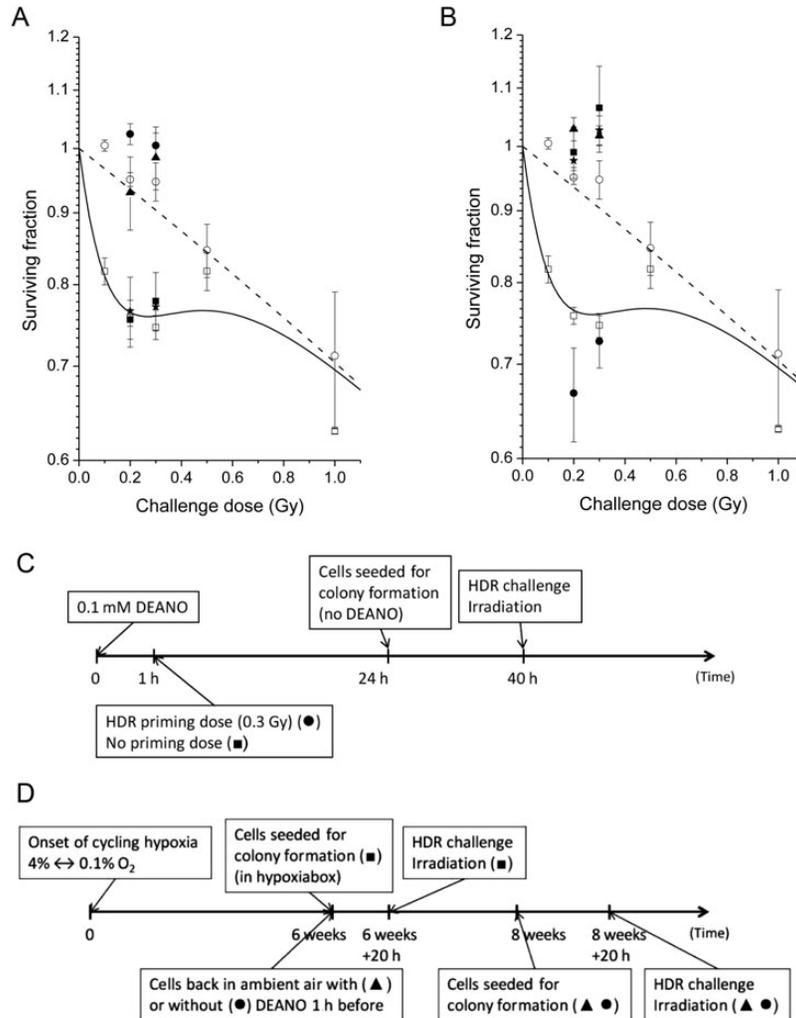


Fig. 2. (A) T98G cells were exposed to 0.1 mM DEANO for 24 h before being plated in fresh medium 20 h before HDR challenge irradiation (closed squares). The results showed that HRS was not removed by NO alone. The effect of NO in combination with high dose-rate priming was then tested: 0.1 mM DEANO (for 24 h) and 0.3 Gy HDR irradiation, 48 h before HDR challenge irradiation (closed circles), 0.1 mM DEANO with 0.1 mM carboxy-PTIO (for 24 h) and 0.3 Gy HDR irradiation, 48 h before HDR challenge irradiation (closed stars), 0.1 mM DEANO (for 24 h) and 0.3 Gy HDR irradiation, two weeks before HDR challenge irradiation (closed triangles). Data from unprimed control cells (open squares) and LDR-primed cells (open circles) (Fig. 1A) are shown as references. The curves represent model-fits to the data from unprimed T98G cells by the IR-model (solid lines) and the LQ-model (dashed lines), respectively. The bars represent standard errors of the mean (SEM) for three individual experiments. (B) T98G cells were grown with 4% oxygen in the gas phase. The cells consume oxygen and the peri-cellular oxygen concentration dropped during growth to below 0.1% with a sudden increase back to 4% O₂ each time the cells were recultured. This resulted in cycling hypoxia. Radiation response was measured after six weeks in such cycling hypoxia with two reoxygenations per week (closed squares) (no HRS) and after six weeks cycling hypoxia followed by two weeks in ambient air (closed circles) (HRS regained). If DEANO was added before transfer to ambient air, HRS was not regained (closed triangles). LDR-primed T98G cells grown two weeks in cycling hypoxia followed by three weeks in ambient air did not regain HRS (closed stars). Data from unprimed control cells (open squares) and LDR-primed cells (open circles) (Fig. 1A) are shown as references. The curves represent model-fits to the data from unprimed T98G cells by the IR-model (solid lines) and the LQ-model (dashed lines), respectively. The bars represent SEM for three individual experiments. (C) Timeline showing the experimental schedules in Fig. 2A. (D) Timeline showing the experimental schedules in Fig. 2B.

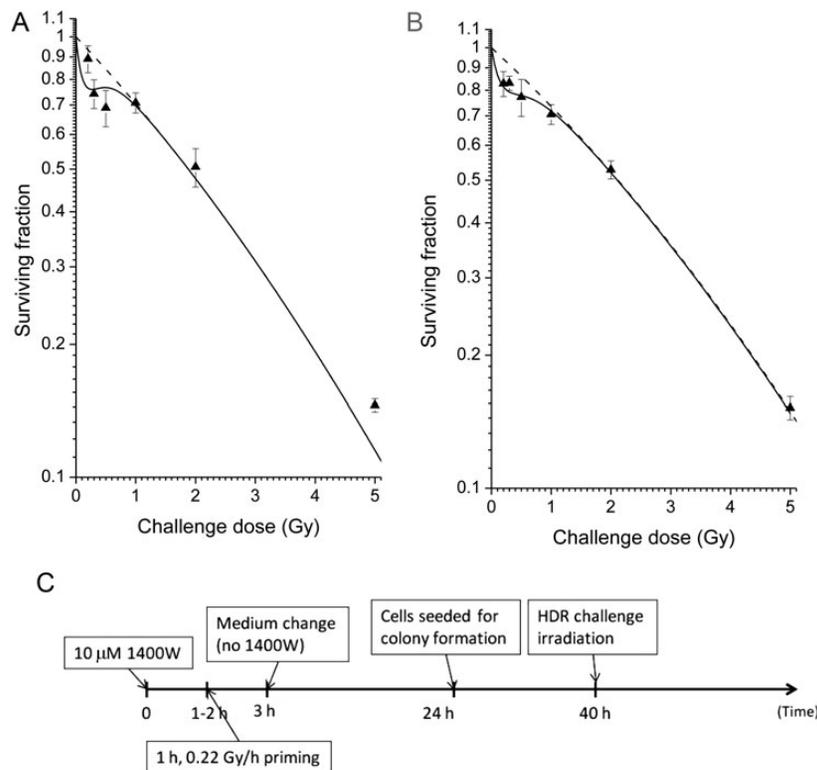


Fig. 3. Data showing that 10 μ M of iNOS inhibitor 1400W added to the cells 1h before LDR priming inhibited the removal of HRS by LDR priming of both T98G (A) and T47D (B) cells. The curves represent model-fits to the data from unprimed cells by the IR-model (solid lines) and the LQ-model (dashed lines), respectively. (C) Timeline showing the experimental schedules.

threshold for downregulated genes. The average values of the three NOS genes are depicted in Table 2. The maximum B-value among the 23 NOS gene transcripts was -3.83 . This illustrates that no NOS genes were found to be significantly differentially expressed between the cells with and the cells without HRS.

The mechanism for removal of HRS in cells by LDR irradiation was also induced by LDR irradiation of cell-conditioned medium

For T-47D cells we have previously shown that medium that had been conditioned by cells and subsequently LDR irradiated without cells present removed HRS in recipient cells. We wanted to test whether iNOS activity and NO production played the same role in LDR irradiation of cell-conditioned medium as in LDR irradiation of cells. Table 3 shows the surviving fractions of T98G cells exposed to two doses in the HRS-range (0.2 and 0.3 Gy). Before irradiation the cells had been exposed to medium harvested from unirradiated cells and subsequently irradiated without cells present. The removal of HRS in cells receiving LDR-irradiated cell-conditioned medium was also seen in T98G cells. When the cell-conditioned medium was supplemented with 0.1 mM DEANO 1 h before it was given an HDR priming dose of 0.3

Gy, HRS was removed in the recipient cells, mimicking the effect of a similar LDR priming dose. Adding 10 μ M 1400W to the cell-conditioned medium 1 h before giving LDR irradiation inhibited the effect of the LDR priming and the recipient cells retained the wild-type HRS-response.

DISCUSSION

Our previous observation that an LDR priming irradiation of 0.3 Gy/h for 1 h removed HRS seemingly permanently in T-47D breast cancer cells [11, 14] was confirmed in another HRS-proficient cell line, T98G glioblastoma, given 0.22 Gy/h for 1 h. Even a priming irradiation of 0.06 Gy/h for 1 h was found to give the same effect of removal of HRS, while 0.19 Gy/h for 15 min did not. Thus, the duration of the irradiation is a determinant parameter for the observed difference in effect of low- and high-dose-rate irradiation with small doses while the total priming dose is not; at least within the range of 0.06 and 0.3 Gy.

The HRS-proficient cell lines used in the present study did not show any cytotoxic bystander effect in response to low-LET radiation (data not shown). This is in agreement with other studies, which show that HRS and cytotoxic bystander effects are mutually exclusive [32]. Instead, both

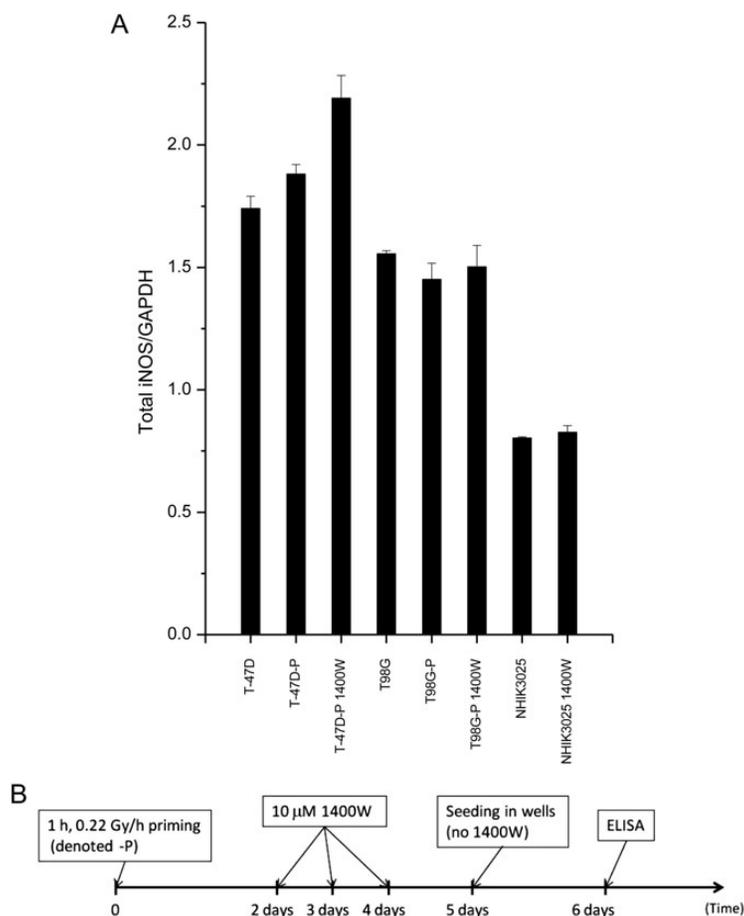


Fig. 4. (A) Cell-based ELISA measurements of the total iNOS protein level normalized to that of GAPDH. The cells were either untreated, LDR-primed three weeks before measurements (LDR priming indicated by P added to the cell name), or LDR-primed and treated with iNOS inhibitor 1400W on three consecutive days ending the day before measurements. The bars represent standard errors of the mean (SEM) for three individual experiments with three parallels in each experiment. (B) Timeline showing the experimental schedules.

radiation-induced adaptive response [32, 33], and adaptive bystander effects [34–38] have been observed in HRS-proficient cells, and both of these have been shown to involve NO produced by inducible iNOS [34–36]. Even if the removal of HRS by LDR priming irradiation is permanent, and thus differs from the adaptive response and adaptive bystander effects, the mechanism inducing the response and transferring the signal through the cell medium might be related.

NO production by iNOS is a slow process [39] and we hypothesized that the mechanism induced by LDR irradiation for 1 h but not for 15 min (and not by 0.3 Gy HDR priming) involved NO. However, exposing the reporter cells to NO from DEANO did not affect the radiosensitivity. Only when the cells were given an HDR priming irradiation with 0.3 Gy after DEANO was added to the cell medium, was HRS removed in the cells at the two time-points when they

were given challenge doses (48 h and two weeks after the priming irradiation). Thus, when given in the presence of NO an acute small dose of 0.3 Gy induced the same effect as the same dose given protracted over 1 h without added NO.

In order to investigate whether the effect was mediated by radiation-induced ROS, we tested the combination of NO with a ROS-generating mechanism, which does not involve radiation. Reoxygenation by air after hypoxia is known to generate a burst of ROS [40–42]. We have previously found that exposing T-47D cells to long-term cycling hypoxia removed HRS in the hypoxic cells, but the wild-type HRS-response was regained within two weeks after transfer to ambient air [26]. The same applied to T98G cells (Fig. 2B). However, by adding DEANO 2 h before the last reoxygenation, the wild-type HRS-response was not regained within three weeks. The effect of LDR irradiation can thus be mimicked by either HDR irradiation or reoxygenation after

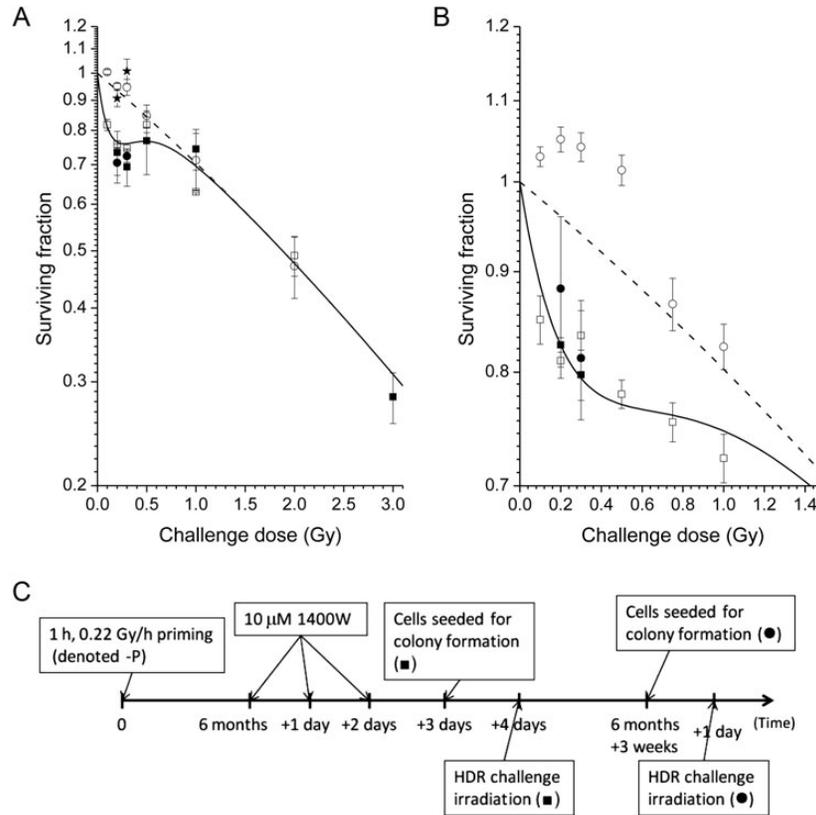


Fig. 5. Data showing that wild-type HRS is regained in three different cell lines after exposure to medium with 10 μ M of iNOS inhibitor 1400W. T98G (A) or T47D (B) cells LDR-primed six months previously were exposed to medium containing 10 μ M 1400W on three consecutive days. The cells regained HRS by this treatment when tested by challenge irradiation one day after removal of 1400W (closed squares) or three weeks after removal of 1400W with 2-weekly reseeds (closed circles). LDR-primed T98G cells exposed to medium containing 10 μ M 1400W for just 4 h did not regain HRS (closed stars). $P < 0.01$ for data-points (closed squares) and (closed circles) compared to LDR-primed cells. Data from unprimed control cells (open squares) and LDR-primed cells (open circles) (Fig. 1A) are shown as references. The curves represent model-fits to the data from unprimed cells by the IR-model (solid lines) and the LQ-model (dashed lines), respectively. (C) Timeline showing the experimental schedules.

hypoxia in combination with NO, suggesting that the effect is due to a cooperation between ROS and NO.

The involvement of NO in mediating the effect of LDR priming was confirmed by the inhibition of removal of HRS by iNOS inhibitor 1400W when present during LDR irradiation (Fig. 5).

The cells that were LDR-primed never regained HRS in contrast to the unprimed cells that were exposed to medium from LDR-primed cells, in which the effect lasted for a few days [14]. The most important question is perhaps how the permanent effect of LDR irradiation is maintained. The present data suggest a role for iNOS. Furthermore the wild-type HRS-response was regained in the LDR-primed cells by adding iNOS inhibitor 1400W to the medium on three consecutive days. Interestingly, a similar treatment with 1400W of cells from an HRS-negative cell line (NH1K 3025) introduced an HRS-response even in these cells. HRS has been

postulated to be the default response to small doses of radiation [6, 7]. Based on the present data a further speculation could be that the absence of HRS in some cases might be due to prior exposure to LDR irradiation or other types of stress with combined ROS and NO production.

In agreement with the ELISA data, in which protein levels of iNOS were the same in the LDR-primed as in the unprimed cells, the microarray data showed no change in iNOS mRNA levels in primed vs unprimed cells. Together this may indicate that the mechanism upholding the HRS-negative phenotype depends on maintaining iNOS activity rather than iNOS protein amount. The activity of the two constitutively expressed NOS isoforms (eNOS and nNOS) is regulated by calmodulin binding in a Ca^{2+} concentration-dependent manner. In contrast, iNOS has calmodulin bound permanently and has been suggested to be regulated through transcriptional control [43]. However,

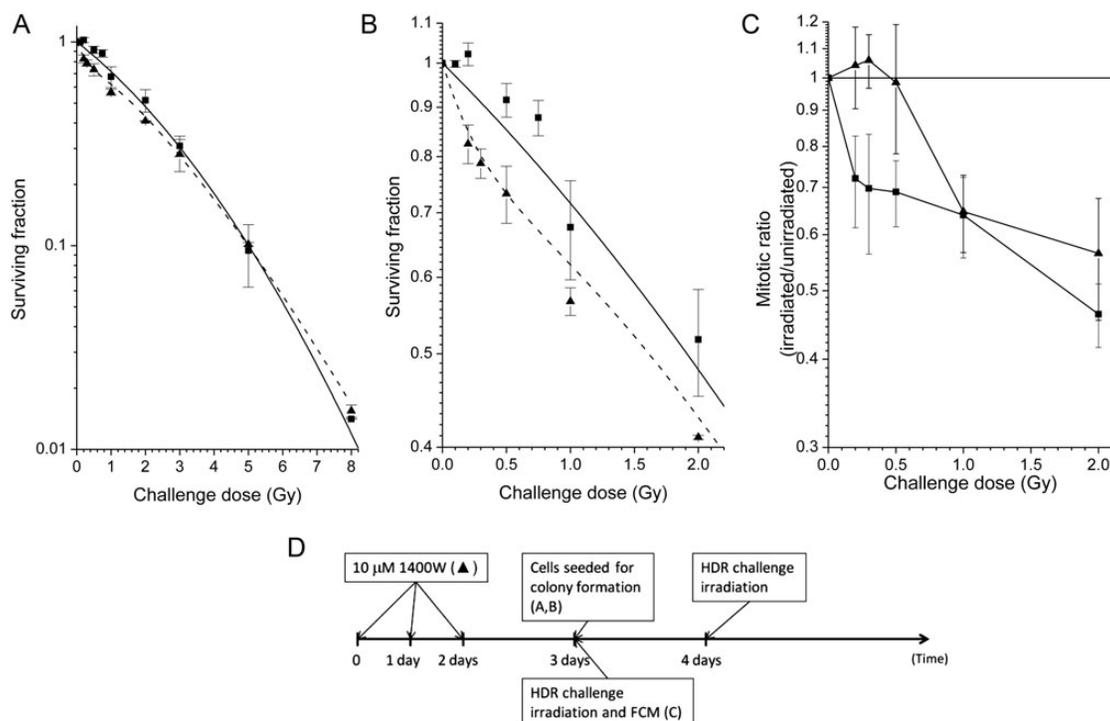


Fig. 6. The effect of iNOS inhibitor 1400W in cells of the HRS-negative line NHIK 3025. (A) and (B): The surviving fraction, measured by the colony assay, as a function of HDR challenge dose (~ 32 Gy/h). NHIK 3025 cells without 1400W (closed squares) and with 1400W (closed triangles). (A) Doses up to 8 Gy. (B) Doses up to 3 Gy. The curves represent model-fits by the LQ-model to the data from unprimed NHIK 3025 cells (solid lines) and by the IR-model to NHIK 3025 treated on three consecutive days with $10 \mu\text{M}$ 1400W (dashed lines). $P < 0.02$ for challenge doses of 0.2 and 0.3 Gy comparing NHIK 3025 cells with and without 1400W treatment. The bars represent standard errors of the mean (SEM) for 3–5 individual experiments. (C) The ratio (irradiated/unirradiated) of mitotic cells (staining positive for phosphorylated histone H3) as a function of radiation dose given 1 h before cell harvest (symbols represent cells as described under (A)). The early G_2 -check point is activated by doses below the HRS-threshold in untreated NHIK 3025 cells but not when they are treated with 1400W. (D) Timeline showing the experimental schedules.

Table 2. Microarray analysis of nitric oxide synthase mRNA comparing HRS-deficient LDR-primed T-47D cells with HRS-proficient HDR-primed cells (HDR) and controls (C)

Gene name	LDR- vs HDR-primed cells			LDR-primed cells vs control		
	logFC	adj. <i>P</i> .Val	B	logFC	adj. <i>P</i> .Val	B
nNOS (neuronal)	0.02	0.83	-6.76	-0.05	0.58	-6.98
iNOS (inducible)	-0.03	0.70	-6.47	-0.03	-0.64	-6.79
eNOS (endothelial)	0.06	0.55	-6.03	0.03	0.61	6.94

logFC = log Fold Change, adj.*P*.Val = adjusted *P*-value (*P*-value after FDR adjustment), B = B-statistic value (differentially expressed genes have $B > 0$).

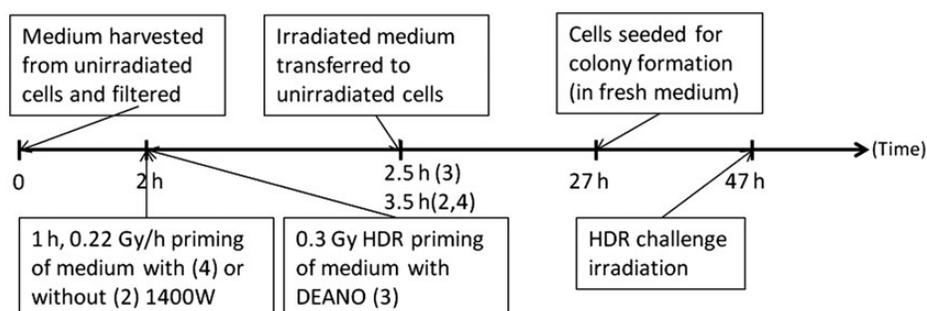
more recent studies have shown that very complex and fine-tuned chemistry is involved in the production of NO from L-arginine, involving several co-factors and intricate electron and proton transfer processes that are yet to be elucidated [44]. Inhibition of iNOS by 1400W given on three consecutive days ending one day before measurements did not reduce protein levels. On the contrary, it seems that the

treatment induced upregulation of iNOS in T-47D cells, perhaps as a countermeasure to compensate for the reduced activity.

The recovery of HRS when the cells were reoxygenated after long-term cycling hypoxia indicated that the self-sustaining mechanism involving iNOS observed in LDR-primed cells was either not activated or became

Table 3. Surviving fractions of challenge irradiated T98G cells exposed (20 h previously for 24 h) to medium conditioned on unirradiated T98G cells and subsequently irradiated without cells present

Pretreatment	Surviving fractions		P-values from two-tailed <i>t</i> -test	
	0.2 Gy	0.3 Gy	0.2 Gy	0.3 Gy
1 T98G, no pretreatment	0.76 ± 0.01	0.75 ± 0.01		
2 Recipients of LDR-primed cell-conditioned medium	1.00 ± 0.03	1.03 ± 0.04	2 vs 1	2.89E-03
3 Recipients of HDR-primed cell-conditioned medium with DEANO	1.02 ± 0.02	1.01 ± 0.05	3 vs 1	2.56E-04
4 Recipients of LDR-primed cell-conditioned medium with 1400W	0.74 ± 0.02	0.76 ± 0.05	4 vs 2	2.98E-03

**Fig. 7.** Timeline showing the experimental schedule for irradiation of T98G cells exposed (20 h previously for 24 h) to medium conditioned on unirradiated T98G cells and subsequently irradiated without cells present (Table 3).

deactivated by reoxygenation. To test if reoxygenation could deactivate the permanent HRS-removal induced in LDR-primed cells, LDR-primed cells (without HRS) were exposed to long-term cycling hypoxia followed by reoxygenation (Fig. 2B). However, these cells did not regain HRS, suggesting that iNOS is not involved in the removal of HRS induced by cycling hypoxia.

Regarding the mechanism inducing the adaptive response, it has been suggested that NO secreted from irradiated cells may either initiate a signaling pathway in recipient cells to induce the adaptive response [19, 20] or act in combination with ROS as initiator of signaling mediated by TGF- β 1, TNF- α , IL-1 β , or estrogen [45]. In the present study an adaptation resulting only in increased resistance to doses in the HRS-range (<0.5 Gy) was investigated. The adaptation could be induced permanently by LDR priming or transiently by medium transferred from LDR-primed cells or LDR-primed cell-conditioned medium. Exposing the unprimed cells to NO from DEANO did not affect the radiosensitivity (Fig. 2A). Thus, it appears that in the process induced by LDR priming of cells, the presence of NO in combination with ROS activates another factor, which in turn removes the HRS-response.

The microarray analysis showed an upregulation of several genes of the MAPK signaling pathway in the LDR-primed cells compared to unirradiated cells and cells exposed to an HDR priming dose (harvested after 24 h when HRS had

returned). Interestingly, the MAPK pathways have been found to be involved both in the cytotoxic bystander response [46] and in the adaptive response to 0.02 Gy (HDR X-rays) [47]. It was proposed that NO acts as an initiator or mediator of cyto-protective bystander responses that are likely supported by MAPK pathways [48, 49]. Seven of the upregulated genes were involved in the JNK/p38 MAPK pathways, which are stress-activated. Interestingly, the p38 MAPK has been reported to induce rapid G₂-delay in response to hypertonic stress [50]. It is tempting to speculate that the continuous activation of iNOS in the LDR-primed cells results in NO-mediated upregulation of the p38 MAPK pathway and thus in abolition of HRS through facilitated activation of the early G₂-check point. This is consistent with our previously published data showing a decrease in mitotic ratio in the HRS dose-range for the LDR-primed cells but not for unprimed cells [13].

The present data show responses to the lowest radiation doses with surviving fractions >1 in cells that were pretreated with LDR irradiation or NO in combination with either HDR irradiation or hypoxia/reoxygenation (Figs 1, 2 and 5). This is consistent with our earlier observations [11, 13, 14, 26], and seems to be more pronounced in T-47D cells than in T98G cells. p38 MAPK has been found to be involved in the activation of quiescent somatic stem cells [51]. If this mechanism is also present in T-47D cells, and to some degree in T98G cells, it could be involved in the higher clonogenicity in

cells given a small challenge dose than in unirradiated (but pretreated) control cells. The control cells in our experiments normally have a plating efficiency of 50–70% and it is possible that some of these cells are quiescent cells that can be activated to resume cell cycling.

While the general view of radiation-induced cell regulatory processes after large doses is that these are associated with or initiated by DNA damage, one may start wondering to what extent DNA damage is involved in regulatory responses to low-dose LDR irradiation. In our experiments, cell-conditioned medium was irradiated either at LDR or at HDR in the presence of NO (Table 3 and Fig. 7), and in both cases unirradiated recipient cells were found to lack HRS on challenge irradiation. In both these cases no irradiation was involved during conditioning of the medium and no cells were present during the priming irradiation of the medium. It is, therefore, difficult to see how DNA damage could be involved in the regulatory processes. Other studies seem to support this view to some extent, and studies using microbeam irradiation have shown that cytoplasmic irradiation can induce bystander effects, as well as induce apoptosis [52] and DNA damage similar to mutations of spontaneous origin [53]. In concordance with this, Maeda *et al.* [54] previously reported that irradiation of nuclei using X-ray microbeams resulted in more pronounced HRS than whole-cell irradiation of V79 Chinese hamster cells, and proposed that cytoplasmic irradiation either suppresses HRS or enhances IRR.

CONCLUSION

In conclusion, the mechanism for the effect on HRS induced by low and not HDR irradiation appears to depend on NO produced by iNOS that cooperates with either radiation- or reoxygenation-induced ROS. The data suggest that LDR irradiation for 1 h, but not 15 min, activates iNOS to produce NO. The mechanism appears to be induced without involving DNA damage. Sustained iNOS activity seems necessary for the permanent removal of HRS by LDR priming to be maintained.

The study shows how resistance against low levels of DNA damage can be turned on and off in cells. HRS has been seen in normal human skin tissues and in renal damage in mice [7, 55–57], as well as in clinical studies on metastatic tumor nodules in the skin [58] and in some tumor xenograft experiments [59, 60]. However, some other tumor xenograft experiments did not show HRS [61–64]. In the present study, we show that HRS can be ‘turned off’ by various pretreatments, which may explain the diverging outcome of attempts to exploit HRS in cancer treatment. However, they also provide a method of sensitizing tumor tissue to doses in the HRS-range by inhibition iNOS activity. In addition, the present study potentially contributes to increased

understanding of the importance of distinguishing between high and LDR irradiation, both in cancer treatment planning and when discussing radiation protection issues.

SUPPLEMENTARY DATA

Supplementary data is available at the *Journal of Radiation Research* online.

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