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TGF- β 3 DEPENDENT MODIFICATION OF RADIOSENSITIVITY IN REPORTER CELLS EXPOSED TO SERUM FROM WHOLE-BODY LOW DOSE-RATE IRRADIATED MICE

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□ Prior findings *in vitro* of a TGF- β 3 dependent mechanism induced by low dose-rate irradiation and resulting in increased radioresistance and removal of low dose hyper-radiosensitivity (HRS) was tested in an *in vivo* model. DBA/2 mice were given whole-body irradiation for 1 h at low dose-rates (LDR) of 0.3 or 0.03 Gy/h. Serum was harvested and added to RPMI (4% mouse serum and 6% bovine serum). This medium was transferred to reporter cells (T-47D breast cancer cells or T98G glioblastoma cells). The response to subsequent challenge irradiation of the reporter cells was measured by the colony assay. While serum from unirradiated control mice had no effect on the radiosensitivity in the reporter cells, serum from mice given 0.3 Gy/h or 0.03 Gy/h for 1 h removed HRS and also increased survival in response to doses up to 5 Gy. The effect lasted for at least 15 months after irradiation. TGF- β 3 neutralizer added to the medium containing mouse serum inhibited the effect. Serum from mice given irradiation of 0.3 Gy/h for 1 h and subsequently treated with iNOS inhibitor 1400W did not affect radiosensitivity in reporter cells; neither did serum from the unirradiated progeny of mice given 1h LDR whole-body irradiation.

Key words: TGF- β 3, low dose-rate irradiation, low dose hyper-radiosensitivity, iNOS, whole-body irradiation

INTRODUCTION

Cells exhibiting low dose hyper-radiosensitivity (HRS) are characterized by a high sensitivity to radiation doses below ~0.5 Gy (cell line dependent) which is followed by a more radioresistant response per unit dose (IRR) in the dose range ~ 0.5-1 Gy.

HRS was first identified *in vitro* in 1993 (Marples and Joiner 1993) after having been observed in mouse skin in 1986 (Joiner and Denekamp 1986) and in mouse kidney in 1988 (Joiner and Johns 1988). HRS has been observed in cells given acute proton and pi-meson irradiation (Marples *et al.* 1994; Marples *et al.* 1996; Schettino *et al.* 2001) as well as in cells given high LET neutrons at a low dose-rate (Dionet *et al.* 2000)

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and appears to be the default response for all radiation qualities in both tumour and normal cell lines (only ~20% of the >50 tested cell lines failed to exhibit HRS (Marples *et al.* 1997; Joiner *et al.* 2001; Krueger *et al.* 2007)). IRR, on the other hand, is only observed after low LET irradiation and only in repair-competent cells lines (Marples and Collis 2008).

Exposing HRS-proficient cell cultures to a high dose-rate (HDR) priming dose of 0.2-0.3 Gy transiently abolishes the HRS-response to subsequent challenge irradiation (Marples and Joiner 1995; Joiner *et al.* 1996; Wouters and Skarsgard 1997; Short *et al.* 2001; Edin *et al.* 2007), however, previous studies from our laboratory have shown that by reducing the dose-rate to 0.3 Gy/h (LDR), the elimination of HRS persisted for a seemingly unlimited period and even if the dose-rate was reduced to 0.06 Gy/h (also 1h of irradiation) the same effect was seen (Edin *et al.* 2007; Edin *et al.* 2009a; Edin *et al.* 2009b).

In certain cell lines radiation causes cells to produce signals released into the environment, which can cause cytotoxic bystander effects in unirradiated cells exposed to medium from the irradiated cells (Mothersill and Seymour 1998; Lyng *et al.* 2000; Seymour and Mothersill 2000; Lyng *et al.* 2002). It appears that repair-deficient cells produce bystander effects after γ -irradiation which result in more cell death than wild-type repair-competent cell lines do (Little *et al.* 2003; Nagasawa *et al.* 2003; Mothersill *et al.* 2004). On the other hand, medium harvested from repair-competent cells γ -irradiated with a small dose seems to be capable of inducing an adaptive response to subsequent irradiation in unirradiated cells (Iyer and Lehnert 2002; Mitchell *et al.* 2004; Mothersill *et al.* 2006; Maguire *et al.* 2007). There also seems to be an inverse relationship between the ability to produce cytotoxic signals and the presence of HRS in cell lines (Mothersill *et al.* 2002).

Our previous studies showed that HRS was removed transiently in unprimed cells by transfer of filtered medium from LDR primed cells or of medium conditioned by unirradiated cells and subsequently given LDR priming without cells present (Edin *et al.* 2009a). Matsumoto *et al.* (2009 and 2011) earlier proposed a mechanism for radiation-induced bystander adaptive response originating in DNA-damaged sites involving nitric oxide (NO), transforming growth factor beta 1 (TGF- β 1) and reactive oxygen species (ROS). The mechanism induced by LDR priming and affecting the presence of HRS was shown to depend on TGF- β 3 and iNOS activity (Edin *et al.* 2013; Edin *et al.* 2014).

Cells directly exposed to LDR priming were found to have a permanently increased level of intracellular TGF- β 3 which seemed to protect the cells against subsequent radiation-damage. When cell cultures were exposed to recombinant TGF- β 3, a concentration of 0.001 ng/ml induced resistance only to doses in the HRS-range in line with observations of LDR primed cells, while increasing the concentration to 0.01 ng/ml resulted in

increased resistance also to doses above the HRS-range (Edin *et al.* 2014). The mechanism of maintaining the non-HRS response in LDR primed cells was found to depend on iNOS activity and HRS was recovered in cells treated on 3 consecutive days with iNOS inhibitor 1400W (Edin *et al.* 2013).

In the present study, it was investigated if the same mechanism could be induced *in vivo*. DBA/2 mice with normal microbial flora were given whole-body irradiation for 1 h at low dose-rates of 0.3 or 0.03 Gy/h. Reporter systems have been used for many years to study clastogenic or other bystander effects of blood or explants from whole body irradiated humans or animals (Goh and Sumner 1968; Emerit *et al.* 1980; Singh *et al.* 2011). We used our well-established *in vitro* model with T-47D and T98G cells as reporter system and it was found that serum from irradiated mice, but not from unirradiated controls, removed HRS and increased radiosensitivity to all doses examined in the reporter cells through a mechanism dependent on TGF- β 3. Inhibiting iNOS activity in the mice reversed the mechanism, which maintained the ability of the mouse serum to affect radiosensitivity in reporter cells.

MATERIALS AND METHODS

Mouse model and *in vivo* irradiation

Inbred nonspecific pathogen free DBA/2 mice (Anver and Haines 2004) were used. After sex segregation at weaning they were kept 8 per cage and fed mouse pellets and water *ad libitum*. The animal room, the animal care and the experimental use of the animals were in accordance with the Slovak Ethical rules.

The animals entered experiments when 3 months old. Animals were placed in a circular pen with wedge shaped individual rooms, giving each animal an identical exposure to the radiation. The dose rates used were 0.3 and 0.03 Gy/h and the duration of irradiation one hour.

A [^{60}Co]-source (Theratron Elite 100, Best Theatronics, Canada) was used. The received dose was measured with total cerrobend filtration in solid PMMA phantom by optimal SSD (-217 cm) for a dose rate of the 0.3 Gy/h with field size 35x35 cm². The time for application of 0.3 Gy was in the interval 59.6-60.3 min. The treatment parameters were corrected at each application to ensure the mice received the demanded dose rate. The applied dose was controlled by a ionization chamber (FC-65G, Wellhöfer, Germany) calibrated by Standard laboratory IAEA Siebersdorf.

Eight mice were LDR irradiated for 1h and a similar number of age matched non-irradiated mice were used as controls in each experiment. Mice LDR irradiated with 0.3 Gy were sacrificed and bled at different time points from one day to 15 months after irradiation, the mice LDR-irradiated with 0.03 Gy were bled after one month.

Furthermore eight mice were LDR irradiated with 0.3 Gy and a week later given the selective iNOS inhibitor 1400W ((N-(3)aminobenzyl)benzyl)acetamine), dihydrochloride, Sigma-Aldrich, Germany), 10mg/kg intraperitoneally in 0.1 ml 0.9 per cent saline every 6th hour for 5 consecutive days. The mice were sacrificed and bled 24 h after the last inoculation. The doses and intervals were based on a study of Nemec et al. (2010).

Reporter cell culture

Human breast cancer cells of the line T-47D (approximate doubling time 30 h) and human glioblastoma cells of the line T98G (approximate doubling time 20 h) (purchased from ATCC, LGC Standards AB, SE-501 17 Borås, Sweden) were grown as monolayer cultures in RPMI 1640 medium (JRH Biosciences, Kansas, USA), supplemented with 10% fetal calf serum, 2mM L-glutamine (Sigma-Aldrich, Germany), 200 units l⁻¹ insulin, and 2% penicillin/streptomycin at 37°C in air containing 5% CO₂.

Mouse serum (4%) was added to RPMI with 6% foetal calf serum and the medium was filtered (0.22 µm) before it was transferred to the reporter cells. The reporter cells were exposed to the mouse serum for 24h before being plated for colony formation in fresh medium (10% calf serum). 200 cells per flask were seeded except for those given 5 Gy, in which 400 cells were seeded. The plating efficiencies for T-47D cells were 0.72 ± 0.10 for cells receiving serum from unirradiated mice and 0.70 ± 0.17 for cells receiving serum from irradiated mice. For T98G cells the plating efficiencies were 0.48 ± 0.07 for cells receiving serum from unirradiated mice and 0.48 ± 0.06 for cells receiving serum from irradiated mice.

2 µg/ml TGF-β3 neutralizer (AF243-NA, R&D systems, Minneapolis, MN, USA) was added to the medium containing mouse serum before transfer to reporter cells.

Irradiation procedures

The cells were seeded for colony formation in fresh medium after 24h with the mouse serum and challenge irradiated 16-20h later in T25 flasks (Nunc, Roskilde, Denmark) from below with a [⁶⁰Co]-source (Theratron 780-C, MDS Nordion, Ottawa, ON, Canada). The irradiation field was 40 × 40 cm² and the source-to-flask distance was 80 cm giving a high dose-rate (HDR) of 30 Gy/h. The cell flasks were placed on a hollow water-filled perspex plate, which was heated to maintain 37°C in the medium of the flasks by circulating water from a water bath (Grant Instruments, Cambridge, England).

Cell Survival

Colonies containing more than 50 cells were scored as survivors. In order to account for the increased multiplicity per colony-forming unit during the interval between seeding and irradiation, an extra flask was seeded for each experiment. This flask was fixed at the time the challenge dose was delivered and the multiplicity was differentially counted. The mean value was calculated and used for correction for single-cell survival in flasks with plating efficiency below 1 according to a formula previously published (Elkind *et al.* 1965). We have previously shown that the measured multiplicity did not depend on cell number seeded for T-47D cells (Edin *et al.* 2009b). These cells also did not show any bystander cell death (Edin *et al.* 2009a). We therefore assume independent survival of the cells in the microcolony and have applied corrections for multiplicity for T-47D cells. We have not tested independent survival for T98G cells and therefore present the data both with and without multiplicity correction (Figure 1 B&C).

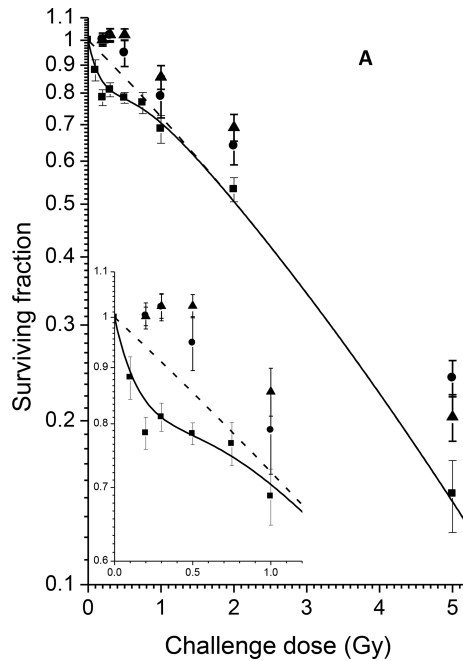


FIGURE 1A. Colony survival as a function of an acute challenge radiation dose measured in reporter T-47D cells that received medium with serum pooled from 8 female mice that were either sham irradiated (■) or exposed to γ -irradiation for 1h at 0.3 Gy/h (●) or 0.03 Gy/h (▲) 4 weeks prior to harvest. The bars represent standard errors of the mean (SEM) for 6-8 individual experiments. The curves represent model-fits to the data from cells exposed to serum from unirradiated mice by the IR-model (solid line) and the LQ-model (dashed line).

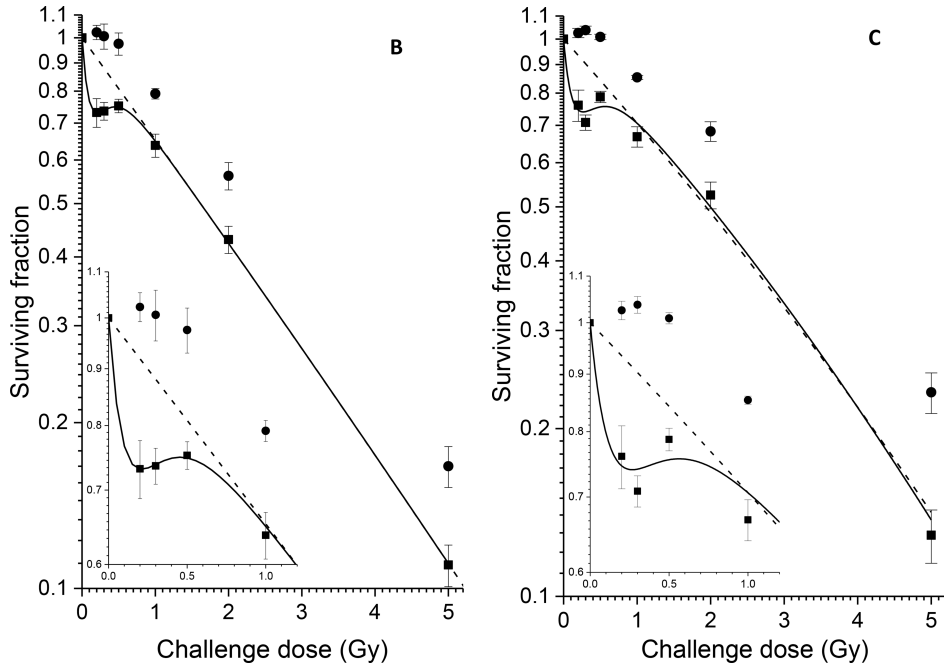


FIGURE 1B & C. Colony survival as a function of an acute challenge radiation dose measured in reporter T98G cells that received medium with serum pooled from 8 mice that were either sham irradiated (■) or exposed to γ -irradiation for 1h at 0.3 Gy/h (●) 1 week prior to harvest. The bars represent standard errors of the mean (SEM) for 3 individual experiments with serum from female mice (●) and 8 individual experiments of which 3 with serum from male and 5 from female mice (■). The curves represent model-fits to the data from cells exposed to serum from unirradiated mice by the IR-model (solid line) and the LQ-model (dashed line). (B) Data with multiplicity correction. (C) Data without multiplicity correction.

Statistical analysis

All experiments were repeated at least 3 times. In the colony assay five flasks for each dose and ten for controls were used. Within each experiment, the arithmetic means were calculated weighing the errors. All error values and bars are standard error.

The curves represent fits (Origin 9.1, OriginLab, Northampton, MA, USA) to data from T-47D cells recipient of serum from unirradiated mice and irradiated with a single acute dose by either the induced repair (IR) model (solid line) or the linear quadratic (LQ) model (dashed line) using the method of least-squares and weighing the errors. For the fit by the LQ-model, only data above 1 Gy were used.

The LQ-model is described by the equation:

$$S = \exp(-\alpha d - \beta d^2) \quad (1)$$

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

In the IR-model (Joiner and Johns 1988), α is replaced by:

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

where d is dose, α_r is the value of α extrapolated from the high dose LQ response (equation 1), 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.

Statistical analyses were performed using paired t -test to compare mean values from each dose point below 1 Gy; P values <0.05 were considered significant.

RESULTS

Serum collected from either whole body irradiated mice (0.3 Gy/h for 1h) or unirradiated mice was tested on 2 HRS-positive reporter cells lines, T98G and T-47D, for the effect on radiosensitivity (Figure 1). No change was observed in the response to challenge irradiation in cells exposed to serum from unirradiated mice, while serum from mice irradiated with 0.3 Gy/h or 0.03 Gy/h for 1h and harvested 4 weeks later removed HRS in T-47D reporter cells (0.3 Gy/h: $p<0.01$; 0.03 Gy/h: $p<0.001$ compared to unirradiated mice for reporter cell doses of 0.2 and 0.3 Gy). The same result was seen when the serum was harvested 18h or 1 week after irradiation with 0.3 Gy/h (data not shown). Cells of another HRS-positive cell line, T98G, were tested as reporter cells and the same effect of mouse serum was seen in these cells (Figure 1 B&C). In contrast to previous observations with irradiated cells, the response to doses above the HRS-range was also affected by the mouse serum, indicating a higher concentration of TGF-β3 in the mouse serum than secreted by LDR primed cells (Edin *et al.* 2014).

That TGF-β3 activity induced in the mouse serum is a vital ingredient for this effect was confirmed by adding TGF-β3 neutralizer to the medium containing serum from irradiated mice before transfer to the reporter cells. The presence of TGF-β3 neutralizer completely inhibited the effect of the mouse serum on HRS in the reporter cells (Figure 2) ($p<0.05$ compared to same serum without TGF-β3 neutralizer for reporter cell doses of 0.2 and 0.3 Gy). This experiment was performed with serum from mice that were LDR irradiated 4 weeks before sacrifice and bleeding. A similar experiment using TGF-β1 neutralizer was done for control. This had no effect on the ability of the mouse serum to remove

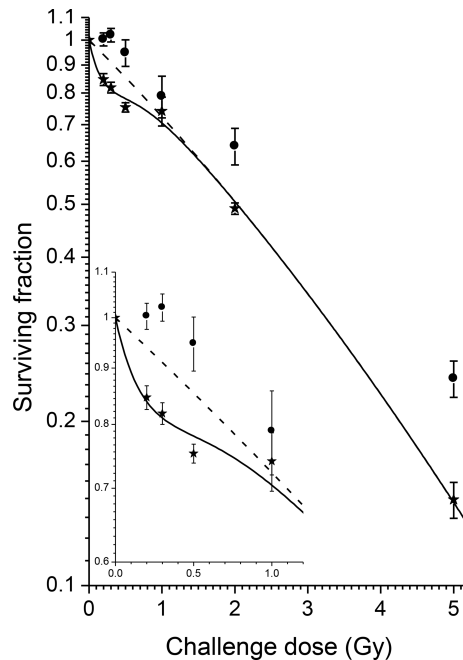


FIGURE 2. Colony survival as a function of an acute challenge radiation dose measured in reporter T-47D cells that received medium with TGF- β 3 neutralizer in addition to serum pooled from 8 female mice that were exposed to γ -irradiation for 1h at 0.3 Gy/h 4 weeks prior to harvest (★). Data without TGF- β 3 neutralizer are replotted from Figure 1 as reference (●). The curves represent model-fits to the data from cells exposed to serum from unirradiated mice by the IR-model (solid line) and the LQ-model (dashed line). The bars represent standard errors of the mean (SEM) for 3 individual experiments.

HRS in the reporter cells (data not shown). Further experiments showed that serum harvested even 15 months after 1h LDR irradiation increased radio resistance and removed HRS in reporter cells (Figure!3) ($p < 0.01$ compared to unirradiated mice of same age for reporter cell doses of 0.2 and 0.3 Gy). Serum from unirradiated control mice of the same age did not remove HRS.

Mice were then treated with 1400W intraperitoneally every 6th hour for 5 days starting 1 week after whole-body LDR irradiation at 0.3 Gy/h for 1h. The serum harvested from these mice did not affect HRS in the reporter cells ($p < 0.02$ compared to untreated mice exposed to same irradiation for reporter cell doses of 0.2 and 0.3 Gy) (Figure!4).

Since increased TGF- β 3 activity appeared to be permanent after LDR irradiation, the offspring of LDR irradiated animals were tested, but serum from these mice did not affect HRS in reporter cells (only reporter cell dose of 0.3 Gy significant compared to irradiated mice from Figure 1 (0.3 Gy/h) $p = 0.005$) (Figure!5).

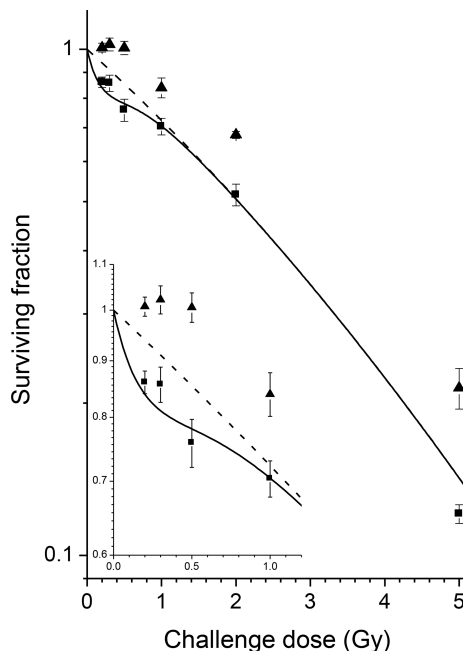


FIGURE 3. Colony survival as a function of an acute challenge radiation dose measured in reporter T-47D cells that received medium with serum pooled from 8 male mice that were either sham irradiated (■) or exposed to γ -irradiation for 1h at 0.3 Gy/h (▲) 15 months prior to harvest. The curves represent model-fits to the data from cells exposed to serum from unirradiated female mice by the IR-model (solid line) and the LQ-model (dashed line). The bars represent standard errors of the mean (SEM) for 3 individual experiments.

DISCUSSION

The experiments presented here indicate a long-lasting activation of TGF- β 3 in mice after whole-body LDR irradiation for 1h, in line with previously findings in 2 cultured human cancer cell lines. Serum collected from unirradiated mice did not affect the radiosensitivity of the reporter cells regardless of the age of the mice. However, just 4%, added to the medium, of serum from mice exposed for 1 h to whole-body irradiation at a dose-rate of 0.3 Gy/h removed HRS and increased radio resistance in the reporter cells even if the serum was harvested 15 months after the whole-body exposure.

Serum harvested 3 months after the mice had been irradiated with 1/10 of the dose-rate (0.03 Gy/h) for 1 h also removed HRS and increased resistance to larger doses. This is in concordance with previous results in which T98G cells were LDR primed and later challenge irradiated. In these cells it was shown that lowering the priming dose-rate to 0.06 Gy/h but still keeping the exposure time to 1h also removed HRS, while (priming) irradiating for 15 min at 0.19 Gy/h did not affect HRS (Edin

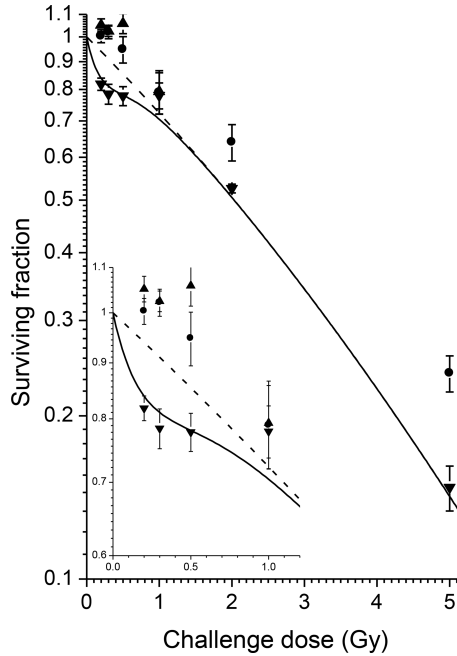


FIGURE 4. Colony survival as a function of an acute challenge radiation dose measured in reporter T-47D cells that received medium with serum pooled from 8 female mice that were exposed to γ -irradiation for 1h at 0.3 Gy/h and a week later given iNOS inhibitor 1400W i.p. every 6th hour for 5 consecutive days prior to sacrifice (▼). Reporter cells given serum from mice irradiated and sacrificed at the same time but without 1400W treatment (only doses up to 1 Gy) (▲) and from mice sacrificed 4 weeks after irradiation (replotted from Figure 1) (●) are shown as references. The curves represent model-fits to the data from cells exposed to serum from unirradiated mice by the IR-model (solid line) and the LQ-model (dashed line). The bars represent standard errors of the mean (SEM) for 3 individual experiments.

et al. 2013). Thus, priming exposure time seems to be important rather than the total dose.

TGF- β 3 was previously found to be a key factor in removal of HRS after LDR irradiation of cells and an increased cytoplasmic level of active TGF- β 3 was demonstrated in LDR primed cells (Edin *et al.* 2014). The present experiments corroborate these findings in an *in vivo* model. The inhibitory effect of adding a neutralizing TGF- β 3-antibody to the mouse serum on the reporter cell response is a strong indication that some (or all) cells in mice exposed to 1h of LDR whole body irradiation secrete active TGF- β 3. The effect of the TGF- β 3 neutralizer in cells exposed to recombinant TGF- β 3 was previously confirmed by ELISA (Edin *et al.* 2014).

We have so far failed to measure TGF- β 3 levels by ELISA both in mouse serum and cell medium. In previous experiments where recombinant TGF- β 3 was added to cells before they were challenge irradiated, it was found that the concentration of TGF- β 3 necessary for the effect was

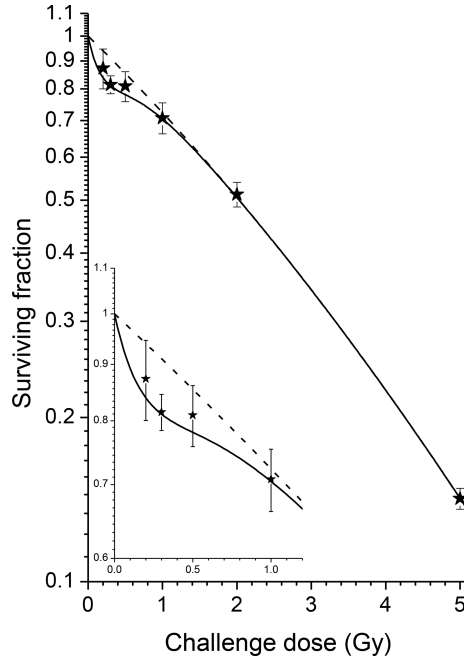


FIGURE 5. Colony survival as a function of an acute challenge radiation dose measured in reporter T-47D cells that received medium with serum pooled from the offspring of mice (both parents) exposed to γ -irradiation for 1h at 0.3 Gy/h (\star). The curves represent model-fits to the data from cells exposed to serum from unirradiated female mice by the IR-model (solid line) and the LQ-model (dashed line). The bars represent standard errors of the mean (SEM) for 3 individual experiments.

only 0.001 ng/ml. This is far below the detection limit of ELISA, which we found to be ~ 0.1 ng/ml (Edin *et al.* 2014).

Whereas only the response to doses in the HRS-range (<0.5 Gy) was affected in LDR primed cells or cells recipient of medium from LDR primed cells (Edin *et al.* 2009a; Edin *et al.* 2009b), the radio resistance of reporter cells exposed to serum from LDR irradiated mice was also increased in response to doses above the HRS-range. However, when the cells in culture were exposed to recombinant TGF- β 3 at a concentration of 0.01 ng/ml prior to challenge irradiation, increased resistance to radiation doses of 2 and 5 Gy was also observed. In contrast, when the cells were exposed to recombinant TGF- β 3 at a concentration of 0.001 ng/ml before challenge irradiation, only the response to doses in the HRS-range was affected (Edin *et al.* 2014). This may be interpreted that the serum concentration of the irradiated mice is in the order of at least 0.01 ng/ml. However, the failure of ELISA measurements of TGF- β 3 in mouse serum indicate a concentration below the sensitivity of the assay (~ 0.1 ng/ml (Edin *et al.* 2014)).

The permanent elimination of HRS in LDR primed cells was found to be reversed by treatment with either iNOS inhibitor 1400W (Edin *et al.* 2013) or peroxynitrite scavenger uric acid (Edin *et al.* 2014). This indicates a mechanism for maintaining the non-HRS response depending on peroxynitrite as a product of NO produced by iNOS and superoxide due to radiation induced hydrated electrons reacting with oxygen (Edin *et al.* 2014). The response in mice to LDR irradiation was seen to last at least 15 months after the exposure but could be reversed by injections of iNOS inhibitor 1400W suggesting that iNOS is involved in maintaining TGF- β 3 activation in LDR irradiated mice as in LDR primed cells. The response to LDR irradiation was not transferred to the progeny of the irradiated mice.

The present data are in line with a study by Cai and Wang (1995) in which male mice were adapted by LDR whole body irradiation at 27.6 mGy/ day for 40 days. Another 40 days later the mice were given a HDR challenge dose of 1.5 Gy. A significant lower number of chromatid breaks and exchanges was observed in sperm cells from the mice given the first LDR irradiation compared to sperm cells from mice not pre-irradiated. However, no effect of paternal LDR irradiation was seen in the offspring when the response to HDR challenge irradiation was measured as chromatid changes in bone marrow cells and spermatocytes or as radiation induced cell killing of splenocytes.

The protective effect of TGF- β 3 against large doses of radiation has previously been seen by Potten *et al.* (1997) and Booth *et al.* (2000) as a four to six-fold increase in intestinal crypt survival in mice irradiated with doses between 8 and 16 Gy of 300 keV x-rays to the abdomen. In their study TGF- β 3 was administered before irradiation as several i.p. injections over a 24h period and animal survival time was measured in response to 15.8 and 17.4 Gy. While no protection was seen in response to the largest dose, TGF- β 3 provided significant protection against gastrointestinal death after 15.8 Gy with 95% animals surviving for more than 30 days against only 35% surviving for 12 days in the untreated group. However, when one dose of TGF- β 3 was given after irradiation, the protection induced by the doses given before irradiation was abrogated and the crypts were sensitized to radiation instead. The effects of TGF- β 3 were attributed to reversible cell cycle arrest.

In our cell model, we have not found any cell cycle arrest in cells exposed to 0.01 ng/ml TGF- β 3 for 24 h and thereafter cultured for another 20h without TGF- β 3 (the treatment of the reporter cells before challenge irradiation) (Edin *et al.* 2014). Potten *et al.* (1997) used 4 or 5 doses of 2.5 μ g TGF- β 3 i.p. For a mouse of 25 g this corresponds to about 100 ng/ml per dose depending of local uptake and distribution.

This study suggests that prior exposure to LDR irradiation may affect the response to subsequent radiation treatment or accidental radiation exposure through a mechanism involving TGF- β 3 and iNOS activity.

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