Identification of genes responsible for radiosensitivity and their mechanism

PhD theses

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1. Introduction

Radiation levels what people receive today consist of background radiation from natural sources from the environment and the surplus from different human activities.

Biological effects of electromagnetic radiations, radiosensitivity

The focus of my thesis are the gamma and X-rays, extensively used both for diagnostic and therapeutic purposes. Following radiotherapy, 5-10 % of the patients develop severe early or late side-effects. The biological effects of RT depends on the type of radiation, the dose received, the type of the target cell (resting or fast dividing) and on the organelles hit and damaged by the radiation beam. The agents which are able to modulate the radiation damage, hence the individual radiosensitivity are mostly the proteins with a role in DNA damage recognition and repair, or in the cell cycle regulation.

1.1. Studied radiation-response genes

In our earlier experiments with fibroblast cells we identified by in vitro experiments several radiation-response genes, which altered their expression both after low and high doses of irradiation. In the current study we have chosen two of them (GDF-15 and TP53INP1) for functional studies and for studies of their role in individual radiosensitivity.

2. Objectives

Our aim was to identify radiation-response genes with a contribution to the survival of cells directly hit by radiation or neighbouring cells; and which affects the individual radiosensitivity.

Specific questions:

1. To determine the response to ionizing radiation of GDF-15 and TP53INP1 genes in fibroblasts by measuring the dose- and time dependency.
2. To measure the effects of inhibition of GDF-15 and TP53INP1 genes on the reactions of the cells to ionizing radiation

3. Methods

3.1. Used cell lines:

The origin of our primary cell lines were fibroblasts cultured from human skin biopsies (F11). From this primary cell culture we created an immortalized cell line (F11-hTERT) by stable transfection of human telomerase gene (hTERT) in the cells.

From this cell line we created several different subclones by introducing different shRNA constructs into the cells: GDF-15 silenced, overexpressing and TP53INP1 silenced lines

3.2. Gene expression studies with qPCR

We measured the functions of genes from (a) genomic DNA by isolating RNA from cells and transcribing into cDNA and (b) the deletions of mitochondrial DNA by isolating total DNA from cells

In case of genomic DNA, cells were subjected to 2 Gy γ-irradiation and following a 2 hours incubation time, RNA was isolated from the cells, cDNA was transcribed. The working solution of PCR reaction was the following for one sample and one gene: (in 25 µl final volume:) 12.5 µl Maxima SYBR Green qPCR Master Mix, 2 µl cDNA, 1,3 µl primer pair (forward and reverse primers in concentration of 12.5 pM) and 9.2 µl water.

In case of mitochondrial DNA: DNA was directly isolated from cells and added to the other components as described above.

In bystander effect studies, the cells to be analysed were not irradiated, instead they were incubated for 2 hours in medium from irradiated cells.

PCR reactions were carried out in Rotor-Gene, Corbett-3000 real-time PCR System machine. The ΔΔCt values were used to calculate relative gene expressions. (CT –cycle threshold)

3.3. Validation of gene silencing efficiency at protein level by ELISA

By ELISA (Enzyme-Linked Immunosorbent Assay) method, the secreted GDF-15 protein was measured from supernatants of GDF-15 mutated cells. One million cells per 25 cm^2 culture dish were seeded, and the medium was collected 24, 30 or 48 hrs after seeding. This medium was further concentrated and used for measurements.

3.4. Studies of radiosensitivity

The survival of cells after irradiation was measured by colony forming tests. The surviving fraction (SF) is the number of colonies of radiated cells compared to colony numbers from non-irradiated numbers.

3.5. Cell division, cell cycle analysis

We have studied how gene silencing affects the radiation induced cell cycle processes. Propidium iodide staining was used, which is bound to DNA by a vibrant signal during flow cytometer testing. Depending on the cell cycle, the actual DNA content of the cells is different, as is indicated by the amount of dye (interlaced) that is bound to it. Cells were collected at 6, 24, 48 and 72 hours after irradiation with 2 Gy, and after dyeing DNA content of the cell cycle was measured with flow cytometer (FACS).

3.6. Studies of autophagy

There were two ways to measure the degree of radiation induced autophagy: 1.) With Acridine Orange staining, single cell suspension was seeded to cover plates and it was irradiated with 0 and 6 Gy and 48 h after they were incubated with complete medium containing 1 μg / ml of Acridine Orange. Fluorescence microscopy was used to calculate the number of orange red autophagic vacuoles.

2.) With the flow cytometer, the difference between irradiated and non-irradiated cells was monitored by immunofluorescence of the LC3 molecule involved in autophagic vacuole formation.

3.7. Study of radiation induced senescence in modified cells

One form irradiation caused cell death is senescence. We measured the senescence by culturing F11-hTERT and shTP53INP1 cells on slides, irradiated them with 6 Gy, and after following washing and fixing steps, we incubated the cells in X-Gal solution containing (1 mg/ml 5-bromo-4-kloro-3-indolil-β-D-galaktoside), a dye staining the dying cells . The stained cells were counted under light microscope.

3.8 Measurement of DNA double strand breaks by γ-H2AX staining

A frequently used method for assessing DNA double strand breaks is the measurements of phosphorylated histone H2AX, a repair protein which is gathered to the sites of double strand breaks. We can measure them by staining with special detectable fluorescent antibodies. We studied the kinetics of the repair of DNA double strand breaks after irradiation with 6 Gy, in six different time points (directly after irradiation, and 1, 2, 4, 6, 24 h after

irradiation. Cells were permeabilized, immunostained and measured by flow cytometry.

3.9 Statistical analysis

The results are presented as mean and standard deviations of at least three independent experiments. Data were analysed using unpaired t-test and one-way ANOVA (to compare multiple groups) with $P<0.05$ conferring statistical significance. The calculations were made with the GraphPad (GraphPad Prism 5.0; Software, USA) software.

4. Results

4.1. Changes in GDF-15 gene function in irradiated fibroblast cells and in bystander cells

The GDF-15 gene expression of directly-irradiated cells increases with a dose and the difference from the control is significant at 0.5 and 2 Gy (1.677 +/- 0.02 and 1.946 +/- 0.49). In the non-irradiated recipient cells there was no difference compared to the control cells at any of the doses.

4.2. Inhibition of GDF-15 protein production by lentiviral vector mediated shRNAs

Using five shRNA constructs, we obtained several fibroblast clones producing varying amounts of GDF-15. The MTP1/4 clone expresses the lowest amount of GDF-15 (33 %) as compared to normal F11-hTERT and the MTP2/3 clone GDF-15 gene expression was (unexpectedly) higher (292 %) compared to the normal control. In parallel, it can be observed that the extent of GDF-15 expression is inversely proportional to cell survival after 2 Gy irradiation. This means that GDF-15 deficient MTP1/4 cells are more and MTP2/3 cells are less (although in this case not significantly) affected by ionizing radiation than unmodified F11-hTERT. Thus, the intensity of GDF-15 gene activity affects the radiosensitivity of the fibroblasts.

4.3. Quantitative change of GDF-15 protein in time in different silenced cells

Silenced cells produced much less GDF-15 protein (8 +/- 2 pg/ml) than normal cells (213.5 +/- 8.5 pg/ml), while in overexpressing cells GDF-15 protein production was more than six fold higher (1366.47 +/- 70.53).

Furthermore, we could also see that, the amount of cytokine production increased over time and then released into the medium is.

4.4. Time dependence of radiation-induced GDF-15 gene expression

In normal fibroblasts, the radiation-induced GDF-15 gene expression increases by irradiation and reaches the maximum 2 hours after irradiation (relative expression value to the control: 2.74 +/- 0.8) and returns to the original level after 48 hours. For gene silenced cell lines, the control group's basal GDF-15 expression is far below normal cell line's (0.33 +/- 0.1), proving the effective gene silencing. At the same time, the radiation-induced GDF-15 expression of these cells was not remarkable, and even the maximum increase of these silenced cell's GDF-15 expression did not reach the baseline of genetically unmodified fibroblasts's (0.76 +/- 0.69).

4.5. The effect of GDF-15 level changes on other irradiation-induced genes

In the normal fibroblast cell line, the expression of TP53INP1 (2.56 +/- 0.21), CDKN1A (2.61 +/- 0.28) and GADD45A (1.433 +/- 0.14) genes significantly increases 2 h after 2 Gy gamma radiation, while levels of TGF-β1 (0.998 +/- 0.09) does not change.

When the expression of the GDF-15 gene was inhibited, the expression of TGF-β1 increased (1.41 +/- 0.21) in fibroblast cells, while expression of the other 3 genes did not significantly change.

The overexpression of GDF-15 has no effect on TGF-β1, neither CDKN1A nor GADD45A gene expression, but the expression of the basic TP53INP1 increased (3.07 +/- 0.75).

The intensity of GDF-15 activity contrasts the function of TGF-β1 and TP53INP1 genes. When we investigated the dependence on GDF-15 of the

radiation-induced gene expression change, we found that for GADD45A, the 2 Gy induced increase in gene expression was not influenced by the absence or overexpression of the GDF-15 gene. However, when GDF-15 is present in a smaller amount (1.36 +/- 0.17 instead of 2.47 +/- 0.57) in fibroblast cells, neither the TP53INP1 nor the CDKN1A gene expression did not increase as much as in case of GDF-15 normally expressing cells after 2 Gy irradiation. (1.36 +/- 0.17 and 1.74 +/- 0.33 instead of 2.47 +/- 0.57). Thus, the GDF-15 gene affected the radiation-induced gene expression alterations in case of CDKN1A and TP53INP1 genes. That is, lack of GDF-15 inhibits the radiation induced stimulation of TP53INP1 and CDKN1A genes.

4.6. The effect of GDF-15 on the radiation sensitivity of fibroblast cells

The mortality rate was lower in GDF-15 overexpressing cells after irradiation compared to control cells. In cells irradiated with 2 Gy the number of colonies fell to 31 % in normal F11-hTERT cells, while in MTP1/4 to 12 %, and MTP 2/3 to 34.9 %. Radiation with 4 Gy resulted in survival of 1.7 % (MTP 1/4) and 8.4 % (MTP2/3) instead of 5.6 % in normal cells.

4.7. Assessment of bystander effects in cells silenced for GDF-15 and normal cells by measuring the mitochondrial DNA damage

Presence or absence of GDF-15 does not influence the amount of deletions in mitochondrial DNA in directly irradiated cells 3 days after irradiation. In bystander cells we found that in normal fibroblast cells the amount of deletions increases with dose, even at low doses. In case of partial deletion of GDF-15, at low doses the number of deletions in Common Deletion region does not differ from the control cells, however, after irradiation with 2 Gy increases significantly.

4.8. Effect of GDF-15 silencing on cell cycle after irradiation with 2 Gy

We could not detect significant changes in different cell cycle phases at different time points. However, the irradiation influenced the cell cycle timing: 6 hours after irradiation the majority of cells were stacked in G2 phase, the cell division has stopped. In case of partial functionality of GDF-15 this G2 stop was delayed, it occurred only after 48 hours and at less extent. In conclusion, the presence of GDF-15 is important for the regeneration of the cells, its absence reduces the time spent in G2 phase which is required for repair

4.9. Changes in expression of TP53INP1 gene expression in directly irradiated and not directly irradiated human fibroblasts

Both in directly irradiated and bystander cells the transcription of the gene increased, the difference was significant even after 0.5 Gy.

4.10. Time dependent changes in expression of TP53INP1 gene after irradiation with 2 Gy

In cells irradiated with 2 Gy the transcription of TP53INP1 gene peaked at 2 hours after irradiation, then decreased gradually, but even after 48 hours the changes compared to non irradiated cells were still notable.

4.11. Silencing of TP53INP1 gene with a lentivirus encoding short hairpin RNA (shRNA), and the changes in the radiation response of resulting cell lines

In this silenced cell line, in non-irradiated cells the expression of TP53INP1 decreased more than 50 % compared to irradiated cells. Furthermore, the

radiation-induced expression increase showed only 0.97 +/- 0.18 instead of in normal cells (2.61 +/- 0.44).

4.12. Study of TP53INP1 dependent radiosensitivity by colony forming assay

In case of decreased TP53INP1 expression in fibroblasts, the cells became more radiosensitive. After irradiation with 2 Gy the surviving fraction decreases from 22 % to 4.9 % and after 4 Gy: from 3 % to 0.3 %.

4.13. Development of IR induced autophagy in fibroblast cells expressing different amount of TP53INP1 protein

The number of autophagic vacuoles in normal fibroblasts increased almost 4 times after irradiation with 6 Gy (from 2.333 +/- 1.589 % to 8.718 +/- 2.66 %) while in case of TP53INP1 silenced cells this increase was just almost double compared to non-irradiated (from 2.608 +/- 0.842 % to 5.501 +/- 1.47 %). We studied the same process also with immunostaining of LC3, a protein characteristic for the autophagic vacuoles, and similarly, we found that in the normal fibroblasts the amount of LC3 molecules was higher.

4.14. Effect of irradiation with 6 Gy on the senescence of TP53INP1 silenced cells.

Following irradiation with 6 Gy, the senescence increased both in normal and in TP53INP1 deficient cells. (72.972 +/- 3.182 % and 76.468 +/- 5.425 %). There were no significant differences in the two cell lines.

4.15. Effect of irradiation on mitochondrial DNA deletions of TP53INP1 silenced cells

The number of mitochondrial DNA deletions increased with the radiation dose. In the shTP53INP1 cells this increase was larger, it was significant both after irradiation with 0.1 Gy and 2 Gy.

4.16. The influence of TP53INP1 protein on the radiation induced changes of GDF-15, CDKN1A and GADD45A gene expression

If we looked at the effects of gene inhibition without irradiation in cell lines, we found that there is no significant difference in the gene expression of GDF-15 and CDKN1A between basic expression in the TP53INP1-normal and TP53INP1 gene-silenced cells, so the TP53INP1 silencing has no effect on gene expression of these two genes. Contrary to this, GADD45A increased when TP53INP1 was inhibited. However, when looking at differences in expression changes due to radiation, GADD45A did not have an effect on the absence of TP53INP1 but the expression of both GDF-15 and CDKN1A was not as high as in the presence of the TP53INP1 gene (2.084 +/- 0.332 instead of 3.788 +/- 0.758 and 4.166 +/- 0.867 instead of only 2.516 +/- 0.226)

4.17. The time-kinetics of repair of ionizing radiation induced DNA double chain breaks

In F11-hTERT and shTP53INP1 cells treated with 6 Gy, the H2AX phosphorylation at double strand breaks was measured by a flow cytometer to estimate the effect of TP53INP1 on the process of DNA repair. We have found that 1 hour after irradiation there are many defective DNAs in both cell lines, but significantly less in F11-hTERT cells than in shTP53INP1. After 2 hours, the gene silenced cell line has significantly more signs than its own

control. After 4 h the gH2AX value started to reach the control value at both cell lines, but after 24 h, the shTP53INP1 cells did still not complete the DNA repair.

5. Conclusion

Both GDF-15 and TP53INP1 genes play an important role in radiotherapy, both genes contribute to the survival of fibroblast cells following irradiation, which can be an important factor in the development of new radiation sensitizing agents and in alleviating adverse reactions due to radiation from normal tissues. This would improve the rate of healing of cancer patients, and the fewer side effects could improve their future quality of life.

5.1. We found that the expression of GDF-15 and TP53INP1 genes has increased with increasing dose of ionizing radiation on fibroblasts. The expression of the GDF-15 gene reached a maximum at 2 h after 2 Gy irradiation and then returned to the normal pre-irradiation value after 48 hours. At the same dose in case of TP53INP1 gene we also measured the maximum increase after 2 hours, which showed higher values than in control cells even 48 h after irradiation.

It is true for both genes that their defective function increased the radiosensitivity of fibroblast cells.

5.2. The function of CDKN1A and GADD45A genes was not influenced by GDF-15 (neither if it was inhibited nor when overexpressed). The expression of TGF-β1, however, increased in fibroblast cells when the expression of the GDF-15 gene was inhibited. The TP53INP1 gene function was enhanced by the overexpression of GDF-15.

The absence of GDF-15 influenced the radioresponse of CDKN1A and TP53INP1 genes 2 hours following 2 Gy irradiation, furthermore on its absence the 2 Gy-induced cell cycle inhibition was time-delayed. In the directly irradiated cells the number of mitochondrial DNA breaks following irradiation of 2 Gy was not influenced by inhibition of GDF-15 but in

bystander cells less DNA deletion could be detected in the GDF-15 gene silenced cell line than in the normal cell line.

5.3. The absence of the TP53INP1 gene in the radiation response of fibroblast cells was found to have no effect on the degree of senescence resulting from the irradiation of 6 Gy. However, the amount of autophagic cells was less after 6 Gy irradiation in shTP53INP1 cells compared to F11-hTERT cells.

Irradiation with 2 Gy resulted in higher amount of mitochondrial DNA damage in TP53INP1 silenced cells, than in normal TP53INP1 expressing control group, and the time-kinetics of genomic DNA repair was slower and prolonged. The TP53INP1 gene has an effect on radiation response of GDF-15 and CDKN1A genes at 2 hours after 2 Gy irradiation, and not on radiation response of GADD45A.

6. Publications

Publications related to the topic of the doctoral dissertation:

1.: Sandor N, Schilling-Toth B, Kis E, Fodor L, Mucsanyi F, Safrany G, Hegyesi H. (2015) **TP53inp1 Gene Is Implicated in Early Radiation Response in Human Fibroblast Cells**. Int J Mol Sci, 16(10):25450-25465.

2.: Sándor N, Schilling-Tóth B, Kis E, Benedek A, Lumniczky K, Sáfrány G, Hegyesi H**.** (2015) **Growth Differentiation Factor-15 (GDF-15) is a potential marker of radiation response and radiation sensitivity**. Mutat Res Genet Toxicol Environ Mutagen, 793:142-149.

3.: Hegyesi H, Sándor N, Schilling B, Kis E, Lumniczky K, Sáfrány G. **Differentially expressed genes associated with low-dose gamma radiation: Growth Differentiation Factor (GDF-15) as a radiation response gene and radiosensitizing target.** In: Gomez Tejedor GG, Fuss MC. (edit.), Radiation Damage in Biomolecular System. Springer, Heidelberg; London; New York: 2012: 359-370.

Publications not related to the topic of the doctoral dissertation:

1. Schilling-Tóth B, Sándor N, Kis E, Kadhim M, Sáfrány G, Hegyesi H. **Analysis of the common deletions in the mitochondrial DNA is a sensitive biomarker detecting direct and non-targeted cellular effects of low dose ionizing radiation**. (2011) Mutat Res. 716(1-2):33-39.

2. Schilling-Tóth B, Sándor N, Walter FR, Bocsik A, Sáfrány G, Hegyesi H. (2014) **Role of GDF-15 in radiosensitivity of breast cancer cells**. Cent. Eur. J. Biol. 9(10):982-992

3. Sándor N, Walter FR, Bocsik A, Sántha P, Schilling-Tóth B, Léner V, Varga Z, Kahán Z, Deli MA, Sáfrány G, Hegyesi H. (2014) **Low Dose Cranial Irradiation-Induced Cerebrovascular Damage Is Reversible in Mice.** PLOS ONE, 9(11).

4. Hegyesi H, Lambert JR, Sándor N, Scilling-Tóth B, Sáfrány G. **Validation of Growth Differentiation Factor (GDF-15) as a Radiation Response Gene and Radiosensitizing Target in Mammary Adenocarcinoma Model.** In: Done SJ (edit.), Breast Cancer - Recent Advances in Biology, Imaging and Therapeutics, InTech, 2011 Chapter 20.