EXPERIMENTAL STUDY OF RADIOGENIC HEART DAMAGE USING *IN VITRO* AND *IN VIVO* ANIMAL MODELS

Ph.D. Thesis

Laura Kiscsatári

Supervisor:

Zsuzsanna Kahán MD, DSc

Department of Oncotherapy

University of Szeged

Doctoral School of Interdisciplinary Medicine

Szeged

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LIST OF PUBLICATIONS

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I. Laura Kiscsatári, Zoltán Varga, Andrew V Schally, Renáta Gáspár, Péter Ferdinandy, Gabriella Fábián, Zsuzsanna Kahán, Anikó Görbe
The protective effect of GHRH agonists against radiation-induced damage of neonatal rat cardiac myocytes
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II. Laura Kiscsatári, Márta Sárközy, Bence Kővári, Zoltán Varga, Nikolett Morvay, István Leprán, Hargita Hegyesi, Gabriella Fábián, Bálint Cserni, Gábor Cserni, Tamás Csont, Zsuzsanna Kahán
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OTHER FULL PAPERS PUBLISHED DURING THE PH.D. FELLOWSHIP

I. Fekete G, Újhidy D, Együd Z, Kiscsatári L, Marosi G, Kahán Z, Varga Z: **Partial breast radiotherapy with simple teletherapy techniques.** Med Dosim. 2015, **40**: 290-295.
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II. Kiscsatári L, Végváry Z, Nagy N, Széll M, Haracska L, Kahán Z: **Kifejezett sugárkárosodás régiós emlőbesugárzás után.** Magyar Belorvosi Archívum 2015, **65**: 184-188.

PUBLISHED CONFERENCE ABSTRACTS RELATED TO THE SUBJECT OF THE THESIS

I. Kiscsatári L,Varga Z, Görbe A, Morvay N, Kővári B, Ferdinandy P, Leprán I, Kahán Zs: ***In vitro* és *in vivo* állatmodellek a szív radiogén sugárkárosodásának vizsgálatára.** Magyar Onkológia 2013, **57**: (Klnsz.) 114–135.

II. Varga Z, Kiscsatári L, Marosi G, Varga L, Kelemen Gy, Kahán Zs: **Egyedüli tumorágy besugárzás teleterápiával.** Magyar Onkológia 2013, **57**: (Klnsz.) 114–135.

III. Kiscsatari L, Varga Z, Gorbe A, Morvay N, Kovari B, LepranI , Ferdinandy P, Kahan Z: P689 **Examination of radiation-induced heart damage using *in vitro* and *in vivo* animal models.** Cardiovascular Research 2014, **103**: S102–S141.

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INTRODUCTION

Radiation-induced heart damage and its clinical importance

Radiotherapy applied with curative or palliative intent, is an important treatment modality for cancer patients. Even though radiotherapy aims to kill tumor cells, it has potential side effects on the surrounding normal tissues. A substantial number of patients treated with radiotherapy suffers from severe acute or late side effects that cause deterioration of quality of life, extra cost to health care, or lead occasionally to fatal outcome. Among these, cardiotoxicity is one of the most important concerns in oncology practice. Radiation-induced heart disease (RIHD) is dose-dependent, and develops many years after the radiotherapy of thoracic tumors including breast, lung, oesophageal and childhood cancers or Hodgkin's lymphoma. Although the application of modern radiotherapy planning and delivery significantly improves the radiation protection of the heart, in many cases, the whole heart or a part of it still receives a dose sufficient to cause RIHD including ischemic heart disease, congestive heart failure, electrical conduct defects or valve abnormalities. The extent of the damage depends on the irradiated volume, the total dose, the dose/fraction, the use of cardiotoxic treatments, the individual's radiation sensitivity and age. The prevention of RIHD is essential.

The pathomechanisms of radiation-induced heart disease

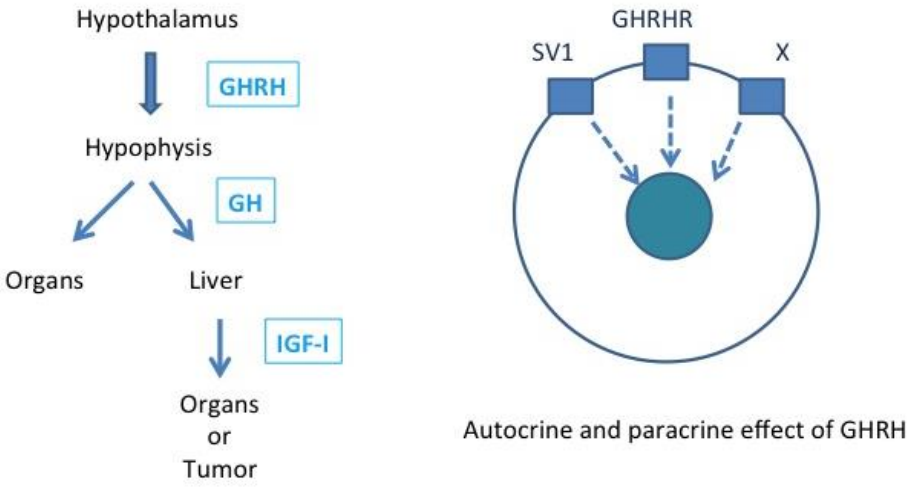
Radiation may deteriorate heart function synergically via the damage to the macrovasculature (i.e. coronary arteries), and the microvessels and cardiomyocytes (diffuse injury). Radiation-induced atherosclerotic changes of the coronary arteries indistinguishable from other etiologies, cause angina or myocardial infarct. Radiation-induced diffuse myocardium injury is time-dependent developing after even a low radiation dose. RIHD first starts with structural changes of the endothelial cells and myocytes inducing cardiac hypertrophy. Later, interstitial inflammation and progressive fibrosis take place ultimately resulting in heart failure. Although much has been learned about the mechanisms of RIHD, the exact pathomechanisms have not been fully elucidated; furthermore, the identification of its early markers and the development of specific therapy for its prevention are needed.

Growth Hormone-Releasing Hormone (GHRH)

Growth hormone releasing hormone (GHRH) is secreted in the hypothalamus, and controls release of growth hormone (GH) in the anterior pituitary section. The neuroendocrine axis plays a role in normal cell and tumor growth via directly autocrine and indirectly by paracrine

mechanisms. Previous studies have shown the presence of GHRH and its receptors in peripheral tissues, including the heart. GHRH exerts cardioprotective effect, however, its mechanism(s) is poorly understood. In many earlier publications the direct GHRH-induced cell activation repair mechanism is suggested in heart damage, independently from the GH / insulin-like growth factor 1 (IGF-1) pathways.

The GHRH and its agonistic analogs have been shown to be involved in the metabolism of reactive oxygen and nitrogen species, and in the proliferation and survival of a series of normal cells including cardiomyocytes. The administration of GHRH or its agonistic analogs (GHRHas) improved contractile recovery, ventricular remodeling during reperfusion and reduced the infarct size. These effects were absent if a GHRH antagonist was co-administered, or in case of sole treatment with growth hormone (GH), which point to the role of a GHRHR-mediated mechanism.



The possible mechanisms of the stimulating effects of GHRH and GHRH agonists on healthy organs and tumors.

AIMS

The aim of this research has been the development of *in vitro* and *in vivo* animal models appropriate for the study of the mechanisms of radiation damage, and the testing of radioprotective agents.

The following specific aims were defined:

I. The development of an *in vitro* model utilizing newborn rat ventricular myocyte (NRVM) cultures appropriate for the study of RIHD

II. The investigation of the presence of GHRH receptors in cardiomyocytes

III. The *in vitro* study of the effects of GHRH and its agonistic analogs, JI-34 and MR-356, in both irradiated and unirradiated cardiomyocytes

IV. The development of an *in vivo* model for the comprehensive study of RIHD after selective heart irradiation, with functional, imaging, biochemical and morphological endpoints

MATERIALS AND METHODS

In vitro study

NRVM were isolated from newborn rats and cultured at 37 °C and 5% CO_2. For the optimization of radiation-induced *in vitro* heart damage model, the 24-hour cultures were exposed to specified doses of radiation, and then the effect was examined at different latency times. After the irradiation, cells were cultured either in a medium containing 1% fetal bovine serum (FBS), or under serum-free conditions. The degree of toxicity of the radiation was evaluated with calcein fluorescence viability assay, cell proliferation was tested by BrdU labeling. After defining the optimal conditions of the irradiation, cells were treated with GHRH or its agonists, JI-34 and MR-356 to test protective effects.

Western blot analysis was performed to detect the presence of GHRH receptors, as well as the relative amount of phosphorylated ERK and Akt before and after the irradiation of the 24-hour cultures.

The presence of superoxide was detected using the oxidative fluorescent dye dihydroethidium (DHE), that of general reactive oxygen species (ROS) was detected by 2', 7'-dichlorofluorescein diacetate (DCFH-DA). All values are presented as mean±SEM. In the *in vitro* viability and proliferation studies one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) or Dunnet's post-hoc tests were used to evaluate differences between the groups. In case of superoxide and ROS determinations two experimental groups were compared with the t-test. Western blot results were analyzed with two-way analysis of variance (ANOVA).

In vivo study

Male Sprague-Dawley rats were divided into two groups. In the treated group selective heart irradiation was carried out with a single 2 cm 6 MeV electron field with 50 Gy dose. Body weight was measured weekly in both groups. At weeks 0, 12 and 19, cardiac morphology and function were assessed by transthoracic echocardiography and ECG. At weeks 0, 3, 8 and 26, 1 ml blood samples were collected and plasma was separated and stored at -70 °C until cytokine (GDF-15 and TGF-beta) measurements. Despite the initial plan to follow up the animals for 24-28 weeks, in the last experiment, at week 19 autopsy was carried out because of status deterioration, and the organs were removed for different examinations. Fibrosis, as a late end-point of radiation injury were visualized by means of picrosirius red staining and

analyzed with a computerized image analysis program. Histological examination of the coronary arteries was performed as well in order to analyze vascular injury. In the *in vivo* study, the statistical analysis was performed using Sigmaplot 12.0 for Windows (Systat Software Inc, Chicago, Illinois, USA). Baseline and different follow-up data including body weight and echocardiographic parameters were compared by means of repeated measures two-way ANOVA among the control and irradiated groups. All other parameters were compared by unpaired t-tests between the control and the irradiated groups. $P<0.05$ was accepted as statistically significant.

RESULTS

In vitro study

To determine optimal experimental conditions, NRVM were exposed to different radiation doses, and viability was measured following varying latency times. A significant cell loss of 50% was obtained after 10 Gy dose of radiation and latency time of 48 hours. This set of conditions was chosen and applied throughout all the experiments.

hGHRH did not significantly influence cell survival as compared to control. hGHRH was tested for its effect on cell proliferation via the BrdU incorporation assay. Cell proliferation of both irradiated and non-irradiated cells was slightly stimulated by hGHRH at a concentration of 50 nM. The effects of the GHRH agonists, JI-34 and MR-356, were first tested in unirradiated NRVM. The administration of JI-34 had no effect on cell viability at concentrations of 1-500 nM. However, in irradiated cells, JI-34 showed a protective effect at concentrations of 10 and 100 nM. Anti-proliferative effect of JI-34 was detected at 50 nM in unirradiated and at 1-50 nM in irradiated cultures. MR-356 did not show protective effect on cell proliferation. We have tested the same parameters under serum-deprived conditions to test the possible effects of the absence of 1% FBS including the avoidance of the binding of the analogs to the plasma proteins. NRVM cultures maintained in serum-free medium for 2 days contained roughly 50% less cells both in the irradiated and unirradiated plates. Again, a strong protective effect of JI-34 was detected after irradiation, at concentrations of 10 and 100 nM.

Western blot analysis was performed using an antibody able to detect pGHRHRs. A 52 kDa protein isoform was readily detected in the samples together with GAPDH used as internal control. This 52 kDa glycosylated GHRHR was expressed in both irradiated and unirradiated

cells. Irradiation caused significant decline in receptor expression after 48 hours. The treatment of cells with JI-34 did not influence the expression of GHRHRs (probably due to low dose of agonist) in irradiated or unirradiated NRVM.

The activation of RISK/SAFE pro-survival kinases was tested. The phosphorylation ratio of both ERK and AKT significantly increased after the irradiation with 10 Gy, which was substantially attenuated by JI-34 treatment.
JI-34 agonist was additionally tested for its possible effect on ROS production. Both the overall level of ROS and that of superoxide significantly increased 48 h after the irradiation. JI-34 treatment significantly decreased overall ROS production after irradiation, while MR-356 decreased its level in both irradiated and unirradiated cultures. Both agonists decreased superoxide level significantly in irradiated cultures.

In vivo study

Based on our preliminary studies, in the animal model, 50 Gy radiation dose and a follow-up time of 24 weeks seemed to be optimal for the detection of the selected biological effects.

The body weight constantly increased throughout the observation period, however, the irradiated animals showed significantly lower weight gain than the control animals. After a 19-week time of monitoring, the termination of the experiment was necessary because of status deterioration of the animals.

With the aim of identifying possible predictive biochemical markers of subsequent morphological changes, blood samples were collected for circulatory cytokine measurements. GDF-15 elevation occurred at weeks 3, 12 and 26, TGF-beta concentrations were continuously elevating during the observation period until the maximum value at week 12.

Transthoracic echocardiography was performed at weeks 0, 12 and 19. Septal, anterior and posterior wall thicknesses were significantly increased in the irradiated group as compared to the control group referring to left ventricular hypertrophy. E/e' was significantly increased and e' velocity was significantly decreased in the irradiated group as compared to the control group referring diastolic dysfunction.

At autopsy, the most common finding was the presence of extensive pleural fluid (11.3±1.7 ml, n=10) in irradiated animals. Neither abnormal macroscopic heart changes (including the

large vessels, the valves, the coronary arteries etc.) nor sign of radiation pneumonitis nor lung fibrosis were visible, confirming the selectivity of heart irradiation.

In the irradiated group, the tibia length and the weight of various organs, including the heart, was significantly smaller than in the control group (1.3±0.06 vs. 1.7±0.07 g, $p<0.05$).

Conventional morphological examinations of the pericardium, myocardium, vessels, perivascular, subendocardial, subepicardial areas and the coronary arteries, or the lungs, failed to reveal abnormalities in irradiated rats. At histological examination 19 weeks after the irradiation, extensive fibrosis was detected in all parts of the myocardium with picrosirius red staining, readily measurable wih our computer-assisted method. However, no significant vascular changes were found in the branches of the coronary arteries or the aorta.

NEW FINDINGS

The agonistic analogs of GHRH, JI-34 and MR-356 reduce radiation-induced cell loss in *in vitro* cultured NRVM, the mechanisms of action include the attenuation of the phosphorylation ratio of ERK and AKT and the reduction of radiation-induced superoxide production. Based on these preliminary findings, the agonistic analogs of GHRH should be tested *in vivo* as potential protective agents against radiogenic heart damage.

An animal model for the comprehensive study of radiation heart damage has been developed. The identification of biochemical, functional and morphological paremeters may serve as predictive markers or study endpoints in future experiments. Already collected samples provide possibility to further investigate the pathomechanisms of radiation damage. The model may be used for testing potential protective agents for the prevention of heart sequelae.

CONCLUSIONS

Our *in vitro* experiments suggest a role for GHRH and its receptors in response to irradiation in cardiomyocytes. The agonistic analogs of GHRH JI-34 and MR-356 exert protective effects in irradiated NRVM cultures hence the study of GHRH analogs in *in vivo* experiments seems justified. Our NRVM radiobiology model provides appropriate system for testing other cardioprotective agents, too.

The reported selective heart irradiation *in vivo* rat model provides various readily measurable end-points for future radiobiology experiments. The short-term (circulatory inflammatory cytokines) and medium-term (heart functional abnormalities) objectives may be used to test protective agents in the same setting, while if comprehensive or long-term measures (fibrosis and other morphology endpoints) are needed, both the radiation dose is to be lowered and the follow-up time is to be extended. Ultimately this radiobiology model should be used for testing cardioprotective agents including the agonistic analogs of GHRH.

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