Recombinant human neuregulin-1β is protective against radiation-induced myocardial cell injury

QIANG ZHOU, WENBING HU, XINXIONG FEI, XUQUN HUANG, XI CHEN, DEQING ZHAO, JUN HUANG, LAN JIANG and GANGSHENG WANG

Department of Medical Oncology, Huangshi Central Hospital, Huangshi, Hubei 435000, P.R. China

Received May 19, 2015; Accepted April 5, 2016

DOI: 10.3892/mmr.2016.5207

Abstract. The aim of the present study was to investigate the role of recombinant human neuregulin-1β (rhNRG-1β) in the repair of the radiation-induced damage of myocardial cells and the underlying mechanism. Rats were divided into the radiotherapy alone group, the rhNRG-1β group (radiotherapy with rhNRG-1β treatment) and the Herceptin group (radiotherapy with Herceptin treatment), and their myocardial cells were analyzed. The morphology of the myocardial cells was observed under an optical microscope, and the expression of γ-H2AX and p53 was analyzed using immunohistochemistry and western blot analysis. Damage to the myocardial cells was identified in the three groups following radiation treatment, which was identified by cell swelling and altered morphology. The integrated optical density values of γ-H2AX in the radiotherapy alone, rhNRG-1β and Herceptin groups were 50.96±5.548, 27.63±10.61 and 76.12±2.084, respectively. The OD of the radiotherapy alone group was significantly higher than that of the rhNRG-1β treated group (P<0.0001), and the value of the Herceptin group was significantly higher than that of the radiotherapy alone group (P<0.0001). The p53 level in the rhNRG-1β group was less than that of the radiotherapy alone group (P<0.001), and was higher in the Herceptin group compared with the radiotherapy alone group (P<0.0001). Thus, rhNRG-1β can ameliorate radiotherapy-induced myocardial cell injury, predominantly by enhancing myocardial cell DNA repair, inhibiting cell apoptosis and improving myocardial function. The results of this study in myocardial cells suggest that patients with thoracic cancer may benefit from treatment with rhNRG-1β for the repair of the radiation-induced damage of myocardial cells.

Correspondence to: Professor Gangsheng Wang, Department of Medical Oncology, Huangshi Central Hospital, 293 Yiyuan Street, Huangshi, Hubei 435000, P.R. China
E-mail: wanggangsheng1@163.com

Key words: recombinant human neuregulin-1β, radiotherapy, radiation-induced damage, myocardial cell injury, epidermal growth factor receptor pathway

Introduction

Radiotherapy is an important treatment for thoracic cancer. With the development in radiotherapy technology and the rise in fractionation dosage, radiotherapy is increasingly precise; however, radiotherapy-induced myocardial cell damage remains inevitable. In addition, as the survival time of patients with malignant tumors is increasing, the problem of radiation-induced heart damage is becoming more apparent. Since the last century, radiation-induced heart damage has been identified in patients undergoing radiotherapy for Hodgkin's lymphoma and breast cancer (1). In a study of radiotherapy in breast cancer, radiation-induced myocardial cell injury was considered to be a primary factor that influences mortality rate (2). Increased cardiovascular mortality following treatment can be avoided by the use of appropriate treatment techniques. Excessive treatment, for example, with large fraction sizes and high total doses, should be avoided, and individual treatment planning should be encouraged so that the dose distribution in the myocardium can be assessed (3). However, the risk of fatality from myocardial infarction following treatment for Hodgkin disease remains high for at least 25 years. The increased risks are associated with supradiaphragmatic radiotherapy but may also be related to anthracycline and vincristine treatment (4). Exposure of the heart to ionizing radiation during radiotherapy for breast cancer increases the subsequent rate of ischemic heart disease. The rate of ischemic heart disease is positively correlated with the mean dose of radiotherapy receive; this effect begins within a few years following radiotherapy exposure and may continue for at least 20 years. Women with preexisting cardiac risk factors have greater absolute increases in risk from radiotherapy than those without (5). Compared with patients that received no treatment, the risk of mortality as a result of cardiac diseases was significantly higher in individuals who had received a cumulative anthracycline dose or an average radiation dose that exceeded 5 Gy to the heart (6). A linear correlation was identified between the average dose of radiation to the heart and the risk of cardiac mortality (7). The relative hazard of morbidity from ischemic heart disease among patients in the radiotherapy compared with the no-radiotherapy group was 0.86, and that for mortality associated with ischemic heart disease was 0.84. The hazard rate of morbidity from ischemic

heart disease in the radiotherapy group compared with the no radiotherapy group did not increase with time from treatment (8). One study demonstrated that irradiation with 2 and 8 Gy induced modest changes in murine cardiac function within 20 weeks, however, cardiac function did not deteriorate further, despite progressive structural and microvascular damage, indicating that heart function can compensate for significant structural damage, although, higher doses of radiation eventually lead to sudden death (9). In addition, premature endothelial senescence, increased oxidative stress, decreased NO availability, and enhanced inflammation were shown to be causes of radiation-induced long-term vascular dysfunction (10). Therefore, decreasing radiation-induced heart damage will improve the quality of life and survival time of patients undergoing thoracic radiation therapy.

A previous study demonstrated that NRG-1β is important in the growth and function of the heart (11). In the adult heart, NRG-1β is expressed in cardiac endothelial cells adjacent to myocardial cells, including in the endocardium and myocardial microvasculature. NRG-1β acts on proximate myocardial cells in a paracrine manner and is critical in the differentiation, survival and damage repair of myocardial cells. An animal experiment of rhNRG-1β drugs demonstrated that neuregulin can significantly improve the cardiac function of mice with heart failure, and reverse heart failure to a degree (12). In addition a previous study indicated that Herceptin, a HER-2 antagonist (ErbB2), can increase cardiac toxicity (13). Thus, these studies demonstrated that ErbB2 downstream pathways are essential in myocardial cell function.

p53 activity is increased following DNA damage, and reduces the tolerance of cells to apoptotic factors through regulation of Bcl-2 and Bax expression, therefore p53 is able to reflect the state of myocardial cell apoptosis, and associate with EGFR pathway (14).

In the present study, immunohistochemistry, then double strand break (DSB) formation and restoration parameter, γ-H2A histone family member X (γ-H2AX), combined with DNA, as well as the cell apoptosis status. Results demonstrated that recombinant human (rh)NRG-1β has a protective effect in radiotherapy-induced myocardial cell damage, and contributes to the repair of radiation-induced DNA breaks and reduces apoptosis to alleviate the radiation-induced myocardial injury. The NRG-1β/ErbBs receptor pathway is important in myocardial cell damage healing, and exogenous rhNRG-1β can improve cardiac function.

Materials and methods

Experimental animals and myocardial cell acquisition. The study was approved by the ethics committee of Huangshi Central Hospital (Huangshi, China). In total, 12 Sprague-Dawley (SD) rats (weight, 180-122 g; age, 3 months) were obtained from the Animal Center of the Second Affiliated Hospital of Harbin Medical University (Harbin, China). Rats were housed in controlled room temperature (22-24˚C) and humidity (55-65%) with free access to food and water, and maintained under a 12:12 h light-dark cycle. Isoflurane inhalation (2%; Tocris Bioscience, Bristol, UK) was used to anesthetize and the rats were sacrificed by an overdose of anaesthesia. Following anesthesia their hearts were obtained, and 3-mm^2 sections were obtained. The tissue samples were added to a centrifuge tube for natural sedimentation. The supernatant was discarded and a combination of collagenase II and collagenase IV (Sigma-Aldrich, St. Louis, MO, USA) was added. The mixture was combined by pipetting, and the contents were then transferred to a culture dish and incubated overnight (12 h). The myocardial cells and fibroblasts were then separated by centrifugation at 500 x g for 2 min, as well as differential velocity adherence. Subsequently, the excess laminin was removed and cells were added to cover slips in 6-well plates. Then 4 μl/ml 5-FU (Sigma-Aldrich) was added to suppress fibroblast cells. Cells were incubated with 5-FU (0.4 ml) per cover slip for 6 h, and then adherent cell morphology was observed . Then, 1 ml oligodendrocyte precursor cell medium (OPCM; ScienCell Research Laboratories, Carlsbad, CA, USA) was added to each well, and culturing was continued for 36 h. When the culture state was steady the cells were then used for further experiments. The cells were divided into 3 groups: i) rhNRG-1β group (Zensun, Shanghai, China), 1 μg/kg rhNRG-1β was added to the culture medium (15) and radiation was conducted; ii) Herceptin group (Hanxiang, Shanghai, China), 0.1 mg/kg Herceptin was added to the culture medium and radiation was conducted; and iii) control, radiotherapy conducted without other treatment. The animal experiment was approved by the Medical Ethics Committee of Huangshi Central Hospital (Huangshi, China).

Cell radiotherapy, immunohistochemistry staining and image analysis. After culturing the 3 groups of myocardial cells for 3 days, the 6-well plate was sealed and cell radiation was performed using a VARIAN linear accelerator (6MeV, 5 Gy each time). The cells were cultured for 6 h for immunohistochemistry staining according to standard protocols. Monoclonal rabbit anti-rat histone γ-H2AX antibody (dilution, 1:800; cat. no. 7631; Cell Signaling Technologies, Inc., Danvers, MA, USA) and 4',6-diamidino-2-phenylindole (DAPI; Gibco; Thermo Fisher Scientific, Inc., Waltham MA, USA) were used in this step. After staining, 3 sections were selected stochastically, and 5 visual fields were randomly selected from each slide. Histone positive reaction to red, localized in the nucleus, and coincided with DAPI. Cells were counted and images were analyzed using ImageJ (version 1.47v; National Institutes of Health, Bethesda, MD, USA) to reveal myocardial nuclear bound histone in each group, and the average optical density values were calculated and analyzed.

Cell lysis and western blotting. Myocardial cells were suspended using cell lysis buffer (Bio-Rad, Richmond, CA, USA) and heated to 95˚C for 5 min to denature the proteins. Then, protein samples were collected and quantified using a Bicinchoninic Acid Protein Assay kit (Pierce, Thermo Fisher Scientific, Inc.) to ensure a consistent volume of each sample. Equal amounts of protein (30 μg) were loaded into the wells of 10% SDS-PAGE gels, along with molecular weight marker, and separated for 1 h at 100 V. The proteins were transferred to a polyvinylidene membrane using semi-dry transfer methods. The membrane was blocked with 5% w/v

bovine serum albumin (Beyotime Institute of Biotechnology, Haimen, China) in Tris-buffered saline Tween 20 (TBST) for 1 h at 4˚C with agitation. The membranes were incubated overnight with primary antibodies at 4˚C. Polyclonal goat anti-rat p53 antibody (dilution, 1:200; cat. no. AF1355) was purchased from R&D systems, Inc. (Minneapolis, MN, USA). Monoclonal mouse anti-actin antibody (dilution, 1:5,000; cat. no. ab8226; Abcam, Cambridge, UK) was used as an internal loading control. The blots were washed 3 times for 10 min with TBST, then incubated with polyclonal goat-anti rat horseradish peroxidase-conjugated secondary antibody (dilution, 1:5,000; cat. no. ab6741; Abcam) for 1 h at room temperature. The immune complexes were detected using the enhanced chemiluminescence reagents (ProteinSimple, San Jose, CA, USA) plus Western Blotting Detection system (Amersham, Aylesbury, UK) and acquired using darkroom development techniques. Band density from triplicate blots was measured using ImageJ software (version 1.47v).

Statistical analysis. All experiments were conducted in triplicate. SPSS 19.0 (IBM, Armonk, NY, USA) was used for statistical analysis and continuous variables are expressed as the mean ± standard deviation. Continuous variables that met the criteria for a normal distribution were determined using a two-tailed Student's t-test. One-way analysis of variance with Student-Newman-Keuls post-hoc test was used for comparison between groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cell myocardial pathology. The morphology of cardiomyocytes was observed by microscopy, and demonstrated that compared with the rhNRG-1β group, myocardial cell lysis was more significant in the radiotherapy group, with increased cardiomyocyte swelling and more irregular morphology. In addition, in the Herceptin group, there was increased lysis compared with the radiation group and cross striation of the myocardial cells could not be observed clearly under the microscope, as shown in Fig. 1.

γ-H2AX immunohistochemical staining of rat cardiomyocytes. Rat cardiomyocyte immunohistochemical staining was performed 6 h after radiotherapy, results showed that compared with the other two groups, the optical density (OD) of nuclear γ-H2AX staining decreased markedly in the rhNRG-1β group compared with the other two groups (Fig. 2), indicating that exogenous rhNRG-1β can enhance DNA break repair at an early stage in myocardial cells. The OD value was significantly decreased in the rhNRG-1β group compared with the control (Table I and Fig. 3; $P<0.0001$). This indicated that rhNRG-1β can improve radiation-induced DNA damage repair. In addition, the OD value of the Herceptin group was significantly increased compared with the control ($P<0.0001$), indicating that blocking the EGFR pathway can increase radiation-induced DNA damage and aggravate myocardial cell injury. This result is in line with the majority of the literature and clinical cases, and further illustrates the important role of the EGFR pathway in the function of cardiomyocytes.

Western blot analysis of p53. p53 protein was detected by western blot analysis and a significant difference in the levels was demonstrated between the 3 groups of cardiomyocytes, as shown in Fig. 4 (compared with β-actin proteins). The p53 expression levels in myocardial cells of the 3 groups after radiotherapy for 6 h are shown in Table II. The expression of p53 in the rhNRG-1β group was lower than that in radiotherapy group ($P<0.001$), however, there was increased p53 expression in the Herceptin group compared with the radiotherapy alone group ($P<0.0001$), as shown in Fig. 4.

Discussion

Radiation-induced heart disease is associated with numerous factors. It is directly correlated with the radiation dose, the irradiated volume of heart and single radiation dose. In addition, chemotherapy drugs affect radiation-induced heart damage. For example, a previous study demonstrated that the use of anthracyclines increased radiation-induced cardiac toxicity (16). Another study indicated that when 240-300 mg/m^2 doxorubicin accumulated, cardiac dysfunction increased after postoperative radiotherapy (17). Endocrine agents also affect radiation-induced heart damage, experiments have demonstrated that Herceptin may block ErbB2 downstream pathways, thereby increasing cardiac toxicity (15). Therefore, in recent years, the role of the ErbB2 pathway in myocardial cells has become a focus of heart disease research.

NRG-1β is a significant ligand in the EGFR family, it forms dimers by activating receptors, and combines with ErbB2 and ErbB4 receptors on the surface of myocardial cells. It then activates the downstream pathways, which are involved in the regulation of proliferation, development and survival of cardiomyocytes (18). NRG-1β is important not only in the course of heart development, but also in the adult cardiovascular system. One study found that the ErbB3 receptor is not expressed on the adult cardiac cell surface (19). In order to establish a similar model to adult cardiomyocytes, the cardiac cells from adult rats were selected to perform primary culture to eliminate interference of the ErbB3 receptor. In the literature, there were few examples of primary cultures from adult rat cardiomyocytes. The majority of studies of NRG-1β used neonatal rat cardiac cells for primary culture; however, neonatal rat cardiomyocytes have self-division and growth capabilities; thus are not the same as human cardiac cells. Therefore, myocardial cells from adult SD rats were selected in the present study.

γ-H2AX is a member of the histone family. Following a DSB, H2AX is phosphorylated forming γ-H2AX, which can be observed under a fluorescence microscope as γ-H2AX focal points in the nucleus. DSBs are caused by radiation therapy and integrated optical density of γ-H2AX is regarded as the gold standard to observe the repair of DSBs (20).

The present study observed the effect of NRG-1β by evaluating the state of γ-H2AX in rat cardiomyocytes after radiotherapy for 6 h. DNA DSB repair (integrated optical density of γ-H2AX indicated breaks) was shown to be increased in the NRG-1β group compared with the control group ($P<0.0001$). This indicated that NRG-1β has a protective role in radiation-induced myocardial injury. In addition, in the radiotherapy alone group, the OD value was lower than

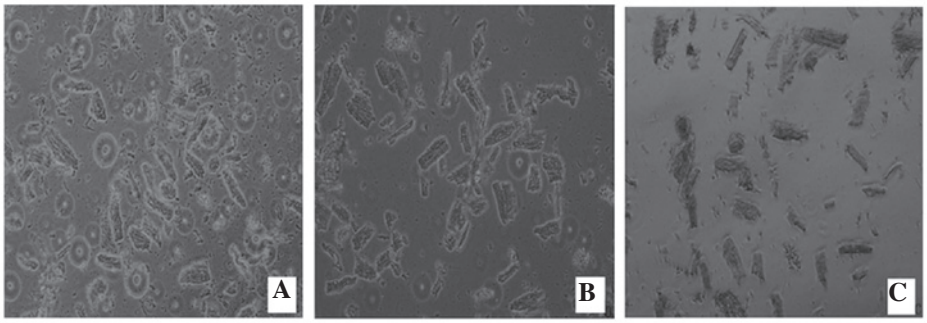

Figure 1. Pathological changes of myocardial cells observed using an optical microscope (S-PX400) in (A) the radiotherapy group, (B) the recombinant human neuregulin-1β (rhNRG-1β) group and (C) the Herceptin group. Cells in radiotherapy group demonstrated more lysis, with increased cardiomyocyte swelling and more irregular morphology compared with NRG-1β group. Cells in the Herceptin group showed more lysis and cardiomyocyte cross striation could not be observed clearly. (magnification, x400).

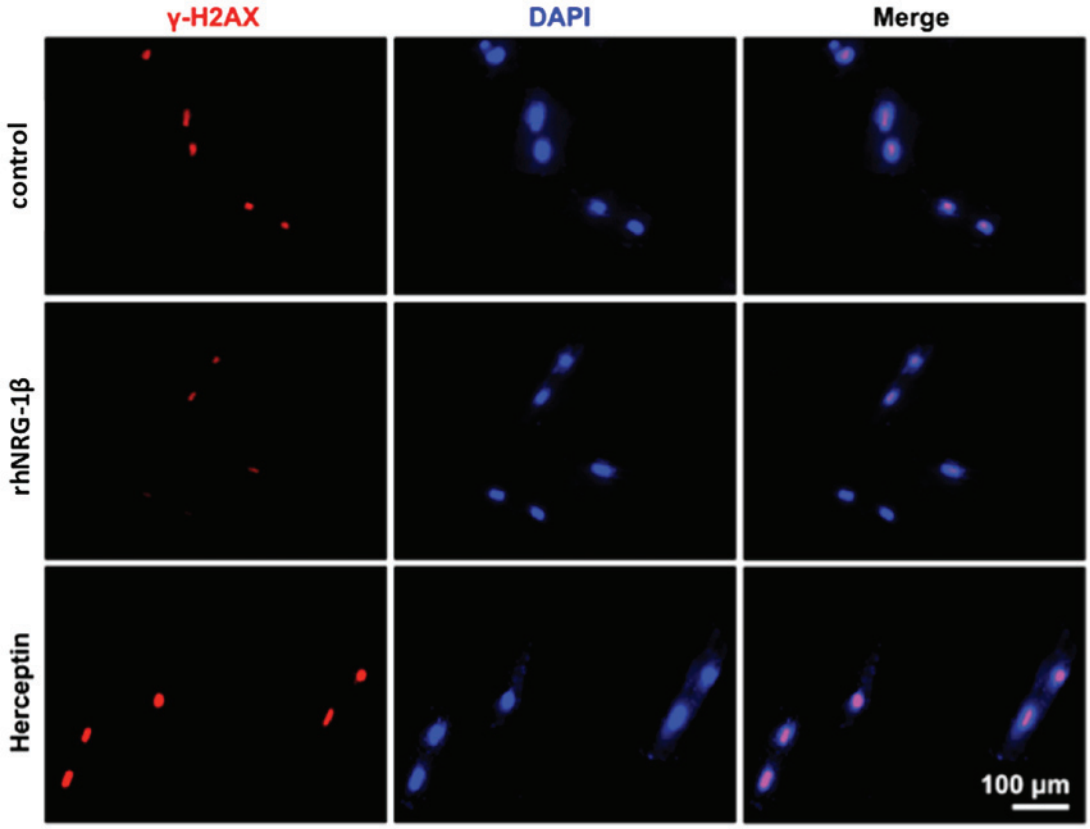

Figure 2. Immunofluorescence for γ-H2AX. After radiotherapy (6 h), the γ-H2AX expression was observed in the radiotherapy group, NRG-1β group and the Herceptin group (S-PX400) by immunohistochemistry. γ-H2AX staining (red) decreased notably in rhNRG-1β group compared with the other two groups. Merged pictures demonstrate that γ-H2AX is localized to the nucleus. γ-H2AX, γ-H2A histone family member X; rhNRG-1β, recombinant human neuregulin-1β.

that in the Herceptin group (P<0.0001), which confirmed that blocking the EGFR pathway may aggravate myocardial cell damage and reduce the ability to repair DSBs, which is consistent with the results of a number of other clinical trials (21,22). The present study and previous investigations demonstrated that the NRG-1β/ErbBs receptor pathway is important in myocardial cell damage repair, and that exogenous NRG-1β can improve cardiac function. Therefore, this suggests that NRG-1β is able to reduce myocardial cell apoptosis induced by radiotherapy, increase DNA damage repair, and regulate recent myocardial cell injury and repair in rats.

Radiotherapy causes DNA damage (23). p53 activity increases after DNA damage, and reduces the tolerance of cells to apoptotic factors through the regulation of the Bcl-2 and Bax expression, therefore p53 is able to reflect the state of myocardial cell apoptosis, and is associated with the EGFR pathway (24). Western blot analysis demonstrated that compared with the radiotherapy alone group, the p53 expression level decreased in the NRG-1β group, indicating that myocardial cell apoptosis was reduced by NRG-1β. In addition, NRG-1β protects against anthracycline-induced apoptosis by Her-4 dependent activation of the phosphoinositide 3-kinase (PI3K)/Akt pathway, and the

Table I. OD for γ-H2AX following radiotherapy for 6 h.

Group	γ-H2AX OD
Radiotherapy alone	50.96±5.548
Radiotherapy with rhNRG-1β	27.63±10.61
Radiotherapy with Herceptin	76.12±2.084

n=15 per group, mean ± standard deviation. OD, optical density; γ-H2AX, γ-H2A histone family member X; rhNRG-1β, recombinant human neuregulin-1β.

Table II. p53 expression in myocardial cells after radiotherapy for 6 h (n=45, mean ± standard deviation).

Group	p53 level
Radiotherapy alone	0.76±0.08
Radiotherapy with rhNRG-1β	0.56±0.04
Radiotherapy with Herceptin	1.09±0.06

n=15 per group, mean ± standard deviation. rhNRG-1β, recombinant human neuregulin-1β.

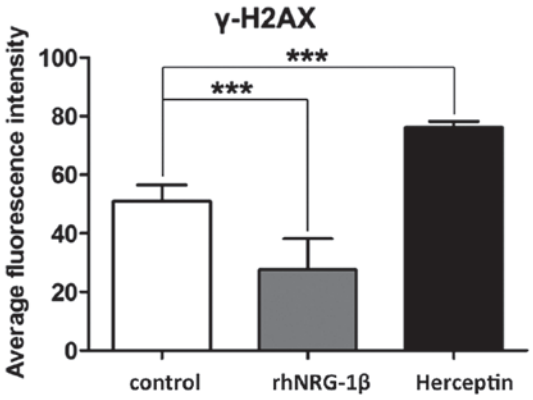

Figure 3. Average fluorescence intensity of the γ-H2AX expression in the radiotherapy, NRG and the Herceptin groups. ***P<0.0001, comparison indicated by brackets. γ-H2AX, γ-H2A histone family member X; rhNRG-1β, recombinant human neuregulin-1β.

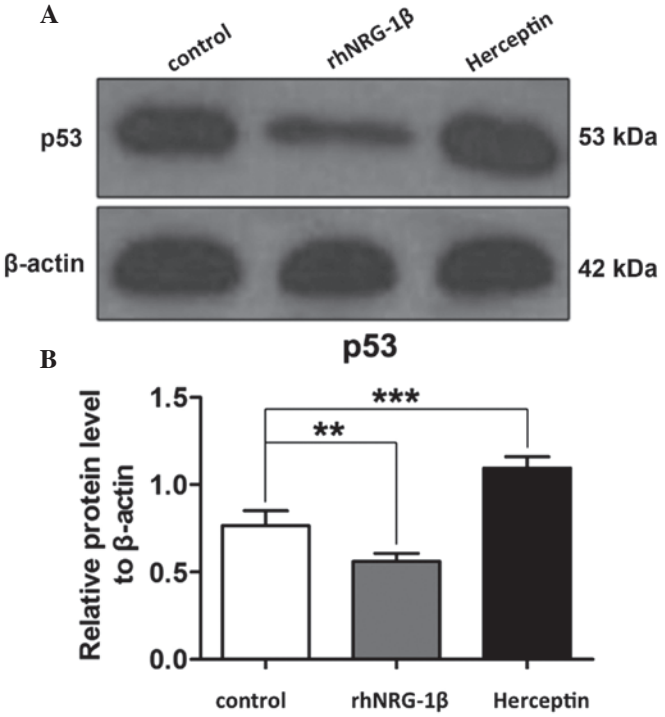

Figure 4. p53 expression in the radiotherapy, NRG and Herceptin groups. (A) p53 levels were analyzed by western blot analysis. (B) Quantification of the western blot. β-actin served as a control. **P<0.01, ***P<0.0001, comparisons indicated by brackets. rhNRG, recombinant human neuregulin-1β.

activity of p53 and PI3K anti-apoptotic signaling pathways are known to be closely associated (25). A previous study demonstrated that p53 is involved in the PI-3K/Akt pathway (26). The present study suggested by detecting changes in p53 that the NRG-1β/ErbBs pathway is activated downstream of the PI-3K/Akt pathway, which can increase Akt expression, augment DNA damage repair, consequently reduce the expression of p53 and decrease cardiomyocyte cell apoptosis. However, the correlation between the PI-3K/Akt pathway and p53 is complex and includes multifaceted feedback regulatory factors. Research shows that chronic radiation-induced DNA damage and oxidative stress result in induction of the p53/p21 pathway, which inhibits the replicative potential of human umbilical vein endothelial cells and leads to premature senescence (27). Participation of the PI3K/Akt/mTOR pathway inhibits premature endothelial senescence triggered by chronic low dose radiation (28). However, to further investigate the role of these pathways, detection of more upstream and downstream proteins is required.

Under certain conditions, NRG-1β opposing effects on the treatment of cancer, a study demonstrated that NRG-1β can promote tumor growth, and is associated with tumor migration particularly in patients with high expression of HER-2 (29). Another study demonstrated that, as a tumor suppression gene, exogenous NRG-1β may inhibit tumor growth and induce apoptosis through the synthesis of apoptotic proteins and stimulation of downstream pathways (30,31). However, these results require confirmation in human studies. Considering ~60% of patients with breast cancer are HER-2 negative, and there are a various other types of thoracic tumors that require radiotherapy, the results of this study indicate that numerous patients may benefit from treatment with rhNRG-1β to repair the radiation-induced damage of myocardial cells.

In conclusion, NRG-1β can ameliorate radiotherapy-induced myocardial cell injury to a certain extent. It predominantly acts by enhancing myocardial cell DNA repair, inhibiting apoptosis and improving myocardial function. In addition, when combining with EGFR, NRG-1β may activate a variety of pathways, including the PI-3K pathway, to reduce the radiation-induced myocardial apoptosis. For patients with radiation-induced damage of cardiomyocytes, NRG-1β may be useful to minimize this damage and potentially applied in the clinic. However, further studies are required to clarify the signaling pathways and mechanisms by which NRG-1β functions.

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