Original Article
Asparaginyl endopeptidase (AEP) regulates myocardial apoptosis in response to radiation exposure via alterations in NRF2 activation

Lu Cao1, Cheng Xu1, Peiqiang Yi1, Huan Li1, Yingying Lin2, Gang Cai1, Shubei Wang1, Dan Ou1, Min Li1, Jiayi Chen1

1Department of Radiation Oncology, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China; 2Department of Neurosurgery, Renji Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China

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Abstract: Radiation-induced heart disease (RIHD) leads to myocardial dysfunction and metabolic abnormalities in patients treated with thoracic irradiation which restricts the long-term survival benefits of radiotherapy. There is no specific or effective manner of intervention currently available. Asparaginyl endopeptidase (AEP) plays a pivotal role in the maintenance of cellular functions through regulating proteolytic cleavage as peptidase enzyme. We aimed to investigate the role of unique cardiac AEP in cardiac function by modulating key signaling elements in the myocardium. The murine heart was exposed to a single dose of 14 Gy radiation. Cellular signaling and apoptosis was analyzed in human and rat cardiomyocytes treated with various doses of radiation, we observed expression of AEP was increased by immunohistochemical staining in murine heart exposed to radiation. The AEP production along with its increased level of mRNA expression was associated with increased doses of radiation (0, 2, 5, 10 Gy) in cardiomyocytes. The myocardial cells transfected with AEP overexpression showed overall cellular viability enhancement, DNA damage inhibition, the foci formation of γ-H2AX suppressed and DNA repair enhancement significantly after radiation exposure. Small interfering RNA-mediated AEP knockdown was with reduced cardiomyocyte viability, elevated apoptotic rate, increased γ-H2AX foci formation and inhibited DNA repair as well after irradiation. After radiation exposure of 10 Gy, the expression of AEP increased in P53 overexpressing cardiomyocytes and decreased in the P53 knockdown cells, indicates that radiation-induced expression of AEP might be regulated by P53. Moreover, treatments with either AEP overexpression or knockdown showed enhanced NRF2 activity in the nuclear or suppressed NRF2 expression in the cytoplasm of myocardial cells after irradiation, respectively, defined a possible regulatory effect of AEP associated with diminished NRF2 translocation and activation by radiation exposure, including impair myocardium and myocardial apoptosis. These findings suggest that increased levels of AEP in failing myocardium after irradiation is mediated by P53 and regulate a novel pathway that involves NRF2 activation. AEP is essential for maintaining cellular redox homeostasis of cardiac function.

Keywords: Radiation-induced myocardial injury, irradiation, asparaginyl endopeptidase (AEP), nuclear factor erythroid 2-related factor 2 (NRF2)

Introduction
Radiotherapy is an important component of multidisciplinary treatments for thoracic tumors. For operable breast cancer, adjuvant radiotherapy has been proved to significantly reduce risk of locoregional recurrence and improve breast cancer-specific survival [1, 2]. In locally advanced lung cancer and esophagus cancer, the role of radiotherapy as definitive therapeutic strategy has also been well established [3, 4]. Radiation-induced heart disease (RIHD) is a clinical syndrome which significantly offsets the efficacy of radiotherapy in patients with thoracic tumors by rising non-cancer mortality rates [5]. Because of cardiac dysfunction, multiple organ systems are affected resulting in malfunction with devastating consequences. Besides functional and structural changes in the radiation exposed myocardium, cardiac enzymatic metabolism is impaired, resulting in energy depletion and oxidative stress [6, 7].
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Despite tremendous advanced technologies of radiation therapy, it is still impossible to spare the whole heart in thoracic irradiation. Several studies have demonstrated that oxidative stress, inflammation, dysregulation of micro-RNA, neuroimmune, and neurohumoral activation attributed to radiation-induced cardiac injury [8-10]. However, the regulatory consequences in enzymatic and oxidative reactions involved in major myocardium apoptotic pathway in response to irradiation are remained to be defined.

AEP is a 32 kDa cysteine protease that is initially found in trematode Schistosoma mansoni and is identified in humans [11, 12] recently. AEP belongs to peptidase family C13 with strict specificity for asparagine bond cleavage [13-16]. There was only one functional AEP isoform in humans, but it has also been found extensively widespread and evolutionarily conserved posttranslational modification of extranuclear protein in a variety of tissues including the heart [17-19]. AEP plays important role in regulating pathophysiological functions, including digestion, immunity, antimicrobial activity and apoptosis [20, 21]. The possible role of AEP in cardiac injury and involvement in a wide variety of physiological functions and diseases has indicated this protease as a potential therapeutic application.

Recent study revealed that AEP may also contribute to oxidative stress [22] which is considered as the most important factor involved in the mechanisms of radiation-induced cardiac damage [8, 23]. However, whether the role of AEP in the process of oxidative stress would also contribute to molecular process of response of cardiomyocytes to irradiation is still largely unknown. Nuclear factor erythroid 2-related factor 2 (NRF2) is a transcription factor that plays a fundamental role in regulating the expression of oxidative stress related proteins [24]. Loss of function of NRF2 is associated with increased sensitivity to oxidative stress induced cytotoxicity [25]. However, the regulation of NRF2 in response to irradiation and its relationship with AEP in this process in cardiomyocytes has not been explored in previous studies.

In this article, we aimed to examine the function of cardiac AEP in regulating cardiomyocyte survival and DNA damage after irradiation in association with NRF2 expression in nuclear and cytoplasm of specific targets on the cardiac function and myocardial apoptosis. It is interesting to note that AEP may play an important role in regulating NRF2 translocation in cultured cardiomyocytes exposed to radiation through myocardial apoptotic pathway, which suggests AEP as a potential biomarker and therapeutic target for RIHD.

Material and method

Animal model and irradiation in vivo

All animal experiments and procedures were approved by Shanghai Jiao Tong University School of Medicine Institutional Animal Care and Use Committee. The cardiac injury model was established using male C57/BL6 mice (20-25 g, 6-8 weeks old). Ten mice were randomly evenly assigned for irradiation (IR) group and control group. The mice heart in IR group was exposed to X-ray irradiation with a medical linear accelerator (Varian Trilogy, FL, USA), operating at beam energy of 6-MV with dose rate of 300 cGy/min, using a single fraction of 14 Gy, setting source-surface distance (SSD) at 100 cm and radiation field at 1 × 1 cm. The control group underwent sham irradiation. Cardiac tissues were collected at day 21 after irradiation.

Histological evaluation

After mice were sacrificed, hearts were immediately removed and fixed in 4% polyformaldehyde. The short-axis paraffin sections with 4 μm thickness were prepared by routine method for subsequent analysis. Cardiac injury after IR was evaluated in hematoxylin and eosin (HE)-stained sections. The expression of AEP in mice heart after irradiation were evaluated in immunohistochemistry (IHC)-stained left ventricular sections and labeled by sheep anti-mouse AEP (R&D Systems, Abingdon, Oxford, UK). The sections were observed and photographed by two investigators who were blinded to the treatment of IR under a microscopy (Zeiss AxioVert A1, Jena, Germany).

Murine echocardiography

The animal visual ultrasound imaging system with mouse probe (Sonic Vevo2100 and MS-400 probe) was used to measure the systolic function at day 21 after irradiation. The detec-
The radiation rate was set at 30 MHz. 2.2% isoflurane gas was used to breathe anesthetized mice. The mice were fixed on a thermostat in supine position and on electrodes coated with conductive agents in limbs. Superficial anesthesia was maintained with 1% isoflurane and oxygen. M-motion curves of left ventricular wall were collected, and at least 3 continuous and stable cardiac cycle images were collected and saved. Left ventricular M-mode motion curves were used to measure ejection fraction (EF) and fractional shortening (FS). All data were averaged for three cardiac cycles.

**Cell culture and irradiation in vitro**

Human ventricular cardiomyocytes (AC16) and rat cardiomyocytes (H9C2) were obtained from the American Type Culture Collection (Bethesda, MD, USA). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotics (streptomycin and penicillin). Cells were cultured at 37°C in a humidified incubator with an atmosphere of 95% air and 5% CO₂. When the cell population reached 70-80% confluence, experiments were performed and repeated at least three times.

Escalated irradiation dose of 0 Gy, 2 Gy, 5 Gy and 10 Gy or single radiation dose of 10 Gy was delivered to cells. The irradiation was conducted using a medical linear accelerator (Varian Trilogy, FL, USA) with beam energy at 6-MV, dose rate of 300 cGy/min, source-surface distance (SSD) of 100 cm and radiation field of 30 × 30 cm with 1 cm solid water build-up and 5 cm solid water back-scatter.

**Lentivirus transfection**

Human and mouse AEP overexpressing or knockdown sequences and P53 overexpressing or knockdown sequences were cloned into the PHY-008 vector by Hanyi Co. (Shanghai, China), respectively. The recombinant lentivirus and negative control (NC) lentivirus (Hanyin Co. Shanghai, China) were prepared and titered to 10⁹ TU (transfection units)/mL. After 48 hours, the overexpression or knockdown efficiency was confirmed via quantitative real-time PCR (qRT-PCR). To obtain stable cell lines, AC16 and H9C2 cells overexpressing or knockdown AEP and AC16 and H9C2 cells overexpressing or knockdown P53 and control cells were seeded in six-well dishes at a density of 2 × 10⁵ cells per well. On the following day, the cells were then infected with virus at the same titer in the presence of 8 μg/mL Polybrene. Approximately 72 hours after viral infection, the culture medium was replaced with DMEM containing 4 μg/mL puromycin. The cells were then cultured for at least 14 days. The puromycin-resistant cells were cultured in medium containing 2 μg/mL puromycin for 7-9 days and then transferred to medium without puromycin.

**Cell apoptosis and viability assays**

Cell apoptosis was quantitatively evaluated by staining cells with FITC-labeled annexin V and DAPI (Invitrogen, Carlsbad, CA, USA) with flow cytometry. Briefly, at 24th hour after irradiation, H9C2 and AC16 cells were trypsinized from confluent monolayer cultures, washed, and resuspended in annexin V binding buffer. Cells (approx. 5 × 10⁴ cells/ml) were incubated with FITC-labeled annexin V and DAPI. Then a flow cytometer (BD Biosciences, San Jose, CA, USA) was used to analyze the apoptosis and the apoptotic percentage was calculated.

Cell viability was determined by Cell Counting kit 8 (CCK-8) assay kit (Beyotime, Shanghai, China). Cells were inoculated in 96-well culture plates overnight and then were exposed to irradiation, respectively. At 24th, 48th, 72th, 96th, 120th hour after irradiation, the cells were assayed for cell viability in a humidified incubator at 37°C following the manufacturer’s instructions. The optical density was measured at 450 nm with a microplate reader (Synergy 2, BioTek, Winooski, VT, USA).

**Quantitative real-time polymerase chain reaction**

Cells were seeded into 6-well culture plates at a density of 1 × 10³ cells/well, respectively. At 24 hours after irradiation, total RNA was isolated and extracted with TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. 1 μg of total RNA was converted to the corresponding cDNA with ReverTra Ace® qPCR RT Kit (Toyobo, Osaka, Japan) and 10 ng from each sample were used for quantitative RT-PCR using the SYBR GREEN Realtime PCR Master Mix Kit (Toyobo, Osaka, Japan). The pairs of primers used for amplification of β-actin, NRF2, AEP and P53 mRNA were
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**Table 1. Primers used in qRT-PCR**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>human NRF2-F</td>
<td>CACATCCAGTCAGAAACCGATGG</td>
</tr>
<tr>
<td>human NRF2-R</td>
<td>GGAATGTGCTCAGCGCCAAAGCTG</td>
</tr>
<tr>
<td>Rat NRF2-F</td>
<td>TCCAAACAGATGGCCTTGACT</td>
</tr>
<tr>
<td>Rat NRF2-R</td>
<td>GCTTAATTACGTCTAGGGCCG</td>
</tr>
<tr>
<td>human AEP-F</td>
<td>CCTGAGATGGAGGCGACACT</td>
</tr>
<tr>
<td>human AEP-R</td>
<td>GTCTGCTCAAGGATCCCAGTGG</td>
</tr>
<tr>
<td>Rat AEP-F</td>
<td>GCTCCAATGGCTGTTAATTAC</td>
</tr>
<tr>
<td>Rat AEP-R</td>
<td>GAGTGGGGTTTCTCTGATGG</td>
</tr>
<tr>
<td>human P53-F</td>
<td>CCTCAGCATCTTATCGAGGTG</td>
</tr>
<tr>
<td>human P53-R</td>
<td>TGGATGTGGCTACAGTCAAGGC</td>
</tr>
<tr>
<td>Rat P53-F</td>
<td>CGTGGGCTCTGTCATTTCC</td>
</tr>
<tr>
<td>Rat P53-R</td>
<td>GCTGCGAAGACAGCTTTAATG</td>
</tr>
<tr>
<td>human Actin-F</td>
<td>CACCATTGGCAATGACGTCCACAGT</td>
</tr>
<tr>
<td>human Actin-R</td>
<td>AGGTCTTTGGCGAGATGCCACCT</td>
</tr>
<tr>
<td>Rat Actin-F</td>
<td>CGTAAAGACCTCTTATGCAACA</td>
</tr>
<tr>
<td>Rat Actin-R</td>
<td>AGCCCAAATCCACACACAG</td>
</tr>
</tbody>
</table>

synthesized based on the reported sequences (Table 1). The fluorescent product was detected during the annealing/extension periods, and a dissociation (melting curve) analysis was used to confirm the specificity of the amplified products on the LightCycler® 96 QPCR System (Roche, Basel, Switzerland). The relative mRNA expression was evaluated by the 2-deltadelta method.

**Western blot analysis**

At 24th after irradiation, cells were collected for western blot analysis, respectively. The nuclear and cytoplasm proteins were extracted using cytoplasmic and nuclear extraction kits (Invent Biotechnologies, USA). The cell lysates were separated by 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride membrane. The membranes were obstructed in 5% non-fat milk with TBST buffer for 1 hour at room temperature and incubated in primary antibody overnight at 4°C and then in a secondary antibody for 2 hours at room temperature. Target proteins were detected by the following antibodies: anti-NRF2 (sc-722, 1:200, Santa Cruz, CA, USA), goat and human-AEP, and sheep anti-mouse AEP (R&D, Minneapolis, MN), anti-BRCA1 (16004-1-AP, Proteintech Group, IL, USA), anti-ATM (27156-1-AP, Proteintech, USA), anti-ATR (19787-1-AP, Proteintech, USA), anti-Ku80 (ab80592, Abcam, UK), anti-P53 (60-283-2-lg, Proteintech, USA), The anti-Lamin B1 (12987-1-AP, 1:500, Proteintech, USA), β-actin (4970s, CST) and β-Tubulin (2146S, CST) were used as internal control. Immunopositive blots were visualized with enhanced chemiluminescence (ECL) Kit (Beyotime Biotechnology, Shanghai, China).

**Immunofluorescence staining**

At one hour after irradiation, cells were permeabilized with 0.3% Triton X-100-PBS for 15 minutes at room temperature. Cells were then blocked with 3% BSA for 60 minutes followed by incubated in γ-H2AX antibody (CY6572, Abcam, 1:100) overnight at 4°C with gentle shaking. After washing with PBS, γ-H2AX antibody was visualized with Alexa Fluor-label antibody. Nuclei were counterstained with DAPI in PBS, and the coverslips were mounted on slides using Prolong Antifade Reagent (Molecular Probes). Images were then acquired using a Nikon Eclipse Ti fluorescent microscope (Nikon Instruments, NY) and imported into Image J (NIH) analysis software.

**Statistical analysis**

Results were expressed as mean ± S.E.M. Probability values of $P < 0.05$ were considered significant. Comparison between 2 groups was calculated and statistically compared using the 2-tailed Student’s t-test and one-way ANOVA with Bonferroni’s multiple comparison test. Statistical analysis was performed using SPSS software version 25.0 (IBM corporation, USA) and GraphPad Prism 5 (GraphPad Software Inc., USA).

**Results**

**Effect of irradiation on cardiac tissue and function**

In HE staining and controls of left ventricular tissue, the slices showed that the arrangement of myocardial cell was disordered at day 21 after irradiation with vacular and adipocyte changes, as well as the loose of structure of myocardial cell in some areas and slight pyknosis in the nucleus (Figure 1A). On day 21, decrease in systolic function parameter EF and FS as observed in the IR group compared with the control group, but without statistical significance (Figure 1B-D). It showed high abundance suggesting that heart irradiation may represent a molecular signature of failing myocardium.
Increased AEP expression in cardiomyocytes after irradiation

To evaluate the effect of irradiation on the expression of AEP in cardiomyocytes in vivo, a single dose of 14 Gy was delivered to the whole heart of C57/BL6 mice. At 21th day after irradiation, the IHC staining of the left ventricular sections showed that the intensity of AEP expression in cardiomyocytes was positive in both the IR group and control group. The AEP expression was increased after irradiation compared with the control group (Figure 2A and 2B).

To further explore the impact of irradiation on the expression level of AEP in cardiomyocytes, we delivered escalated irradiation dose of 0 Gy, 2 Gy, 5 Gy and 10 Gy to cardiomyocytes. Cells were collected at 24th hour after irradiation. The RT-PCR showed that the relative mRNA expression of AEP was significantly up-regulat-
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Figure 2. Increased AEP expression in cardiomyocytes after irradiation. The whole heart of mice was irradiated with a single dose of 14 Gy using 6-MV X-ray. Cardiac tissue was sampled at day 21 after irradiation. Three representative photomicrographs (× 20, scale bar = 100 μm) of left ventricular sections with immunohistochemical staining for AEP were shown in mice treated with irradiation (IR) and without irradiation (Control) (A and B). Escalated irradiation dose of 0 Gy, 2 Gy, 5 Gy and 10 Gy was delivered to cardiomyocytes. Cells were collected at 24th hour after irradiation. The RT-PCR was used to determine the relative mRNA expression level of AEP (C and D). Western blot analysis was used to determine the AEP protein expression after irradiation in normal control group after irradiation (E and F). Each value represents the mean ± S.E.M of three determinations. Student's t-test was used to detect the difference between groups. ***P < 0.0001, **P < 0.001, *P < 0.01 vs. Normal Control group treated with sham irradiation.

ed dose-dependently after irradiation compared with normal control group (Figure 2C and 2D). Consistently, the Western blot analysis also confirmed that expression of AEP protein
was up-regulated after irradiation in both human and rat myocardial cells (Figure 2E and 2F). These results affirmed that the level of AEP was highly expressed and positively regulated in response to irradiation in cardiomyocytes.

Overexpression and knockdown of AEP in cardiomyocytes

To further investigate the role of AEP expression in cardiomyocytes after irradiation, we constructed stably transfected AEP overexpressing and knockdown vector in human AC16 and rat H9C2 myocardial cells. The relative mRNA expression level of AEP was significantly higher in AEP overexpressing cells (AC16-AEP-OE and H9C2-AEP-OE) compared with the control cells (AC16-NC and H9C2-NC) (Figure 3A and 3B) as determined by quantitative real-time RT-PCR (4.7-fold for AC16-AEP-OE and 3.5-fold for H9C2-AEP-OE). The relative mRNA expression level of AEP in AEP knockdown cells (AC16-AEP-KD and H9C2-AEP-KD) was consistently significantly lower than that in the control cells (AC16-NC and H9C2-NC) (Figure 3C and 3D) (0.35-fold for AC16-AEP-KD1, 0.81-fold for AC16-AEP-KD2, and 0.61-fold for AC16-AEP-KD3; 0.33-fold for H9C2-AEP-KD1, 0.13-fold for H9C2-AEP-KD2 and 0.73-fold for H9C2-AEP-KD3). The relative mRNA expression level of AEP was lowest in the AC16-AEP-KD1 cells (0.35-fold change) and H9C2-AEP-KD2 cells (0.13-fold change), these two cell lines were selected for sequent experiments. Moreover, western blotting analysis showed that the protein level of AEP displayed overexpressed (281.1%, 109.3%, 70.0% and 23.6% for AC16; 849.0%, 226.7%, 354.0% and 188.5% for H9C2, at the dose of 0 Gy, 2 Gy, 5 Gy and 10 Gy, respectively) or decreased (75.5%, 76.1%, 45.5% and 33.8% for AC16; 67.8%, 49.7%, 29.3% and 12.2% for H9C2, at the dose of 0 Gy, 2 Gy, 5 Gy and 10 Gy, respectively) sharply in both AC16 and H9C2 cells in presence or absence of radiation exposure (Figure 3E and 3F), suggesting that the expression level of AEP was potential candidate marker for cardiomyopathy.

Overexpression of AEP enhanced cardiomyocyte survival after irradiation

To investigate the impact of AEP on the survival of cardiomyocytes after irradiation, we delivered escalated irradiation dose of 0 Gy, 2 Gy, 5 Gy and 10 Gy to AEP overexpressing cells and evaluated cell viability using CCK-8 assay at 24th, 48th, 72th, 96th, 120th day after irradiation. The results of CCK-8 assay showed that cell viability was enhanced in AEP overexpressing cells compared with the control group at the dose point of 0 Gy, 2 Gy and 5 Gy (Figure 4A and 4D). To further explore the underlying mechanism of enhanced survival observed in AEP overexpressing cells, we conducted flow cytometry to quantify the myocardium apoptosis rate after irradiation. The results showed that apoptosis rate was significantly lower in AEP overexpressing cells compared with the control group. This finding was observed a trend toward an increase of cell viability in AEP overexpressed myocardial cells in comparison with wild cells, at different doses of radiation exposure (0, 2, 5 and 10 Gy) (Figure 4B, 4C, 4E and 4F).

To further confirm the impact of AEP expression level on cell survival after irradiation, escalated radiation dose of 0 Gy, 2 Gy, 5 Gy and 10 Gy was delivered to AEP knockdown cells. The results of CCK-8 assay showed that cell viability was suppressed in both AC16-AEP-KD cells and H9C2-AEP-KD cells compared with the corresponding control cells (Figure 5A and 5D). Consistently, the apoptosis rate after irradiation was also significantly higher in H9C2-AEP-KD cells compared with H9C2-NC (Figure 5E and 5F) at various doses of 0 Gy, 5 Gy and 10 Gy. The apoptosis rate was also higher in AC16-AEP-KD cells compared with AC16-NC. However, no statistically significant difference was detected (Figure 5B and 5C). These results further revealed that myocardial AEP enhanced cell survival in response to irradiation.

AEP reduced DNA damage in cardiomyocytes after irradiation

To explore the potential mechanisms of AEP in response to irradiation, we determined the γ-H2AX foci formation in cardiomyocyte after irradiation. As shown in Figure 6, markedly elevated γ-H2AX foci cell formation in AEP knockdown cells of AC16-AEP-KD (1.78-fold, 1.63-fold and 1.33-fold at the dose of 2, 5, 10 Gy) and H9C2-AEP-KD (2.43-fold, 1.33-fold, and 1.81-fold at the dose of 2, 5, 10 Gy) was found compared with the corresponding control cells (Figure 6A, 6E, 6C, 6G). Furthermore, weaken
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Figure 3. Construction of AEP overexpressing and knockdown cardiomyocytes. Lentivirus transfection was used to transfer AEP overexpressing or knockdown plasmid into the cardiomyocytes to build AEP overexpressing or knockdown cell lines using AC16 cells and H9C2 cells. The RT-PCR was used to determine the relative mRNA expression level of AEP in AC16 cells (A and C) and H9C2 cells (B and D), respectively. Western blot analysis was used to confirm the effect of expression of AEP overexpression and knockdown in AC16 cells (E) and H9C2 cells (F) with or without irradiation. Each value represents the mean ± S.E.M of three determinations. Student’s t-test was used to detect the difference between groups.
Figure 4. Overexpression of AEP enhanced cardiomyocyte survival after irradiation. Escalated irradiation dose of 0 Gy, 2 Gy, 5 Gy and 10 Gy was delivered to AEP overexpressing cells. Cell viability was evaluated by CCK-8 assay (A and D). Myocardium apoptosis was quantified with flow cytometry. The apoptosis rate was
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presented as the percentage of cells with FITC-Annexin V positive/PI negative in (B and E). The representative photomicrographs of flow cytometry detecting myocardium apoptosis were shown in (C and F). Each value represents the mean ± S.E.M of three determinations. Student’s t-test was used to detect the difference between groups. ***P < 0.0001, **P < 0.001, *P < 0.01 vs. Normal Control group at every dose point.
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Figure 5. Knockdown of AEP reduced cardiomyocyte survival after irradiation. Escalated irradiation dose of 0 Gy, 2 Gy, 5 Gy and 10 Gy was delivered to cardiomyocytes. Cell viability was evaluated by CCK-8 assay (A and D). Myocardium apoptosis was quantified with flow cytometry (B and E). The representative photomicrographs of flow cytometry were shown in (C and F). Each value represents the mean ± SD of three determinations. Student’s t-test was used to detect the difference between groups. ***P < 0.0001, **P < 0.001, *P < 0.01 vs. Normal Control group at every dose point. NS = not significantly (P≥0.05).
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Figure 6. AEP reduced DNA damage significantly in cardiomyocyte after irradiation. Escalated irradiation dose of 0 Gy, 2 Gy, 5 Gy and 10 Gy was delivered to cardiomyocytes. Cells were collected at one hour after irradiation. The representative images of γ-H2AX staining of AC16 and H9C2 cells was showed in the left column as in (A-D); Quantitative γ-H2AX staining assays was showed in the corresponding row as in (E-H). Each value represents the mean ± S.E.M of three determinations. Student’s t-test was used to detect the difference between groups. ***P < 0.0001, **P < 0.01, *P < 0.05 compared with normal control group, NS = not significantly (P≥0.05).
γ-H2AX foci cell formation was observed in AEP overexpressing cells of AC16-AEP-OE (0.29-fold, 0.80-fold, and 0.79-fold at the dose of 2, 5, 10 Gy) and H9C2-AEP-OE (0-fold, 0.63-fold and 0.58-fold at the dose of 2, 5, 10 Gy) compared with the corresponding control cells (Figure 6B, 6F, 6D, 6H), indicating that AEP is highly associated with signaling of DNA damage in cardiomyocyte after irradiation.

**AEP promotes the DNA repair after radiation**

To further explore the underlying mechanism of how AEP reduced the DNA damage in cardiomyocytes after irradiation, we examined the effect of level of AEP on the expression of molecules that involved in the repair process of radiation-induced DNA damage and response in cardiomyocyte after irradiation. Irradiation dose of 10 Gy was delivered to the AEP overexpressing or knocked down H9C2 cells (H9C2-AEP-OE and H9C2-AEP-KD) as well as control cells (H9C2-NC), respectively. The western blot analysis showed that, the expression level of BRCA1, ATM, ATR, KU80 significantly increased in H9C2-AEP-OE cells, but sharply decreased in the H9C2-AEP-KD, after irradiation compared with control cells (H9C2-NC) (Figure 7). Thus, the protective effect of AEP on radiation-induced DNA damage might be mediated by up-regulating the molecules that participating in the DNA repair and then promote the DNA repair after radiation.

**AEP alters NRF2 activity in cardiomyocytes upon irradiation**

We expanded this study and investigated whether AEP expression has altered NRF2 status and activation in radiation exposed myocardium. In AEP knockdown cells, Western blot showed that the expression of NRF2 markedly attenuated (0.26-fold, 0.03-fold, 0.04-fold and 0.04-fold for AC16; 0.25-fold, 0.02-fold, 0.02-fold, 0.03-fold for H9C2, at the dose of 0, 2, 5, 10 Gy) in the cytoplasm while no obvious change in the nuclear compared with normal cells (Figure 8A and 8C). However, the activation of NRF2 was markedly elevated (1.45-fold, 2.13-fold, 3.58-fold and 5.90-fold for AC16; 2.20-fold, 1.94-fold, 2.00-fold and 0.69-fold for H9C2, at the dose of 0, 2, 5, 10 Gy) in the nuclear of AEP overexpressing cells (Figure 8B and 8D). RT-PCR analysis represented that the transcripts of NRF2 was suppressed after irradiation (Figure 8E-H). Together, these results indicate that the alteration in NRF2 activation regulated by AEP protease expression correlates with changes in both basal expression level and activation status of NRF2 contributing to myocardial dysfunction and cardiomyopathy.

**P53 regulates the expression of AEP after radiation**

To explore whether impact of radiation on the level of AEP is regulated by P53, we constructed stably expressing P53 or knocking down P53 in human AC16 and rat H9C2 myocardial cells. The relative mRNA expression level of P53 was significantly higher in P53 overexpressing cells (AC16-P53-OE and H9C2-P53-OE) compared with the control cells (AC16-NC and H9C2-NC) (Figure 9A and 9E) as determined by quantitative real-time RT-PCR (37.4-fold for AC16-P53-OE and 11.8-fold for H9C2-P53-OE). The relative mRNA expression level of P53 in P53 knockdown cells (AC16-P53-KD and H9C2-P53-KD) was consistently significantly lower than that in the control cells (AC16-NC and H9C2-NC) (Figure 9B and 9F) (0.33-fold for AC16-P53-KD1, 0.54-fold for AC16-P53-KD2, and 0.78-fold for AC16-P53-KD3; 0.23-fold for H9C2-P53-KD1, 0.62-fold for H9C2-P53-KD2 and 0.83-fold for H9C2-P53-KD3). The relative mRNA expression level of P53 was lowest in the AC16-P53-KD1 cells (0.33-fold change) and H9C2-P53-KD1 cells (0.23-fold change), these two cell lines were selected for subsequent experiments.
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Figure 7. AEP promotes the DNA repair after irradiation. Radiation dose of 10 Gy was delivered to AEP overexpressing or knocked down H9C2 cells as well as control cells. Cells were collected at 24th hour after irradiation. Western blot analyses were conducted to examine the protein expression of BRCA1, ATM, ATR, Ku80. Each value represents the mean ± S.E.M of three determinations. Student’s t-test was used to detect the difference between groups.
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Figure 8. AEP alters NRF2 activity in cardiomyocytes upon irradiation. Escalated irradiation dose of 0 Gy, 2 Gy, 5 Gy and 10 Gy was delivered to AEP overexpressing and knockdown cardiomyocytes. Cells were collected at 24th hour after irradiation. Western blot analyses of NRF2 expression in the cytoplasm and nuclear were conducted, respectively (A-D). RT-PCR was also conducted to determine the relative mRNA expression of NRF2 as shown in (E-H). Each value represents the mean ± S.E.M of three determinations. Student’s t-test was used to detect the difference between groups. ***P < 0.0001, **P < 0.001, *P < 0.01 vs. sham irradiation in normal control group, ###P < 0.0001, ##P < 0.001, #P < 0.01 vs. sham irradiation in AEP overexpressing and knockdown cells.
Figure 9. P53 regulates the expression of AEP after radiation. Lentivirus transfection was used to transfer P53 overexpressing or knockdown plasmid into the cardiomyocytes to build P53 overexpressing or knockdown cell lines using AC16 cells and H9C2 cells. The RT-PCR was used to determine the relative mRNA expression level of P53 in AC16 cells (A and B) and H9C2 cells (E and F). Irradiation dose of 10 Gy was delivered to cardiomyocytes. Cells were collected at 24th hour after irradiation. RT-PCR was also conducted to determine the relative mRNA expression of AEP as shown in (C, D, G and H). Western blot analyses of AEP expression were conducted, as shown in (I and J). Each value represents the mean ± S.E.M of three determinations. Student’s t-test was used to detect the difference between groups.
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decreased in the AC16-P53-KD and H9C2-P53-KD cells, compared with the control cells (AC16-NC and H9C2-NC) (Figure 9I and 9J). These findings suggested that impact of radiation on the expression level of AEP can be mediated by P53.

**Discussion**

Cardiac dysfunction including metabolic abnormalities, oxidative stress and energy depletion develop in radiation exposed myocardium and contribute to cardiac remodeling. The major finding of our study is that heart irradiated increase of cardiac protease AEP expression in myocardium manifested by increasing cell viability, inhibiting apoptosis and DNA damage. We demonstrated that AEP was up-regulated by myocardial irradiation both in vivo and in vitro, probably through the activation and translocation of NRF2 between the nuclear and cytoplasm. This effect leads to apoptotic pathway of AEP protease cleavage in radiation induced damaged myocardium and contributes to altered cardiac oxidative reaction and energy metabolism that is associated with cardiac dysfunction in humans.

We investigated the underlying mechanisms that AEP functions as a mediator of response to irradiation in cardiomyocytes. Loss of AEP was recently reported to induce oxidative stress in cells [22]. The STAT3 activation triggered by such lysosomal oxidative stress plays a crucial role in the splenomegaly and hyperproliferative kidney disease in AEP-deficient mice [22]. Oxidative stress is also considered as the most direct and critical factor among mechanisms involved in radiation-induced cell apoptosis, which could cause oxidative damage of biological macromolecules (DNA, protein, and lipids) and a series of molecular signaling pathways mediated by Reactive Oxygen Species (ROS) through the excessive production of ROS [8, 23]. First, we detected that the expression level of protein and transcript of AEP was up-regulated significantly by in vitro experiments in human cardiomyocyte-like AC16 cells and rat H9C2 cells at 24 hours after irradiation. Moreover, AEP was consistently expressed in human and rat myocardial cell cultures, in which we also observed increased radiation dose dose-dependently promoted AEP enzymatic activity. The results suggest that AEP plays a regulatory role in the process of radiation induce cardiac injury. The finding links AEP-mediated oxidative stress in RIHD.

To further clarify the specific role of AEP in the process of radiation-induced myocardial damage, we established both AEP overexpressing cell lines and AEP knockdown cell lines using a lentivirus transfection system. After delivery of escalated radiation dose, overexpression of AEP was found to alleviate the radiation-induced damage response in cardiomyocytes, including cardiomyocytes death, apoptosis, DNA damage and DNA repair. In contrast, knockdown of AEP sensitized cardiomyocytes to radiation. Regarding to cardiomyocytes survival, the protective effect of AEP overexpressing was consistently observed at a variety of radiation doses (0, 2, 5 and 10 Gy). These results demonstrate an undescribed function of AEP in radiation-induced myocardial injury. This finding indicates that AEP has the potential to serve as an indicator of radiation-induced cardiac injury and therapeutic target for RIHD. AEP regulates myocardial cell apoptosis through direct targeting at the upstream mRNA untranslated region needs to be evaluated in further studies.

Most studies have shown that AEP-mediated ubiquitination contributes to the activation of metabolic enzymes and pathways [26, 27]. Our data demonstrate that knockdown of AEP leads to the cellular DNA and myocardial damage after irradiation that is associated with decreased enzymatic and impaired cardiomyocyte activities. However, we did not observe to exhibit marked AEP expression levels associated with a global increase in total protein by immunocytochemical-positive staining in the myocardial tissue of C57/BL6 mice after irradiation in comparison with normal heart (Figure 2). In the present study, we collected the cardiac tissue at day 21 after radiation. It might be too early to observe the myocardial tissue change. The expression of AEP in the myocardial tissue at a period of time after irradiation, which will be helpful to clarify the association between AEP expression and radiation-induced myocardial damage in vivo.

Oxidative activity are the main cellular reaction responsible for energy balance and metabolism homeostasis [28]. The cardiac NRF2 plays a pivotal role in the maintenance of microvascular and myocardial cellular function of heart [29]. Several recent studies revealed AEP and NRF2 as major regulators of the apoptotic pathways controlling dynamics of metabolic reprogramming and antioxidant defense mecha-
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For example, NRF2 knockout mice display more sensitivity to acute toxic agents, hyperopia or carcinogens [30]. Activation of the NRF2 signaling plays a crucial role in reducing cell damage caused by radiation-induced oxidative stress [31, 32]. Our previous study found that upregulation of NRF2/HO-1-dependent signaling activation involved in cardioprotective effect in the acute stage of radiation-induced myocardial damage [33]. Regulating translocation of NRF2 between nuclear and cytoplasm is a fundamental mechanism by which cells respond to oxidant stress [34]. In nucleus, NRF2 undergoes heterodimeric combinations with other transcription factors and induces expression of stress-preventing genes, such as c-Jun, Jun B, Jun D, ATF3, ATF4, and small Mafs, and induces expression of stress-preventing genes [35]. Several signals have been reported to be involved in the translocation and activity of NRF2 in response to oxidative stress, including NRF2-ARE pathway, Smad pathway, TAK1/MKK3/p38 pathway, ERK signaling, PI3K pathway, and MAPK pathway [29, 36-38]. In this study, we found the association between nuclear NRF2 and AEP expression in the response of cardiomyocyte to radiation exposure. The results showed that the NRF2 was translocated to nucleus after irradiation in AEP overexpressing cardiomyocytes (Figure 8). We also showed that AEP knockdown cells exhibit reduced NRF2 expression levels in the cytoplasm while no obvious change in the nuclear (Figure 8). The expression of NRF2 mRNA was suppressed after irradiation in AEP overexpressing cells (Figure 8). However, conflicting results suggesting a positive correlation between NRF2 activation and AEP enzymatic activity have also been observed. The cardiac protective effects of AEP might play its role for modulating oxidative stress through the activation of NRF2 in myocardial cells after radiation exposure.

P53 is a well-known transcription factor that modulates the expression of numerous genes and is also an important molecule in cellular response to DNA damage caused by radiation [39, 40]. Yamane et al. [41] reported that the expression and enzyme activity of AEP was regulated by P53 in HCT116 cells. In this study, we found that the mRNA and protein expression of AEP increased sharply in P53 overexpressing cardiomyocytes, but decreased significantly in the P53 knockdown cardiomyocytes, compared with control cells. Based on these findings, we speculate that radiation-induced the expression of AEP might be regulated by P53. Further studies are needed to verify the underlying mechanism of how P53 regulated the expression of AEP after radiation.

Taken together, our data suggest a novel pathway of combined epigenetic, transcriptional, and posttranslational regulation of proteins that are involved in cardiac energy metabolism and oxidative reaction during radiation-induced cardiac injury development and progression. It remains to be explored whether the opposite effect of AEP-mediated NRF2 alterations is associated with heart disease type specificity, cellular conditions, or microenvironment. Further studies are necessary to examine the dual regulatory roles of AEP on its targets in different animal models of cardiac injury, and discuss its potential translation into therapy of RIHD.

Conclusion

In conclusion, we here show a novel regulatory pathway in radiation-induced myocardial injury that involves myocardium AEP-modulated activation of NRF2 leading to increasing cell viability, inhibiting apoptosis and DNA damage, as well as promoting DNA repair and recovering cardiac dysfunction. These findings might provide novel targets for selective therapeutic interventions in the syndrome of RIHD.

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Disclosure of conflict of interest

None.

Address correspondence to: Jiayi Chen and Min Li, Department of Radiation Oncology, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China. E-mail: chenjiayi0188@aliyun.com (JYC); lm11866@rjh.com.cn (ML)

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