Injured pancreatic β cells enhance the release of miR-375-3p into the extracellular space

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Abstract. miR-375-3p is a highly expressed microRNA in pancreatic β cells. We have previously reported that when mice were exposed to 7 Gy X-ray irradiation, miR-375-3p was increased in the serum and there was cytotoxicity in pancreatic β cells. However, it was unknown whether miR-375-3p is then released from injured pancreatic β cells to the extracellular space. The present study investigated the effect of ionizing radiation and streptozotocin (STZ) treatment on the expression of extracellular miR-375-3p into culture supernatants using the rat pancreatic β cell line RIN-5F. Cell growth was reduced, and cell death was increased at 24 h following exposure to 7 Gy irradiation as well as 24 h following treatment with 30 mM STZ compared with the control. Expression levels of miR-375-3p were significantly increased 24 h after 30 mM STZ treatment, yet this was only observed at 48 h following exposure to 7 Gy compared with the control. This suggests that the mechanism of cell death in RIN-5F is different between 7 Gy irradiation and 30 mM STZ treatment. The results of the present study suggest that injured pancreatic β cells enhance the release of miR-375-3p from cells into extracellular space.

Introduction

MicroRNAs (miRNAs) are small (18-25 nucleotides) non-coding RNA harboring a post-transcriptional gene regulatory function (1). Currently, miRNAs have been detected in various body fluids, including serum (2), plasma (3), urine (4), cerebrospinal fluid (5), breast milk (6), saliva (7), bronchoalveolar lavage fluid (8), ascites (9), and pleural effusion (10). Noteworthy, miRNAs are stable under non-physiological conditions, whether in body fluids or culture media. Taylor *et al* (11) reported that plasma miR-21 is stable for at least 28 days at -30˚C. We have reported that extracellular small RNAs are stable for 4 weeks at room temperature, after 20 freeze-thaw cycles and exposure to pH 2.0, and are resistant to ribonuclease A degradation (12). In body fluids, miRNAs are present in extracellular vesicles (EVs) (13) or high-density lipoproteins (14) and bind RNA-binding proteins (15). They are proposed to become novel biomarkers of disorders including some cancers and neurodegenerative diseases.

miR-375-3p is expressed in pancreatic β cells (16), where this miRNA is involved in pancreatic development, β cell proliferation, and insulin secretion via gene regulation (17). Overexpression of miR-375-3p suppresses insulin secretion (18), whereas inhibition of endogenous miR-375-3p increases insulin secretion (19). Streptozotocin (STZ) is a nitrosourea alkylating agent that induces tumor shrinkage and hypoglycemia and causes the selective destruction of pancreatic β cells via a glucose transporter 2 (20). Therefore, STZ have been used as a therapeutic drug for the treatment of neuroendocrine tumors in Japan (21). In mice and rats, the administration of STZ induces diabetes after pancreatic β cells are injured (22,23). Erener *et al* (24) reported that blood miR-375-3p increased in STZ-treated mice.

We have previously shown that mice irradiated with a lethal X-ray dose of 7 Gy present a significant serum increase of miR-375-3p at 72 h after exposure (2). Since miR-375-3p is expressed the highest in the pancreas among 20 types of cells and organs examined, it was inferred that it derived from the pancreas. This research suggested that radiation-induced death of pancreatic β cells is associated with the release of EVs containing miR-375-3p. Although miR-375-3p is expected to be released from injured pancreatic β cells, no evidence has been obtained. Therefore, it is necessary to investigate whether miR-375-3p is released from cells by STZ treatment and 7 Gy X-ray irradiation, which is a different mechanism to injure pancreatic β-cells. In this study, we investigate the expression level of extracellular miR-375-3p released from an insulinoma cell line exposed to 7 Gy X-ray irradiation or STZ treatment.

Materials and methods

Cell line and culture. The rat pancreatic β cell line (RIN-5F) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). RIN-5F cells were cultured

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in RPMI-1640 medium (Wako, Tokyo, Japan) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml of penicillin, and 100 μg/ml of streptomycin (Wako). Cells were cultured at 37˚C in a humidified atmosphere with 5% CO_2.

X-ray irradiation. RIN-5F cells were exposed to X-rays (MBR-1520R-3 X-ray machine, Hitachi Medical Corporation, Tokyo, Japan) at a dose rate of 1.0 Gy/min (150 kVp, 20 mA, 0.5-mm aluminum, and 0.3-mm copper filters).

STZ treatment. STZ and Dulbecco's phosphate-buffered saline [D-PBS(-), pH 7.2] were purchased from Wako. STZ was diluted in D-PBS(-).

RNA extraction. Total RNAs from RIN-5F cells were extracted using Isogen II reagents (Nippongene, Tokyo, Japan) according to the manufacturer's instruction. Cell culture medium samples were centrifuged at 300 x g at 4˚C for 3 min and floating cells removed. Total RNAs from 200 μl culture supernatants added to 5 μl cel-miR-39 (1 nM) were extracted using Isogen II reagents and ethachinmate (Nippongene).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). The expression of rat insulin 1 (*Ins 1*) and β actin (*Actb*) mRNAs in RIN-5F cells were determined by RT-qPCR. Briefly, total RNAs were isolated from RIN-5F cells. cDNA was synthesized from total RNA using the Applied Biosystems™ High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. qPCR was performed using a FastStart Universal SYBR Green Master (Roche Diagnostics, Basel, Switzerland), 10 μM of forward and reverse primer pairs (Table I), and the StepOne Plus Real-time PCR system (Thermo Fisher Scientific, Inc.) in the following conditions: 10 min at 95˚C, followed by 40 cycles each of 95˚C for 15 sec, and 60˚C for 60 sec. *Actb* was used as internal control. The PCR products were separated by electrophoresis on 4% agarose gel and detected by ethidium bromide staining.

RT-qPCR was used to determine the expression levels of miR-375-3p in culture supernatants. The cDNAs were synthesized using the TaqMan™ miRNA RT kit and the prescribed 5 x RT primer (both from Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. qPCR for miRNAs was performed using a FastStart TaqMan probe master (Roche Diagnostics), a 20 x probe, and the StepOne Plus Real-Time PCR system (Thermo Fisher Scientific, Inc.) under the following conditions: 10 min at 95˚C, followed by 45 cycles at 95˚C for 15 sec, and 60˚C for 60 sec. Cel-miR-39 was used as an external control. The comparative Ct method was used to determine expression levels.

Immunofluorescence staining. Immunofluorescence staining was performed for detection of insulin in RIN-5F cells. RIN-5F cells were plated in 8-well chamber slides at a density of $2x10^4$ cells per well in culture media. After 48 h, the cells were washed three times with D-PBS(-) and treated with 0.2% Triton X-100 in D-PBS(-) at room temperature for 5 min. After washing three times in D-PBS(-), the cells were incubated in an Image-iT FX signal enhancer solution (Thermo Fisher Scientific, Inc.) at room temperature for 30 min. Next, the cells were incubated with a primary rabbit monoclonal antibody raised against insulin (C27C9) (no. 3014; Cell Signaling Technology, Inc., Danvers, MA, USA) at 1:100 dilution in Immunostain Solution A (Toyobo, Osaka, Japan) at room temperature for 60 min. After five times washing with D-PBS(-), cells were incubated at room temperature for 60 min with an anti-rabbit IgG Alexa Fluor 488-conjugated secondary antibody (no. 4412; Cell Signaling Technology, Inc.) at 1:200 dilution in Immunostain Solution B. The cells were washed five times with D-PBS(-), and the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) in ProLong Gold Antifade Reagent (Thermo Fisher Scientific, Inc.). The cells were visualized under a confocal laser scanning microscope LSM710 (Carl Zeiss, Oberkochen, Germany).

Flow cytometry. RIN-5F cells were plated on 3-cm dishes at a density of $2x10^6$ cells per dish in culture medium. After 96 h, culture medium was discarded, the cells were washed three times in D-PBS(-), and 10 ml culture medium was added to each dish. Then, RIN-5F cells were exposed to none or 7 Gy of X-rays at a dose rate of 1.0 Gy/min and treated with STZ of a concentration of 0, 1, 3, 10, or 30 mM. The RIN-5F cells were collected and were stained by propidium iodide (PI) solution. PI-positive cells were detected using a Cytomics FC500 (Beckman Coulter, Brea, CA, USA).

Cell proliferation assay. RIN-5F cells were seeded at the density of $2x10^4$ cells per well of a 96-well flat-bottomed microplate. After pre-culture for 48 h, media were discarded and the cells washed with D-PBS(-). The cells were incubated with STZ diluted in medium at various concentrations (0, 1, 3, 10, or 30 mM) for 0, 24, or 48 h. The cells irradiated with 7 Gy of X-rays and control cells not irradiated were cultured for 0, 24, 48, or 72 h after irradiation. Viable cells were detected using the alamarBlue® cell viability reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The fluorescence intensity was measured after 6 h at an excitation wavelength of 544 nm and an emission wavelength of 590 nm using a Fluoroskan Ascent™ system (Thermo Fisher Scientific, Inc.).

Statistical analysis. Statcel 3 software (OMS Publishing Inc., Saitama, Japan) was used to perform all statistical analyses. Student's t-test was performed to compare the results of the two groups. One-way analysis of variance (ANOVA) was performed followed by Tukey-Kramer multiple comparisons test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

RIN-5F cells express insulin. To confirm the pancreatic β cells characteristics of RIN-5F cells, the expression of insulin mRNA was determined by RT-qPCR. Both *Ins1* and *Actb* mRNAs were detected (Fig. 1A). In addition, insulin proteins were detected in RIN-5F cells by immunofluorescence analysis using confocal laser scanning microscopy (Fig. 1B). These results indicate that RIN-5F cells expressing insulin process characteristics of pancreatic β cells.

Table I. Primers for reverse transcription-polymerase chain reaction.

Primer name	Accession no.	Sequence (5'-3')	Size (nt)	Amplicon size (bp)
Ins1 forward primer	NM_019129.3	TCATAGACCATCAGCAAGCAG	21	95
Ins1 reverse primer		CTTGGGCTCCCAGAGGAC	18	
Actb forward primer	NM_031144.3	CCCGCGAGTACAACCTTCT	19	72
Actb reverse primer		CGTCATCCATGGCGAACT	18	

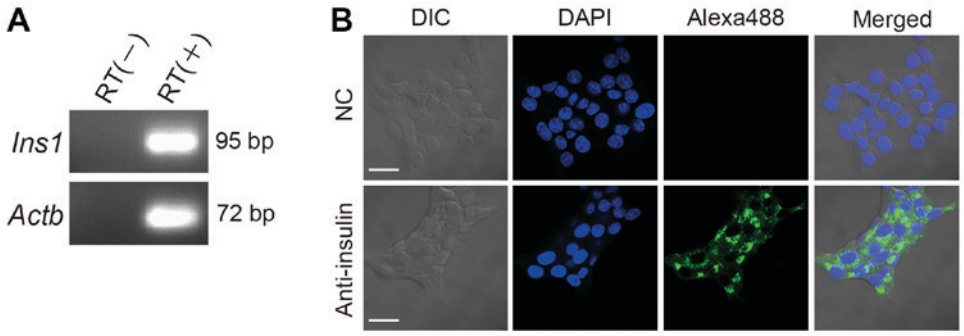

Figure 1. The expression of insulin in RIN-5F cells. (A) The expression of *Ins1* and *Actb* mRNAs determined by RT-qPCR. PCRs with or without reverse transcription (RT) were performed in the RT-qPCR reaction mixture for amplification of *Ins1* and *Actb* mRNAs using total RNA from RIN-5F cells and the primer pair that does not allow amplification of fragments from the genomic DNA. The PCR products were separated by electrophoresis on 4% agarose gel and detected by ethidium bromide staining. RT(-) was performed to examine the amplification from template RNA and/or genomic DNA. (B) Representative immunofluorescence image of insulin in RIN-5F cells. Green, Insulin; Blue, Nuclei. Scale bar represents 100 μm. RT, reverse transcription; DIC, differential interference contrast; DAPI, 4',6-diamidino-2-phenylindole; NC, negative control.

X-ray irradiation and STZ treatment suppress cell proliferation in RIN-5F cells. To assess the sensitivity of RIN-5F cells to irradiation and STZ treatment, a cell proliferation assay was performed. Cell proliferation of RIN-5F cells was significantly suppressed at 24, 48, and 72 h after 7 Gy irradiation (Fig. 2A) and by treatment of 10 or 30 mM STZ at 24 h (Fig. 2B) compared with controls. These results indicate that cells exposed to 7 Gy irradiation and high concentration of STZ cannot further proliferate.

X-ray irradiation and STZ treatment induce cell death in RIN-5F cells. To evaluate cell death induced by 7 Gy irradiation or STZ treatment in RIN-5F cells, we performed a flow cytometry analysis in PI-stained RIN-5F cells. The number of PI-positive cells significantly increased at 24, 48, and 72 h after 7 Gy irradiation compared with non-irradiated (0 Gy) cells (Fig. 3A). PI-positive cells were significantly increased at 24 h after treatment with 30 mM STZ and at 48 h after treatment with 3, 10, or 30 mM STZ compared with control cells (Fig. 3B). These results indicate that within 24 h, cell death is induced in RIN-5F cells either exposed to 7 Gy irradiation or 30 mM STZ treatment.

X-ray irradiation and STZ treatment enhance the release of miR-375-3p in RIN-5F cells. To examine the impairment-associated release of miR-375-3p from RIN-5F cells, we performed RT-qPCR on culture supernatants of RIN-5F cells that were exposed to 7 Gy irradiation or STZ treatment. The expression of miR-375-3p increased significantly at 48 or 72 h after 7 Gy irradiation (Fig. 4A) and at 24 or 48 h after treatment of 30 mM STZ compared with control supernatants (Fig. 4B). These results suggest that 7 Gy irradiation and STZ treatment trigger the release of miR-375-3p from RIN-5F cells into cell culture supernatants.

Discussion

We have previously shown that miR-375-3p increases in the serum of mice exposed to 7 Gy X-rays causing cytotoxicity in pancreatic β cells (2). However, the direct relationship between miR-375-3p level increases in the blood and the cytotoxicity caused by 7 Gy irradiation remained to be clarified. In this study, employing an *in vitro* cell culture model of pancreatic β cells, we investigated the direct release of miR375-3p from damaged cells.

First, we confirmed that RIN-5F cells have traits characteristics of pancreatic β cells. Pancreatic β cells are insulin-producing cells and are known to express miR-375-3p (16). The expression of insulin mRNA was confirmed by RT-PCR, and insulin was detected by fluorescent immunostaining (Fig. 1). In this study, RIN-5F cells had traits of pancreatic β cells including expression of insulin.

Next, we investigated the effects of 7 Gy X-ray irradiation on cell proliferation and cell death in RIN-5F cells. The suppression of cell proliferation and the increase of PI-positive cells were observed at 24 h after irradiation (Figs. 2A and 3A). Although the expression of extracellular miR-375-3p was not significantly different after 24 h of irradiation, it was significantly increased after 48 and 72 h (Fig. 4A), suggesting that miR-375-3p requires over 24 h after irradiation to be released in the medium. On the other hand, 30 mM STZ treatment inhibited cell proliferation and increased cell death at 24 or 48 h, when treated with 30 mM STZ (Figs. 2B and 3B), and the expression of extracellular miR-375-3p was concomitantly and

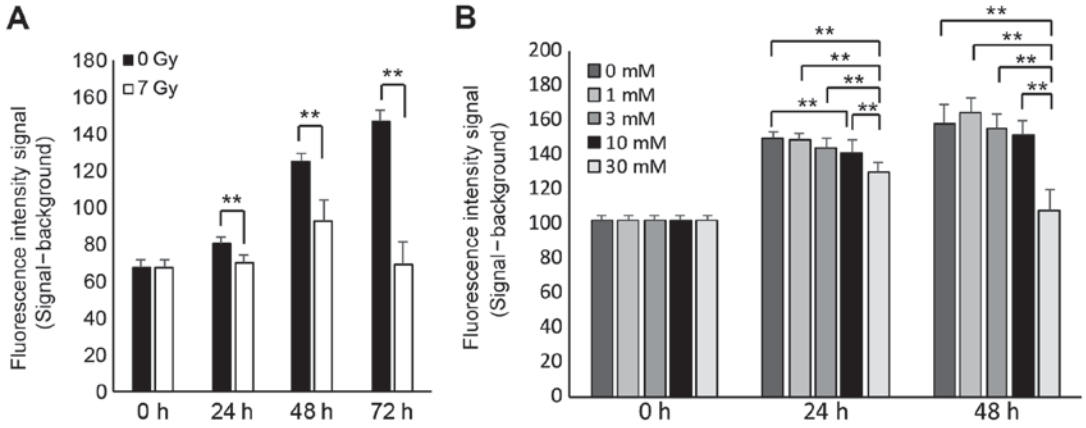

Figure 2. The effects of X-ray irradiation and STZ treatment on cell proliferation in RIN-5F cells. Cell proliferation assay was performed using an alamarBlue cell viability reagent. (A) Cell proliferation profiles after 0 and 7 Gy irradiation. Statistical comparisons were determined using Student's t-test. **P<0.01. (B) Cell proliferation profiles after treatment with 0, 1, 3, 10, and 30 mM STZ. The horizontal axis represents time after irradiation or treatment (h), whereas the vertical axis represents fluorescence intensity (Excitation, 544 nm; Emission, 590 nm). Statistical comparisons were determined using the one-way ANOVA with Tukey's multiple comparisons. **P<0.01. All bars, mean ± standard deviations. Each 8 sample was used.

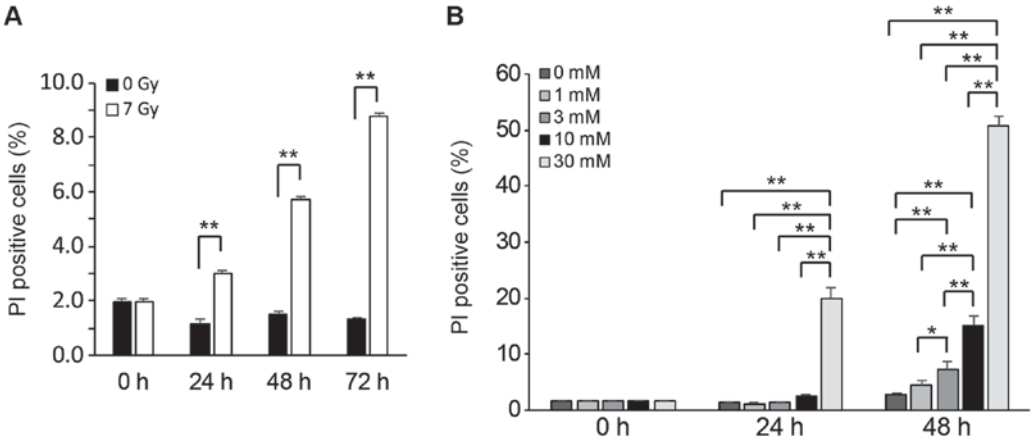

Figure 3. The effects of X-ray irradiation or STZ treatment on cell death in RIN-5F cells. (A) Cell death detection by PI-positive RIN-5F cell quantification following 0 and 7 Gy irradiation. Statistical comparisons were determined using Student's t-test. **P<0.01. (B) Cell death detection by PI-positive RIN-5F cell quantification following 0, 1, 3, 10 and 30 mM STZ treatment. Statistical comparisons were determined using the one-way ANOVA with Tukey's multiple comparisons. *P<0.05 and **P<0.01. All bars, mean ± standard deviations. Each 4 sample was used. PI-positive RIN-5F cells were quantified using Cytomics FC500 (Beckman Coulter). The horizontal axis represents time after irradiation or treatment (h), whereas the vertical axis represents the rate of PI-positive cells within a gate (%).

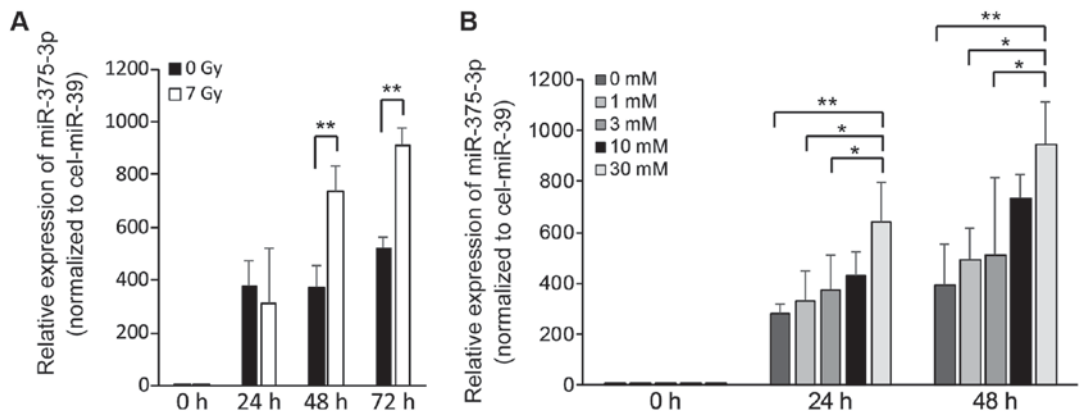

Figure 4. The effects of X-ray irradiation and STZ treatment on miR-375-3p released from RIN-5F cells. (A) The expression of miR-375-3p in culture supernatants of RIN-5F cells after 0 and 7 Gy irradiation. Statistical comparisons were determined using Student's t-test. **P<0.01. (B) The expression of miR-375-3p in culture supernatants of RIN-5F cells treated with 0, 1, 3, 10, and 30 mM STZ. Statistical comparisons were determined using the one-way ANOVA with Tukey's multiple comparisons. *P<0.05 and **P<0.01. All bars, mean ± standard deviations. Each 4 sample was used. The expression of miR-375-3p in culture supernatants of RIN-5F cells were determined by RT-qPCR. The horizontal axis represents time after irradiation or treatment (h), whereas the vertical axis represents relative expression of miR-375-3p. Cel-miR-39 was used for as an external control.

significantly increased at these times (Fig. 4B). Therefore, the mechanism of miR-375-3p release may be different after X-ray irradiation compared with after STZ treatment.

STZ causes the selective destruction of pancreatic β cells via a glucose transporter 2 (20). The release of miR-375-3p from cellular to extracellular space is enhanced by the treatment of STZ (22,23). Therefore, STZ have been used as a therapeutic drug for the treatment of insulinoma (21). We propose that the miR-375-3p increase in blood is due to leakage from damaged pancreatic β cells caused by STZ. High-dose irradiation induces a programmed cell death called apoptosis (25). Apoptotic cell death minimizes leakage of cell contents by forming EVs such as apoptotic bodies (26). In Fig. 4A, extracellular miR-375-3p did not increase at 24 h after 7 Gy irradiation compared with control probably due to cell death by apoptosis. To clarify whether extracellular miR-375-3p is contained in EVs such as apoptotic bodies of culture supernatant after X-ray irradiation and STZ treatment, we aim to analyze in detail apoptosis and EV contents following X-ray irradiation and STZ treatment in the future.

In this study, we clarified that pancreatic β-cell injury induced an extracellular miR-375-3p increase. PI is only taken up by the cell when the membrane is damaged and decreased metabolism, and binds to the nuclear DNA. Therefore, cells undergoing late apoptosis or necrosis can be detected with PI. In the future, it is necessary to assess the degree of membrane injury using Annexin V staining binding to membrane phospholipid.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MC was a major contributor in performing experiments and writing the manuscript. IN, HU, and HK helped perform experiments. MC was the leader of this study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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