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Letter

Relationship between micronucleus formation and oxidative stress in human vascular endothelial cells under low dose rate irradiation

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ABSTRACT — Acute irradiation stimulates oxidative stress and DNA damage responses. However, it is unknown whether chronic irradiation (IR) at a low dose rate causes similar responses, and epidemiological studies of radiation-exposed people with low doses have reported effects on cardiovascular diseases. Therefore, we investigated the cellular effects under low dose rate of IR in human vascular endothelial cells as a model for cardiovascular diseases. We demonstrated that a low dose rate of IR induces phosphorylation of p38MAPK and STAT1, which is related to cGAS, and increases p21, a cellular senescence-regulatory factor. A low dose rate of IR also causes a remarkable formation of micronuclei in human vascular endothelial cells. DIA proteome analysis in human vascular endothelial cells indicated an increase in oxidative stress- and inflammation-related protein levels, and a decrease in protein levels related to the repression of micronuclei formation following exposure to low dose rate of IR. These results suggest that a low dose rate of IR might induce oxidative stress and micronuclei formation, which could activate the cGAS pathway and subsequently lead to cellular senescence.

Key words: Oxidative stress, Micronuclei, ATM, Low dose rate radiation, Mitochondria

INTRODUCTION

Genomic DNA records genetic information, and along with several proteins including histones, forms chromatin which maintains genomic stability. However, various endogenous and exogenous genotoxic stresses threaten genomic stability through the generation of various types of DNA damage. Among them, the most serious damage to mammalian cells is DNA double-strand breaks (DSB), often generated due to exposure to ionizing radiation (IR), and can lead to cell death or tumorigenesis. Hence, cells have developed DNA damage responses (DDRs), including cell cycle checkpoints and DNA repair mechanisms. DNA repair for DSB damage is mainly regulated by non-homologous end-joining, and homologous recombination (HR) in eukaryotes (Saito *et al*., 2016). Base excision repair (BER) can repair the oxidative damage to genomic DNA caused by reactive oxidative species (ROS)

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and induced endogenous and exogenous stresses like IR (Halliwell and Aruoma, 1991). An unrepaired oxidative DNA damage could lead to tumorigenesis via base substitution during DNA replication (Friedberg *et al*., 2005). Exposure of mammalian cells to 1 gray (Gy) of acute irradiation is estimated to generate ~50 nucDNA DSBs (Goodhead, 1994). Exposure to < 10 mGy of γ-rays generates < 1 DSB per nucleus. However, 1 Gy of acute irradiation induces more than one hundred thousand ionizations to various molecules (mainly water) in the nucleus. Hence, 10 mGy of γ-rays might generate more than one thousand ionizations in the nucleus, which could lead to ROS generation through water radiolysis and mitochondrial dysfunction (Kawamura *et al*., 2018). As ROS cause oxidative DNA damage, the generation of ROS with low dose (rate) irradiation may be a threat to genomic stability.

Radiation hypersensitivity disorders have been useful in identifying the mechanisms of DSB damage responses following IR, with ataxia-telangiectasia (AT) being the most common disorder. Cells derived from AT patients commonly show radiation hypersensitivity, radio-resistant DNA synthesis (RDS), and chromosome aberrations (Paull, 2015; Kobayashi, 2019). The gene responsible for AT is *ATM*, and the protein product, ATM, is a protein kinase (Paull, 2015; Kobayashi, 2019). ATM is an inactive homodimer without DSB damage, however, generation of DSB damage stimulates auto-phosphorylation of ATM, leading to dimer dissociation. MRE11/RAD50/NBS1 (MRN) is an important DDR complex that binds to the ATM monomer, and facilitates its recruitment to the DSB damage sites. Defects in these MRN genes also cause genetic radiation hypersensitivity disorders (Kobayashi, 2019). ATM then phosphorylates several DDR factors that regulate cell cycle checkpoints and DNA repair (Kobayashi, 2019). Although ATM kinase activation is dependent on DSB damage generation, it can also occur following oxidative stress (Kobayashi, 2019; Guo *et al.*, 2010). Guo *et al*. (2010) reported that oxidative stress by hydrogen peroxide treatment caused ATM activation *in vitro* and *in vivo*, likely due to the formation of disulfide bonds, causing conformational changes in the ATM dimers. Neural stem cells are sensitive to oxidative stress, and neural cells from ATM-deficient mice show decreased viability. However, radical scavenger treatment recovered the viability of ATM-deficient cells (Guo *et al*., 2010; Stern *et al*., 2002), suggesting that ATM could function to assert against oxidative stress, and might be dispensable for the viability of neural cells in the cerebellum.

Humans, such as residents in high background radiation areas or space station astronauts, are continuously exposed to low dose rates of IR for long periods. Residents in the vicinity of the evacuated areas of Fukushima Daiichi nuclear power plant may have been exposed similarly due to the disaster. Exposure to low dose rates of IR may induce excess ROS, and subsequent oxidative DNA damage, which may be related to cancer risk. Hence, it is important to clarify biological responses toward low dose rates of IR, and the subsequent biological fates. Several researchers have reported the biological effects of exposure to low dose rate of IR in human fibroblasts (Nakamura *et al*., 2005, 2006), however its effects on other types of cells, including vascular endothelial cells, are not known. Epidemiological studies in people exposed to low doses of radiation, have revealed carcinogenic effects at exposures of 100 mGy or more (Grant *et al*., 2017). Cardiovascular diseases have also been suggested from the epidemiological studies (Ozasa *et al.*, 2017), with the involvement of inflammatory cytokines in these cardiovascular diseases (Kusunoki *et al*., 2010). However, the actual mechanism of radiation effects on the circulatory system is unknown. It has been recently reported that cGAS, induced by high doses of IR, accumulates and activates in some of the micronuclei, inducing the expression of inflammatory cytokines; the extracellularly secreted cytokines induce cellular senescence around non-irradiated cells (Mackenzie *et al*., 2017; Harding *et al*., 2017). Micronuclei form due to oxidative stress induced by treatment with hydrogen peroxide, and also with irradiation using low doses of IR (Widel *et al*., 2003; Dreosti *et al.*, 1990). These reports suggest that the formation of micronuclei in vascular endothelial cells may lead to cardiovascular diseases through inflammation via the oxidative stress/micronuclei/cGAS pathway under low dose rate of IR. Therefore, we investigated whether a low dose rate of IR induces oxidative responses and micronuclei formation in human vascular endothelial cells, and the contribution of cGAS. We found that a low dose rate of IR activated oxidative stress responses, such as the phosphorylation of p38MAPK, and cGAS-related pathways, and a reduced induction of DSB-related responses. A low dose rate of IR also remarkably caused the formation of micronuclei; however, a high dose rate of IR did not. Inhibition of ATM increased oxidative damage following IR. DIA proteome analysis indicated the possible activation of oxidative stress- and inflammation-related responses, following exposure to a low dose rate of IR. Thus, a low dose rate of IR might induce micronuclei formation in human vascular endothelial cells, and we discuss the relationship between micronuclei formation and other cellular responses under low dose rate of IR.

MATERIALS AND METHODS

Cells and culture

Human vascular endothelial cells, HUE101-1, were cultured with HuMedia-EG (Kurabo, Osaka, Japan) on collagen I-coated dishes (Anno *et al.*, 2007). hTERT-immortalized human fibroblasts (48BR) and U2OS cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and antibiotics (Kawamura *et al*., 2019).

Gamma-ray irradiation and drug treatment

High dose rate irradiation was performed using the Gammacell 40 Exactor (Nordion Inc., Kanata, Canada). The radioisotope source was Cs-137 (132.2 TBq), and the dose rate was 0.9 Gy/min. Low dose rate irradiation was performed using a low dose and low dose-rate irradiation system (Sangyo Kagaku Co., Ltd., Tokyo, Japan). The radioisotope source was Cs-137 (1.85 TBq), and the dose rate was 1 mGy/min (Nakamura *et al*., 2005).

Antibodies

The following antibodies were used in this study: mouse monoclonal anti-γH2A histone family X (H2AX) [#05-636], and rabbit polyclonal anti-H2B (#07-371) (Merck Millipore, Burlington, MA, USA); anti-phospho-KAP1 (A300-767A; Bethyl Laboratories, Inc., Montgomery, TX, USA); rabbit polyclonal anti-phospho-p38MAPK (#4511), anti-phospho-pHSP27 (#2405), anti-phospho-pAMPKα (#2535), anti-cGAS (#15102), and phospho-STAT1 (#9167) (Cell Signaling Technology, Danvers, MA, USA); mouse monoclonal anti-KAP1 (GTX49179; GeneTex, Irvine, CA, USA); mouse monoclonal anti-beta-actin (A53169; Sigma-Aldrich); mouse monoclonal anti-p53 (sc-126), and anti-cyclin A (sc-751) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). KU-55933 (Sigma-Aldrich) was used to inhibit ATM kinase activity.

Western blotting and immunofluorescence

Western blotting of whole cell extract was performed as described previously (Kawamura *et al*., 2019). Target proteins were detected with the primary antibodies listed above, and secondary horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies (GE Healthcare, Chicago, IL, USA), and were visualized using the ECL plus chemiluminescence system (GE Healthcare).

Immunofluorescence staining was carried out as described previously (Kawamura *et al*., 2019), using Alexa-488-conjugated anti-mouse IgG (Molecular Probes, Inc., Eugene, OR, USA) to visualize the localization of target proteins.

DIA proteome

Unirradiated or irradiated (30 min after high or low dose rate of IR) HUE101-1 cells were collected after washing with PBS. DIA proteome analysis of the cells was performed by Kazusa Genome Technologies Inc. (Kawashima *et al*., 2019). The relative expression of identified proteins was analyzed using the Cystoscape 3.8/string APP software.

RESULTS

Activation of the cGAS-dependent pathway in human vascular endothelial cells

We first investigated the continuous effect of radiation exposure on human vascular endothelial cells, HUE101-1, and the role of the cGAS-related pathway, using western blot analysis. A high dose rate of IR caused an increase in the phosphorylation of histone H2AX (γ-H2AX), a DSB damage marker, while a low dose rate of IR did not, indicating generation of few DSB damage under low dose rate of IR (Fig. 1A). KAP1, phosphorylated by ATM in response to DSB damage also showed a similar tendency. cGAS was very low in HUE101-1 cells with or without irradiation, however, phosphorylation of STAT1 which is dependent on cGAS activation, increased after 1 day as well as 15 min following low dose rate of IR. Furthermore, p21, an important regulator of cellular senescence (Naka *et al.*, 2004), increased following a low dose rate of IR, however morphological changes in HUE101-1, like senescent human fibroblasts were not observed (Fig. 1B) (Naka *et al.*, 2004).

We next compared cellular responses after irradiation among HUE101-1, human fibroblast 48BR, and tumor-derived U2OS cells (Figs. 1C, D). Phosphorylation of STAT1, and of p38MAPK, which is stimulated in response to oxidative stress, were induced remarkably in HUE101-1 cells following a low dose rate of IR, whereas γ-H2AX was not (Fig. 1C). Pyocyanin is an inhibitor of the electron transfer chain of mitochondria and induces mitochondria-dependent ROS (Managò *et al.*, 2015). As shown in Fig. 1C, pyocyanin treatment increased phosphorylation of STAT1 and p38MAPK, suggesting that increase in their phosphorylation following low dose rate IR might be due to ROS induction. In the case of 48BR fibroblasts, phosphorylation of STAT1 with or without IR and treatment was not observed (data not shown), although phosphorylation of p38MAPK was high after low dose rate of IR and pyocyanin treatment (Fig. 1D). DNA damage and oxidative stress-dependent signal transducer, p53 increased markedly following low dose rate

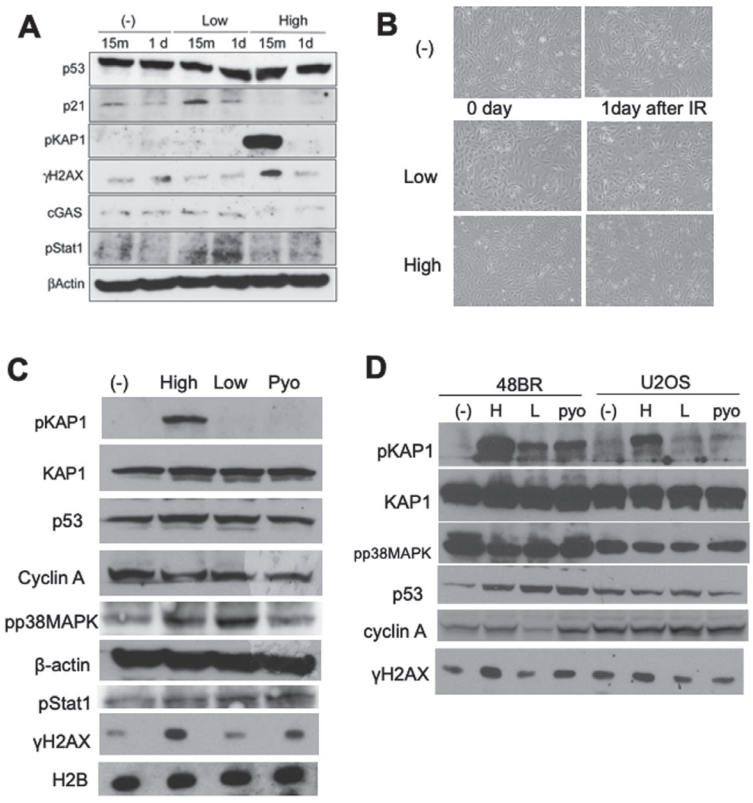

Fig. 1. Low dose rate of IR caused both cGAS- and oxidative stress-dependent responses in human vascular endothelial cells. (A) Extracts from HUE101-1 cells, irradiated by high or low dose rate γ-rays following incubation for 15 min or 1 day, were analyzed by western blot analysis using the indicated antibodies. (B) Morphology of HUE101-1 with or without irradiation with phase-contrast microscope. (C and D) Extracts from HUE101-1 (C) or 48BR and U2OS (D) cells, irradiated by high or low dose rate γ-rays following incubation for 15 min or treated with 10 μM of pyocyanin for 2 hr, were analyzed by western blot analysis using the indicated antibodies.

of IR and pyocyanin treatment in 48BR cells (Fig. 1D), while a slight increase in p53 was observed in HUE101-1 cells (Fig. 1D). Cyclin A, a marker for cell proliferation, decreased in 48BR cells following a low dose rate of IR (Fig. 1D), in agreement with continuous arrest in the G1 phase as observed by flow cytometry analysis (data not shown). However, a remarkable change in cyclin A in HUE101-1 cells with or without IR or pyocyanin treatment was not observed (Fig. 1C). Phosphorylation of KAP1 increased in U2OS cells after a high dose rate of IR, however, no other changes were caused (Fig. 1D). These results suggest that a low dose rate of IR might induce an oxidative response in both human vascular endothelial cells and fibroblasts, while the activation of the cGAS-dependent pathway, including STAT1 phosphorylation, may be a vascular endothelial cell-specific phenomenon.

Induction of γ-H2AX-positive micronuclei in human vascular endothelial cells

As the results suggest the activation of the cGAS-dependent pathway (Figs. 1A, B, C), we investigated the formation of micronuclei, which could cause cGAS activation after IR in HUE101-1 cells, by immunofluorescence of γ-H2AX and DAPI (4',6-diamidino-2-phenylindole) staining (Fig. 2A). Approximately 10% of HUE101-1 cells contained γ-H2AX-positive micronuclei in each cytoplasm, and that the percentage increased significantly to more than 20%. Pyocyanin treatment also induced γ-H2AX-positive micronuclei, suggesting micronuclei formation due to ROS-dependent oxidative stress. These micronuclei may activate cGAS and the dependent pathways in response to a low dose rate of IR.

We also examined micronuclei formation in 48BR cells, and observed that 48BR cells did not form both γ-H2AX-positive or -negative micronuclei (data not shown).

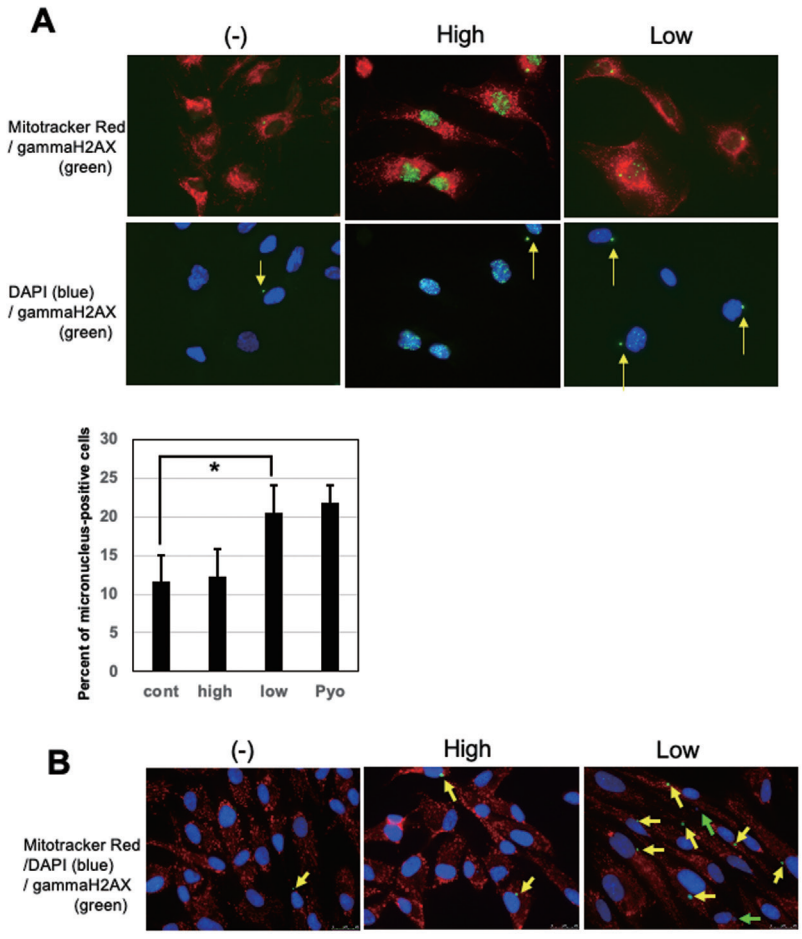

Fig. 2. Low dose rate of IR causes γ-H2AX-positive micronuclei. (A) HUE101-1 cells, irradiated by high or low dose rate γ-rays following incubation for 30 min, fixed and then immunostained with γ-H2AX antibodies and stained using Mitotracker Red for mitochondria and DAPI. The percentages of micronuclei-positive cells are shown lower graph. [* $p < 0.05$]. (B) 48BR cells, irradiated by high or low dose rate γ-rays with ATM-specific inhibitor (KU-55933, 10 μM) following incubation for 30 min, fixed and then immunostained with γ-H2AX antibodies and stained using Mitotracker Red for mitochondria and DAPI.

As ATM can be activated in response to ROS, and possess anti-oxidative stress function (Guo *et al.*, 2010; Stern *et al.*, 2002; Kamsler *et al.*, 2001), we speculated that inhibition of ATM kinase activity could increase ROS, and subsequent oxidative stress. Hence, we investigated the formation of γ-H2AX-positive micronuclei in the presence of the ATM-specific inhibitor KU-55933 in 48BR cells (Fig. 2B). KU-55933 treatment induced γ-H2AX-positive micronuclei in non-irradiated cells (5% of cells), while a low dose rate of IR remarkably increased the number of micronuclei-positive cells to 43.8%, and a high dose rate of IR induced less micronuclei-positive cells (11.8%). We also observed the formation of γ-H2AX-negative-miclonuclei (green arrows) following a low dose rate of IR, however, such micronuclei may not contain any remarkable DNA double-strand break damage. Taken together, 48BR cells might resist oxidative stress more than HUE101-1 cells, however, disturbance of anti-oxidative stress function by ATM may cause micronuclei in response to IR.

ATM can participate in cGAS-dependent pathway as well as oxidative stress responses

ATM plays an important role as an anti-oxidative stress mediator particularly in 48BR fibroblasts, to repress γ-H2AX-positive micronuclei with oxidative stress under low dose rate of IR. Hence, we investigated whether inhibition of ATM kinase with KU-55933 amplifies the oxidative stress response and the cGAS-dependent pathway. Inhibition of ATM kinase caused a remarkable decrease

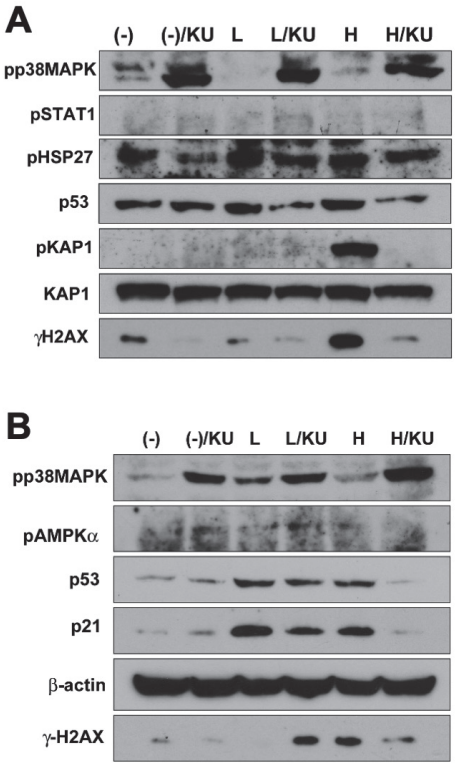

Fig. 3. Inhibition of ATM causes oxidative stress responses. Extracts from HUE101-1 (A) or 48BR (B) cells irradiated with high or low dose rate γ-rays (total dose: 3 Gy) with or without KU-55933, and incubated at the indicated time points post-irradiation, were analyzed by western blot analysis using the indicated antibodies.

in cell viability in non-irradiated HUE101-1 (data not shown), suggesting that ATM activity might be essential for the survival of human vascular endothelial cells. However, treatment with KU-55933 for 2 days did not influence cell viability in 48BR cells (data not shown).

We examined oxidative stress or cGAS-dependent phosphorylation with an ATM-specific inhibitor in HUE101-1, and the inhibitor treatment remarkably increased the phosphorylation of p38MAPK with or without IR, while p53 and phosphorylation of HSP27 were not influenced (Fig. 3A). The inhibitor treatment could repress phosphorylation of KAP1 and H2AX by ATM, following a high dose rate of IR. However, cGAS-dependent phosphorylation of pSTAT1 increased mildly. In the case of 48BR cells, ATM inhibition also increased the phosphorylation of p38MAPK and AMPKα, indicating an increase in oxidative stress (Fig. 3B). The inhibitory effect on γ-H2AX in 48BR cells also showed a similar tendency in HUE101-1. Although responses of p53 and p21 following high dose rate of IR are dependent on the generation of DSB damage and subsequent ATM activation, their responses did not disturb the ATM-specific inhibitor. A low dose rate of IR may induce ATM-independent responses of p53 and p21.

DIA proteome analysis reveals activation of oxidative stress and inflammation-dependent responses

To clarify the details of the activation of the oxidative response and the cGAS-dependent pathway, extracts from non-irradiated and irradiated cells were analyzed using the DIA proteome method. The DIA analysis clarified that there was a two times increase in 131 proteins at a low dose rate of radiation in HUE101-1 cells compared with non-irradiated cells (data not shown). These proteins contain factors related to growth factor signaling, inflammation, oxidative stress response, p53 pathway, or integrin signaling, and most of them increased after a high dose rate of IR as well. There was an increase in 33 proteins after a low dose rate of IR alone (Table 1), however the Cystoscape 3.8/string APP analysis did not show marked gene (protein) interaction (Fig. 4A), and no relationship between the cGAS pathway and micronuclei formation was found.

The DIA analysis also identified decrease by half in 145 protein levels due to low dose rate of IR, the irradiated cells compared to non-irradiated cells (data not shown). These proteins were factors related to DNA replication, chromatin/chromosome regulation, ubiquitin/SUMO pathway, or DNA repair, with a decrease in 83 proteins (Table 2), only due to a low dose rate of IR. Analysis was performed using the Cystoscape 3.8/string APP software, and a marked interaction of 21 proteins was observed (Fig. 4B). Among them, the kinesin superfamily proteins and DNMT1 are reported to be related to micronuclei formation (Wei and Yang, 2019; Tan and Porter, 2009). Moreover, the identified SMC proteins are important for chromatin maintenance (Hassler *et al.*, 2018). Reduction of these proteins may facilitate micronuclei formation in human vascular endothelial cells.

DISCUSSION

Epidemiological studies of radiation-exposed people with low doses have revealed carcinogenic effects at exposures of 100 mGy or more (Grant *et al.*, 2017). However, studies have also reported effects on cardiovascular diseases, related to inflammatory cytokines in radiation-exposed individuals (Ozasa *et al*., 2017; Kusunoki *et al.*, 2010). cGAS, activated by micronuclei is able to activate inflammatory pathways, and cause subsequent cellu-

Table 1. Increased proteins after low dose rate of IR.

Protein name	Gene name	Ratio of protein amount (Low/control)
Sulfiredoxin-1	SRXN1	3.22
Hornerin	HRNR	4.94
Ferritin light chain	FTL	3.62
Ubiquitin-conjugating enzyme E2 Q1	UBE2Q1	2.51
Scavenger receptor class B member 1	SCARB1	2.21
ADP-ribosylation factor-like protein 8A	ARL8A	2.40
Filaggrin-2	FLG2	3.18
CD63 antigen	CD63	2.71
GDP-fucose transporter 1	SLC35C1	2.36
Signal peptide peptidase-like 2B	SPPL2B	2.17
Golgi-associated kinase 1B	GASK1B	2.27
FGGY carbohydrate kinase domain-containing protein	FGGY	3.76
Sentrin-specific protease 1	SENP1	2.40
Ferritin heavy chain	FTH1	3.25
DNA replication complex GINS protein SLD5	GINS4	3.01
Zinc finger protein 644	ZNF644	2.05
Calcium signal-modulating cyclophilin ligand	CAMLG	3.26
Probable JmjC domain-containing histone demethylation protein 2C	JMJD1C	2.10
Son of sevenless homolog 2	SOS2	7.27
Desmoglein-1	DSG1	3.32
Collagen alpha-1(XXII) chain	COL22A1	3.03
Unconventional myosin-If	MYO1F	3.10
Mitogen-activated protein kinase kinase kinase 4	MAP3K4	4.20
EGF-like repeat and discoidin I-like domain-containing protein 3	EDIL3	5.64
Beta-secretase 2	BACE2	3.41
Uncharacterized protein C1orf131	C1orf131	2.67
Tumor necrosis factor receptor superfamily member 21	TNFRSF21	3.82
Nuclear factor 1 A-type	NFIA	3.32
DET1 homolog	DET1	12.4
Pleckstrin homology domain-containing family O member 1	PLEKHO1	3.49
DNA-directed RNA polymerase III subunit RPC10	POLR3K	2.25
Ras-related protein Rab-37	RAB37	535
MLX-interacting protein	MLXIP	2.84

lar senescence following high doses of IR (Mackenzie *et al*., 2017; Harding *et al*., 2017; Widel *et al*., 2003; Dreosti *et al.*, 1990). Hence, we investigated cellular effects under a low dose rate of IR, and the contribution of cGAS in human vascular endothelial cells. We demonstrate that a low dose rate of IR activates oxidative stress responses such as phosphorylation of p38MAPK and cGAS-related pathways, like phosphorylation of STAT1, and increase in p21, a cellular senescence marker (Fig. 1A, C). A low dose rate of IR also causes remarkable formation of micronuclei in human vascular endothelial cells, however, a high dose rate of IR does not (Fig. 2A). Inhibition of ATM increases oxidative damage following IR (Fig. 3A). Low dose rate of IR does not cause formation of micronuclei in human fibroblast, but inhibition of ATM causes formation of micronuclei (Fig. 2B) and increases oxidative stress (Fig. 3B). DIA proteome analysis in human vascular endothelial cells suggests that oxidative stress and inflammation-related responses are activated following exposure to low dose rate of IR. These results indicate that a low dose rate of IR might induce micronuclei particularly in vascular endothelial cells, which could activate the cGAS pathway, and subsequently lead to cellular senescence (Fig. 5). ATM has anti-oxidative roles, which might contribute to repress micronuclei formation under low dose-rate of IR.

DIA analysis indicates a two times increase in 131 proteins, and a decrease in 145 proteins by half, in low dose rate of IR-exposed HUE101-1 cells compared with non-irradiated cells. In particular, 21 remarkably decreased

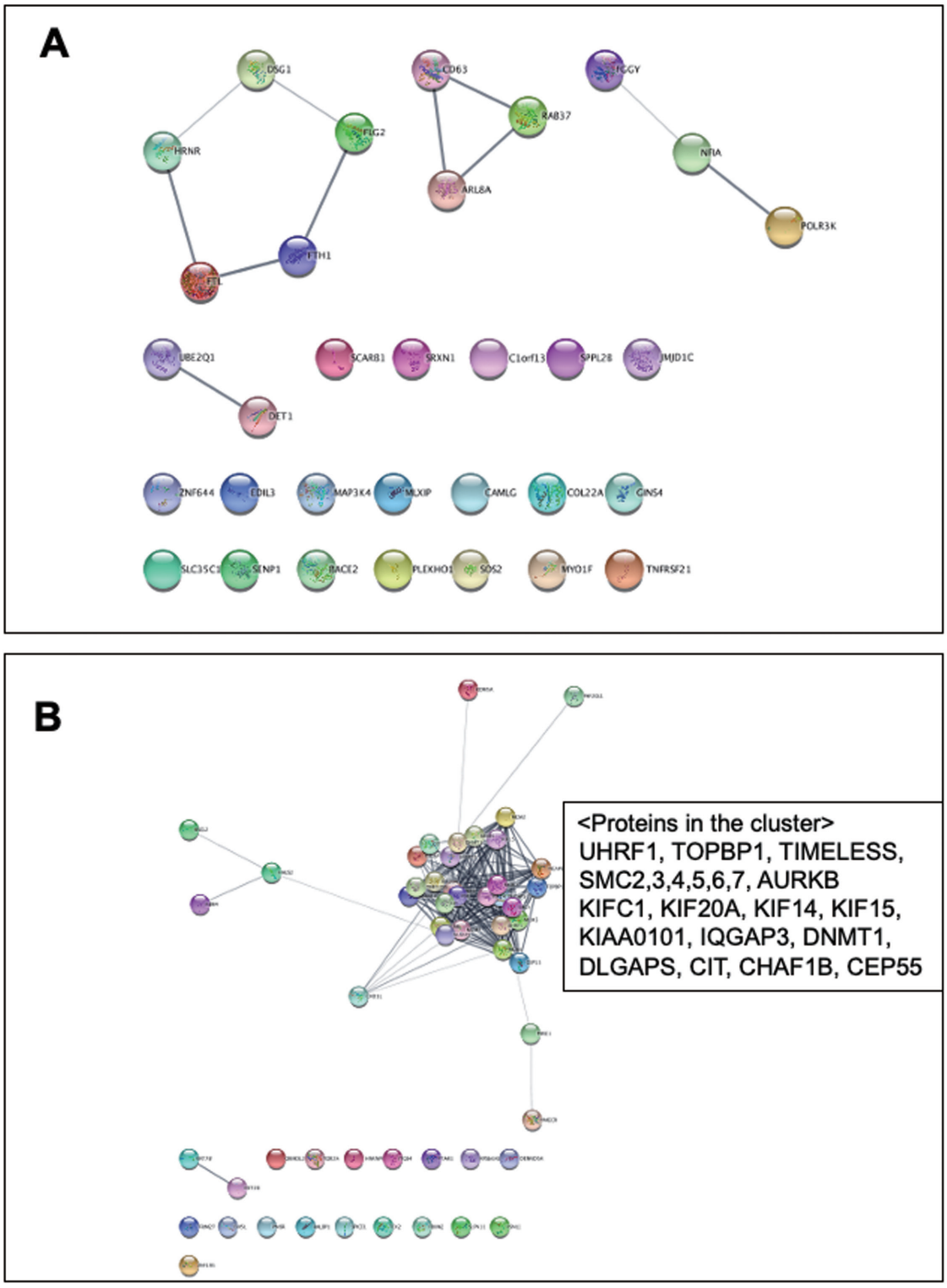

Fig. 4. DIA proteome analysis in non-irradiated and irradiated HUE101-1 cells. DAI proteome analysis of extracts from cells, irradiated, and then harvested at 30 min after IR. Low dose rate of IR-specific increased proteins (A) and decreased proteins (B) were analyzed using Cytoscape 3.8/string APP software for gene interaction.

Table 2. Decresed proteins after low dose rate of IR.

Protein name	Gene name	Ratio of protein amount (Low/control)
DNA replication licensing factor MCM7	MCM7	0.425
DNA replication licensing factor MCM6	MCM6	0.476
DNA (cytosine-5)-methyltransferase 1	DNMT1	0.389
DNA replication licensing factor MCM3	MCM3	0.437
Torsin-2A	TOR2A	0.474
Cyclin-dependent kinase 1	CDK1	0.462
DNA replication licensing factor MCM4	MCM4	0.445
Structural maintenance of chromosomes protein 4	SMC4	0.482
DNA replication licensing factor MCM2	MCM2	0.437
Deoxyuridine 5'-triphosphate nucleotidohydrolase, mitochondrial	DUT	0.388
Ribonucleoside-diphosphate reductase subunit M2	RRM2	0.454
DNA replication licensing factor MCM5	MCM5	0.429
DNA primase small subunit	PRIM1	0.328
Thymidylate synthase	TYMS	0.399
Chromatin assembly factor 1 subunit B	CHAF1B	0.435
Condensin-2 complex subunit D3	NCAPD3	0.399
E3 ubiquitin-protein ligase UHRF1	UHRF1	0.307
Proliferation marker protein Ki-67	MKI67	0.305
Dihydrofolate reductase	DHFR	0.394
Schlafen family member 11	SLFN11	0.385
Lymphokine-activated killer T-cell-originated protein kinase	PBK	0.308
ORM1-like protein 2	ORMDL2	0.308
Ras GTPase-activating-like protein IQGAP3	IQGAP3	0.424
Disks large-associated protein 5	DLGAP5	0.317
Chromosome-associated kinesin KIF4A	KIF4A	0.385
Intraflagellar transport protein 81 homolog	IFT81	0.237
F-box/WD repeat-containing protein 2	FBXW2	0.350
Mimecan	OGN	0.253
Arginine/serine-rich protein PNISR	PNISR	0.203
Testis-expressed protein 2	TEX2	0.310
CD320 antigen	CD320	0.313
Ubiquitin-conjugating enzyme E2 C	UBE2C	0.228
Condensin complex subunit 3	NCAPG	0.449
Kinetochore protein Spc24	SPC24	0.416
Integrin beta-4	ITGB4	0.422
Zinc finger protein RFP	TRIM27	0.474
Fanconi anemia group I protein	FANCI	0.159
Kinesin-like protein KIF15	KIF15	0.304
Nucleolar and spindle-associated protein 1	NUSAP1	0.336
Citron Rho-interacting kinase	CIT	0.341
3-hydroxy-3-methylglutaryl-coenzyme A reductase	HMGCR	0.471
SHC SH2 domain-binding protein 1	SHCBP1	0.208
Cystathionine beta-synthase-like protein	CBSL	0.435
Kinetochore protein NDC80 homolog	NDC80	0.263
Angiomotin-like protein 2	AMOTL2	0.430
PAN2-PAN3 deadenylation complex subunit PAN3	PAN3	0.489
Proline/serine-rich coiled-coil protein 1	PSRC1	0.503
Protein timeless homolog	TIMELESS	0.493
HAUS augmin-like complex subunit 2	HAUS2	0.209
DENN domain-containing protein 5A	DENND5A	0.262
ATPase family AAA domain-containing protein 2	ATAD2	0.139

Table 2. (Continued).

Protein name	Gene name	Ratio of protein amount (Low/control)
Phosphatidate phosphatase LPIN2	LPIN2	0.449
Spindle and centriole-associated protein 1	SPICE1	0.424
Centrosomal protein of 55 kDa	CEP55	0.321
Heterogeneous nuclear ribonucleoprotein C-like 1	HNRNPCL1	0.263
Chromodomain-helicase-DNA-binding protein 1-like	CHD1L	0.231
EVI5-like protein	EVI5L	0.343
Zinc finger protein 64	ZFP64	0.269
Protein prenyltransferase alpha subunit repeat-containing protein 1	PTAR1	0.150
Nitric oxide synthase, inducible	NOS2	0.487
Aurora kinase B	AURKB	0.478
Kinesin-like protein KIFC1	KIFC1	0.450
Aldose 1-epimerase	GALM	0.233
Vang-like protein 1	VANGL1	0.497
Kinesin-like protein KIF20A	KIF20A	0.173
Kinesin-like protein KIF14	KIF14	0.417
Angiomotin-like protein 1	AMOTL1	0.395
Visinin-like protein 1	VSNL1	0.0868
Lysine-specific demethylase 5A	KDM5A	0.400
RNA-binding protein 4	RBM4	0.295
Seipin	BSCL2	0.316
Putative Ras-related protein Rab-1C	RAB1C	0.347
RalA-binding protein 1	RALBP1	0.468
PCNA-associated factor	PCLAF	0.447
DNA topoisomerase 2-binding protein 1	TOPBP1	0.256
Ribosomal protein S6 kinase alpha-5	RPS6KA5	0.327
Zinc finger protein 195	ZNF195	0.409
Decaprenyl-diphosphate synthase subunit 2	PDSS2	0.486
Keratin, type I cytoskeletal 20	KRT20	0.304
Keratin, type II cytoskeletal 78	KRT78	0.310
PHD finger protein 20-like protein 1	PHF20L1	0.305
E3 ubiquitin-protein ligase RAD18	RAD18	0.461
SOSS complex subunit B2	NABP1	0.212

proteins possess a marked interaction (Fig. 4B). Among them, KIF14 and KIFC1-deficient cells show a defect in metaphase mitotic spindle assembly, abnormality of chromosome, and subsequent formation of micronuclei in daughter cells (Wei and Yang, 2019). Inhibition of kinesin-related pathway by an anti-cancer drug causes micronuclei formation (Mitchison *et al*., 2017). Defects in Aurora kinase B (AURKB), important for chromosome assembly in metaphase, shows chromosome miss-segregation and micronuclei formation (Ma and Poon, 2020). This evidence indicates that, remarkable decrease in chromosome assembly regulatory proteins such as kinesin-related proteins (K1FC1, KIF20A, KIF14, KIF15, CIT), and AURKB under low dose of IR may lead to micronuclei formation. Furthermore, DNA methyltransferase I and DNMT1 are reported to localize to micronuclei and mediate their formation, suggesting a role in genome stability (Tan and Porter, 2009). Moreover, their identified SMC proteins are important for chromatin maintenance and genome stability (Hassler *et al*., 2018). Defects in *NSMCE3* (NSE3 homolog, SMC5-SMC6 complex component) causes chromosome breakage syndrome associated with severe lung disease in early childhood, and the patient cells show disrupted interactions within the SMC5/6 complex, leading to destabilization of the complex, also causing chromosome rearrangements and micronuclei formation (van der Crabben *et al.*, 2016). Such reductions in DMNT1 or SMC proteins may facilitate micronuclei formation in human vascular endothelial cells. We reported that mitophagy-related proteins such as PINK1, MFN1 and MFN2 remarkably reduced in human normal fibroblasts (Meng *et al*., 2021), while this

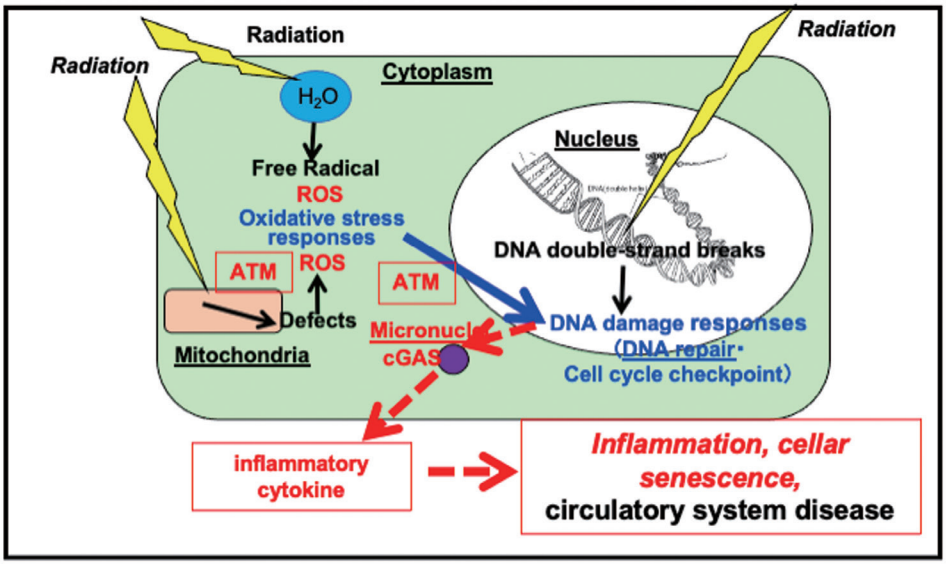

Fig. 5. Possible cellular responses in human vascular endothelial cells under low dose rate of IR. Low dose rate of IR might induce micronuclei, which could activate the cGAS pathway, and subsequently lead to inflammation, cellular senescence and other circulatory diseases-related responses. ATM has an anti-oxidative roles, which might contribute to repress micronuclei formation under low dose-rate of IR.

current DIA proteome analysis did not find such reduction in human vascular endothelial cells. As dysfunction of mitophagy pathway is related with an accumulation of oxidative stress, we have to investigate whether the reduction of mitophagy-related proteins with low dose-rate irradiation is specific to fibroblasts.

ATM is activated in response to oxidative stress such as treatment with hydrogen peroxide, or in oxidative stress-rich organelles (Kobayashi, 2019; Guo *et al.*, 2010; Valentin-Vega *et al.*, 2012; Zhang *et al.*, 2015). In our study, ATM inhibition increases the oxidative stress response in both, vascular endothelial cells and fibroblasts (Figs. 3A, B), and causes cell death in vascular endothelial cells (data not shown), and remarkable micronuclei formation fibroblasts exposed to low doses of IR (Fig. 2B). It is reported that AT-patient cells show remarkable micronuclei formation in response to oxidative stress due to TPA treatment (Ward *et al.*, 1994). In the case of low dose rate of IR, AT patient cells may form oxidative stress-related micronuclei (Nakamura *et al.*, 2006). Anti-oxidative stress function may be important to repress micronuclei formation in normal human fibroblasts. Human vascular endothelial cells under low dose rate of IR demonstrate remarkable micronuclei formation without ATM inhibition (Fig. 2A), suggesting that these cells may express ATM at a lower level than fibroblasts, and may be sensitive to oxidative stress, leading to marked cell death following treatment with ATM inhibitor without irradiation.

Our study demonstrates that a low dose rate of IR causes activation of both, oxidative stress and cGAS-dependent pathways such as STAT1 phosphorylation, subsequent cellular senescence, and micronuclei formation in human vascular endothelial cells (Figs. 1A, C, 2A). Oxidative stress as well as IR, cause micronuclei (Dreosti *et al.*, 1990), and oxidative stress induced by exposure to a low dose rate of IR might cause micronuclei formation and subsequent activation of cGAS. Activation of cGAS is related to inflammation and cellular senescence (Mackenzie *et al.*, 2017; Harding *et al.*, 2017), which agrees with an increase in p21 (Fig. 1A) and increase in several inflammation-related proteins as indicated by DIA proteome analysis (data not shown). No cGAS-related factors were observed among the low dose rate of IR-specific proteins (Fig. 4A), but these 33 proteins (Table 1) may have unknown roles related to cGAS. In order to clarify the effects of chronic exposure of low dose rate of IR in circulatory organs including the heart, there is need to clarify the functional relation of cGAS and the 33 proteins in a circulatory organs model, and in human vascular endothelial cells.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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