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研究課題名（和文）低線量・低線量率放射線被曝による心血管疾患誘発の機構解明

研究課題名（英文）Mechanisms of low-dose/low-dose-rate radiation-induced cardiovascular disease

研究代表者
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交付決定額（研究期間全体）：（直接経費）　　3,300,000円

研究成果の概要（和文）：本研究の目的は、低線量放射線被曝による心血管系疾患リスクとそのメカニズムを解明することである。本研究結果から、血管の内皮最内層が放射線の重要な標的である可能性が明らかとなった。低・中程度の線量（率）の放射線被曝後に、内皮細胞には細胞老化が誘導され、顕著な老化関連分泌表現型（SASP）を示した。in vitro共培養実験によって、照射後の内皮細胞の表面に単球が局所的に集積した。また、アテローム性動脈硬化症のモデルマウスを用いたin vivo実験でも、照射後のSASPと大動脈形態に変化が生じた。細胞老化による内皮-単球の親和性は、放射線誘発アテローム性動脈硬化症の開始に重要である可能性を示唆する。

研究成果の学術的意義や社会的意義
心血管疾患（CVD）は、世界的に主要な死因である。これまでの疫学研究から、放射線被曝が、しきい値無し直線的関係でCVDを誘発する可能性が強く示唆されている。福島原発事故後の健康への社会不安を踏まえ、放射線被曝による心血管系への放射線のリスクを評価し、そのメカニズムを解明することは急務であると言える。本研究は、内皮細胞の老化誘導と機能障害が放射線によるアテローム性動脈硬化症の発症の根本的原因である可能性を示唆する実験結果を提供している。本研究成果は、心血管系への放射線リスクを評価し、CVD予防の標的を特定するための将来的な研究の起点となるものと考える。

研究成果の概要（英文）：The purpose of the current project is to advance the assessment of radiation-associated cardiovascular risk at low doses and dose rates, and to elucidate its molecular mechanisms.
Our data imply that the endothelial lining of blood vessels is a critical target of radiation. Following low and moderate dose/dose rate radiation exposure, endothelial cells exhibited pro-inflammatory changes and cellular senescence with acquisition of robust Senescence Associated Secretory Phenotype (SASP). By using an in vitro co-culture method, we provide evidence for focal accumulation of monocytes on the surface of senescent endothelial cells, which was dependent on both the growth arrest and SASP components of the senescence program. In vivo experiments in a mouse model of atherosclerosis also suggest changes in the SASP profile and aortic morphology after irradiation. We propose that senescence-facilitated endothelial-monocyte affinity is an important step in atherosclerosis initiation by radiation.

研究分野：Radiation biology

キーワード：chronic radiation　low dose/low dose rate　endothelial cells　cardiovascular disease　atherosclerosis　monocyte adhesion

１．研究開始当初の背景（Background at the beginning of research）

Cardiovascular diseases (CVD) are the leading cause of death in the developed World and the second largest in Japan. In recent years it has been recognized that radiation exposure can provoke the development of CVD, especially atherosclerosis, and epidemiological studies in A-bomb survivors and nuclear power plant personnel provide evidence for a linear non-threshold dose dependency[1,2]. Yet, the risk at low doses and dose rates, as well as the mechanisms underlying radiation-induced CVD remain unknown.

In addition to epidemiological data, animal experiments have demonstrated accelerated formation of atherosclerotic plaques in irradiated hypercholesterolemic ApoE$^{-/-}$ mice[3]. However, since such animal models develop extensive spontaneous atherosclerosis, these studies could not shed light on the initial stages of radiation-induced CVD. Outside the radiation context, it is known that atherosclerotic plaques are initiated when monocyte-derived macrophages accumulate within the vascular wall, and that the innermost lining of the blood vessels, which consists of a single layer of highly specialized endothelial cells, plays a major role in this process[4]. Endothelial cells bind and traffic immune cells in response to inflammatory stimuli, but if endothelial damage occurs, dysregulated monocyte trafficking may lead to the initiation of an atherosclerotic plaque. Irradiation of endothelial cells at high doses has been shown to affect expression of adhesion molecules and cytokines and to alter endothelial affinity to immune cell binding[5]. Our preliminary work prior to this project suggests that radiation even at low doses and dose rates can increase the adhesion of monocytes to the endothelial surface.

This project is based on the hypothesis that enhanced endothelial-monocyte interaction can contribute to the formation of atherosclerotic plaques after irradiation (Fig. 1). The underlying molecular pathways may in the future represent promising therapeutic targets.

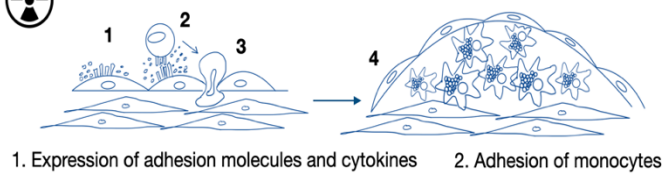

Fig. 1. Scheme of radiation-induced atherosclerotic plaque formation

２．研究の目的（Purpose）

1. To advance cardiovascular risk assessment by interrogating different aspects of the vascular response to radiation at low and moderate doses and dose rates.

2. To elucidate the molecular mechanisms of radiation-induced CVD by investigating the role of inflammatory and senescence related molecular factors in the vascular response to radiation, and particularly in radiation-facilitated endothelial-monocyte interaction.

３．研究の方法（Methods）

***In vitro* experiments:** *In vitro* experiments were carried out using primary adult human aortic endothelial cells (HAEC) and human umbilical vein endothelial cells (HUVEC) obtained from a

single donor (Lonza, USA). Human immortalized U937 cells were used as a monocyte model in functional adhesion assays. Acute irradiation was delivered at 0.8Gy/min and doses between 0.1Gy and 4Gy. Chronic irradiation was delivered over a time interval of 4 days at dose rates between 0.1Gy/d and 1Gy/d and total doses between 0.1Gy and 4Gy.

***In vivo* experiments:** Young adult Ldlr knockout mice were exposed to whole body gamma irradiation with 2Gy acutely (0.8Gy/min) or under chronic conditions at 0.1mGy/d for 20 days (total dose 2Gy). Animals were fed high fat diet for the duration of the experiment and were sacrificed 4 months after irradiation.

Immunofluorescence analysis: Markers of cell proliferation, DNA damage, cell adhesion molecules, etc., in cell cultures or mouse aortic specimens were detected by indirect immunofluorescence using a high-throughput imaging system Opera Phenix® and analyzed by Harmony® software package (Perkin Elmer, Germany).

Quantitative analysis of soluble inflammatory factors in cell culture supernatants and mouse serum samples was performed by Cytometric Bead Array immunoassay using custom made human multiplex soluble factor panels and mouse multiplex pre-defined inflammatory cytokine panels (BioLegend, USA).

Cell senescence was detected by SA-ß-Gal staining, using Senescence detection kit (Abcam, UK). Samples were visualized by wide-field light microscopy and scored manually.

４．研究成果（results）

Experiment 1. Morphological and inflammatory changes in irradiated endothelial cells.

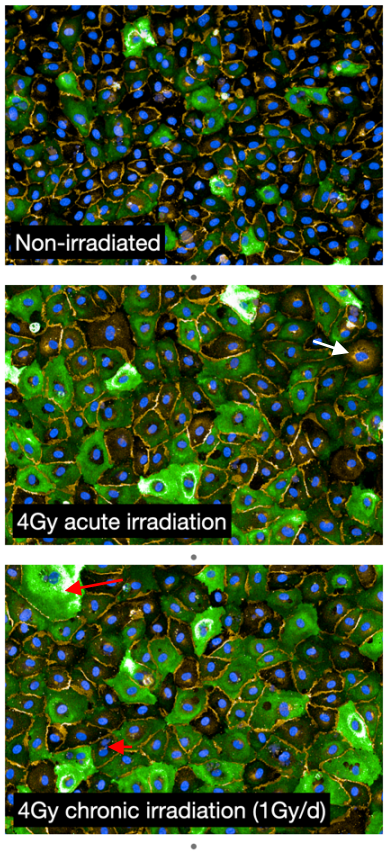

Fig. 2. Expression of ICAM-1 (green) and VE-Cadherin (orange) in irradiated endothelial cells

Adhesion molecules such as ICAM-1, V-CAM-1 and E-Selectin are expressed on the surface of endothelial cells to mediate the interaction with different types of immune cells. Here we observed that **following acute or chronic gamma radiation**, there was a **significant increase in the percentage of endothelial cells expressing high levels ICAM-1,** and to a lesser extend VCAM-1, on the cell surface (Fig. 2, green signal). **The effect was dose dependent**, providing evidence that **low and moderate radiation doses and dose rates can induce a pro-inflammatory response in endothelial cells**. We noted that this upregulation persisted for 10 days after irradiation, indicating that the cells maintained a **chronically upregulated inflammatory status**. Surprisingly, a significant dose rate effect was not observed, i.e., acute and chronic radiation were comparably effective in inducing ICAM-1 upregulation.

In addition, at later time-points (day 8 and day 10 after radiation), notable morphological changes had occurred,

such as decreased cell density, grossly enlarged cell area (Fig. 2., red arrow), bi-nucleated (Fig. 2., red arrowhead) and micro-nucleated cells. Similar morphological changes have long been known to persist in human endothelium and are associated with aging and disease.

Loss of VE-Cadherin mediated cell junctions (Fig. 2., orange signal and white arrow) also was observed in a dose dependent manner, suggesting that irradiated endothelial cells may exhibit increased permeability which *in vivo* might facilitate monocyte penetration into the vascular wall. **Thus, the experiment provided evidence for radiation-induced persisting upregulation of inflammatory molecular mediators and loss of tight cell-to-cell contacts in a dose dependent manner without a significant dose rate effect.**

Experiment 2. Radiation-induced endothelial cell senescence.

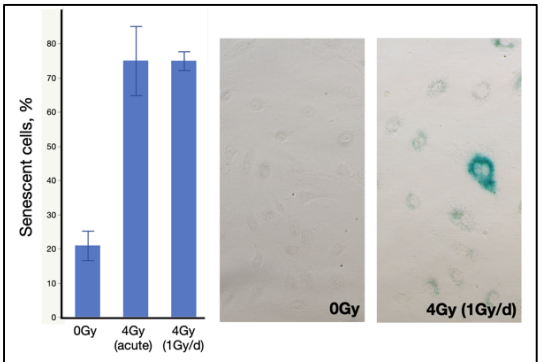

Fig. 3. SA-ß-Gal staining (blue) for analysis of senescence in irradiated endothelial cells

Prompted by the observations of morphological changes, persisting DNA damage and inflammatory signaling, we proceeded to investigate whether irradiated endothelial cells enter a state of senescence. It was revealed that **endothelial cells are highly sensitive to radiation-induced senescence induction**, as even a low dose rate of 0.1Gy/d (total dose 0.4Gy) could increase the percentage of senescent cells, and the majority of cells were senescent at a dose of 4Gy. Again, no significant difference was observed between acute and chronic irradiation (Fig.3).

Senescent cells are known to exhibit a sustained increased secretion of 40-80 soluble factors, known by the common term Senescence Associated Secretory Phenotype (SASP). Here we revealed a robust SASP acquisition by irradiated endothelial cells, manifested mainly as enhanced secretion of **Interleukin-6, Interleukin-8 and Monocyte Chemoattractant Protein-1,** among others. These soluble factors have important roles in intercellular signaling and are required for the binding of immune cells on the vascular wall. **Thus, the experiment provided evidence for the close relationship between endothelial senescence and persistent inflammation, including the secretion of soluble factors which are potent mediators of endothelial-monocyte binding.**

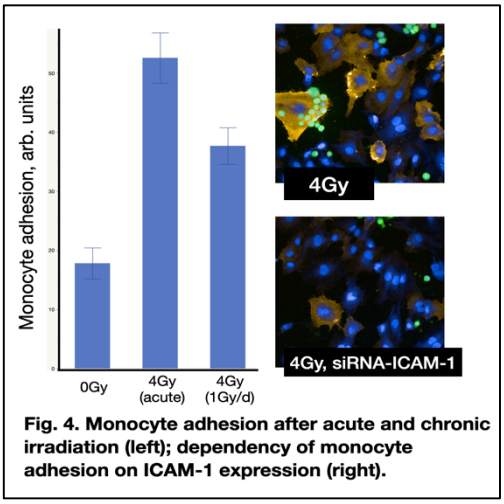

Fig. 4. Monocyte adhesion after acute and chronic irradiation (left); dependency of monocyte adhesion on ICAM-1 expression (right).

Experiment 3. Endothelial-monocyte interaction.

We developed a system for assessment of endothelial-monocyte affinity *in vitro*, and we revealed that in agreement with inflammatory factors expression, radiation effects on endothelial-monocyte binding persist for the whole duration of the experiment (10 days after irradiation). In general, radiation causes an increase in monocyte adhesion (Fig. 4, bar plot) in a dose dependent

manner without a significant dose rate effect. A special focus on cells exhibiting senescence morphology revealed that a subpopulation of these cells shows high ICAM-1 expression and high affinity to monocyte binding (Fig. 4, top image, monocytes – green, ICAM-1 – orange). Monocyte adhesion, ICAM-1 expression and SASP acquisition were abrogated by inhibition of the NF-κB pathway, which did not affect the growth arrest and senescence. On the other hand, siRNA knockdown experiments indicate that bypassing the radiation-triggered growth arrest was able to also prevent the inflammatory upregulation. These results confirm that the growth arrest and SASP are two distinct but inter-related components of the senescence program.

Experiment 4. Inflammatory response in *in vivo* irradiated Ldlr knockout mice.

Radiation effect in Ldlr-/- mice	Genetic background effect (Ldlr-/- vs WT)	No difference
IL-1a	IFN-g	IL-12p70
IL-23	TNF-a	IL-1b
MCP-1	Eotaxin	IL-10
IL-17A	KC	IL-6
IFN-b	MCP-1	IL-27
Eotaxin	MIG	GM-CSF
KC (IL-8 analog)	Mip-1b	RANTES
MIG	BLC	Mip-3a
Mip-1a	LIX	TARC
MDC		IP-10

Table 1. List of soluble factors analyzed in peripheral blood from Ldlr-/- and wild type mice

Ldlr knockout mice are a useful model of human atherosclerosis. Here, in order to evaluate the relevance of our *in vitro* observations, we explored the SASP profile in serum and the endothelial morphology in aortas of mice subjected to *in vivo* irradiation. A total of 25 candidate SASP factors were analyzed, of which 10 showed some radiation effect (Table 1), among them MCP-1 and IL-8, which also showed radiation changes *in vitro*. In regard to both SASP expression and aortic morphology, the effect of high fat diet and the genetic background of Ldlr-/- mice overshadowed the radiation effect.

Conclusions: We demonstrate here that the endothelial lining of blood vessels is highly sensitive to radiation-induced senescence in response to both acute and chronic radiation exposure. Endothelial senescence is accompanied by persisting upregulation of soluble and cell surface inflammatory factors. We provide direct evidence that a subpopulation of senescent endothelial cells exhibits high affinity to monocyte binding. We propose that senescence-associated endothelial-monocyte interaction may be a critical event in the initiation of radiation-induced cardiovascular disease.

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５．主な発表論文等

〔雑誌論文〕　計5件（うち査読付論文　5件／うち国際共著　3件／うちオープンアクセス　3件）

１．著者名 Aneva, N., Zaharieva, E., Katsarska, O., Savova, G., Stankova, K., Djounova, J., Boteva, R.	４．巻 60
２．論文標題 Inflammatory profile dysregulation in nuclear workers occupationally exposed to low-dose gamma radiation	５．発行年 2019年
３．雑誌名 J. of Radiation Research	６．最初と最後の頁 768-779
掲載論文のDOI（デジタルオブジェクト識別子） 10.1093/jrr/rrz059	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

１．著者名 Sasatani M.、Zaharieva E.K.、Kamiya K.	４．巻 42
２．論文標題 The in vivo role of Rev1 in mutagenesis and carcinogenesis	５．発行年 2020年
３．雑誌名 Genes and Environment	６．最初と最後の頁 1-5
掲載論文のDOI（デジタルオブジェクト識別子） 10.1186/s41021-020-0148-1	査読の有無 有
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１．著者名 Cao L.、Wang M.、Dong Y.、Xu B.、Chen J.、Ding Y.、Qiu S.、Li L.、Zaharieva E.K.、Zhou X.、Xu Y.	４．巻 11
２．論文標題 Circular RNA circRNF20 promotes breast cancer tumorigenesis and Warburg effect through miR-487a/HIF-1alpha/HK2	５．発行年 2020年
３．雑誌名 Cell Death & Disease	６．最初と最後の頁 1-10
掲載論文のDOI（デジタルオブジェクト識別子） 10.1038/s41419-020-2336-0	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

１．著者名 Ding Yu、Cao Lili、Chen Ju、Zaharieva Elena Karamfilova、Xu Yanbin、Li Liang	４．巻 26
２．論文標題 Serial image changes in ultrasonography after the excision of benign breast lesions by mammotome biopsy system	５．発行年 2019年
３．雑誌名 Saudi Journal of Biological Sciences	６．最初と最後の頁 178～182
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.sjbs.2018.08.016	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

1．著者名 Zaharieva Elena, Sasatani Megumi, Matsumoto Ryoga, Kamiya Kenji	4．巻 in print
2．論文標題 Formation of DNA damage foci in human and mouse primary fibroblasts chronically exposed to gamma radiation at 0.1 mGy/min	5．発行年 2021年
3．雑誌名 Radiation Research	6．最初と最後の頁 1-15
掲載論文のDOI（デジタルオブジェクト識別子） なし	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

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1．発表者名 Zaharieva, E., Sasatani, M., Kamiya, K.
2．発表標題 Effects of dose-rate on the radiation response of primary fibroblasts
3．学会等名 16th International Congress of Radiation Research
4．発表年 2019年

1．発表者名 Zaharieva, E., Sasatani, M., Kamiya, K.
2．発表標題 Response of primary murine fibroblasts to low-dose-rate versus acute gamma radiation exposures.
3．学会等名 Joint QST-NIRS Workshop (to advance France-Japan collaboration in Radiation Research)
4．発表年 2019年

1．発表者名 Zaharieva, E., Sasatani, M., Kamiya, K.
2．発表標題 DNA damage and oxidative stress in primary murine fibroblasts subjected to acute or chronic gamma radiation.
3．学会等名 The 60th Annual Research Conference of Atomic Bomb Disease
4．発表年 2020年

１．発表者名 Zaharieva, E., Sasatani, M., Kamiya, K.
２．発表標題 Oxidative stress and DNA damage in primary murine fibroblasts subjected to acute or chronic gamma radiation
３．学会等名 3rd International Symposium of the Network-type Joint Usage/Research Center for Radiation Disaster Medical Science
４．発表年 2018年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関