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Effects of chronic restraint-induced stress on radiation-induced chromosomal aberrations in mouse splenocytes

Takanori Katsube[a,*], Bing Wang[a], Kaoru Tanaka[a], Yasuharu Ninomiya[a], Guillaume Varès[b], Taiki Kawagoshi[a], Naoko Shiomi[a], Yoshihisa Kubota[a], Qiang Liu[c], Akinori Morita[d], Tetsuo Nakajima[a], Mitsuru Nenoi[a,*]

[a] National Institute of Radiological Sciences, National Institutes for Quantum and Radiological Science and Technology, Chiba, 263-8555, Japan
[b] Advanced Medical Instrumentation Unit, Okinawa Institute of Science and Technology Graduate University, Okinawa, 904-0495, Japan
[c] Institute of Radiation Medicine, Chinese Academy of Medical Sciences & Peking Union Medical College, Tianjin, 300192, PR China
[d] Department of Biomedical Science and Technology, Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima, 770-8503, Japan

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ABSTRACT

Both ionizing radiation (IR) and psychological stress (PS) cause detrimental effects on humans. A recent study showed that chronic restraint-induced PS (CRIPS) diminished the functions of Trp53 and enhanced radiocarcinogenesis in *Trp53*-heterozygous (*Trp53*$^{+/-}$) mice. These findings had a marked impact on the academic field as well as the general public, particularly among residents living in areas radioactively contaminated by nuclear accidents. In an attempt to elucidate the modifying effects of CRIPS on radiation-induced health consequences in *Trp53* wild-type (*Trp53*$^{+/+}$) animals, investigations involving multidisciplinary analyses were performed. We herein demonstrated that CRIPS induced changes in the frequency of IR-induced chromosomal aberrations (CAs) in splenocytes. Five-week-old male *Trp53*$^{+/+}$ C57BL/6J mice were restrained for 6 h per day for 28 consecutive days, and total body irradiation (TBI) at a dose of 4 Gy was performed on the 8th day. Metaphase chromosome spreads prepared from splenocytes at the end of the 28-day restraint regimen were painted with fluorescence *in situ* hybridization (FISH) probes for chromosomes 1, 2, and 3. The results obtained showed that CRIPS alone did not induce CAs, while TBI caused significant increases in CAs, mostly translocations. Translocations appeared at a lower frequency in mice exposed to TBI plus CRIPS than in those exposed to TBI alone. No significant differences were observed in the frequencies of the other types of CAs (insertions, dicentrics, and fragments) visualized with FISH between these experimental groups (TBI + CRIPS *vs.* TBI). These results suggest that CRIPS does not appear to synergize with the clastogenicity of IR.

1. Introduction

Ionizing radiation (IR) and psychological stress (PS) have a number of detrimental effects on humans. IR represents mutagenicity, carcinogenicity, teratogenicity, and organ system toxicity. DNA damage resulting from the deposition of energy in the cell nucleus is the main cause of the detrimental effects of IR. Based on the findings of epidemiological studies, PS has been suggested to increase the risk of various conditions, such as vision disorders, hypertension, cardiovascular diseases, diabetes, metabolic syndrome, Alzheimer's disease, and cancer development [1–7]. The dysregulation of the immune and other body systems mediated by the hypothalamic-pituitary-adrenal axis and sympatho-adrenomedullary system in response to PS is the main physiopathological process underlying the detrimental health consequences of PS [8–10].

Abbreviations: IR, onizing radiation; PS, psychological stress; CRIPS, chronic restraint-induced PS; CAs, chromosomal aberrations; TBI, total body irradiation; FISH, fluorescence *in situ* hybridization; DCs, dicentrics.

* Corresponding authors at: National Institute of Radiological Sciences, National Institutes for Quantum and Radiological Science and Technology, 4−9−1, Anagawa, Inage−ku, Chiba 263−8555, Japan.
E-mail addresses: katsube.takanori@qst.go.jp (T. Katsube), wang.bing@qst.go.jp (B. Wang), tanaka.kaoru@qst.go.jp (K. Tanaka), ninomiya.yasuharu@qst.go.jp (Y. Ninomiya), guillaume.vares@oist.jp (G. Varès), kawagoshi.taiki@qst.go.jp (T. Kawagoshi), shiomi.naoko@qst.go.jp (N. Shiomi), kubota.yoshihisa@qst.go.jp (Y. Kubota), liuqiang@irm-cams.ac.cn (Q. Liu), morita@tokushima-u.ac.jp (A. Morita), nakajima.tetsuo@qst.go.jp (T. Nakajima), nenoi.mitsuru@qst.go.jp (M. Nenoi).

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Nuclear power plant accidents represent a significant threat and major health concern. The release of multiple radioisotopes and subsequent radiological contamination of the surrounding environment result in exposure to IR. In addition to the potential health effects directly caused by IR, the nuclear accident itself is a cause of social PS due to the fear of health effects [11–13]. Furthermore, radioactive contamination in the environment often prohibits people, particularly children, from doing outdoor activities, which may result in physiological stress as well as PS. Thus, some people will be simultaneously exposed to radiation and develop PS due to nuclear power plant accidents. Therefore, whether PS alters the responses of humans to IR, particularly radiocarcinogenesis, needs to be determined in more detail.

Relationships have been reported between PS and the incidence, progression, and mortality of various cancer types in clinical and epidemiological studies, and have been corroborated by *meta*-analyses [6,14–16]. The stress-induced suppression of immune responses is suspected to impact on the promotion, progression, and metastasis of existing premalignant cells as well as the virus-mediated onset of cancer [8,9,17]. A recent study using a well-established typical mouse model, in which chronic physical restraint was applied to mice to induce PS, demonstrated that chronic restraint-induced PS (CRIPS) promoted radiocarcinogenesis in *Trp53*-heterozygous (*Trp53*$^{+/-}$) mice irradiated with a 4 Gy dose of X-ray radiation [18]. It was also shown that CRIPS decreased Trp53 protein levels and function as well as apoptotic responses in the spleen following total body irradiation (TBI) in *Trp53*$^{+/+}$ mice, and that CRIPS also promoted the growth of human xenograft tumors in a largely Trp53-dependent manner in *Trp53*$^{+/+}$ athymic nude mice [18]. The attenuation of Trp53-mediated cellular responses to IR, such as apoptosis and cell cycle checkpoint regulation, was proposed as a causal mechanism to promote radiocarcinogenesis by CRIPS.

Psychosocial consequences of disasters have been studied for more than 100 years. However, investigations after the nuclear power plant accidents are neither complete nor comprehensive [19]. Psychosocial sequelae and mental health effects were intense and long lasting, occurred independently of the actual exposure received, and were the most significant health consequence following large-scale nuclear power plant disasters (i.e. the Chernobyl and Fukushima accidents) [11–13,20–22]. Experiencing PS repeatedly over a long period of time (i.e., chronic) or exposure to PS in the early time of life may strongly impact on health including increased cancer risk [2,23]. Especially in children, there is a wide range of mental and behavioral sequel due to PS, which could last a long time [24] and cause alterations of mitochondrial DNA copy number and shortening of telomere length [25–27]. This work highlights the effects of health at a young age. However, such studies are still rare, and the documented works have limited information [11]. It is important to note that in radiobiology, IR itself, as the assault that induces various effects, is also called “a stress” or “a stressor”. To avoid any confusion, the term “stress” used herein refers to PS unless otherwise specified.

To elucidate the possible effects of PS on IR-induced health consequences in children, we started a series of investigations in which the biological responses of young *Trp53*$^{+/+}$ mice to TBI under CRIPS were examined using the same experimental setup and conditions (6-h restraint per day for 28 consecutive days, and IR at 4 Gy on the 8th day) as those described by Feng et al. [18]. Measurements included changes in body weight gain and the weights of immune organs, alterations in the levels of blood cytokines and stress hormones, changes in the peripheral blood hemogram and the anti-oxidative activity of blood cells, chromosome aberrations (CAs) in splenocytes and micronuclei in bone marrow erythrocytes, and epigenetic variations (DNA methylation and miRNA expression) and protein expression profiles in the liver. In the present study, we investigated the modifying effects of CRIPS on IR-induced CAs in splenocytes 3 weeks after TBI. CAs are well-recognized biological markers of IR exposure and cancer risk [28–30]. Below we describe experimental conditions and results suggesting the limited impact of CRIPS on carcinogenesis in the splenocytes of *Trp53*$^{+/+}$ mice exposed to TBI.

2. Materials and methods

2.1. Animals and experimental design

Four-week-old C57BL/6 J Jms male mice were purchased from SLC, Inc. (Japan) and maintained in a clean conventional temperature- and humidity-controlled animal facility under a 12-h light/12-h dark photoperiod (lights on from 7:00 A.M. to 7:00 P.M.). Animals housed in autoclaved cages (3 mice per cage) with sterilized wood chips were allowed free access to standard laboratory chow (MB-1, Funabashi Farm Co., Japan) and acidified water (pH 3.0 ± 0.2). Animals were acclimatized to the laboratory conditions for 1 week before use. Five-week-old mice were randomly assigned to 4 experimental groups with 6 mice in each group: the “control group (C Gr)”, receiving neither restraint nor TBI, the “restraint group (R Gr)”, receiving only chronic restraint, the “TBI group (IR Gr)”, receiving only TBI, and the “restraint and TBI group (R + IR Gr)”, receiving chronic restraint and TBI. Chronic restraint, a well-established typical mouse model [18] to induce PS, was applied to mice as described in our previous study [31]. In brief, the mouse restraint system (Flat Bottom Rodent Holder, RSTR541, Kent Scientific Co., USA) was used for chronic periodic restraint on a daily basis of 6 h for 28 consecutive days. Individual five-week-old mice were placed in the restrainer and maintained horizontally in their home cage during the 6-h restraint session (9:30 A.M.–3:30 P.M.) daily. Animals were then released into the same cage and allowed access to food and water during the free session (3:30 P.M.–9:30 A.M.). C Gr and IR Gr received no restraint, but were abstained from food and water at the same time as R Gr and R + IR Gr during the 6-h restraint session each day. TBI with 4 Gy X-ray radiation was delivered at a dose rate of 0.25 Gy/min to IR Gr and R + IR Gr on the 8th day of the 28-day restraint regimen. The 4 Gy is a higher dose than that the nuclear and radiation workers, patients under medical diagnosis or residents living in areas radioactively contaminated by nuclear accidents would be exposed to, while comparable to the dose received in the normal tissues for some cases in the cancer radiotherapy. X-rays were generated with an X-ray machine (Pantak-320S, Shimadzu, Japan) operated at 200 kVp and 20 mA, using a 0.50 mm Al + 0.50 mm Cu filter. An exposure rate meter (AE–1321 M, Applied Engineering Inc., Japan) was used for dosimetry. Mice held in acryl containers were exposed to TBI at room temperature without anesthesia. All experimental protocols involving mice were reviewed and approved by The Institutional Animal Care and Use Committee of the National Institute of Radiological Sciences (NIRS). Experiments were performed in strict accordance with the NIRS *Guidelines for the Care and Use of Laboratory Animals*.

2.2. Isolation of splenocytes and determination of splenocyte numbers

At the end of the 28-day restraint regimen, mice were anesthetized by the inhalation of gaseous isoflurane (2-chloroo-2-(difluoromethoxy)-1,1,1,-trifluoro-ethane) (CDS019936, Sigma-Aldrich, Japan) and then euthanized by cervical dislocation. Spleens were removed aseptically. Splenocytes were isolated by gently rubbing the spleen with a frosted slide glass in RPMI1640 medium (R8758, Sigma-Aldrich, Japan) supplemented with 10% fetal bovine