A laser-plasma–produced soft X-ray laser at 89 eV generates DNA double-strand breaks in human cancer cells

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ABSTRACT

While it has been expected that X-ray laser will be widely applied to biomedical studies, this has not been achieved to date and its biological effects such as DNA damage have not been evaluated. As a first step for its biological application, we developed a culture cell irradiation system, particularly designed for a plasma-driven soft X-ray laser pulse, to investigate whether the soft X-ray laser is able to induce DNA double strand breaks (DSBs) in living cells or not. The human adenocarcimona cell line A549 was irradiated with the soft X-ray laser at a photon energy of 89 eV and the repair focus formation of the DSBs was assessed by immunofluorescence staining with antiphosphory-lated DNA-PKcs (p-DNA-PKcs), ATM (p-ATM) and γ-H2AX antibody. The p-DNA-PKcs, ATM, and γ-H2AX foci were clearly identified after soft X-ray laser irradiation. Furthermore, the increase in the X-ray laser shot number, even from a single shot, results in the increase in p-DNA-PKcs foci. These results are the first evidence that the 89 eV soft X-ray laser is able to induce DSB in living cells. Our study demonstrated that this irradiation system is a useful tool for investigating the radiobiological effect of soft X-ray laser.

KEYWORDS: laser-plasma soft X-ray laser, DNA double strand break

INTRODUCTION

X-ray lasers, including X-ray free-electron lasers and laser-plasma X-ray lasers, can now be generated using the larger accelerators and high-intensity lasers [1–3]. Since the X-ray laser has particular physical properties, such as high intensity, monochromaticity, ultra-short pulse and excellent coherency, its biomedical application allows us to analyze protein structures without crystallization and to take X-ray micrographs on the nanometer scale [4].

The X-ray laser, the energy of which is in the soft X-ray region, is also likely to be useful for investigation of DNA damage induction. It is known that the energy of secondary electrons and ions are mainly distributed around the several tens of electronvolts, even though the

energy of primary radiation is measured in mega-electronvolts [5]; a large part of DNA damage, including DNA single- and double-strand breaks (SSB and DSB), is induced by these low-energy secondary species [6–9]. In addition, the photon absorption cross-section of DNA peaks at ~15 eV [10]. Therefore, low-energy electron and photon irradiation is useful for investigating the basic mechanisms by which DNA is damaged. To study the initial process of DNA damage induction, electron gun [11] and synchrotron radiation sources [10, 12] have commonly been used for the generation of electron and photon beams, respectively, the energy of which are modulated from several electronvolts to kilo-electronvolts. In these studies, plasmid DNA has been adopted as an appropriate material for the analysis of DNA strand breaks [10–12]. Although these methods are advantageous for analyzing the dose–response relationship and the energy dependence of DNA damage, dried plasmid DNA under vacuum conditions is not sufficient to reflect the native reactions of DNA damage in cells. Therefore, to elucidate the intricate mechanisms of DNA damage induction, it is necessary to develop an irradiation system that can verify not only dried biological samples but also living cells using a variety of methods. In addition, if the irradiation system is equipped with a soft X-ray laser, some novel phenomenon might be elucidated, such as the evaluation of temporal electron dynamics during DNA strand break induction, and combination of nano-scale microbeam irradiation with high-resolution imaging. A pulse width as short as several femtoseconds and the focusing ability of the soft X-ray laser are preferred for these techniques— difficult to be achieve using conventional radiation sources.

Although biological applications of the soft X-ray laser have been predicted, a cell irradiation system using a soft X-ray laser has not been developed. Shinohara *et al.* [13] and Devidkova *et al.* [14] recently reported the biological effect of a high-intensity laser-produced X-ray pulse, but their X-rays were not coherent. Therefore, the biological effects of the soft X-ray laser have not been assessed yet. Before launching the biological application of the soft X-ray laser, the radiation effects of soft X-ray lasers on living cells need to be verified. In this study, we aimed to establish the basis of biological application of the X-ray laser. We developed a laser-plasma soft X-ray laser irradiation system for radiobiological experiments and assessed whether the soft X-ray laser induces DNA damage in a human culture cell line. We demonstrated for the first time that a single shot of soft X-ray laser was able to induce DNA DSBs.

MATERIALS AND METHODS
Soft X-ray laser generation
A soft X-ray laser with a photon energy of 89 eV was generated by the Ng:glass laser installed at the Japan Atomic Energy Agency. The details of the optical configuration have been described elsewhere [15, 16]. The output energy, flux, repetition ratio, and pulse width of the soft X-ray laser are ~0.3 μJ, 0.5×10^{10} photons per single X-ray pulse, 0.1 Hz, and 7 ps, respectively. The fluctuation of the output energy is ±15%. The spot size is 121.5 and 135 μm along the horizontal and vertical direction, respectively (Supp. Fig. 1A and B). The estimated absorbed energy is 0.05 μJ.

Soft X-ray laser irradiation system
It is difficult to transmit soft X-ray photons at 89 eV through air and water, because the transmission length for which the photon number

is decreased to 0.1% is ~1 mm and 1 μm for air and water, respectively. Therefore, X-ray attenuation should be prevented as much as possible in order to effectively irradiate the cells with a soft X-ray laser. To prevent X-ray attenuation by air and culture medium, a cell culture dish specifically designed for soft X-ray laser irradiation was used in this experiment (Fig. 1A and B). A square pit was established at the bottom of the dish, and a round hole was opened at the center of the square pit. By attaching a silicon nitride (SiN) membrane to the square pit, the dish can be used as an X-ray incident window separating the air and the vacuum. In addition, the cells could be irradiated with a soft X-ray laser, regardless of the X-ray attenuation by the culture medium, because the living cells were directly adhered onto the SiN membrane used as the X-ray incident window.

Cell line, culture methods, and the soft X-ray laser irradiation procedure
Human adenocarcinoma cell line A549 was used, and the cells were cultured as described in a previous report [17]. The cells were maintained with 10% fetal bovine serum (BioWest, Nuaillé, France) containing D-MEM (Wako, Osaka, Japan) in an incubator set at 37°C, 95% air and 5% CO_2. SiN membranes 100 nm thick (Silson Ltd, Northampton, England) were adopted as the cell culture substrate, and the membranes were coated with collagen type-I (Type-IC, Nitta Gelatin, Osaka, Japan) two days before the irradiation to promote cell adhesiveness. One day before irradiation, 1.5×10^4 cells/cm^2 of A549 cells were seeded onto the SiN membrane (Fig. 1C and D). Just before irradiation, the SiN membrane was transferred to the irradiation dish described in the previous section.

The soft X-ray laser irradiation experiment was performed at 24°C. The cells were irradiated with 1, 5 and 10 shots of the soft X-ray laser, the corresponding photon numbers were 0.5×10^{10}, 2.5×10^{10}, and 5.0×10^{10}, respectively. At most, 15 min was needed for the irradiation experiment to be completed, including installation and removal of the irradiation dish. Immediately after irradiation, the SiN membrane was removed from the irradiation dish, and the cells were incubated using a common culture dish in an incubator at 37°C, 95% air and 5% CO_2. After 30 min of incubation, immunofluorescence staining was performed.

The control study for the soft X-ray laser irradiation was performed using the TITAN-320 (Shimazu, Kyoto, Japan), which is conventional X-ray irradiation equipment. The tube voltage, current, distance from X-ray focus to incident surface, and dose rate were 200 kV at peak (kVp), 20 mA, 60 cm and 1 Gy/min, respectively.

Immunofluorescence staining
The staining protocol is described elsewhere [17]. The anti-phosphorylated DNA-PKcs (Abcam, Cambridge, MA), anti-phosphorylated ATM (Abcam), and anti-γ-H2AX (Cell Signaling Technology, Denver, MA) antibodies were used as primary antibodies. Cy3-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and Cy2-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories) antibodies were used as secondary antibodies.

RESULTS
Soft X-ray laser irradiation induces DNA double-strand breaks repair
To evaluate whether DSBs are induced by soft X-ray laser irradiation, we assessed the DSB foci formation by means of immunofluorescence
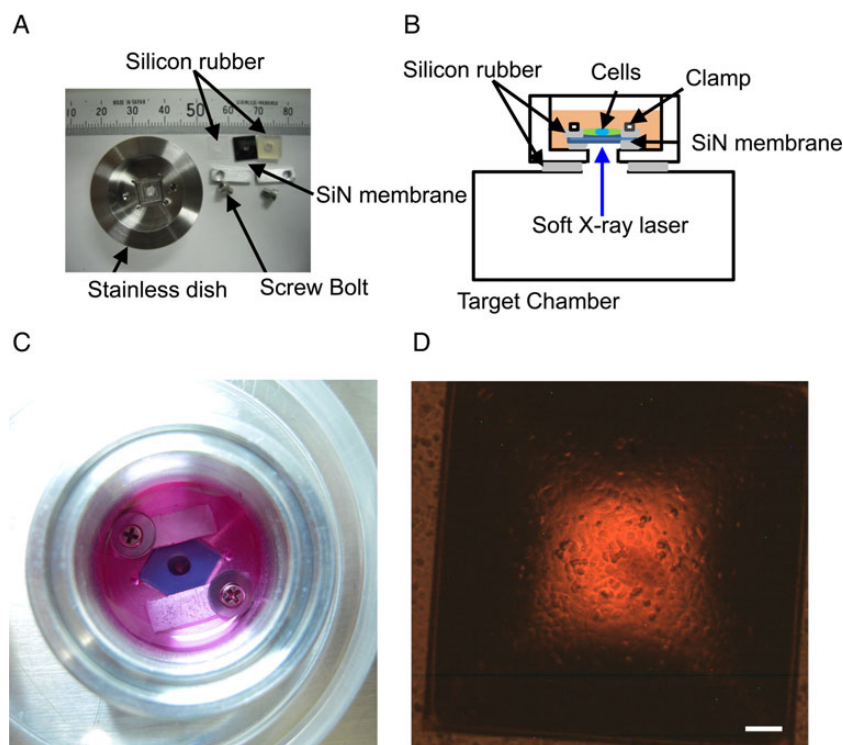
Fig. 1. The framework of the irradiation dish. Panels A, B and C: The irradiation dish made of stainless steel served as both cell culture dish and vacuum window of the target chamber. An X-ray incident window was opened at the center of the dish. The silicon nitride (SiN) membrane on which the cells are cultured was attached to the X-ray incident window. To prevent leakage of culture medium into the target chamber, the SiN membrane was clipped with silicon rubbers. The silicon rubbers were cut into 7.5-mm squares with 3-mm holes in the center. The silicon rubbers and SiN membrane were finally clasped with screw bolts. The SiN membranes were set just before the soft X-ray irradiation experiment (A, B). After the setting, 2 ml of the cell culture medium was added into the irradiation dish (C). Panel D: the cells on the SiN membrane. The photograph was taken using the upright microscope that was installed in the target chamber before the irradiation. The cells were laid in the SiN membrane. The scale bar shows 100 μm.

staining of the DSB repair proteins, phosphorylated DNA-PKcs (p-DNA-PKcs), ATM (p-ATM), and histone H2AX (γ-H2AX). At 30 min after irradiation with five shots of the soft X-ray laser, the p-DNA-PKcs, p-ATM, and γ-H2AX-positive cells were clearly identified in the center of the X-ray incident window (Fig. 2). Interestingly, these foci were unevenly distributed in the nucleus (lower panel in Fig. 2C and F and Supp. Fig. 2) in comparison with the conventional X-ray irradiation (lower panel in Fig. 2B and E and Supp. Fig. 2). These results clearly demonstrated that an 89-eV soft X-ray laser is able to induce DSBs in living cells.

Shot number–dependent increase in DSB repair foci

We subsequently assessed whether the single shot of X-ray laser is enough to induce DSBs. We observed that cells with p-DNA-PKcs foci were clearly detected 30 min after single shot soft X-ray laser. To assess the actual region irradiated by the single shot of soft X-ray laser, we measured the region containing cells with p-DNA-PKcs foci. Our results indicated that the region ranged between 100 and 140 μm in the short and long axis, respectively (Fig. 3A and Supp. Fig. 3A). These dimensions approximately corresponded to the size of the soft X-ray laser spot (Supp. Fig. 1C). In addition, these foci were collectively distributed in a part of the nucleus (Fig. 3D), which is

similar to the results presented in Fig. 2. When the cells were irradiated with five or ten shots of soft X-ray laser, the number of the foci and also the area containing the focus-positive cells increased accordingly with shot number (Fig. 3B–C, E–F and Supp. Fig. 3).

DISCUSSION
Transmission of soft X-ray laser photons in culture cells

In this study, we showed that p-DNA-PKcs, p-ATM and γ-H2AX foci formation could be induced by soft X-ray laser irradiation (Fig. 2). In addition, p-DNA-PKcs foci were induced, even by a single shot of X-ray laser, and these foci increased correspondingly with an increased number of laser shots (Fig. 3 and Supp. Fig. 3). These results indicate that an 89-eV soft X-ray laser is enough to induce DSBs, even though the photon energy is extremely low.

To understand the reason for DSB induction by the 89-eV soft X-ray laser, the X-ray absorption by cellular components, such as the plasma membrane, cytoplasm and nucleus, must be taken into account. If the usual biological experimental conditions, such as vertical irradiation using common cell culture dishes, were adopted in soft X-ray laser irradiation, X-ray photons could not reach the nucleus, because the $1/e$ attenuation length, which is the depth from the X-ray incident surface to the point at which the X-ray photon number is
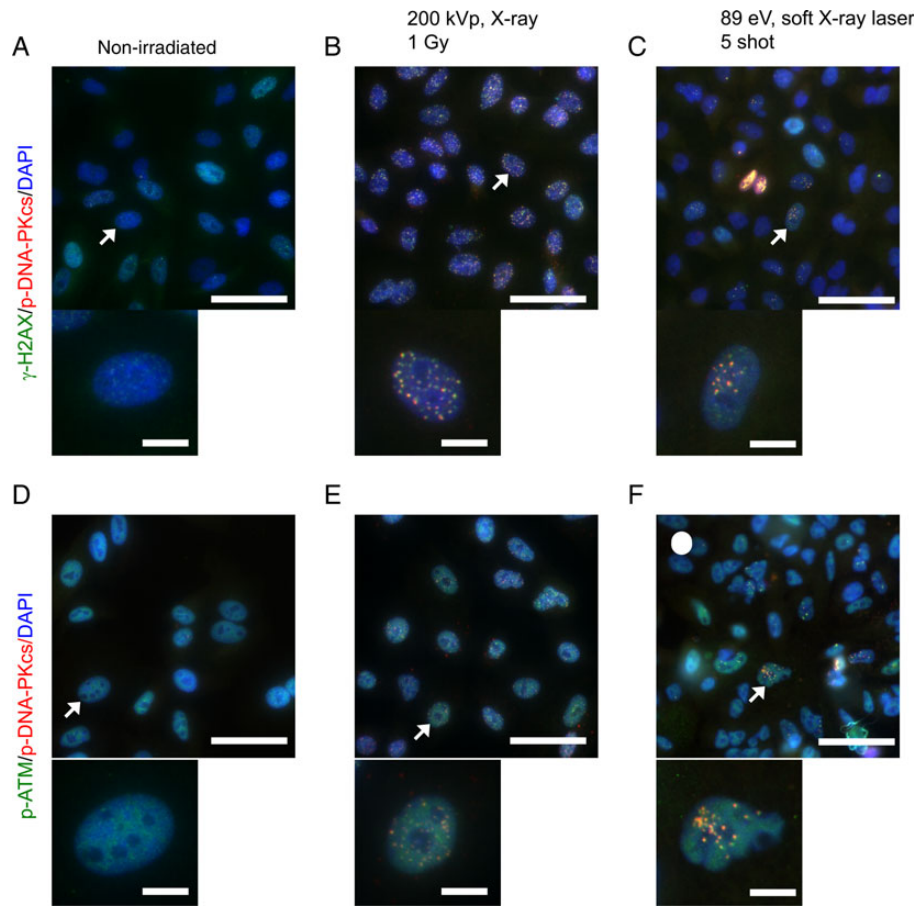
Fig. 2. Induction of DNA double-strand breaks by soft X-ray laser irradiation. Panels A, B and C: Colocalization of γ-H2AX (green) and p-DNA-PKcs (red) foci in non-irradiated cells (A), after 1 Gy of conventional X-ray (B), and five shots of soft X-ray laser (C) irradiation. Panels D, E and F: Colocalization of p-ATM (green) and p-DNA-PKcs (red) in non-irradiated cells (D), after 1 Gy of conventional X-ray (E), and five shots of soft X-ray laser (F) irradiation. The nuclei (blue) are stained with DAPI. The small panel below each large panel shows the magnified image of the cells indicated by the white arrow. The scale bars shown in the large and small panels indicate 50 and 10 μm, respectively.

decreased to 36.8%, of the SiN penetrating X-ray photons is only 53 nm. Furthermore, >99.9% of these photons were absorbed by water at ∼1 μm depth when these X-ray photons were incident (Supp. Fig. 4). However, in our experimental conditions, cells were directly adhered onto the X-ray incident window. Moreover, the photon number of our soft X-ray laser [3, 14] is significantly higher than that of the conventional X-ray tube [18]. When 0.5×10^{10} photons were incident at the X-ray incident window, it is estimated that at least 70 photons were transmitted 2 μm in depth through the SiN membrane (Supp. Fig. 4). Given these geometrical and physical features of our experimental set-up, these photons are likely to reach the nucleus. Since these photons effectively ionize the surrounding cellular components and generate a lot of low-energy secondary electrons, DNA strand breaks, the mechanisms for which are described later, can be generated along the beam path. It is estimated that the mean free path (the mean distance of the photons, the electrons, or the ions traveling in the material) of these secondary electrons is ∼ 0.1–10 nm [19]. Given the DNA strand and the nucleosome are ∼2 and ∼11 nm in diameter, respectively, the energy of these secondary electrons was

largely transferred into the DNA and the nucleosome, and this might efficiently damage the DNA. Thus, our results clearly demonstrated that the 89-eV soft X-ray laser could induce DSBs (Figs 2 and 3).

Mechanistic insights for DSB induction by soft X-ray laser irradiation

SSBs and DSBs were induced by photons produced by the soft X-ray laser, although the photon energy was quite low. Prise *et al.* reported SSBs and DSBs produced by irradiation at photon energies ranging from 7 to 150 eV. They showed that the SSB and DSB yields were steeply increased from 7 to 11 eV, but that the yields almost plateaued after 20 eV [10, 12]. The yield of SSBs and DSBs had no apparent dependence on the incident photon energy. Their results indicate that 89 eV is enough to break the DNA strand, and suggest that the DSB induction in our experiments might have been partially caused by the photons of the 89-eV X-ray laser that could penetrate the DNA.

It is also essential to consider the mechanisms for DNA strand breaks by low-energy electrons in order to understand our results. Since the ionization potential of liquid water and DNA is ∼7–10 eV
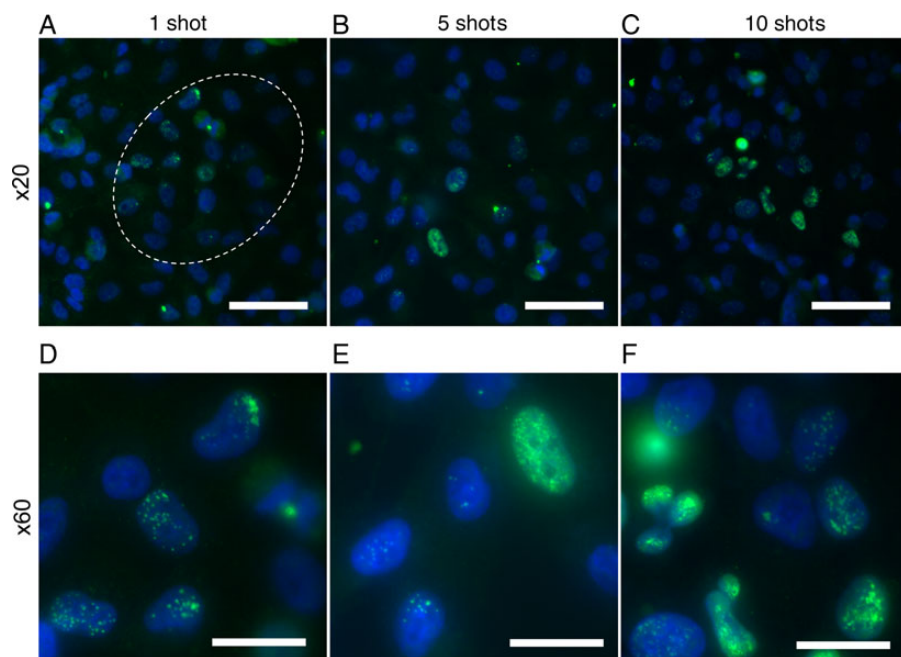
Fig. 3. Laser shot-dependent increase in phosphorylated DNA-PKcs focus number. Phosphorylated DNA-PKcs (p-DNA-PKcs)
foci and nuclei are shown in green and blue, respectively. Panels A, B and C: p-DNA-PKcs focus formation after 1, 5 and 10
shots of soft X-ray laser irradiation. The dashed circle in panel A is the region that contained the cells with p-DNA-PKcs focus.
The distances along the short and long axes are 120 and 140 μm, respectively. Panels D, E and F: The magnified images of
panels A, B, and C, respectively. The scale bars indicate 50 μm for panels A, B and C, and 20 μm for panels D, E, and F.

[6, 20], an 89-eV soft X-ray laser is enough to ionize the DNA and
the surrounding medium. Thus, secondary electrons can be generated
around the DNA when photons of the soft X-ray laser reach the
nucleus. The energy of these electrons is mainly distributed in the
range from several electronvolts to several tens of electronvolts [5].
The mechanism of SSB and DSB induction by low-energy electrons
below ∼20 eV is caused by dissociative electron attachment (DEA),
which is the resonant reaction between low-energy electrons and
molecules. The yielding of SSBs and DSBs via DEA is largely depend-
ent on the energy of incident electrons: there is a broad peak at
∼10 eV [6–9]. In brief, low-energy electrons are captured by the
bases and phosphate backbones in DNA. Then, they transiently load
the negative charge into the DNA. This induces the dissociation of
DNA into anion fragments and radical species including O^-, H^-, and
OH^-, and eventually this dissociation generates SSBs at the electron-
attached site. Meanwhile, the reactions between these DEA products
and the opposite DNA strand result in DSBs. For electrons above
20 eV, the inelastic collision contributes to generating SSBs and
DSBs. In particular, it has been demonstrated that the yield of mul-
tiple DSBs (MDSBs) is steeply increased with increasing energy of
the incident electrons [9]. This might be attributed to clustered DNA
damage induction. The induction ratio of DSBs to SSBs for the low-
energy electrons was approximately one-third to one-fourth [6, 11],
whereas the ratio for low-energy photons was less than one-tenth
[9, 12, 21, 22]. These data indicate that low-energy electrons can
induce DSBs more effectively compared with low-energy photons.
Given these reports, the DSBs detected in our experiments are likely
to be largely caused by interactions between low-energy secondary
electrons and DNA, such as DEA and inelastic collisions.

On the other hand, many of the photons were certainly absorbed
by the cytoplasm. While DSB induction is mainly caused by energy
transfer into the nucleus, it might be possible that energy transfer to
the cytoplasm and plasma membrane also contribute to the induction
of DSBs. In fact, induction of DNA damage (such as genetic muta-
tion [23, 24] and 53BP1 phosphorylation [25]) due to cytoplasmic
irradiation using an α-ray beam have previously been reported. Inter-
estingly, Tarter *et al.* reported that 53BP1 foci after cytoplasmic α-ray
irradiation were dispersedly formed over the nucleus [25]. In contrast
to their report, our results showed that the p-DNA-PKcs foci were
collectively distributed in a part of the nucleus (Fig. 3). Therefore,
these spatial distributions of foci might strongly indicate that the DSB
induction was due to the effective energy transfer of low-energy sec-
ondary electrons and of some of the 89-eV photons that reached the
nucleus.

CONCLUSION

We demonstrated for the first time that a single shot of the soft X-ray
laser, the photon energy of which is 89 eV, could induce p-DNA-
PKcs, p-ATM and γ-H2AX foci formation in irradiated living culture
cells. Furthermore, the focus number of p-DNA-PKcs was increased
concomitantly with increase of the laser shots. These results mean
that the 89-eV soft X-ray laser has enough capacity to induce DSBs,
even with a single laser shot.

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