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p53 binding protein 1 foci as a biomarker of DNA double strand breaks induced by ionizing radiation

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ABSTRACT

Foci of p53 binding protein 1 (53 BP1) have been used as a biomarker of DNA double-strand breaks (DSBs) in cells induced by ionizing radiations. 53 BP1 was shown to relocalize into foci shortly after irradiation, with the number of foci closely paralleling the number of DNA DSBs. However, consensus on criteria in terms of the numbers of 53 BP1 foci to define cells damaged by direct irradiation or by bystander signals has not been reached, which is partly due to the presence of 53 BP1 also in normal cells. The objective of the present work was to study the changes in the distribution of cells with different numbers of 53 BP1 foci in a cell population after low-dose ionizing irradiation (<0.1 Gy) provided by alpha particles, with a view to propose feasible criteria for defining cells damaged by direct irradiation or by bystander signals. It was proposed that the change in the percentage of cells with 1–3 foci should be used for such purposes. The underlying reasons were discussed.

1. Introduction

The p53 binding protein 1 (53 BP1) is a member of the BRCT (BRCAI C-terminal) repeat family, which consists of many members, including the DNA damage response proteins NBS1 and BRCA1. Ionizing radiation produces a broad spectrum of DNA damages, including base lesions and strand breaks [1]. 53 BP1 is involved in repair and checkpoint signal transduction, and is required for the phosphorylation of numerous ataxia-telangiectasia-mutated substrates during the double-strand break (DSB) response [2,3]. Shortly after an exposure to ionizing radiation, 53 BP1 was shown to relocalize into foci, with the number of foci closely paralleling the number of DNA DSBs [4]. As such, fluorescent detection of these foci of 53 BP1 protein was used as a marker of DNA damages [5–8].

However, consensus on criteria in terms of the numbers of 53 BP1 foci to define cells damaged by direct irradiation or by bystander signals has not been reached, which is partly due to the presence of 53 BP1 also in normal cells. Different criteria have been proposed in previous works, including the use of a "critical" number of foci per cell (fpc; namely, a positive expression when the fpc is larger than the "critical" number and a negative expression when the fpc is smaller than or equal to that "critical" number) [9–11]; the use of an average number of fpc in a population of cells [12] and the use of different kinds of distributional grouping of fpc (such as examining the percentage of cells in the groups with 0–5 fpc, 6–30 fpc, … <90 fpc) [13].

In the present work, we studied the effects of low-dose (<0.1 Gy) alpha-particle irradiation as well as the associated radiation-induced bystander effect (RIBE) on HeLa cells in terms of changes in the distribution of HeLa cells with different numbers of 53 BP1 foci, with a view to propose feasible criteria for defining cells damaged by direct irradiation or by bystander signals. Immunostaining of 53 BP1 protein was used to characterize the DNA DSBs. RIBE has been widely studied since its revelation by Ref. [14], and refers to the phenomena that irradiated cells might release some stress signal factor(s) to affect the cells nearby or to affect the cells that have received the medium conditioned by the irradiated cells.

2. Methodology

2.1. Fabrication of substrates

In the present work, thin polyallyldiglycol carbonate (PADC) films with a thickness of 17 μm were fabricated using the method devised by Chan et al. [15], and were used as our cell culture substrates. PADC substrates can record positions of alpha-particle hits and give information on the energy of the alpha particles

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through the optical appearance of the etched alpha-particle tracks [16,17]. Thick PADC films (with thickness > 100 μm) are commercially available as CR-39 detectors and are commonly used solid-state nuclear track detectors (SSNTDs) [18]. Thin PADC films (with thickness < ~20 μm) were successfully employed as PADC substrates in alpha-particle radiobiological experiments involving cell cultures [19–22] and those involving zebrafish embryos [23–27]. In the present work, we prepared thin PADC films with a size of 1.5×1.5 cm^2 from 100 μm thick CR-39 detectors (Page Moldings) by chemical etching in 1 N NaOH/ethanol at 40 °C until the desired thickness of 17 μm was reached.

2.2. Experimental setup and cell culture

Handling of HeLa cells was described in detail by Ng et al. [28]. The cells were trypsinized, adjusted to 1.2×10^5 cell/ml cells for a total of 120 μl medium and plated out on PADC substrates for 1 d. The cells were then irradiated for 20 min with 5 MeV alpha particles having traveled a distance of 5 mm in air from an ^{241}Am alpha-particle source (main alpha particle energy of 5.48 MeV) with incident angles between 70.8° and 90° (normal incidence) controlled by a collimator. As irradiation was performed from the bottom of the substrates using the SRIM program [29], the residual energy of the 5 MeV alpha particles after traversing the 17 μm PADC film was found to be ~2.5 MeV [30].

2.3. Detection of 53 BP1 foci and hit positions

Immunochemical staining of cells was performed largely following the procedures described by Aten et al. [31] with some modifications. Optical and fluorescence images of cells were captured by a fluorescence microscope (Nikon ECLIPES 80i). The cells on PADC substrates were then removed by running water before the substrates were etched in a 14 N KOH solution at 37 °C for 3 h to reveal visible tracks corresponding to alpha particles striking the substrates. The epoxy remained intact at this etching temperature [20]. Optical images of these tracks were captured, which were then superimposed onto images of cells described above, from which the positions of alpha-particle hits on cells could be determined. Due to the short irradiation time, very few cells were hit by more than one alpha particle. In addition, from analyzing the captured fluorescent images with the help of the ImageJ software (http://rsbweb.nih.gov/ij/), the distribution of gray levels of the pixels in the 53 BP1-stained cell nuclei of the normal (unirradiated) cells was determined, in terms of the percentage standard deviations of average gray levels, as described in more details in Section 3 below.

3. Results

The distribution of gray levels of 53 BP1 signals in normal unirradiated HeLa cell nuclei was first determined to provide a basis for determining changes due to radiation effects including RIBE. The percentage standard deviations of average gray levels were required. For example, if scanning the image pixels for one particular cell nucleus gave an average gray level of 18 ± 4.6, where 4.6 was the standard deviation, the percentage standard deviation was calculated as 4.6/18 = 21.1%. By scanning the nuclei of 40 normal unirradiated HeLa cells, we obtained the average percentage standard deviation as 27.4 ± 15.1%. In the present work, a 53 BP1 focus was defined as a particular area in a cell nucleus with a gray level that was 100% larger than the average gray level of that nucleus. Fig. 1 shows representative fluorescence images for 53 BP1 foci in control, as well as irradiated and bystander cells, with superimposed optical images for irradiated and bystander cells.

To take into account the possible variations among different experiments, normalization was also carried out on the percentages in each data set as shown in Figs. 2 and 3. Our experimental results were shown in terms of the percentage of cells (comparing with the corresponding control (Ctrl) in each set) in irradiated (Irr) cells (Fig. 2) and bystander (By) cells (Fig. 3) cultured on

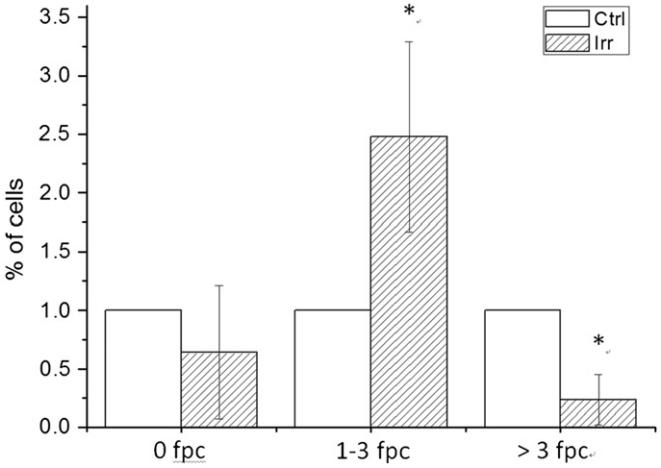

Fig. 2. Normalized percentage of control (Ctrl) and irradiated (Irr) cells cultured on PADC substrates with number of 53 BP1 foci in the nuclei shown in three groups: (1) with 0 fpc, (2) with 1–3 fpc and (3) with > 3 fpc. Error bars represent one standard deviation. The Irr cells had significantly higher and lower normalized percentages in the groups of 1–3 fpc and > 3 fpc, respectively, when compared with the Ctrl cells (* represents $p < 0.05$, $n = 3$).

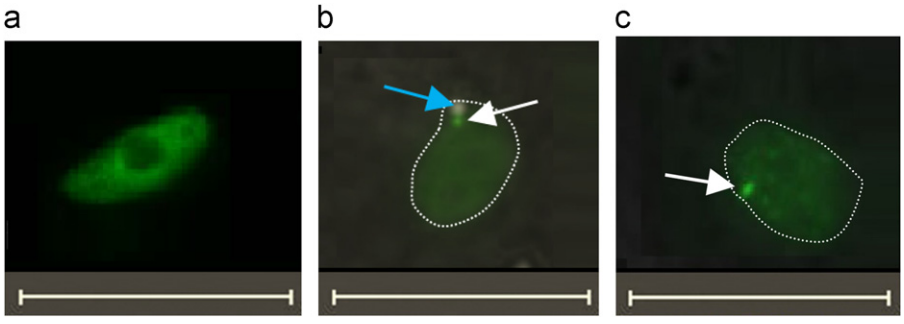

Fig. 1. Representative fluorescent images for 53 BP1 foci in different cells. (a) A control cell. (b) An irradiated cell. The fluorescence image has been superimposed with the optical image. Dashed line: outline for the cell nucleus; White arrow: 53 BP1 focus shown in the fluorescence image; blue arrow: etched alpha-particle track shown in the optical image. (c) A bystander cell. The fluorescent image has been superimposed with the optical image. Dashed line: outline for the cell nucleus; white arrow: 53 BP1 focus shown in the fluorescent image. Bar = 25 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

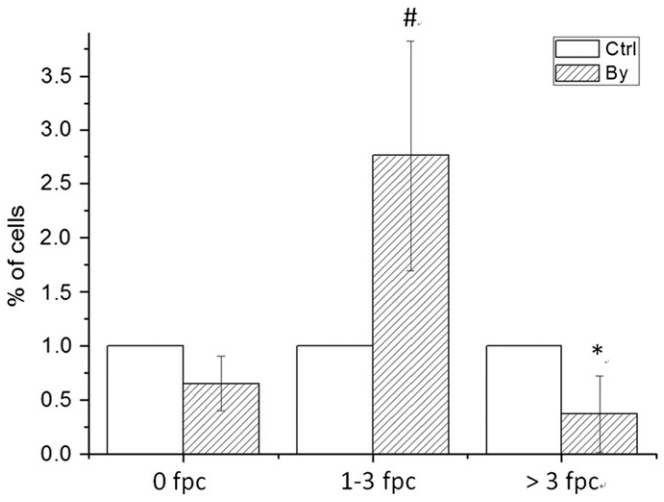

Fig. 3. Normalized percentage of control (Ctrl) and bystander (By) cells cultured on PADC substrates with a number of 53 BP1 foci in the nuclei shown in three groups: (1) with 0 fpc, (2) with 1–3 fpc and (3) with >3 fpc. Error bars represent one standard deviation. The By cells had marginally higher (# represents $p=0.05$, $n=3$) and significantly lower (* represents $p<0.05$, $n=3$) normalized percentages in the groups of 1–3 fpc and >3 fpc, respectively, when compared with the Ctrl cells.

PADC substrates with different numbers of 53 PB1 foci in the nuclei in three groups: (1) with 0 fpc, (2) with 1–3 fpc and (3) with >3 fpc. Irradiated cells in the present studies referred to those with their cell nuclei hit by only 1 alpha particle. Figs. 2 and 3 show that the normalized percentages of irradiated and bystander cells with >3 fpc were significantly lower when compared to the corresponding control samples ($p<0.05$, $n=3$). Furthermore, the normalized percentages of irradiated and bystander cells with 1–3 fpc were significantly higher ($p<0.05$, $n=3$) and marginally higher ($p=0.05$, $n=3$), respectively, when compared to the corresponding control samples.

4. Discussion

To the best of our knowledge, there are no universally accepted objective criteria for defining a 53 BP1 "focus". In the present work, a "focus" in a cell nucleus is defined objectively by referring to the gray levels of image pixels of the cell nucleus. If a particular area in the nucleus had pixels with gray levels significantly above the average gray level of that nucleus, that particular area is defined as a focus. The distribution of gray levels was determined from normal unirradiated HeLa cell nuclei. Our results showed that the average percentage standard deviation for the gray levels was $27.4\pm15.1\%$. In the present work, a 53 BP1 focus was defined as a particular area in a cell nucleus with a gray level 100% larger than the average gray level of that nucleus, so the p value (of claiming by mistake a focus at a position with gray levels merely coming from a fluctuation of the background) is less than 0.001.

As described in the Introduction section, there has also not yet been a universal consensus on the criteria in terms of the number of 53 BP1 foci to prove the presence of effects on cells subjected to direct irradiation or to bystander signals. In the present work, very low-dose irradiation of the cells was used. The distribution of 53 BP1 foci was shown by dividing the cells into three groups, namely, those with 0, 1–3 and >3 fpc. The rationale for choosing these three groups will be discussed below.

Figs. 2 and 3 show that the percentages of irradiated and bystander cells with 1–3 fpc were significantly larger and marginally larger, respectively, than that of unirradiated cells. At the same time, the percentages of irradiated and bystander cells with >3 fpc were significantly lower than that of the corresponding controls. This is explained by re-localization of the highly mobilized 53 BP1.

Anderson et al. [13] found that 53 BP1 (*Xenopus* homolog) was localized to a few large foci in the cell nucleus under normal conditions. They confirmed that the induction of 53 BP1 foci in response to the DNA damages induced by an X-ray exposure was not due to an increased level of protein expression, as no difference between the 53 BP1 levels was observed by quantitative immunoblotting before and after the X-ray exposure. On the other hand, the damage-induced 53 BP1 foci appeared as rapidly as within 30 min post-irradiation, and *de novo* synthesis was not required for localization of 53 BP1 to DNA damage sites after irradiation. Richie et al. [32] referred to the small and the larger nuclear structures of 53 BP1 as "foci" and "bodies", respectively. At 90 min after the X-ray exposure, 53 BP1 bodies were no longer observed and the number of 53 BP1 foci had increased. In addition, Pryde et al. [33] studied the dynamics of 53 BP1 in living cells by transient expression of GFP fused to full-length mouse 53 BP1 [34] in HeLa cells. Pryde et al. [33] found that the GFP-53 BP1 protein was homogenously distributed over chromatin in untreated cells, and at 1 h after exposure to X-rays, the diffuse 53 BP1 had redistributed and concentrated to form foci. They hypothesized that 53 BP1 did not diffuse freely in the inter-chromatin space. 53 BP1 protein may have some affinity for chromatin in normal cells, repeatedly binding to and dissociating from the chromatin, which supported surveillance of DNA integrity and the fast recruitment of 53 BP1 to the DNA damage sites.

In fact, many proteins were found to be recruited and relocated to the damaged DNA break rapidly [35–38]. Scully et al. [38] found the percentages of cells with the dispersal of BRCA1 foci were decreased after UV irradiation. After 25 min, significant dispersals of BRCA1 foci were no longer observed. BRCA1 and PCNA pattern changed from distinct to co-localized. In addition, BRCA1 associated protein Rad 51 showed relocation to DNA damaged sites and became co-localized with PCNA (diffuse pattern) after UV irradiation. The accumulation of BRCA1 and Rad 51, focally, in PCNA implied an interaction of BRCA1/Rad 51 containing complexes with damaged, replicating DNA. Ha et al. [35] found that DNMT1 was rapidly and actively recruited to DSBs, showing clear accumulation by $\sim$1 min. Kim et al. [36] found immediate recruitment of ATM (ataxia-telangiectasia-mutated) and ATR (ATM- and Rad3-related) to the laser-induced damage site within 20 min.

With the above findings, we could explain in the following the decrease in the percentage of irradiated cells with >3 fpc when compared to controls observed in the present study as a result of re-distribution of some 53 BP1 foci of cells after alpha-particle irradiation. As mentioned above, the definition of a focus was based on its gray value being 100% higher than the average value in the nucleus, while the size was not taken into account as was performed by Anderson et al. [13]. Thus, for those cells with >3 fpc in their nuclei before irradiation, after irradiation, the 53 BP1 protein would re-localize and formed foci at the positions of DNA strand breaks. For one alpha-particle hit, the dose absorbed by each cell (by taking into account the cell thickness) was as low as 0.0356 ± 0.0099 Gy (see Ng et al. [30] for calculations).

Aten et al. [31] found that after exposure of a HeLa cell to a single alpha particle (irradiation onto the side of the cell, i.e., near-horizontal irradiation), a linear track (consisting of many γ-H2AX foci) was formed in the cell nucleus, which closely followed the trajectory of the alpha particle through the nucleus. At 1 h post-irradiation, there were two main groups of changes

due to the mobility of DSB containing chromosomes. In one group, γ-H2AX foci grouped together to form several clusters along the track. In another group, γ-H2AX foci did not group together to form clusters and these foci could move up to only ~2 μm away from the linear track.

It was reasonable to expect the same behavior for 53 BP1 as Schultz et al. [4] found that 53 BP1 foci co-localized with radiation-induced γ-H2AX foci. In the present work, the studied irradiated cells were only those with their cell nuclei hit by 1 collimated alpha particle from the bottom of the cells. The alpha particle passed through only a short path in the cell nucleus and the positions of DSBs should be shown as very close to the track path as described by Aten et al. [31], so the 53 BP1 foci localized at the DSB positions were considered very close to one another. Since the images were captured from the top of the cells, the probability of observing >3 distinct foci of the 53 BP1 protein in a cell nucleus was very low. Indeed, cell nuclei with >3 fpc were rarely observed in the present study for irradiated cells. When an alpha particle hit a cell nucleus already with several foci (including large bodies), the 53 BP1 protein tried to relocalize and form foci at the DSB sites only, so the total number of foci seemed to have decreased. In fact, in the published figure series in Cao et al. [39], during the tracking of the behavior of 53 BP1 foci over time, we observed that the background foci in general disappeared shortly after exposure to radiation. This explains the decrease in the total number of foci in the cell nuclei after irradiation. Consequently, the number of cells with 1–3 foci increased, since the cells which had >3 background foci before irradiation would have 1–3 foci at the DSB sites after irradiation.

These demonstrated that for low-dose irradiation, simplified ways such as the average 53 BP1 fpc or critical number of 53 BP1 fpc might not be able to characterize the irradiation effect, since increases in the percentage of cells with "1–3 foci" and decreases in the percentage of cells with ">3 foci" may change the measured average fpc in an unexpected fashion. It is therefore suggested here that for low-dose irradiation, a decrease in the percentage of cells in the group with larger values of fpc (>3 fpc) should not be considered synonymous with a decrease of DSBs in the irradiated cells, and that studies on the percentage of cells with 1–3 foci may actually be needed to correctly characterize the radiation effect. Certainly, if the radiation dose becomes high, the number of foci will generally increase as the 53 BP1 foci are localized at the DNA DSBs sites, i.e., more foci will result in a higher dose [13,40].

On the other hand, Sokolov et al. [41] demonstrated that DSB induction was an early step in the bystander response. DSB formation induces the phosphorylation of the histone protein H2AX, and the phosphorylated γ-H2AX forms foci at DSB sites. In the present investigation (also in the studies of Ng et al. [30]), the bystander cells showed the same trend as the irradiated cells (Fig. 3). However, the similarity or difference between the mechanisms for the increase or decrease in the 53 BP1 foci, e.g., so that these can be explained by the re-localization behavior of 53 BP1 proteins, is still unclear. Nevertheless, the present observation in the distribution of fpc in bystander cells illustrates that low-dose RIBE can induce only a very small number of foci (1–3 foci) in bystander cells, which in turn decreases the percentage of cells with >3 fpc.

5. Conclusion

The percentages of irradiated and bystander cells with >3 fpc were significantly lower when compared with the corresponding control. These phenomena were explained by the re-localization characteristics of the highly mobilized 53 BP1. As such, for low-dose irradiation (0.0356 ± 0.0099 Gy), simplified ways such as the average 53 BP1 fpc or the critical number of 53 BP1 fpc to signify positive radiation effect might not be the most accurate. In fact, it is suggested here that for low-dose irradiation, a decrease in the percentage of cells in the group with larger values of fpc (>3 fpc) should not be considered synonymous with a decrease in DSBs in irradiated cells. Studies on the percentage of cells with 1–3 foci may be needed to characterize the radiation effect more accurately. The bystander cells also had low probability of having >3 foci per cell, as was the case for irradiated cells.

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