



Evaluation of circulating endothelial cells in the rat after acute and fractionated whole-body gamma irradiation

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Abstract. *Purpose:* Damage to vascular endothelial cells is a well recognised complication of the irradiation. Our objective was to determine the gamma-irradiation effect on the rat circulating endothelial cells (CEC). *Material and methods:* Eight-week old rats were divided into four groups: group 1 – rats were exposed to acute whole-body gamma irradiation with a wide range of single doses (0.5, 1, 2, 4 and 8 Gy), group 2 – rats were exposed to fractionated low doses of irradiation (0.1, 0.5 and 1 Gy) every three days for two months, group 3 as group 2, but followed by two months of rest, group 4 were control animals. CEC (CD146 positive cells) in group 1 were counted following CD146-based immuno-magnetic separation after one day and one week, as well as at the end of experiment in the other groups. *Results:* Quantified CEC showed that there was a dose-dependent reduction in CEC count in group 1 (one week after irradiation) and group 2. A partial re-population of CEC was observed at the end of experiment in both group 1 and group 2 compared to control group. Group 3 showed a significant increase in CEC levels as compared with group 2 without reaching the control level. *Conclusion:* The number of CEC (CD146 positive cells) in rats exposed to whole-body gamma irradiation was reduced in a dose-dependent manner and it partly recovered during the two-month interval after irradiation. We suggest that CEC count may be an indicator of the radiation-induced vascular damage.

Key words: whole-body gamma irradiation • acute irradiation • fractionated irradiation • biomarkers • circulating endothelial cells • endothelial damage

Introduction

Recently, acute or chronic radiation exposure, such as that of A-bomb survivors or nuclear industry workers, has been revealed to be strongly associated with circulatory diseases [1, 2]. However, with few exceptions, little is known about how ionizing radiation exposure induces cardiac or circulatory diseases [3, 4]. Vascular endothelium is implicated as one of radiation targets, leading to the development of cardiovascular diseases.

In this context, the endothelial dysfunction plays an important role in promoting fibrotic and inflammatory processes after irradiation [5]. Radiation-induced endothelial injury has been described as a crucial event in initiation of normal tissue damage [6]. Endothelial dysfunction is mainly characterised by the loss of thrombo resistance, which is accompanied by a decreased expression of the adhesion molecules and of the Von-Willebrand factor. Previous studies have reported endothelial dysfunction in both human [7] and animal subjects [8–10] exposed to ionizing radiation.

Endothelial cell dysfunction after irradiation can be recognised by morphological changes, such as cell swelling [11], increased leukocyte-endothelial

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cell adherence and infiltration of leukocytes into tissues [12–15]. Also, it can be recognised by the adhesion of platelets to the vascular wall [16], the loss in vascular wall integrity resulting from reduced endothelial cell numbers and the formation of tortuous vessels [17].

During the last years, it has been evidenced that endothelium is a highly dynamic tissue in equilibrium with a circulating compartment composed of various sub-populations offering important opportunities for a noninvasive exploration. Among these, the circulating endothelial cells (CECs) are noninvasive markers of vascular damage and dysfunction [18]. CECs are mature cells shed from blood vessel walls during the natural process of endothelial cell turnover and renewal. These cells are heterogeneous in size (15–50 μm), carry the markers of endothelial cells (e.g.: Von Willebrand factor, CD144, and CD146), and do not have leukocyte markers (CD45) [19]. Changes in CEC levels in peripheral blood possibly reflect endothelial cell dysfunction, which may have occurred due to some environmental conditions such as altitude [20]. So, CEC evaluation in peripheral blood may be used as research tool to determine the effect of exposure to various external factors, including ionizing radiation.

Recent studies have reported that total body irradiation initiates rapid endothelial injury and that CEC in venous blood are a marker of endothelial damage [21, 22]. Moreover, in our recent study, we have demonstrated that chronic exposure to low dose of ionizing radiation leads to significant changes in CEC level [23].

The aim of the present study was to investigate whether whole-body irradiation with a wide range of single doses (0.5–8 Gy) or fractionated radiation schedule (0.1–1 Gy, every three days for two months) could affect the CEC level in the blood of rats.

Materials and methods

Animals

Eight weeks of age Wistar rats were used. This strain was initially obtained from Charles Rivers Laboratories (France) and then it was raised in our animal facility. The animals were handled according to the recommendations of the Declaration of Helsinki and the internationally accepted principles for the care and use of experimental animals. Both control ($N = 8$) and experimental animals ($N = 64$) were kept under stable microenvironment conditions ($22 \pm 1^\circ\text{C}$), with alternating 12 h light and dark cycles and received standard laboratory food and water.

Irradiation

Acute and fractionated whole-body gamma irradiation (116 mGy/m) was administered to rats using gamma ray apparatus (Theratron 80 Canadian

design machine, Co-60, focal distance of 100 cm) located in our laboratories. During irradiation, rats were restrained in special irradiation-cages containing from 5 up to 10 rats at the same time. There was no change in housing, standard food or drinking water following irradiation. The animals were closely observed for unwanted effects and there was no visible sign of discomfort or illness. Age-matched, non-irradiated rats served as controls.

Experimental design

Rats were randomly divided into four groups; one group served as control and the other three groups served as experimental groups. The first group of rats was exposed to acute whole-body gamma irradiation with 0.5, 1, 2, 4 and 8 Gy, respectively, (dose rate 116 mGy/min). Animals in the second group were exposed to fractionated whole-body gamma irradiation of 0.1, 0.5 Gy and 1 Gy, respectively, every three days for two months. The third group was fractionally exposed to low dose of 0.1, 0.5 and 1 Gy every three days for two months, followed by two months without any irradiation for observation of late repair of the vascular endothelium in the irradiated rodents.

Blood collection

Under general anesthesia, the abdomen was opened up through a midline incision; a 25 G needle was inserted in the inferior vena cava and blood was drawn into a citrate containing tube for the immunomagnetic separation (IMS) analysis of CEC; then, the animal was killed via cervical dislocation.

Detection and quantification of circulating endothelial cells

For immuno-magnetic isolation and quantification of endothelial cells, we used monodispersed magnetisable particles (Dynal M450 IgG1, Dynal AS, Oslo, Norway). The 4.5 μm diameter polystyrene beads were coated with affinity-purified Pan-anti-mouse immunoglobulin G1 covalently bound to the surface. The beads were washed according to the manufacturer's protocol, with a strong magnet (MPC6, Invitrogen, CA, USA) used to remove sodium azide. Typically, 100 μL of microbead suspension was coated non-covalently with 10 $\mu\text{g}/\text{mL}$ EPR3208, a rabbit monoclonal antibody against CD146 (Abcam, Cambridge, UK), a pan-rat endothelial cell-specific monoclonal antibody diluted 1:10 in phosphate buffered saline (PBS) with 0.1% bovine serum albumin (BSA) and 0.1% sodium azide, by overnight incubation at 4°C with head-over-head agitation. After three washes with PBS-BSA- NaN_3 to remove excess antibody, the beads were resuspended in buffer until use. One millilitre (ml) of the blood was diluted 1:3 with the isolation buffer and incubated for 1.5 hours at 4°C on a rotator with 15 μL (7×10^7 microbeads)

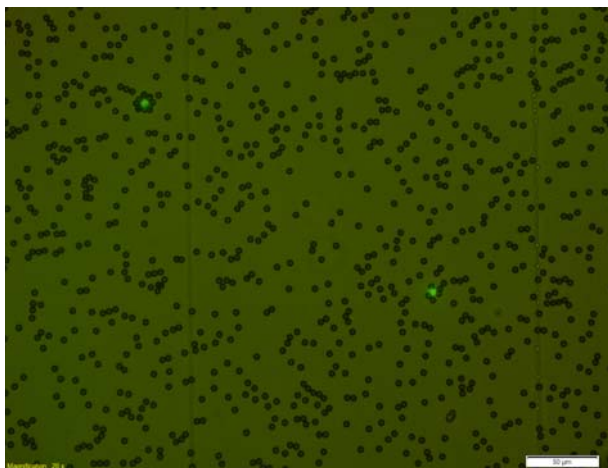


Fig. 1. Quantification of circulating endothelial cells in blood; typical aspect of fluorescent bead-rosetted endothelial cells. CEC stained with acridine orange, photographed under fluorescent microscope, magnification $\times 20$.

of a preparation of anti-CD146-coupled magnetic beads. Separation of beads and rosetted cells from the blood samples required a minimum of 2 minutes exposure to the magnet.

Three washes were performed to completely remove non-rosetted cells. After the third wash, the rosetted cells were recovered in a 100 μ L solution of acridine orange (at a final concentration of 5 μ g/mL in PBS), and observations were made in a 0.5 mm. Nageotte chamber cytometer (Hecht-Assistent, Sondheim, Germany) under fluorescent microscope (BX51, Olympus, Japan) equipped with a 500/20 nm excitation filter. The quality of images was improved by software Deltapix version 1.6 (Deltapix, Måløv, Denmark).

CEC seen under ultraviolet light after staining with acridine orange were identified as 15–50 μ m diameter cells bearing five beads, or as cells with five beads and with a well-preserved and recognisable morphology (clear nucleus in a well-defined cytoplasm, and a size compatible with that of endothelial cells) [24]. Representative photographs of CEC are shown in Fig. 1.

Results

Survival

All animals in the control and irradiated groups survived the whole experimental period. Body weight gain and food intake in the irradiated rats were not significantly different from those in the control group.

CEC in normal rats

The median number of circulating endothelial cells in normal rats was 161 cells/mL (range 102–218 cells/mL). There were no differences in CEC count between the male and female normal rats (mean \pm SEM: 175 ± 39 cells/ml vs. 154 ± 17 cells/ml, $p = 0.9$).

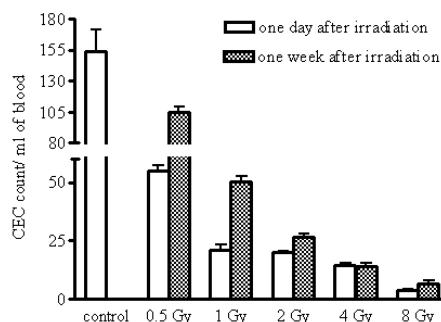


Fig. 2. Dose-response for early radiation-induced reduction of the CEC count and a partial re-population of CEC one week after acute irradiation. CEC numbers were scored one day (open) and one week (dashed) after acute irradiation. Compared to control group, rats irradiated with 0.5, 1, 2, 4 and 8 Gy radiation had significantly lower CEC number. Each data column represents the mean CEC count in four rats, except for control group which contains eight rats. Error bars are standard error of the mean (SEM).

Radiation dose-dependent changes in CEC number following single acute dose

Rats were irradiated with a single gamma ray dose of 2, 4 and 8 Gy, operated at a dose-rate of 116 mGy/min. The acute whole-body gamma irradiation of rats resulting in significant reduction in CEC number at all three doses studied compared with those in the control group (Fig. 2). The CEC count was significantly decreased in a dose-dependent manner one day and one week after single dose of 2, 4, and 8 Gy. Also, single doses of irradiation less than 2 Gy (0.5 and 1 Gy) were applied to rats. A single dose of 0.5 and 1 Gy caused a significantly lower CEC count compared to control (Fig. 2). Results of the present investigation show that low and moderate single doses applied for whole-body irradiation affect the endothelial detachment balance.

Radiation dose-dependent changes in CEC number following repeated fractionated dose

All irradiated rats had significantly lower number of CEC compared to control group (Fig. 3). A dose-dependent decrease in CEC count was observed in the rats following two months fractionated low dose whole-body irradiation.

Partial re-population of CEC count in rats after discontinuation of irradiation

One week after single dose of irradiation, we observed a significant increase in the number of CEC at doses less than 2 Gy compared to the results found one day after irradiation (at 0.5 Gy $p = 0.0017$, at 1 Gy $p = 0.002$, at 2 Gy $p = 0.04$). This increase, one week after irradiation, does not reach normal levels of CEC in non-irradiated rats. However, no significant difference was found in CEC count one

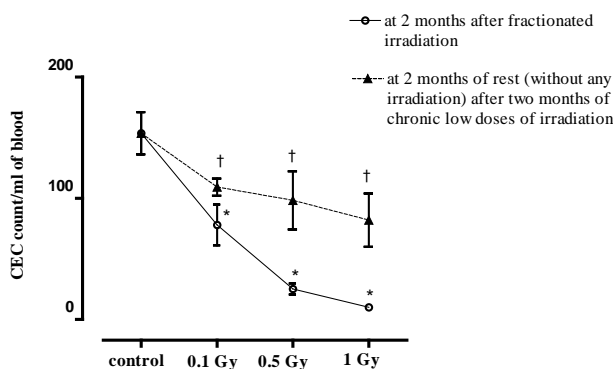


Fig. 3. Dose-response for early radiation-induced reduction of CEC count and partial re-population of CEC after two months of stopping of fractionated irradiation. CEC numbers were scored immediately (solid line) after completing the 2 months fractionated irradiation and after 2 months of rest (without any irradiation) (dashed line). Compared to control group, rats irradiated with 0.1, 0.5 and 1 Gy radiation had significantly lower CEC number. Each data point represents the mean CEC count in four rats, except for control group which contains eight rats. Error bars are standard error of the mean (SEM). (* $p < 0.05$ with respect to the control, † $p < 0.05$ with respect to the rats irradiated every 3 days for 2 months).

week after irradiation compared to those found one day after irradiation in rats irradiated acutely with 4 Gy and 8 Gy (see Fig. 2).

In the group of rats exposed to low gamma ray doses every three days for two months followed by two months of rest, a significant increase in the number of CEC took place at each radiation dose after two months of irradiation, followed by two months of rest, as compared with that found immediately after two months of fractionated irradiation (see Fig. 3).

Discussion

To the best of our knowledge, this is the first experimental study, which investigates the early effect of acute irradiation with moderate and high dose of radiation and the late effect of fractionated irradiation with low and moderate dose of radiation on the circulating endothelial cells (CD146 positive) in blood of rats. The vascular endothelium is a strong target candidate for radiation-induced damage due to its high radio-sensitivity [25].

Endothelial cells are recognised as target of the early radiation-induced apoptosis and are likely involved in delayed vascular necrosis. Accumulating evidences indicate that CEC in venous blood serve as a biomarker of endothelial damage [26, 27]. Vascular injury is associated with increase or decrease of CECs [20, 24, 28]. So, we assessed the endothelium damage inflicted by gamma radiation by measuring CEC levels. We chose *in vivo* model in order to study a complete system, where all cell types and physiological responses are included.

Originally, we found a dose-dependent marked reduction in CEC numbers one day after acute single

dose of irradiation with 0.5, 1, 2, 4 and 8 Gy compared to those in controls. This is consistent with the notion of the very short duration of the apoptotic process *in vivo* [29]. This early loss in CECs number was maintained for up to seven days after irradiation. The alterations observed in cell numbers were expected as irradiation diminishes the clonogenic capacity of endothelial cells *in vitro* and causes their depletion *in vivo* from the vasculature [30, 31]. These results conflict with the hypothesis of dysfunctional endothelial cells being detached. Possibly, the reduced CEC numbers in irradiated rats may point to different mechanisms of endothelial damage and repair, rather than to detachment of dysfunctional endothelial cells. It has been showed that CD146+ circulating cells, beside CECs, contain a subpopulation of progenitor endothelial cells (EPC) [32]. Hence, the decreased CEC in blood of irradiated rats might indicate inadequate EPC numbers, which impair endothelial cell replacement and thereby compromise integrity of endothelium in irradiated rats.

The early decrease in the number of endothelial cells after irradiation has been postulated to be due partly to apoptosis of the endothelial cell population [33, 34]. Our results demonstrate a gradual decline in CEC count after irradiation and are consistent with this notion.

Radiation doses ranging from 2 Gy to 50 Gy have been shown to induce endothelial cell apoptosis *in vitro* [35–37] or *in vivo* [33]. Accordingly, ionizing radiation was shown to induce ceramide-mediated endothelial cell apoptosis *in vivo* [33, 38, 39]. The vulnerability of the endothelium to radiation-induced apoptosis appears to be related to the high level of acid sphingomyelinase expression in endothelial cells (approximately 20-fold higher than in macrophages [40]), and to its preferential trafficking to the plasma membrane.

Also, several previous studies have demonstrated that the early vascular changes following irradiation within 24 h include increased vascular permeability, endothelial cell swelling, neutrophil adhesion to the endothelium [41] and apoptosis [33, 35, 36, 42, 43]. Langley and coworkers [44] has been found that apoptotic microvascular endothelial cells in suspension were particularly fragile after irradiation with 2–10 Gy. We assume that CEC count decrease after whole-body irradiation of rats is due to early damage of the cytoplasmic membranes of endothelial cells. It is caused by irradiation, as proposed by Salovsky [45] presumably by disruption of the cytoskeleton [46] or by CD146 loss after irradiation. We have yet to confirm this directly. It should be noted that Klein-Soyer and coworkers observed that the endothelial cell layer after irradiation was always continuous in their experimental system, without signs of desquamation [47]. Zhou and coworkers [48] noted that cell detachment 24 h after irradiation of the bovine aortic endothelial cells (BAEC) with high doses of radiation (5 Gy or 10 Gy) was significantly lower than that in the unirradiated cells [48]. Therefore, further studies are needed to verify the hypothesis of the increased adhesion of endothelial cells after irradiation.

Whereas the present study provided evidence for the radiation-induced CECs loss, prior investigation by Zeng *et al.* [22] showed an increase in CECs (CD31⁺, CD133⁻, and CD45^{low}) count at five and seven days after irradiation in mice exposed to lethal-intensity (8.5 Gy) and reduced-intensity (5 Gy) irradiation. Differences in the CEC count assay may have accounted for this apparent discrepancy. We explain this difference by the use of CD31 as an endothelial marker by Zeng *et al.* [22]. CD31 is not specific for the endothelium and can be detected on leukocyte subsets or platelet/leukocytes aggregates. Indeed, on the basis of CD31-bright/CD45-negative labeling as a combination to identify CEC, the amount of cells recorded per milliliter of blood is about 1000- to 100 000-fold higher than the level of CEC consensually reported in normal and pathological settings using CD146 based immunomagnetic separation that was used in our study. This huge discrepancy raises the question of the nature of CD31-bright/CD45-negative population in the study of Zeng *et al.* [22].

Recently, the biological effect of low dose radiation on cellular response has received attention, because low radiation doses also could induce serious health effects [49, 50]. Here, we used lower radiation doses in order to more closely mimic the clinical setting. However, no studies have been reported on the effects of doses lower than 1 Gy on CEC *in vivo*. Interestingly, our study shows the influence of fractionated low and moderate doses of irradiation on CEC levels in rats irradiated every three days for two months. We found a dose-dependent significant reduction in CEC numbers at two months after fractionated irradiation with 0.1, 0.5 and 1 Gy compared to those in controls.

This finding demonstrates the importance of CEC enumeration in the evaluation of vascular damage caused by irradiation even with doses less than 2 Gy, that have a minimal lethal effect in endothelial cells *in vitro*, as reported in previous studies [51, 52].

We further examined the recovery from endothelial damage. To investigate this issue, rats were exposed to fractionated low doses of radiation every three days for two months followed by two months recovery period. It is truly amazing that we found a significant increase in the CEC count two months after the end of fractionated irradiation, although it did not reach the control level (i.e. in the unirradiated rats).

Vascular endothelial cells have a slow turnover *in vivo*. It has been reported that ionizing radiation also may have dual roles with respect to endothelial cells, angiogenesis and the microenvironment: whereas radiation has a dominant direct anti-endothelial effect, it may also convey an indirect pro-angiogenic effect to endothelium [53]. Re-population of CEC that occurs after two months of rest of the following fractionated irradiation with low doses (<2 Gy) may be due to endothelial cell proliferation after irradiation. Newly generated endothelial cells have been shown to migrate within capillaries to replace the damaged endothelial cells [54]. This result suggests that the damage caused by fractionated low doses of gamma rays can be repaired and that the endothelial

cell injury possibly plays a role in early, but not late, vascular injury. More detailed studies are required for elucidation of the radiation injury at the cellular level and of the re-population process.

In summary, we provide an evidence of the important role for the circulating (CD146 positive) endothelial cell enumeration in monitoring the endothelial damage in rats after acute and fractionated gamma irradiation.

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