THE QUIESCENT AND MITOGEN STIMULATED PERIPHERAL BLOOD MONONUCLEAR CELLS AFTER GAMMA IRRADIATION AND THEIR P53, P21 AND H2AX EXPRESSION

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Introduction

The aim of this study was to compare reaction of quiescent and proliferating PHA (mitogenic lectin phytohemagglutinin)-stimulated human peripheral blood mononuclear cells (PBMCs) to γ-irradiation and analyze changes of proteins related to repair if DNA damage and apoptosis, such as γH2A.X, p53 and its phosphorylations on serine 15 and 392, and p21

Materials and methods

Cell cultures and culture conditions

The human non-proliferative peripheral blood mononuclear cells (PBMC) were acquired from venous blood of healthy volunteers by centrifugation over Histopague. The lymphocytes were cultured in Iscove's modified Dulbecco's medium (IMDM, Sigma) supplemented with a 20% fetal calf serum (FCS) in a humidified incubator at 37oC and a controlled 5% CO_2 atmosphere in 50 ml tissue culture flask (NUNC). For mitogenic stimulation of T cells was added 10μg/ml phytohemaglutinin (PHA, Sigma).

Gamma irradiation

The cells were irradiated using a 60Co γ-ray source with a dose-rate of 1,342 Gy/min.

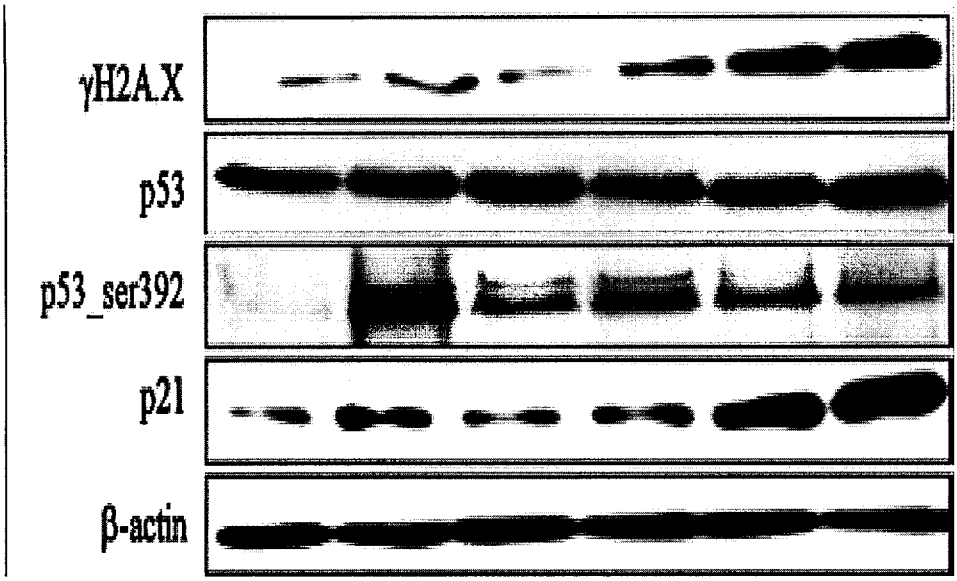
Western Blotting

Western blotting was used for p53, p53 serine392, H2AX serine139 and p21 detection. At various time after irradiation lymphocytes and MOLT-4 cells were lysed. The lysates containing an equal amount of protein (30 mg) have been loaded into a each lane of a polyacrylamide gel. Acquired proteins were separated by sds-page. After electrophoresis, the protein has been transferred to PVDF membranes. The membranes were blocked in Tris-buffered saline containing 0.05% Tween 20 and 5% non-fat dry milk After blocking procedure, membranes were probed with a mouse monoclonal antibody recognizing p53, p53 serine392, H2AX serine139 and with a rabbit polyclonal detecting p21. After washing, the blots were incubated with secondary antibody (Dako) and the signal was developed with a chemiluminiscence (ECL) detection kit (Roche).

Elisa

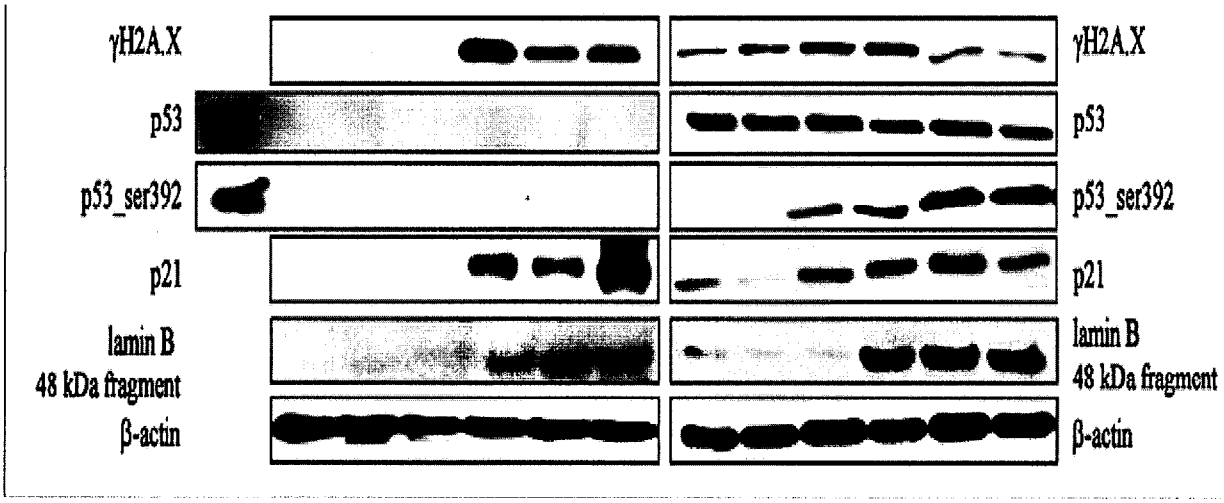
To enhance detection senzitivity the Total p53, PathScan p53 ser15, and Totalp21 Sandwich ELISA Kits were used

Figure 1. Dose dependence of changes in γH2A.X, p53, p53ser392 and p21 4 h after irradiation of PHA-stimulated PBMCs.



Isolated PBMCs were stimulated with PHA (72 h, 10 μg/ml), irradiated with the indicated dose, incubated for 4 h, lysed and analyzed by western blot. A dose-dependent response was observed in the dose range 0.5–4 Gy for the phosphorylation of H2A.X at serine-139 and for the increase in p21. Neither p53 nor p53 phosphorylated at serine-392 exhibited a dose-dependent response. β-actin was used as a loading control.

Figure 2. Comparison of changes in γH2A.X, p53, p53 ser392, p21 levels and apoptosis during 72 h after irradiation by a dose of 4 Gy in quiescent and PHA-stimulated PBMCs.



I. Freshly isolated quiescent PBMCs. The cells were isolated from peripheral blood, irradiated by the dose of 4 Gy and then incubated for 1–72 h. MOLT-4 cells (4 h after the irradiation by the dose of 4 Gy) were used as positive control for p53 detection.

II. PHA-stimulated proliferating PBMCs. Isolated PBMCs were stimulated with PHA irradiated by the dose of 4 Gy and then incubated for 1–72 h. After irradiation, the level of protein p53 (and its phosphorylation) was below detection limits of western blot analysis at quiescent PBMCs, but p53 responded to γ-radiation in proliferating PHA-stimulated PBMCs. PHA-stimulation also caused an increase in γH2A.X and p21. These proteins increase after IR in both quiescent and PHA-stimulated PBMCs, but with different dynamic. Apoptosis was

detected by analysis of lamin B cleavage - a 68-kDa nuclear protein is cleaved into a 48-kDa fragment by active caspases when apoptosis is induced. β-actin was used as a loading control.

Figure 3.Dose dependence of changes in p53 and p53 phosphorylated at serine 15 over the dose range 0.5–7.5 Gy, 4 h after irradiation of PHA-stimulated PBMCs

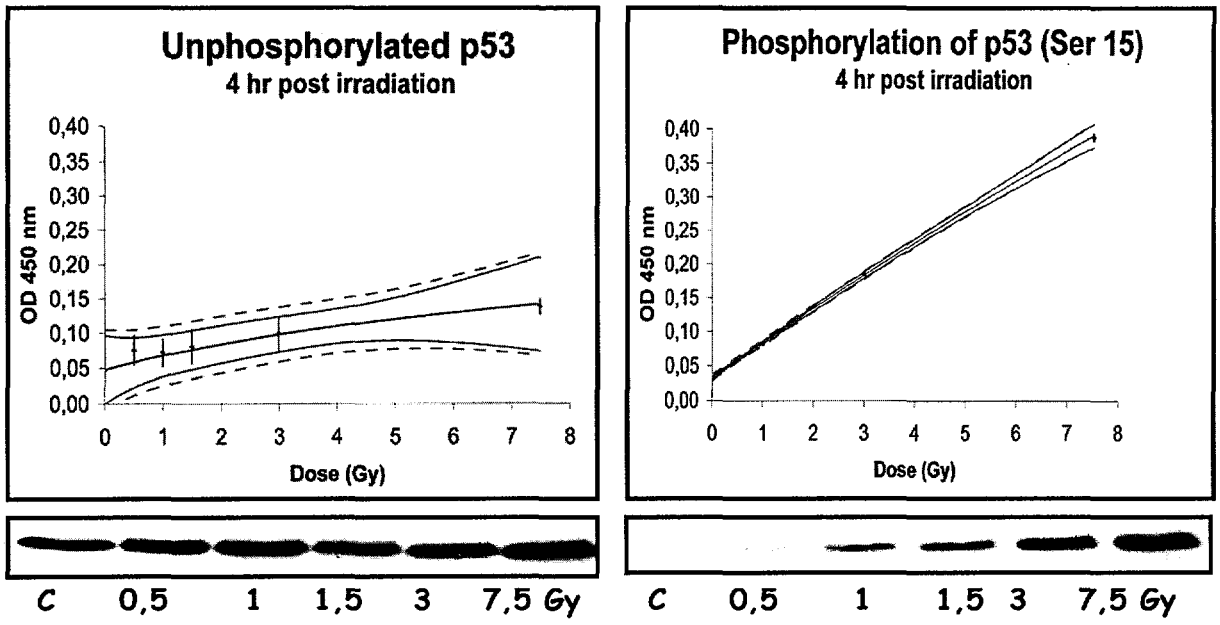
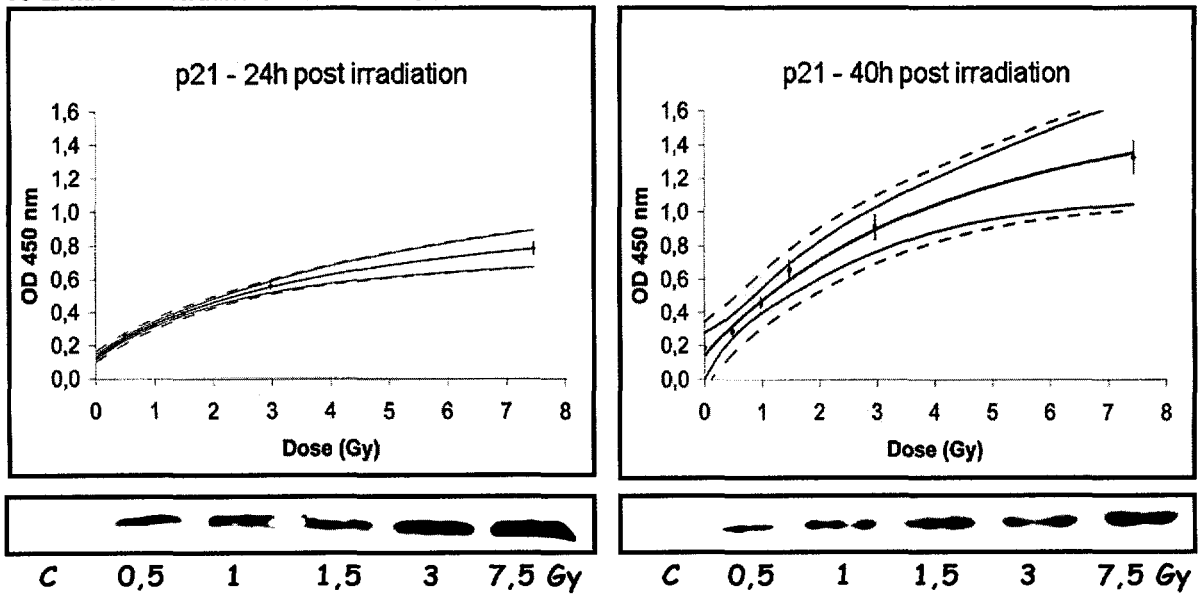


Figure 4. Dose dependence of changes in p21 over the dose range 0.5–7.5 Gy, 24 h and 40 h after irradiation of PBMCs



Isolated PBMCs were irradiated with the indicated dose, incubated for 4 h, 24 h, 40 h and analysed by ELISA. The optical density OD at 450 nm was plotted against dose D (in Gy) and the resulting curve turned out to be compatible with Michaelis–Menten kinetics

Conclusion

Protein changes induced by radiation are different in quiescent and stimulated PBMCs. We analyzed changes in proteins related to DNA damage repair and apoptosis using the western blot method in quiescent and stimulated PBMCs. Western blot technique can detect γH2A.X increase only at later times, when the phosphorylation of H2A.X is related to the

onset of apoptosis (24–72 h after irradiation by the dose of 4 Gy). The level of H2A.X phosphorylation increased after stimulation of PBMC by PHA (72 h, 10 μg/ml) and as shown here it was detectable by western blot analysis. The increase in γH2A.X that we detected by western blot 4 h after irradiation of stimulated lymphocytes was dose dependent. It can be concluded that measurement of γH2A.X during the first hours after the irradiation is a good marker of the received dose of radiation. We compared the dynamics of p53 induction after irradiation by IR in both quiescent and stimulated lymphocytes. p53 increase was observed only in stimulated lymphocytes, as was p53 phosphorylation at serines-392 and -15. The increase in the amount of p53 was not dose-dependent 4 h after the irradiation. On the other hand, phosphorylation of p53 at serine-15 analyzed 4 h after the irradiation is dose-dependent over the studied dose range. Despite the fact that p53 was not detected in quiescent lymphocytes and a reaction to irradiation was not observed either, p21 levels increased after irradiation in both quiescent and stimulated lymphocytes in a dose-dependent manner. IR induces phosphorylation of p53 at both serines-15 and -392 in PHA stimulated human lymphocytes. However, phosphorylation at serine-15 was dose-dependent, while phosphorylation at serine-392 was not. We have shown that irradiation of stimulated lymphocytes causes an increase in phosphorylation of histone H2A.X, an increase in p53 and its phosphorylation, and an increase in p21. ELISA measurement of p53 phosphorylated on serine-15 and p21 can be used as indicator of received dose of IR.

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