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Dynamics of apoptosis in γ-irradiated lymphocytes and the $CD21^{+}CD27^{+}$ B-cell subset as a potential biodosimetric marker

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A B S T R A C T

The dynamics of apoptosis progression has been studied in human peripheral blood mononuclear cells (PBMC) irradiated with selected doses (0.5, 1, 2, 4, 6, 8 and 10 Gy) of 60Co γ rays and subsequently cultivated for 16 h in vitro. A dose-dependent increase and decrease of shrunk and intact cells, respectively, has been observed in the range of 0.5–4 Gy while higher doses have not affected the relative numbers of intact and apoptotic cells. The relative number of early apoptotic cells did not change within the dose range studied. Immunophenotyping of intact and very early apoptotic cells have indicated that the remaining intact cells represent the most convenient population for radioresistance studies in vitro. The $CD21^{+}CD27^{+}$ subset of peripheral B-cells has thus been suggested as a putative biodosimetric marker in vitro.

1. Introduction

Living organisms are continuously exposed to naturally occurring ionizing radiation, which is mostly composed of γ rays with the exception of local exposure to alpha particles from radon decay. Accidentally human beings can experience higher dose exposure due either to radiation disasters or the use of nuclear weapons. Physical dosimetry cannot always be relied upon as, especially in case of accidental irradiation, the affected individuals frequently do not wear personal dosimeters. Therefore, dose estimates and determination of past exposure must often be based on changes occurring within the exposed organism upon irradiation. Such indicators are called radiobiological indicators or radiological biomarkers. Reliable biomarkers must be specific for ionizing radiation and provide sufficient information on radiation type and dose assessment (Blakely et al., 2010a).

Blood cell counts as well as changes in the relative numbers of blood cell populations in irradiated individuals represent an attractive tool in biodosimetry. Blood leukocytes are highly radiosensitive, easily obtainable from circulation and can be repeatedly sampled and evaluated in terms of their numbers and surface phenotype. It is not surprising that one of the earliest and most direct methods of dose determination following radiation exposure involves charting daily counts of different cells circulating in the peripheral blood (Hertveldt et al., 1997).

It has been documented in Chernobyl accident sufferers that total leukocyte counts decline rapidly in the first week following radiation exposures exceeding 1 Gy and both the extent and duration of the decline and subsequent recovery have been shown to correlate well with the received dose (Vorobiev, 1997). The ratio of neutrophils to lymphocytes increases by 44, 12, 8 and 5 fold at 1, 2, 3, and 4 days after 6.5 Gy total body irradiation, respectively, in non-human primates (Blakely et al., 2010b). Lymphocytes represent one of the most radiosensitive populations in the mammalian body (Anderson, 1976). Upon irradiation they undergo the process of programmed cell death also called apoptosis during which swift removal of damaged cells from circulation is mediated by scavenging mechanisms including radiation-resistant phagocytes. Similar processes have been observed in vitro where apoptotic lymphocytes are ingested by a more radioresistant mononuclear phagocyte population (Hertveldt et al., 1997). Differences in radiosensitivity of peripheral blood lymphocyte subpopulations and subsets have been used for biodosimetric studies in vitro. Thus, B-cells have been identified as the most radiosensitive lymphoid population in vitro (Philippe et al., 1997), while natural killer (NK) cells have been claimed to represent the most radiosensitive population (Vokurková et al., 2006).

In our previous work we have characterized radiosensitive lymphocyte subpopulations and their subsets by comparing the surface phenotype of Annexin V-negative cells in irradiated and subsequently cultivated Peripheral blood mononuclear cells (PBMC) (Vokurková et al., 2006). We have also observed that gating for the population of small lymphocytes with the typical lymphoid scatter characteristics can be used to discriminate apoptotic

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lymphocytes from their intact counterparts in vitro (Rehakova et al., 2008; Sinkorova et al., 2009). We have thus decided to test a simplified (light scatter based) experimental setup in the attempt to introduce a fast, simple and cost-effective approach for large scale screening of patients or soldiers exposed to ionizing radiation. In humans, the $CD8^+$ positive peripheral NK subset and $CD27^+$ B lymphocytes have been identified as the most promising biodosimetric candidates. In this work we have tried to characterize the dose dependence of the dynamics of apoptosis in human PBMC in a fixed experimental setup (16 h cultivation after irradiation). In parallel, the dose dependence of the B compartment composition has been determined in intact and very early apoptotic population where unaltered surface marker expression has been observed.

2. Materials and methods

2.1. Cell isolation, irradiation and cultivation

Heparin-treated blood obtained from ten healthy volunteer donors was diluted 1:1 with phosphate buffered saline (PBS) and PBMC were isolated by a standard technique using the discontinuous Ficoll-Histopaque (density 1077, Sigma, St. Luis, MO) gradient (20 min sedimentation at 400 g and 20 °C). PBMC were collected from the Ficoll/plasma interface, washed twice in sterile RPMI 1640 (GIBCO Life Technologies, Grand Island, NY) supplemented with 20% FCS, 2 mM glutamine, 100 UI/ml penicillin and 0.1 mg/ml streptomycin (all products from Sigma) and the cell suspension density was set to 10^6 cells/ml.

6-well polystyrene cultivation plates with 500 μl of PBMC suspension per well were sham-treated or irradiated with the doses of 0.5; 1; 2; 4; 6; 8 or10 Gy; delivered with a rate of 1.29 Gy/min at a distance of 1.23 m from the ^{60}Co source at room temperature. After 16 h cultivation in humidified atmosphere at 37 °C the cells were harvested and centrifuged (400 g for 10 min at 4 °C). PBMC pellets were resuspended and washed twice in ice-cold washing and staining buffer (WSB, PBS containing 0.2% gelatin from cold water fish skin and 0.1% sodium azide, all reagents from Sigma) and the suspension density was set to 5×10^6/ml.

2.2. Flow cytometry

Three color cocktail of mouse anti-human monoclonal antibodies: anti-CD21/APC (clone B-ly4, BD Pharmingen), anti-CD27/PEDy590 (clone LT27, Exbio, Prague, Czech Rep.) and anti-CD38/PEDy747(clone HIT2, Exbio, Prague, Czech Rep.) together with fluorescein isothiocyanate conjugated Annexin V (Annexin V-FITC) from the Apoptest kit (Dako, Denmark) were used for immunophenotyping of B-cell subsets at different stages of apoptosis. Cells were stained with monoclonal antibodies and Annexin V-FITC in parallel in the Annexin V staining buffer for 30 min on ice. After one washing step in the ice-cold washing and staining buffer data were acquired on a CyAn ADP (Beckman Coulter) flow cytometer. Acquisition and analysis were performed using the Summit (Beckman Coulter) software.

2.3. Statistical analysis

Mean value and standard deviation were calculated for all data sets. The significance of differences was evaluated by the Students' test. The Irradiated Versus Non-Irradiated Ratio (IVNIR, Rehakova et al., 2008; Sinkorova et al., 2009) was used for comparison of lymphocyte subset proportion and their relative radiosensitivity determination.

3. Results

Irradiation-induced apoptosis can be visualized by Annexin V-FITC due to its specific binding to superficially exposed phosphatidyl serine occurring in many cell types including lymphocytes upon programmed cell death initiation (Louagie et al., 1998). Fig. 1A

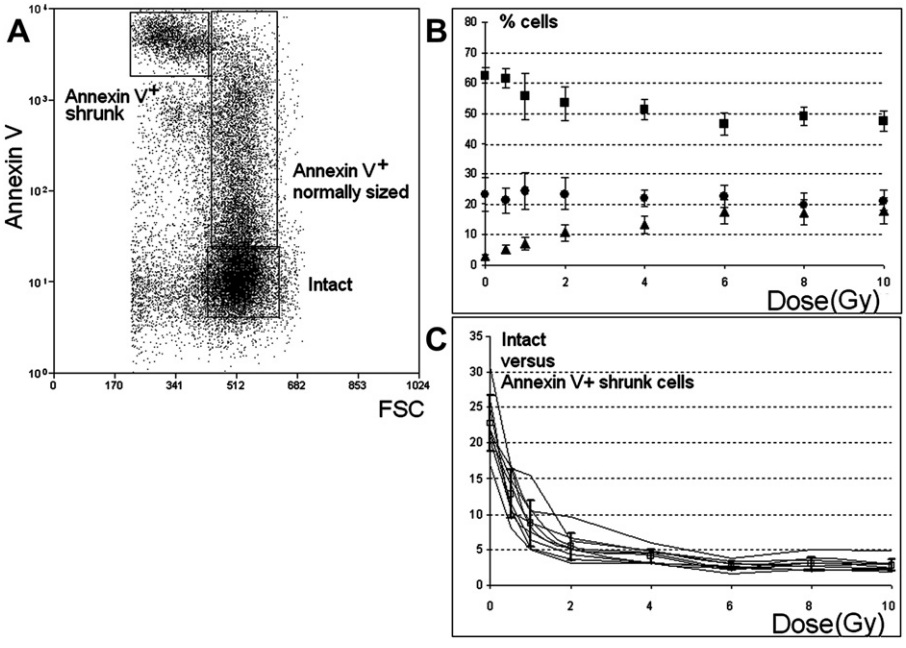

Fig. 1. Analysis of apoptosis progression in PBMC upon 0–10 Gy irradiation followed by 16 h in vitro cultivation. A: Relative size versus Annexin V binding capacity of cells in a 10 Gy irradiated and subsequently cultivated (16 h) human PBMC sample. The three populations of intact (normally sized, Annexin V^-), normally sized Annexin V^+ and shrunk cells with high Annexin V binding capacity are depicted by rectangular regions. B: Dose dependence of the relative proportions of intact (■), normally sized Annexin V^+ (●) and shrunk Annexin V^+ (▲) PBMC. Data are shown as mean values +/− standard deviation of 10 samples for each irradiation dose. C: Dose dependence of the ratio of intact versus shrunk Annexin V^+ cells 16 h after irradiation for 10 individual samples (solid lines) as well as the average values +/− SD (black symbols) for each irradiation dose are shown.