

3-2014

SIMULTANEOUS ANALYSIS OF P53 PROTEIN EXPRESSION AND CELL PROLIFERATION IN IRRADIATED HUMAN LYMPHOCYTES BY FLOW CYTOMETRY

Rafael de Freitas e Silva

Universidade Federal de Pernambuco-Brasil, Universidade de Pernambuco-Brasil, Centro de Pesquisas Aggeu Magalhães, FIOCRUZ-Brasil

Neyliane Frassinetti Gonçalves dos Santos

Universidade Federal de Pernambuco-Brasil

Valéria Rêgo Alves Pereira

Centro de Pesquisas Aggeu Magalhães, FIOCRUZ-Brasil

Ademir Amaral

Universidade Federal de Pernambuco-Brasil

Follow this and additional works at: https://scholarworks.umass.edu/dose_response

Recommended Citation

de Freitas e Silva, Rafael; Frassinetti Gonçalves dos Santos, Neyliane; Rêgo Alves Pereira, Valéria; and Amaral, Ademir (2014) "SIMULTANEOUS ANALYSIS OF P53 PROTEIN EXPRESSION AND CELL PROLIFERATION IN IRRADIATED HUMAN LYMPHOCYTES BY FLOW CYTOMETRY," *Dose-Response: An International Journal*: Vol. 12 : Iss. 1 , Article 7. Available at: https://scholarworks.umass.edu/dose_response/vol12/iss1/7

This Article is brought to you for free and open access by ScholarWorks@UMass Amherst. It has been accepted for inclusion in Dose-Response: An International Journal by an authorized editor of ScholarWorks@UMass Amherst. For more information, please contact scholarworks@library.umass.edu.

SIMULTANEOUS ANALYSIS OF P53 PROTEIN EXPRESSION AND CELL PROLIFERATION IN IRRADIATED HUMAN LYMPHOCYTES BY FLOW CYTOMETRY

Rafael de Freitas e Silva^{1,3}, Neyliane Frassinetti Gonçalves dos Santos¹, Valéria Rêgo Alves Pereira³, and Ademir Amaral¹ □ ¹ Laboratório de Modelagem e Biodosimetria Aplicada (LAMBDA), Universidade Federal de Pernambuco-Brasil; ² Universidade de Pernambuco-Brasil; ³ Centro de Pesquisas Aggeu Magalhães, FIOCRUZ-Brasil

□ P53 protein has an intrinsic role in modulating the cellular response against DNA radioinduced damages and has been pointed out as an indirect indicator of individual radiosensitivity. The rate of cell proliferation is also a parameter that has been related to tissue sensitivity to radiation. However, this feature is yet understudied. In this context, the aim of this work was to employ Flow Cytometry (FC) for simultaneously assessing of p53 protein expression levels together with cellular proliferation rate of irradiated human lymphocytes. From in vitro irradiated human blood samples, mononuclear cells were isolated and labeled with *Carboxylfluorescein Diacetate Succinimidyl Ester* (CFSE) prior to phytohaemagglutinin (PHA) stimulation in culture for 96 hours. Cells were also labeled with anti-p53 monoclonal antibody PE-conjugated in order to analyze either proliferation rate or p53 expression levels by FC. It was verified a reduction in the proliferation rate of irradiated lymphocytes and, in parallel, a rise in the p53 expression levels, similar for quiescent and proliferating lymphocytes. The results emphasize the importance of the use of CFSE-stained lymphocytes in assays associated to proliferation rate and the use of this methodology in several studies, such as for evaluating individual radiosensitivity.

Key words: P53 protein, Proliferation rate, Flow cytometry, CFSE, Radiosensitivity

1. INTRODUCTION

The role of some molecules in the repair of radio-induced DNA damage has been studied and such molecules have been proposed as bioindicators of individual radiosensitivity. The achievement of this goal would be especially important in cancer radiotherapy effectiveness, since individuals differ widely in their responses to ionizing radiation exposures (Borgmann *et al.* 2002; Schnarr *et al.* 2007; Pinto *et al.* 2010). The characteristic of cancer implies that the target volume to be irradiated is generally outlined beyond the tumor boundaries, leading to side effects of radiotherapy that are issues of damages to normal (healthy) tissues. Therefore, normal connective tissue tolerance has an important role in the protocol definition as well as for the effectiveness of radiotherapy

Address correspondence to Rafael de Freitas e Silva, Universidade Federal de Pernambuco, Departamento de Energia Nuclear, Avenida Professor Luiz Freire, 1000, 50740-540, Cidade Universitária, PE – Brasil; Tel. +55 81 21 26 79 77; rafa.freitass86@gmail.com

P53 and Proliferation on Irradiated Lymphocytes

(Barnett *et al.* 2009). In this context, for example, bioindicators of individual radiosensitivity would act as prognostic parameter in cancer radiotherapy.

Due to its intrinsic role in modulating the cellular response against DNA damages, p53 protein levels have been pointed out as an indirect indicator of individual radiosensitivity (Dahm-Daphi 2000; Mayer *et al.* 2002; Komuro *et al.* 2003; Cavalcanti *et al.* 2008; Lu and El-Deiry 2009; Cavalcanti *et al.* 2011). The most advanced investigations concerning this protein expression and its correlation with radiosensitivity use blood-based assays with stimulated cells, mainly lymphocytes. As human lymphocytes are typically in resting phase (G₀), they can reenter the cell cycle if appropriately stimulated to mitosis. However, culture conditions, cell cycle position and differentiation stages are factors that can influence the lymphocyte radiosensitivity in terms of cell death. For example, on actively proliferating cells, apoptosis may occur during the interphase right before mitosis or after some rounds of cell divisions (Carloni *et al.* 2001). Despite of this fact, little information has shed light on how mitogen stimulation and proliferation are affected and influences p53 protein expression levels in human irradiated lymphocytes.

One of the technologies used to quantify bioindicators, flow cytometry (FC) has been widely applied in investigation of antigens located on the cell surface, cytoplasm or nucleus, in a large number of cells in short time (Andrzej *et al.* 1998; Nolan and Yang, 2007). The FC has been also used to assess cell proliferation status or capacity, by labeling with intracellular fluorescent dyes. Among the commercially available dyes, *Carboxylfluorescein Diacetate Succinimidyl Ester* (CFSE) stands out as one of the most used and versatile in terms of homogeneity of lymphocyte staining, membrane stability, optimal concentration and cost-efficiency (Banks *et al.* 2011). The CFSE staining approach can be associated with fluorochrome-labeled monoclonal antibodies, enabling simultaneous analysis of protein expression level and cell proliferation (Parish 1999; Tario *et al.* 2007; Parish *et al.* 2009).

In this scenario, this work was designed to use FC in rapid simultaneous determination of p53 protein expression and the proliferation rate of circulating human lymphocytes irradiated *in vitro*.

2. MATERIAL AND METHODS

2.1 Studied population and ethical aspects

Blood samples from 5 volunteers (25.6 ± 3.9 years old, coded as A, B, C, D and E) were studied, being 3 females and 2 males. All individuals signed a written informed consent before blood collection, following recommendations of ethics committee from Federal University of Pernambuco (project 297/09).

2.2 Sampling and irradiation

From each donor, a total of 20 mL of peripheral blood was collected by venipuncture in sodium-heparin tubes (Vacuette, USA) and divided into two 10 mL aliquots. One aliquot was irradiated with 4 Gy (Cobalt Irradiator, dose-rate of 4.312 Gy/h) and the remaining aliquot was used as control (non-irradiated).

2.3 Isolation of peripheral blood mononuclear cells

After irradiation, blood was diluted (1:1) with phosphate-buffered saline (PBS, pH 7.2) and deposited onto the Ficoll-Paque PLUS density (1.077 g/mL) gradient (GE Healthcare, USA). After centrifugation, peripheral blood mononuclear cells (PBMC) were individually removed and washed twice with PBS.

2.4 CFSE labeling

4×10^6 cells in 1 mL of PBS were resuspended with PBS containing 0.25 μ M of CFSE (Invitrogen, USA) and incubated at 37 °C for 10 min. CFSE concentration was carefully titrated in order to establish the best concentration to avoid inhibition of cell proliferation or even cell death. After incubation, the labeling was quenched with 1 mL of ice cold (4 °C) RPMI 1640 containing 2 mM of L-glutamine, 50 mg/L of gentamicin sulfate and 2 mg/L of amphotericin B, supplemented with 10 % fetal bovine serum (both Cultilab reagents, Brazil). The cells were pelleted and washed once with PBS followed by resuspension with 1 mL of RPMI supplemented at density of 2×10^6 cells/mL.

2.5 Cell culture

Cells were plated in 96-well bottom plates (TPP, Switzerland) at density of 2×10^5 cells/well with 10 μ g/mL phytohemagglutinin (PHA). From each subject, one part of cells was cultivated without stimulation in order to set-up the flow cytometry. Plates were incubated in a humidified atmosphere of 5 % CO₂ at 37 °C for 96 hours.

2.6 Cell labeling with antibodies

Following 96 hours of culture, viability of CFSE labeled cells was assessed by the trypan blue dye exclusion method, which stains non-viable cells. Then, cells were harvested, washed once with RMPI supplemented, resuspended with 1 % paraformaldehyde and incubated for 20 min. After incubation, cells were washed once with RMPI supplemented, centrifuged for 5 min at 300 x g and, then, cells were resuspended with permeabilizing solution (50% FACS lysing solution 1X [BD Pharmingen, USA], 50 % paraformaldehyde 4 % in PBS) and incubated for 10 min at

P53 and Proliferation on Irradiated Lymphocytes

room temperature. The fixation and permeabilization methods employed in this work have been previously established and tested as published elsewhere (Cavalcanti *et al.* 2008, Cavalcanti *et al.* 2011). After this, cells were washed twice with PBS 0.5 % Tween-20 solution (Merck, Germany). Cell pellet was resuspended with 200 μ L of PBS 0.5 % Tween-20 solution and stained with monoclonal antibodies fluorochrome-conjugated, for 20 min at room temperature. Non-irradiated cells were incubated with mouse IgG₁ phycoerithrin (PE)-conjugated (Clone MOPC-21 - BD Biosciences, USA), the isotype control antibody. For detection of p53, irradiated and non-irradiated cells were incubated with anti-p53 antibody PE-conjugated (Clone G59-12 - BD Biosciences, USA). After labeling, cells were washed twice with PBS 0.5 % Tween-20 solution and fixed with 500 μ L of 1 % paraformaldehyde in PBS.

2.7 Flow cytometry analysis

The analyses were performed on a FACScalibur flow cytometer (Becton Dickinson Company, USA) equipped with an argon laser (wavelength 488 nm). Fluorescence of 50,000 lymphogated events, based on scatter parameters of size and granularity was acquired. Data were analyzed and treated with FlowJo 7.6 (Tree Star Inc., USA). Non stimulated cells were used during analysis for setting quadrant parameters, and the levels of p53 protein were subtracted from the value of isotype control (less than 1%).

2.8 Statistical analysis

The results were presented as mean and standard deviation. Differences between means were evaluated by Student's t-test, and they were considered statistically significant when $p < 0.05$.

3. RESULTS

For each subject studied, control of labeling and proliferation was done with non-stimulated cells. Figure 1 shows dot-plots from lymphocytes non-stimulated with mitogen, non-irradiated (A) and irradiated with 4 Gy (B).

As expected, non-stimulated lymphocytes kept in culture were not able to proliferate and showed high levels of CFSE identified for its high fluorescence.

Figure 2 presents dot-plots obtained during analysis of non-irradiated (0 Gy) and irradiated (4 Gy) mitogen-stimulated samples from five subjects (coded as A, B, C, D and E). In each graphic, lower right quadrant displays quiescent lymphocytes (CFSE high fluorescence) non-positive for p53; upper right quadrant displays quiescent cells positive for p53 expression; lower left quadrant displays proliferating lymphocytes (CFSE

R. de Freitas e Silva and others

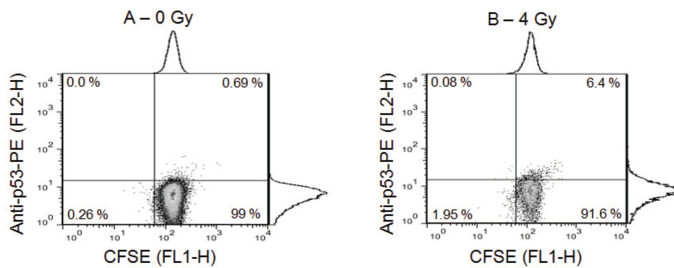


FIG. 1. Dot-plots obtained during analysis of lymphocytes non-stimulated with mitogen, non-irradiated (A) and irradiated with 4 Gy (B). The upper quadrants allows the visualization of p53 expression (left, negative; right, positive) and the lower quadrants, the pattern of proliferation (left, proliferating; right, quiescent).

low fluorescence) non-positive for p53; upper left quadrant displays proliferating cells positive for p53 expression.

The exposure of blood samples to 4 Gy resulted in an increase of quiescent lymphocytes, from $(39.06 \pm 19.07)\%$ to $(47.16 \pm 12.83)\%$, and the consequent decreasing of proliferating lymphocytes, from $(57.88 \pm 18.33)\%$ to $(42.54 \pm 11.91)\%$. However, this radioinduced effects was statistically significant only for proliferating cells ($p < 0.05$).

About p53 protein expression, Table 1 presents expression levels values for quiescent and proliferating lymphocytes from unexposed and 4 Gy-exposed peripheral blood samples of each subject.

Regarding this data, for each group (control or irradiated lymphocytes) there is no statistical difference in p53 expression between quiescent and proliferating cells. However, it is observed a significantly increase of this protein expression levels after irradiation ($p < 0.05$), either for quiescent or proliferating lymphocytes.

4. DISCUSSION

In this research, lymphocytes were chosen due to the ease of obtaining and handling such cells in laboratory and as a result of the well-established method of labeling them with CFSE, especially for studies of immunology. Although PHA is widely employed on researches aimed to investigate molecular parameters in stimulation of lymphocytes, this mitogen is mainly effective in the stimulation and activation of T cells, acting differently for B lymphocytes that are negligibly stimulated (Vilasová *et al.* 2008; Moghbeli-Nejad *et al.* 2012). Henceforth, all results presented in this report are related to T lymphocytes (O'Donovan *et al.* 1995).

This research employs the same CFSE concentration successfully used in similar investigation (Brenchley and Douek 2004; Seggewiss *et al.* 2005). However, in proliferation rate studies, there is a discussion whether CFSE concentration can keep cells alive or not. Some authors highlight that high concentrations of CFSE can inhibit cell proliferation

P53 and Proliferation on Irradiated Lymphocytes

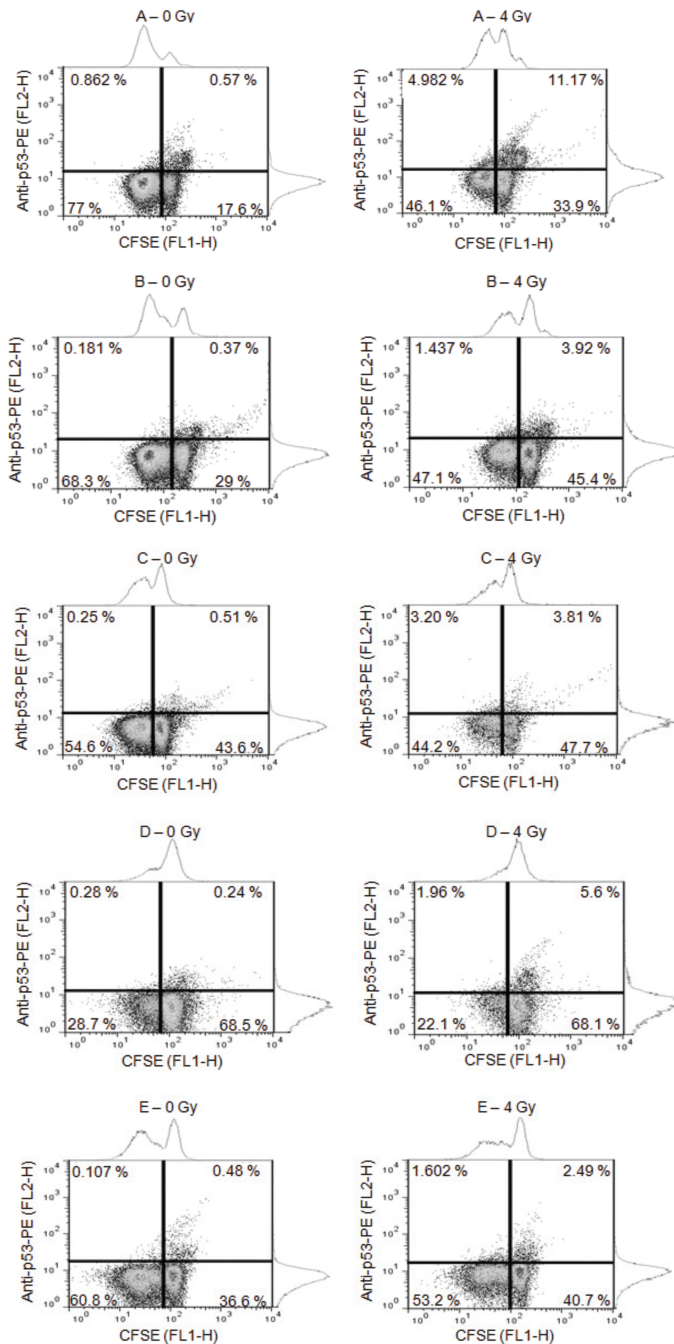


FIG. 2. Dot-plots obtained during the analysis of non-irradiated (0 Gy) and irradiated (4 Gy) proliferating samples from subjects A, B, C, D and E. Lower right quadrant displays quiescent lymphocytes (CFSE high fluorescence) non-positive for p53 and upper right quadrant displays quiescent cells positive for p53 expression; lower left quadrant displays proliferating lymphocytes (CFSE low fluorescence) non-positive for p53 and upper left quadrant displays proliferating cells positive for p53 expression.

R. de Freitas e Silva and others

TABLE 1. p53 expression levels in all samples analyzed before (control non-irradiated) and after *in vitro* irradiation.

Subject	p53 expression levels (%)			
	Control (non-irradiated)		4 Gy-irradiated	
	Quiescent	Proliferating	Quiescent	Proliferating
A	0.57	0.86	11.17	4.98
B	0.37	0.18	3.92	1.44
C	0.51	0.25	3.81	3.20
D	0.24	0.28	5.60	1.96
E	0.48	0.11	2.49	1.60

(the cellular event to which this dye is made to detect) (Quah *et al.* 2007) or even kill the cells. Lašt'ovička *et al.* (2009) have described a range of concentrations in which CFSE can damage lymphocytes enough to kill them. To avoid this drawback, in this study CFSE concentration was carefully titrated with a wide range of concentrations to achieve the best concentration able to keep cells alive and normally proliferating, as confirmed by the use of trypan blue dye method. The choice of the trypan blue dye method was a practical decision due to either the low costs or its efficiency in the evaluation of cell viability.

CFSE is a very versatile dye which permits to monitor *in vivo* and *in vitro* lymphocyte division (Lyons 1999; Parish 1999; Lyons 2000; Veiga-Fernandes *et al.* 2000). At the molecular level, CFSE is non-fluorescent with ability to penetrate in the cell and couples to cellular proteins. Once in the cell, esterases remove acetate groups of the molecule that from this point becomes highly fluorescent. CFSE has the advantage of labeling cells regardless of their stage in the cell cycle (Asquith *et al.* 2006) and its fluorescence intensity is progressively halving after each cell division (Quah *et al.* 2007). Actually, flow cytometric evaluation allows the detection and quantification of several different molecules produced by a single cell by using markers with different light emission properties.

However, the analyses employing probes with similar CFSE light emission are not straightforward (Tario *et al.* 2007). The use of CFSE dye for evaluating cell proliferation is cost-efficient in comparison with other techniques, such as the gold standard tritiated thymidine (3H-TdR). 3H-TdR is a radioactive molecule that labels the DNA of proliferating cells, but due to its cost and need of dedicated lab facilities and special procedures, this methodology has been progressively replaced (Evrard *et al.* 2010).

Our results showed an increase in the level of quiescent lymphocytes and a decrease of proliferating lymphocytes, which might indicate a direct consequence of the apoptotic phenomenon. One of the effects of

P53 and Proliferation on Irradiated Lymphocytes

cellular irradiation is cell cycle arrest, a phenomenon commonly seen as a time-lapse to evoke proteins involved in the repair of damages or even to induce cell death (Wood and Chen, 2008, Landsverk *et al.* 2011).

In normal conditions it is not expected an increase in the levels of p53 protein due to its short average lifetime. On the other hand, after cell irradiation an increase of this protein concentration occurs, which is accompanied by an increase in its mean life span (Fuchs *et al.* 1998; McVean *et al.* 2000; Fei and El-Deiry 2003; Cavalcanti *et al.* 2008). As expected, the results of this study showed an increase of p53 expression at different levels for each subject. However, it was not expected that p53 could be expressed for both quiescent and actively proliferating cells. P53 is a central molecule in the response to radioinduced damages on the DNA, which are followed by activation of a complex process that acts to arrest the cell cycle and allows the identification and repair of damages (Stehg 2012). The role of p53 in the response to DNA damages in lymphocytes actively proliferating has yet to be well determined. These results about the diminishment of lymphocyte proliferation in irradiated samples are indicative of a relationship between p53 expression and cell death. Nevertheless, the exactly mechanism by which p53 and survivin interact to induce cellular apoptosis is not well established.

The results of this research are suggestive that the levels of p53 expressed by proliferating lymphocytes are related with either the cellular apoptotic process to eliminate cells carrying chromosomal damages induced by IR, or the prolonged time-culture and its consequences to the cell survival. Hence, in order to correlate the expression of p53 and the radiosensitivity of tissues, one may postulate that in case the studied subjects are accidentally exposed to IR, at the same conditions, all of them will respond similarly to the irradiation, with exception of perhaps the subject A.

Observing the Table 1, the levels of p53 expression of subject A after irradiation are considerably higher when compared to the others. Moreover, the levels of p53 expressed by the quiescent population irradiated (11.17 %) were approximately two fold higher than their proliferating counterparts (4.98 %). These high p53 expression levels of the subject A would indicate that this individual will probably overreact to the radiation exposure differently, when compared to the others.

Researchers have shown that some tissues from humans and mice expressing high levels of p53 have in common an elevated sensitivity to IR (MacCallum *et al.* 1996; Dainiak 2002). Cavalcanti *et al.* (2011) emphasized a dose-dependent elevation of p53 levels in human lymphocytes. Even being of extreme importance, p53 is not the only factor which might contribute to the cellular fate after exposure to IR.

The assessment of proliferative status of cells from other tissues can also be done in combination with the staining for other intracellular or

R. de Freitas e Silva and others

extracellular proteins. In this sense, we recommended assays with CFSE-labeled lymphocytes to identify differences on cell proliferation rate and p53 protein expression. By this methodology, it was identified a rise of p53 expression levels in irradiated lymphocytes, either quiescent or actively proliferating, and a reduction of the proliferation rates of irradiated lymphocytes. This latter is likely a consequence of activation of a cell death program, eliminating cells by apoptosis even when they are under mitotic stimulation. This approach was completely original in the field of p53 studies since, as far as we know, there is no data in the literature contrasting, at the same time, the expression of p53 and the proliferation status by the use of CFSE dye in human irradiated lymphocytes. The results presented in this report suggest the use of this methodology for more accurate evaluation of protein dose-response from irradiated cells. Naturally, to demonstrate the usefulness of this methodology, further studies with a greater number of subjects are needed in order to support the findings.

ACKNOWLEDGMENTS

The authors would like to thank the Centro de Pesquisas Aggeu Magalhães (Fiocruz-CPqAM-Brazil) for providing laboratory facilities; Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-Brazil) for financial support. Also, we warmly acknowledge Maria Carolina Accioly Brelaz de Castro for technical contributions. The authors declare that there are no conflicts of interest.

REFERENCES

- Andrzej D, Elzbieta B, Wojciech G, and Zbigniew D. 1998. Activation of nuclear factor kappa B (NF- κ B) assayed by laser scanning cytometry (LSC). *Cytometry* 33:376–382
- Asquith B, Debaq C, Florins A, Gillet N, Sanchez-Alcaraz T, Mosley A, and Willems L. 2006. Quantifying lymphocyte kinetics in vivo using carboxyfluoresceindiacetatesuccinimidyl ester (CFSE). *Proc R Soc Lond B Biol Sci* 273:1165–1171
- Banks HT, Sutton KL, Thompson WC, Bocharov G, Doumic M, Schenkel T, Argilaguet J, Giest S, Peligero C, and Meyerhans A. 2011. A new model for the estimation of cell proliferation dynamics using CFSE data. *J Immunol Methods* 373:143-160
- Barnett GC, West CM, Dunning AM, Elliott RM, Coles CE, Pharoah PD, and Burnet NG. 2009. Normal tissue reactions to radiotherapy: towards tailoring treatment dose by genotype. *Nat Rev Cancer* 9:134-142
- Borgmann K, Röper B, El-awady R, Brackrock S, Bigalke M, Dörk T, Alberti W, Dikomey E, and Dahm-Daphi J. 2002. Indicators of late normal tissue response after radiotherapy for head and neck cancer: fibroblasts, lymphocytes, genetics, DNA repair, and chromosome aberrations. *Radiother Oncol* 64:141-152
- Brenchley JM and Douek DC. 2004. Flow Cytometric analysis of human antigen-specific T-cell proliferation. *Method Cell Biol* 75:481-496
- Carloni M, Meschini R, Ovidi L, and Palitti F. 2001. PHA-induced cell proliferation rescues human peripheral blood lymphocytes from X-ray-induced apoptosis. *Mutagenesis* 16:115–120
- Cavalcanti MB, Amaral AJ, Fernandes TS, Melo JA, and Machado CGF. 2008. p53 protein expression levels as bioindicator of individual exposure to ionizing radiation by flow cytometry. *Mol Cell Biochem* 308:127–131

P53 and Proliferation on Irradiated Lymphocytes

- Cavalcanti MB, Silva APG, Silva RF, and Amaral A. 2011. p53 protein expression and cell viability in irradiated peripheral blood mononuclear cells as bioindicators of radiosensitivity. *J Biophys Chem* 2:63-67
- Dahm-Daphi J. 2000. p53: biology and role for cellular radiosensitivity. *Strah Onkol* 176:278–285
- Dainiak N. 2002. Hematologic consequences of exposure to ionizing radiation. *Exp Hematol* 30:513-528
- Evrard B, Dosgilbert A, Jacquemot N, Demeocq F, Gilles T, Chassagne J, Berger M, and Tridon A. 2010. CFSE flow cytometric quantification of lymphocytic proliferation in extracorporeal photopheresis: use for quality control. *Transfus Apher Sci* 42:11–19
- Fei P and El-Deiry W. 2003. P53 and radiation responses. *Oncogene* 22:5774–5783
- Fuchs SY, Adler V, Buschmann T, Yin Z, Wu X, Jones SN, and Ronai Z. 1998. JNK targets p53 ubiquitination and degradation in nonstressed cells. *Genes Dev* 12:2658–2663
- Komuro Y, Watanabe T, Hosoi Y, Matsumoto Y, Nakagawa K, Saito S, Ishihara S, Kazama S, Tsuno N, Kitayama J, Suzuki N, Tsurita G, Muto T, and Nagawa H. 2003. Prediction of tumor radiosensitivity in rectal carcinoma based on p53 and Ku70 expression. *J Exp Clin Canc Res* 22:223–228
- Landsverk KS, Patzke S, Rein ID, Stokke C, Lyng H, De Angelis PM, and Stokke T. 2011. Three independent mechanisms for arrest in G2 after ionizing radiation. *Cell Cycle* 10:819–829
- Lašťovička J, Budinský V, Špišček R, and Bartůňková J. 2009. Assessment of lymphocyte proliferation: CFSE kills dividing cells and modulates expression of activation markers. *Cell Immunol* 256:79-85
- Lu C and El-Deiry WS. 2009. Targeting p53 for enhanced radio- and chemo-sensitivity. *Apoptosis* 14:597–606
- Lyons AB. 2000. Analysing cell division in vivo and in vitro using flow cytometric measurement of CFSE dye dilution. *J Immunol Methods* 243:147–154
- Lyons AB. 1999. Divided we stand: Tracking cell proliferation with carboxyfluoresceindiacetatesuccinimidyl ester. *Immunol Cell Biol* 77:509–515
- MacCallum DE, Hupp TR, Midgley CA, Stuart D, Campbell SJ, Harper A, Walsh FS, Wright EG, Balmain A, Lane DP, and Hall PA. 1996. The p53 response to ionizing radiation in adult and developing murine tissues. *Oncogene* 13:2575-2587
- Mayer C, Popanda O, Zelezny O, von Brevern MC, Bach A, Bartsch H, and Schmezer P. 2002. DNA repair capacity after γ -irradiation and expression profiles of DNA repair genes in resting and proliferating human peripheral blood lymphocytes. *DNA Repair* 1:237–250
- McVean M, Xiao H, Isobe K, and Pelling JC. 2000. Increase in wild-type p53 stability and transcriptional activity by the chemopreventive agent apigenin in keratinocytes. *Carcinogenesis* 21:633-639
- Moghbeli-Nejad S, Mozdarani H, and Aleyasin A. 2012. Increased frequency of micronuclei in lymphocytes of infertile males after exposure to gamma irradiation: a possible sign of genomic instability. *J Assist Reprod Gen* 29:89-94
- Nolan JP and Yang L. 2007. The flow of cytometry into systems biology. *Brief Funct Genomic Proteomic* 6:81-90
- O'Donovan MR, Johns S, and Wilcox P. 1995. The effect of PHA stimulation on lymphocyte sub-populations in whole-blood cultures. *Mutagenesis* 4:371–374
- Parish CR. Fluorescent dyes for lymphocyte migration and proliferation studies. 1999. *Immunol Cell Biol* 77:499–508
- Parish CR, Glidden MH, Quah BJC, and Warren HS. 2009. Use of the intracellular fluorescent dye CFSE to monitor lymphocyte migration and proliferation. In: Colegan JE, Bierer BE, Margulies DH, Shevach EM, Strober W, Coico R, Brown P, Donovan JC and Kreisbeek A (ed), *Current Protocols in Immunology*, pp 4.9.1-4.9.13. John Wiley & Sons, Australia
- Pinto MM, Santos NF, and Amaral A. 2010. Current status of biodosimetry based on standard cytogenetic methods. *Radiat Environ Biophys* 49:567-81
- Quah BJC, Warren HS, and Parish CR. 2007. Monitoring lymphocyte proliferation in vitro and in vivo with the intracellular fluorescent dye carboxyfluoresceindiacetatesuccinimidyl ester. *Nat Protoc* 2:2049–2056
- Schnarr K, Dayes I, Sathya J, and Boreham D. 2007. Individual radiosensitivity and its relevance to health physics. *Dose-Response* 5:333–348
- Seggewiss R, Loré K, Greiner E, Magnusson MK, Price DA, Douek DC, Dunbar CE, and Wiestner A. 2005. Imatinib inhibits T-cell receptor-mediated T-cell proliferation and activation in a dose-dependent manner. *Blood* 105:2473-2479

R. de Freitas e Silva and others

- Stehg AH. 2012. Targeting the p53 signaling pathway in cancer therapy-the promises, challenges and perils. *Expert Opin Ther Targets* 16:67-83
- Tario JDJ, Gray BD, Wallace SS, Muirhead KA, Ohlsson-Wilhelm BM, and Wallace PK. 2007. Novel lipophilic tracking dyes for monitoring cell proliferation. *Immunol Invest* 36:861-885
- Veiga-Fernandes H, Walter U, Bourgeois C, Mclean A, and Rocha B. 2000. Response of naïve and memory CD8+ T cells to antigen stimulation in vivo. *Nat Immunol* 1:47-53
- Vilasová Z, Rezáčová M, Vávrová J, Tichý A, Vokurková D, Zoelzer F, Reháková Z, Osterreicher J, and Lukášová E. 2008. Changes in phosphorylation of histone H2A.X and p53 in response of peripheral blood lymphocytes to gamma irradiation. *Acta Biochim Pol* 55:381-390
- Wood JL and Chen J. 2008. DNA-damage checkpoints: location, location, location. *Trends Cell Biol* 18:451-455