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Proteome analysis of proliferative response of bystander cells adjacent to cells exposed to ionizing radiation

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Abstract

Recently (*Cytometry* 2003, 56A, 71–80), we reported that direct cell-to-cell contact is required for stimulating proliferation of bystander rat liver cells (WB-F344) cocultured with irradiated cells, and neither functional gap junction intercellular communication nor long-range extracellular factors appear to be involved in this proliferative bystander response (PBR). The molecular basis for this response is unknown. Confluent monolayers of WB-F344 cells were exposed to 5-Gray (Gy) of γ -rays. Irradiated cells were mixed with unirradiated cells and co-cultured for 24 h. Cells were harvested and protein expression was examined using 2-DE. Protein expression was also determined in cultures of unirradiated and 5-Gy irradiated cells. Proteins were identified by MS. Nucleophosmin (NPM)-1, a multifunctional nucleolar protein, was more highly expressed in bystander cells than in either unirradiated or 5-Gy irradiated cells. Enolase- α , a glycolytic enzyme, was present in acidic and basic variants in unirradiated cells. In bystander and 5-Gy irradiated cells, the basic variant was weakly expressed, whereas the acidic variant was overwhelmingly present. These data indicate that the presence of irradiated cells can affect NPM-1 and enolase- α in adjacent bystander cells. These proteins appear to participate in molecular events related to the PBR and suggest that this response may involve cellular defense, proliferation, and metabolism.

Keywords

Enolase- α ; Gamma radiation; Mass spectrometry; Nucleophosmin 1; Proliferative bystander response

1 Introduction

Increased proliferation of unirradiated cells in the presence of irradiated cells is one of the manifestations of radiation-induced bystander effects. Evidence that cells targeted with ionizing radiation (IR) can induce a proliferative bystander response (PBR) was reported by Iyer and Lehnert [1]. They showed that monolayers of cells exposed to a low dose of α -particles (1 cGray (cGy) where only about 7% of the cells are hit) exhibited enhanced cell growth. A similar response was elicited when unirradiated cells were treated with

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supernatants from irradiated ones [1]. It has also been reported that cells that were irradiated with heavy ions caused an increase in cell proliferation of bystander cells that were co-cultured at a distance from irradiated cells [2]. These data indicate that high linear energy transfer (LET) IR can induce PBR, and the magnitude of PBR is dependent on the LET [2]. Gerashchenko and Howell [3,4] showed that low-LET γ -rays and intracellularly emitted ^3H β particles could also induce PBR. In their experiments, in which unirradiated WB-F344 rat liver epithelial cells were co-cultured with an equal number of irradiated WB-F344 cells, it was shown that direct cell-to-cell contact appears to be a prerequisite for the proliferative response of bystander cells [5]. Moreover, when irradiated cells are co-cultured with unirradiated cells, proliferation of unirradiated bystander cells is modulated by the number of adjacent irradiated cells [6]. A similar dependence was found by Wang *et al.* [7] using embryonic limb bud cells, however, they observed an inhibition of bystander cell proliferation rather than an enhancement. Interestingly, Liu *et al.* [8] showed that low doses of X-rays stimulated the proliferation of bystander cells, whereas high doses inhibited bystander cell proliferation.

Several reports provide firm evidence that bystander effects can be mediated *via* gap junctional intercellular communication [9–12]. Others have shown that long-range extracellular factors are responsible for transmitting bystander signals [1,13–17]. Neither of these mechanisms appear to play a role in our experimental model [5]. To gain insight into the molecular basis of the PBR observed in our previous studies [3–5], it is important to identify those genes whose expression is modified in the bystander cells as a consequence of signaling initiated by adjacent IR-exposed cells. For this purpose, a proteomic approach was used, which provides not only a quantitative assessment of the expression levels of cellular proteins in response to a particular stimulus, but also provides an important quantitative information on PTMs occurring in each protein. A comparative proteome analysis using MS/MS was performed on samples from the following 24-h cultures of WB-F344 cells: (i) unirradiated, (ii) 5-Gy γ -irradiated, and (iii) 95% bystanders co-cultured with 5% 5-Gy γ -irradiated cells.

2 Materials and methods

2.1 Cell line

The rat liver epithelial cell line WB-F344 [18] was generously provided by Dr. J. E. Trosko (Michigan State University, East Lansing, MI). Cells were asynchronously grown in D-medium (formula No. 78-5470EF; Gibco-BRL, Grand Island, NY) [3] in a 37°C humidified incubator containing 2% CO₂ and 98% air.

2.2 Irradiation

Confluent cell monolayers cultured in 100 × 20 mm² (P100) dishes (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) were irradiated at room temperature with ¹³⁷Cs γ -rays at a dose of 5 Gy. The γ -rays were delivered by a J. L. Shepherd Mark I irradiator (San Fernando, CA) operating at a dose rate of 3.33 Gy/min.

2.3 Co-culture

Details regarding the culture conditions can be found in our previous publications [3–5]. Briefly, monolayers of irradiated and unirradiated cells were washed twice with 10 mL of Dulbecco's PBS (DPBS; Gibco-BRL), trypsinized with Trypsin-EDTA (Gibco-BRL) containing 0.05% trypsin and 0.53 mM EDTA, and suspended at 4.0×10^6 cells/mL in culture medium. Eight hundred eighty microliters of cell suspension containing unirradiated cells and 120 μL of cell suspension containing irradiated cells were added to the culture medium in P100 dishes (705 cells/mm², 88% unirradiated cells and 12% γ -irradiated cells).

(Density of plated cells is critical for inducing PBR. Bystander WB-F344 cells have been reported to proliferate faster when they were plated together with WB-F344 cells exposed to IR at a ratio of 1:1 and plating density ≥ 400 cells/mm² [3–5]. Under these plating conditions, cell confluence reached 80–100%, 24 h after initiating the co-culture of unirradiated cells and irradiated cells.) Cells were mixed well by gentle shaking of the dishes, and were co-cultured for 24 h at 37°C in a CO₂ incubator. At the same time, 1 mL of unirradiated (sham-irradiated) cells and 1 mL of irradiated cells were each plated separately into P100 dishes followed by 24-h incubation in a CO₂ incubator. These latter two cultures constituted the 0 Gy unirradiated and 5 Gy γ -irradiated cultures.

2.4 Flow cytometry (FCM)

To quantify the resulting populations of unirradiated and irradiated cells in the 24-h co-cultures, unirradiated cells were stained with a membrane-permeant reactive tracer Vybrant™ 5- (and -6)-carboxyfluorescein diacetate, succinimidyl ester (CFDA SE; Molecular Probes, Eugene, OR) prior to plating with irradiated cells [5]. After a 24-h co-culture in a CO₂ incubator, cells were harvested and the percentages of unirradiated and irradiated cells were determined using a FCM approach described previously [3]. Cells were analyzed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA), equipped with a 15-mW argon-ion laser (488 nm) [3,5].

2.5 Sample preparation and 2-DE

Monolayers of 24-h cell cultures were washed three times with DPBS, and cells were homogenized in 0.5 mL of lysis buffer consisting of 5 M urea, 2 M thiourea, 2% w/v CHAPS, 2% w/v SB3-10, and 1% w/v DTT. All of these chemicals were purchased from Sigma (St. Louis, MO). Protein concentration in cell lysates was measured with a protein assay kit purchased from BioRad (Hercules, CA). After the samples were desalted, the first-dimension separation of proteins was performed on IPG strips in accordance with published procedures [19,20]. Briefly, the samples (100 μ g of total protein) were applied overnight to Immobiline DryStrips (pH 3–10; Amersham Pharmacia Biotech, Uppsala, Sweden) by in-gel rehydration. The rehydrated gels were then gently dried with tissue paper to remove excess of fluid, and IEF was performed in a Pharmacia Hoefer Multifor II electrophoresis chamber, according to the manufacturer's instructions. The SDS-PAGE second-dimension separation of proteins was performed in 9–18% acrylamide gradient gels using a Pharmacia Hoefer IsoDalt electrophoresis chamber. The 2-D gels were stained with fluorescent dye SYPRO Ruby (BioRad), according to the manufacturer's protocol. The SYPRO Rubystained proteins were detected with a Molecular Imager FX (BioRad). Image analysis and database management were performed with ImageMaster Platinum image analysis software (Amersham Pharmacia Biotech). To compare the intensities of protein spots between the samples (*i.e.* unirradiated cells, irradiated cells, and unirradiated cells co-cultured with irradiated cells), Progenesis software (Nonlinear Dynamics, Newcastle upon Tyne, UK) was used.

2.6 In-gel digestion and mass spectrometric analysis of peptides

These procedures were performed mainly in accordance with the previously published procedures [21]. Briefly, the protein spots of interest were excised from the dried silver stained 2-D gels, and rehydrated in 100 mM NH₄HCO₃ for 20 min. After rehydration, the spots were destained in solution of 15 mM potassium ferricyanide and 50 mM thiosulfate for 20 min [22], rinsed twice in Milli-Q water, and finally dehydrated in 100% ACN until they turned opaque white. The spots were then dried in a vacuum centrifuge followed by overnight rehydration in digestion solution consisting of 50 mM NH₄HCO₃, 5 mM CaCl₂, 0.1 μ g/ μ L modified sequence-grade trypsin (Promega, Madison, WI), and 50% H₂¹⁸O (Aldrich, Milwaukee, NJ) at 37°C. The digestion was terminated by treating the spots in 5%

TFA for 20 min. Peptides were extracted from the spots three times (each extraction took 20 min) with 5% TFA in 50% ACN. The extracted peptides were pooled and dried in a vacuum centrifuge followed by their collection/concentration with Zip-Tip pipette tips (Millipore, Bedford, MA), according to the manufacturer's protocol. The peptide mass fingerprints were measured by MALDI-TOF/TOF MS/MS using a Bruker Daltonics Ultraflex machine (Billerica, MA). Database analysis of peptides was performed with MASCOT search engine (Matrix Science, London, UK), indicating that the peptide mass fingerprint was derived from the protein identified in the search.

2.7 Western analysis

Monolayers of 24-h cell cultures were washed three times with DPBS, and cells were homogenized in 0.5 mL of lysis buffer consisting of 5 M urea, 2 M thiourea, 2% w/v CHAPS, 2% w/v SB3-10, and 1% w/v DTT. Mouse nucleophosmin (NPM) primary antibody (Cat# 32-5200, Zymed) and Ku70 (sc-1486, Santa Cruz Biotechnologies) were used in the analyses. Secondary antibodies conjugated with HRP (Santa Cruz Biotechnologies) and the ECL system from Amersham Biosciences were used for protein detection. Levels of Ku70 were determined as loading controls.

3 Results

3.1 FCM

According to FCM analysis, the co-culture that was initially plated with 88% unirradiated cells and 12% irradiated cells, and subsequently incubated for 24 h, resulted in 95% unirradiated cells and 5% irradiated cells. This apportionment of cells was considered as satisfactory for a proteomic study of PBR because the unirradiated (bystander) cells were “contaminated” with only a very small amount of irradiated cells (*i.e.* 5%). Nevertheless, careful attention was paid to the presence of the irradiated cells when interpreting the proteomic data (see below).

3.2 2-DE

Figure 1 presents a representative 2-D electropherogram of proteins from 24-h cultures of unirradiated (0 Gy (100%)), 5-Gy γ -irradiated (5 Gy (100%)), and the 24-h co-culture containing 95% bystander cells with 5% 5-Gy irradiated cells (0 Gy (95%) & 5 Gy (5%)). Three such 2-D electropherograms were prepared, one for each of three independent experiments. Intersample comparison of protein maps revealed substantial fluctuation in the intensities of several spots, the proteins of which were subsequently identified by MS. The intensity of spot 1 in the sample 0 Gy (95%) & 5 Gy (5%) (Fig. 1A) was greater than the intensities of spot 1 in the samples 0 Gy (100%) (Fig. 1B) and 5 Gy (100%) (Fig. 1C). At the same time, this spot was slightly more intense in the sample 0 Gy (100%) (Fig. 1B) than in the sample 5 Gy (100%) (Fig. 1C). In two electropherograms, spot 2 was clearly present only in the 0 Gy (100%) sample (Fig. 1B). Spots 3 and 4 in the sample 5 Gy (100%) (Fig. 1C) were intense to a greater extent than those in the samples 0 Gy (95%) & 5 Gy (5%) (Fig. 1A) and 0 Gy (100%) (Fig. 1B). Since the protein corresponding to spot 5 appeared to be a variant of the protein corresponding to spot 2 (similar molecular weights, but different pI/s) (Fig. 1B), this protein was also subjected to MS/MS analysis.

3.3 MS analysis of peptides

MASCOT search of the National Center for Biotechnology Information (NCBI) database revealed that the peptides derived from spot 1 corresponded to NPM 1 (Table 1). Spots 2 and 5 corresponded to enolase- α (or enolase 1) (Table 1). The peptides derived from spots 3 and 4 corresponded to BSA precursor (two variants with nominal mass of 71 221 Da each),

which apparently was present in the FBS supplemented culture medium. The elevated level of BSA precursor in the 5 Gy (100%) sample may have been due to increased adhesiveness of plasma membrane surfaces of irradiated cells with respect to this protein. Densitometric analysis of spot 1 showed the marked differences in the values of NPM 1 content between 0 Gy (100%) and 5 Gy (100%) samples, and between 0 Gy (100%) and 0 Gy (95%) & 5 Gy (5%) samples (Fig. 2).

As indicated above, the 0 Gy (100%) control sample revealed two spots that corresponded to enolase- α , namely spots 2 and 5 (Fig. 1D). The 5 Gy (100%) sample showed relocation of this protein from spot 2 (corresponding to basic protein species) to spot 5 (corresponding to acidic protein species) (Fig. 1D). Similar redistribution of enolase- α was observed in the 0 Gy (95%) & 5 Gy (5%) sample (Fig. 1D).

3.4 Western analyses

To confirm the findings obtained by means of proteome analysis, the cell lysates from 0 Gy (100%), 5 Gy (100%), and 0 Gy (95%) and 5 Gy (5%), cultures were subjected to Western blotting. Western analyses were carried out only for NPM 1 because commercially available anti-enolase- α antibodies were unable to produce clear bands. Figure 3 illustrates that among the three independent experiments, two experiments showed the distribution of NPM 1 to be similar to that obtained by means of proteome analysis (*i.e.* NPM in 0 Gy (95%) & 5 Gy (5%) sample is more abundant than in the 0 Gy (100%) and 5 Gy (100%) samples, and NPM in 0 Gy (100%) sample is more abundant than in the 5 Gy (100%) sample). Furthermore, intersample quantitative comparison of NPM 1 (Fig. 4) showed the data to be consistent with the data obtained by means of proteome analysis (Fig. 2).

4 Discussion

4.1 Role of NPM in PBR

Our MS/MS-based comparative proteome analysis revealed that NPM 1 is likely to be involved in the radiation-induced PBR observed in WB-F344 cells. According to Figs. 2 and 4, NPM 1 is less abundant in irradiated cells (5 Gy (100%)) than in unirradiated cells (0 Gy (100%)) and bystander cells that were co-cultured with the irradiated ones (0 Gy (95%) & 5 Gy (5%)). Furthermore, this protein was more abundant in bystander cells that were cocultured with irradiated ones (0 Gy (95%) & 5 Gy (5%)) compared to unirradiated cells (0 Gy (100%)). NPM 1, also known as NPM or B23, is a multifunctional nucleolar protein whose abundance correlates with proliferative activity of cells [23–25]. Therefore, it is not surprising that NPM/B23 is upregulated in bystander cells participating in PBR.

NPM/B23 is known to participate in a variety of cellular processes. This protein is mainly localized in granular regions of the nucleolus and is associated with ribosome biogenesis. It also plays a substantial role in cell-cycle progression and cell division [26–28]. NPM/B23 binds to retinoblastoma protein (pRb) and synergistically stimulates DNA polymerase- α [26]. Down-regulation of NPM/B23 mRNA delays the entry of cells into mitosis [27]. Unduplicated centrosomes have been found to bear NPM/B23, phosphorylation of which by CDK2-cyclin E appears to be required for the initiation of centrosome duplication during mitosis [28,29]. The fact that NPM/B23 shuttles between nucleoli and cytoplasm suggests that its function is not restricted within the nucleolus [30]. NPM/B23 is also associated with neoplastic growth [24].

Several communications have reported that NPM/B23 up-regulation also occurs as a consequence of stress (*e.g.* UV radiation and H₂O₂) [31–34]. In the present study, irradiated cells may be a source of stress factor(s) for adjacent bystander cells. Accordingly, PBR may be linked to bystander stress response *via* NPM/B23 activation. Among the possible factors

capable of triggering a stress response in our experimental model, and ultimately PBR, could be membrane-permeant reactive oxygen species (ROS) generated in irradiated cells and directly delivered to the adjacent bystander cells [5]. While oxidative stress is well known to be detrimental for normal cell function and can even lead to cell death, very low levels of ROS have been reported to stimulate cell proliferation [35,36]. Notably, NPM/B23 cooperates with p53 in response to DNA damage [37–39]. Activation of p53 blocks cell-cycle progression to allow time for the repair of DNA damage [40]. Because p53 can trigger cell-cycle arrest or apoptosis, its activation is tightly controlled. NPM/B23, along with HDM2 [37,39], stabilizes p53 in response to genotoxic stress. Kurki *et al.* [41] have shown that NPM/B23 participation in cellular damage responses is limited to transcriptional stress and is absent in direct double strand DNA breaks. Furthermore, NPM/B23 coexpresses with the proliferating cell nuclear antigen (PCNA) after UV irradiation [42]. PCNA, one of the p53 downstream target gene products [40], is an essential component of DNA repair and replication machinery [43]. The induction of protein modification *via* ADP-ribosylation has also been recognized as an important event in DNA repair [44,45]. Cells exposed to γ -rays (6 Gy) exhibited an increased expression of ADP-ribosylated and phosphorylated species of NPM/B23, 4 h after exposure [46]. However, in the present study, the irradiated cell population (5 Gy (100%)) did not show any obvious fluctuations in pI values for NPM/B23 compared to unirradiated cells (0 Gy (100%)). It is possible that by 24 h postirradiation, the process of NPM/B23 modification was diminished.

There is experimental evidence to suggest that ROS are involved in PBR [1]. Therefore, it is interesting to note that H₂O₂ exogenously added to human epithelial lens CD5A cells at cytotoxic concentrations (500 μ M) has been reported to substantially increase NPM/B23 expression (five–six-fold) as early as 30 min after H₂O₂ exposure [34]. Rapid stimulation of NPM/B23 expression in response to oxidative stress appears to be in concert with the fact that NPM/B23, in cooperation with nuclear factor NF- κ B, induces the *SOD2* gene [47]. Superoxide dismutases (SODs), including MnSOD, are the first line of cellular defense against the damaging effects of superoxide anion radicals [48]. SODs convert superoxide anion radicals into less reactive H₂O₂ and O₂, which is indicative of a cytoprotective function of SODs against oxidative injury. Since superoxide anion radicals produced in irradiated cells (presumably with NAD(P)H-oxidase involvement [49,50]) may diffuse into adjacent bystander cells, it is possible that supra-basal levels of superoxide anion radicals in bystander cells stimulate NPM/B23 expression which in turn mobilizes SODs.

Based on the above discussion, one can conclude that NPM/B23 is a key participant in cell defense and proliferation. Interestingly, both cell defense and proliferation appear to be involved in our bystander studies involving co-cultures of irradiated and unirradiated bystander cells [3–5]. It is possible that the WB-F344 cells in these studies are striving to maintain cell population homeostasis in response to IR [51]. That is, accelerated proliferation of unirradiated (bystander) cells may be playing a compensatory role directed toward repopulation of irradiated (damaged) cells. Such compensatory repopulation may be directed toward protecting the functional integrity of the entire cell community. In this scenario, NPM/B23 could be viewed as an integrator of cell defense and proliferation mechanisms. However, this is speculative. The role of NPM/B23 function in radiation-induced bystander responses is unclear and much work remains to be done.

4.2 Role of enolase- α in PBR

Enolase- α , which plays a critical role in the glycolytic pathway, was observed in our experiments in the form of both acidic and basic species. Both species were observed in the 0 Gy (100%) control culture (Fig. 1D, spots 5 and 2). However, the acidic variant was overwhelmingly present in the 0 Gy (95%) & 5 Gy (5%), and 5 Gy (100%) cell cultures (spot 5 in Fig. 1D). Surprisingly, the presence of even a small fraction of irradiated cells in

the 0 Gy (95%) & 5 Gy (5%) cell culture caused a remarkable relocation of almost all enolase- α pools in bystander cells from the basic species (spot 2) to the acidic species (spot 5). The persistence of the acidic species of α -enolase in the 0 Gy (95%) & 5 Gy (5%) and 5 Gy (100%) 24-h cultures suggests that this protein species is not likely to readily recover from modifications. The reasons for this are unclear.

It should be mentioned that enolase- α has been demonstrated to be an early and specific target for oxidative damage by carbonylation in different cell systems, ranging from yeast [52,53] to humans [54,55]. However, the fact that carbonylation of enolase- α appears not to alter its *pI* [55] suggests that this process may not be directly responsible for the observed charge shifts for enolase- α in the 0 Gy (95%) & 5 Gy (5%), and 5 Gy (100%) samples. Nevertheless, there are other types of protein modifications that can potentially change the *pI* of enolase- α . Enolase- α is a phosphorylatable enzyme, phosphorylations of which at specific sites are believed to directly contribute to its glycolytic function [56–58]. However, the duration and extent of these phosphorylations, and their capacity to lead to charge changes of enolase- α under our experimental conditions, are unknown.

4.3 Cooperation of NPM and enolase- α in PBR

One may ask the question, how do these two apparently unrelated gene products (*i.e.* NPM/B23 and enolase- α), which play such different functional roles in the cell, interrelate in the promitogenic/prosurvival bystander response? It has been reported that enolase- α also plays an important role in the regulation of *c-myc* promoter activity in the form of alternative translation product MBP-1 (37–40 kDa), which is distinct from its role as a glycolytic enzyme [59–62]. The *c-myc* proto-oncogene is known as an essential part of normal cell proliferative machinery [63,64]. MPB-1, structurally very similar to enolase- α , binds to the *c-myc* P2 promoter and down-regulates *c-myc* expression, and therefore, suppresses cell proliferation [60–62]. Unlike enolase- α , which is mainly localized in the cytoplasm, MPB-1 has been primarily found in the cell nuclei [61]. Since under stress conditions, enolase- α is an easy target for protein modifications, there is a good probability that MPB-1 could be readily modified as well which could inhibit down-regulation of *c-myc* expression. Protein modifications, specifically oxidation-induced modifications, appear to reduce their DNA-binding properties of several types of DNA-binding proteins [65–67]. Oxidative stress has been reported to activate *c-myc* along with other “immediate early response” proto-oncogenes such as *c-fos* and *c-jun* [68–70]. As regards NPM/B23, the finding that the expression of *c-myc* correlates with NPM/B23 expression [71–73] is further supplemented by the evidence that NPM/B23 is one of the direct target genes of *c-myc* [73,74]. Thus, NPM/B23 and enolase- α , differently oriented in their functions, seem to interrelate *via c-myc*. Interestingly, by using a rat cDNA microarray, enolase- α has been identified among the many genes up-regulated by *c-myc* [72]. However, in our proteomic study, intersample comparison of expression levels of total enolase- α did not reveal any apparent differences.

4.4 Co-culture

Finally, it should be pointed out that the co-cultures used in these studies consist of a mixture of 95% unirradiated cells and 5% irradiated cells (0 Gy (95%) & 5 Gy (5%)). This ratio was chosen to minimize the amount of protein from irradiated cells in the samples, thereby enabling us to focus on proteins related to the PBR. The results in Fig. 1 attest to the appropriateness of this approach. However, subsequent to submission of these samples for proteomic analysis, we carried out additional experiments designed to assess the impact of changing the percentage of irradiated cells on the PBR [6]. We found that a statistically significant increase in the proliferation ratio was only observed when at least equal numbers of unirradiated and irradiated cells were co-cultured (*i.e.* 50% (0 Gy) $\leftrightarrow$ 50% (5 Gy)). The numbers of unirradiated cells in co-cultures of unirradiated and irradiated cells that have

received a dose D compared with the number of unirradiated cells in a co-culture of unirradiated and irradiated cells that received zero dose is expressed as a proliferation ratio [3]. No statistically significant increase in the bystander proliferation ratio was observed with lower percentages of irradiated cells. Therefore, ideally one would like to have carried out proteomic analyses in bystander cells isolated from a 50% (0 Gy) ↔ 50% (5 Gy) co-culture. Nevertheless, the absence of a statistically significant PBR in the 0 Gy (95%) & 5 Gy (5%) co-culture does not necessarily imply that the proliferation machinery of bystanders has not been initiated. In fact, the results in Fig. 1 support this argument. For example, in the case of NPM 1 (Fig. 2), the highest amounts are found in the 0 Gy (95%) & 5 Gy (5%) coculture, not in the 0 Gy (100%) or 5 Gy (100%) cultures. Furthermore, the redistribution of enolase- α occurs in both the 0 Gy (95%) & 5 Gy (5%) co-culture and the 5 Gy (100%) culture. These observations are consistent with the activation of a bystander response and should not, in principle, be observed in the absence of a bystander response.

In conclusion, by means of a comparative proteome analysis we were able to identify NPM 1 and enolase- α as being involved in regulating the response of bystander WB-F344 cells that were adjacent to γ -irradiated cells of the same type and co-cultured for 24 h. In order to fully understand the sequence of signaling events involved in this response, further investigations are needed. A major challenge for further investigations is also to determine whether the type of radiation (*i.e.* γ -rays, β -particles, α -particles) influences the regulation of the PBRs. There is evidence to suggest that, at least in irradiated cells, radiation type can have a significant impact on cellular responses not only with respect to the number of genes induced, but also with respect to the level of gene induction [75].

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Abbreviations

FCM	flow cytometry
Gy	Gray
IR	ionizing radiation
LET	linear energy transfer
NPM	nucleophosmin

PBR	proliferative bystander response
SOD	superoxide dismutase

Figure 1.

Representative patterns of protein expression after 2-DE separation of protein samples obtained from a 24-h culture of: (A) unirradiated cells (95% of the total cell population) with irradiated cells (5% of the total cell population), (B) unirradiated (100% population), and (C) 5-Gy irradiated cells (100% population). The horizontal axis represents the range of pI values of proteins between 3 and 10. The vertical axis represents the molecular weight of proteins in kDa. The arrows indicate spots, the proteins of which were identified by MS/MS (the names of identified proteins are presented in Table 1). The decision to select these protein spots for MS/MS analysis was based on intersample comparison of 2-D gel images obtained from three independent experiments. The regions enclosed within rectangles are shown enlarged in (D).

Figure 2.

Results of densitometric analysis of changes in expression of protein spot 1 (NPM 1) shown in Fig. 1. For intergel comparisons, the spot volume was normalized to give a fraction value of the total spot volume *per gel*. Data presented are the mean $\pm$ SEM of three independent experiments.

Figure 3.

Western blot analysis of changes in NPM 1 content of protein samples obtained from 24-h cultures of unirradiated cells (100% population), 5-Gy irradiated cells (100% population), and unirradiated cells (95% of the total cell population) with irradiated cells (5% of the total cell population). Two of three independent experiments (marked as Exp. #2 and Exp. #3) showed similar results.

Figure 4.

Results of densitometric analysis of changes in expression of NPM 1 shown in Fig. 3. These results are expressed as a “fold increase” of densitometric values of samples 0 Gy (95%) 5 Gy (5%) and 5 Gy (100%) compared to the densitometric value of sample 0 Gy (100%) considered as a control sample. Data presented are the mean $\pm$ SEM of three independent experiments. The photographic films were analyzed by scanning densitometry with ImageJ software.

Table 1
Identities of proteins corresponding to the spot locations shown in Fig. 1

	Spot 1	Spot 2	Spot 5
Protein name	NPM 1 ^{b)} (<i>Mus musculus</i>)	Enolase 1 alpha (<i>Rattus norvegicus</i>)	Enolase 1 alpha (<i>R. norvegicus</i>)
Accession (NCBIInr)	gi 6679108	gi 6978809	gi 6978809
MW (Da)	32 711	47 428	47 428
Coverage (%)	16	9	11
MOWSE score ^{a)}	226	208	221
Matched peptides (in bold)	1 MEDSMDMDMSPLRPQNYLFGCELKA DKDYHFKVDNENEHQLSLRTVSLG 51 AGAKDELHIVEAEAMNYEGSPIKVTL ATLK MSVQPTVSLGGFEITPPVVL 101 RLKCGSGPVHISGQHLVAVEEDAE SEDEDEEDVKLLGMSGKRS APGGGK 151 VPOKKVK LDEDEDEDEDEDEDEDE DDDDDDFDEEETEEKVPVKKSVRDTPA 201 KNAQKSNQNGKDLKPSTPRSGQE SFKKQEKTPTKPGPSSVEDIKAKMQ 251 ASIEKGGSLPKVEAKFINYVK NCFR MTDQEAIQDLWQWRKSL	1 MSILKIHAREIFDSRGNPTVEVDLY TAKGLFR AAVPSGASTGIYEALRL 51 DNDKTRFMGKGVSKAVEHINKTIA PALVSKKLNVEQEIDQLMIEMDGT 101 ENSKKFGANAILGVSLAVCKAGA VGKGVPLYRHIADLAGNPEVTLPVPAF 151 NVINGGSHAGNKL AMQEFMILPVGAS SFR EAMRIGAEVYHNLKNVIKEY 201 GKDATNVGDEGGFAPNILENKEALE LLKSAIAKAGYTDQVVIGMDVAASE 251 FYRAGKYDLDFKSPDDASRYITPD QLADLYKSFIDYPVVSIEDPFDQDD 301 WDAWQKFTATAGIQVVGDDLTVTN PKRIKAAGEKSCNCLLLKVNQIGSV 351 TESLQACKLAQSNWGWVMVSHRSE ETEDTFIADLVVGLCTGQIKTGAPCR 401 SERLAKYNQILRIEELGSKAKF AGR SFRNPLAK	1 MSILKIHAREIFDSRGNPTVEV DLYTAKGLFR AAVPSGASTGIYEALRL 51 DNDKTRFMGKGVSKAVEHINKTIAPA LVSKKLNVEQEIDQLMIEMDGT 101 ENSKKFGANAILGVSLAVCKAGAV GKGVPLYRHIADLAGNPEVTLPVPAF 151 NVINGGSHAGNKL AMQEFMILPVG ASSFREAMRIGAEVYHNLKNVIKEY 201 GKDATNVGDEGGFAPNILENKEALEL LKSAIAKAGYTDQVVIGMDVAASE 251 FYR AGKYDLDFKSPDDASRYITP DQLADLYKSFIDYPVVSIEDPFDQDD 301 WDAWQKFTATAGIQVVGDDLTVTNP KRIKAAGEKSCNCLLLKVNQIGSV 351 TESLQACKLAQSNWGWVMVSHRSEE TEDTFIADLVVGLCTGQIKTGAPCR 401 SERLAK YNQILRIEELGSKAKF AGRSFRNPLAK

^{a)}Probability-based MOWSE scores were estimated by comparing experimental dataset against each sequence database entry and were reported as $-10\log_{10}(P)$, where P is the absolute probability [76]. All reported proteins showed significant matches, typically much greater than 40 ($P < 0.05$).

^{b)}According to MASCOT search results, NPM 1 of *R. norvegicus* (gi|7242160) matched the same set of peptides.