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REVIEW PAPER

The bystander effect: is reactive oxygen species the driver?

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Abstract The paper reviews selected examples of the bystander effect, such as clonogenic survival decrease, chromosomal aberrations and mutations. The similarities and differences between the biological effects in directly targeted and bystander cells are briefly discussed. Also reviewed are the experimental data which support the role of reactive oxygen species (ROS), especially $^{*}O_2^-$, as mediators of the bystander effect. Endogenously generated ROS, due to activation of NAD(P)H oxidases, play a key role in the induction of DNA damage in bystander cells. All the observed effects in bystander cells, such as alterations in gene expression patterns, chromosomal aberrations, sister chromatid exchanges, mutations, genome instability, and neoplastic transformation are the consequence of DNA damage.

Key words bystander effect • reactive oxygen species • ionising radiation • DNA repair • signalling pathways

Introduction

For several decades it was believed that a cell, to be damaged by ionising radiation, must be traversed by at least one particle or photon, directly targeting the nucleus. It seemed to be a rational view; nevertheless, it has recently been challenged (for review, see [7, 17, 23, 45]). It has been documented that all manifestations of radiation damage exhibited by the directly irradiated cells are shared by the unirradiated ones, called bystander cells. DNA damage and the resulting alterations in gene expression patterns, chromosomal aberrations, sister chromatid exchanges, mutations, apoptosis, genome instability, and neoplastic transformation, all have been documented in bystander cells [4, 7, 10, 18, 20, 23, 26, 27, 33, 34, 45, 47].

The bystander effect was examined with the use of various experimental systems; the most convincing results were obtained in microbeam charged particle experiments, where distinct cells were targeted, consisting a defined fraction of the total cell number of cells under examination [6, 31, 36]. Moreover, medium from irradiated epithelial cell cultures applied to unirradiated cell cultures caused a similar damaging effect; this was taken as indication of the presence of factors secreted into the medium by the directly irradiated cells [21, 22, 37, 38]. Figure 1 diagrammatically presents the family of bystander effects. It should be added that the effects were found [1, 15, 48] to be both independent of and dependent on cell–cell contact (the latter, through gap junctions [1, 49]).

This paper presents examples of bystander effects and hypotheses concerning the mechanism of transmission of the damaging effects from the directly targeted cells to the bystander cells. An excellent summary of earlier facts

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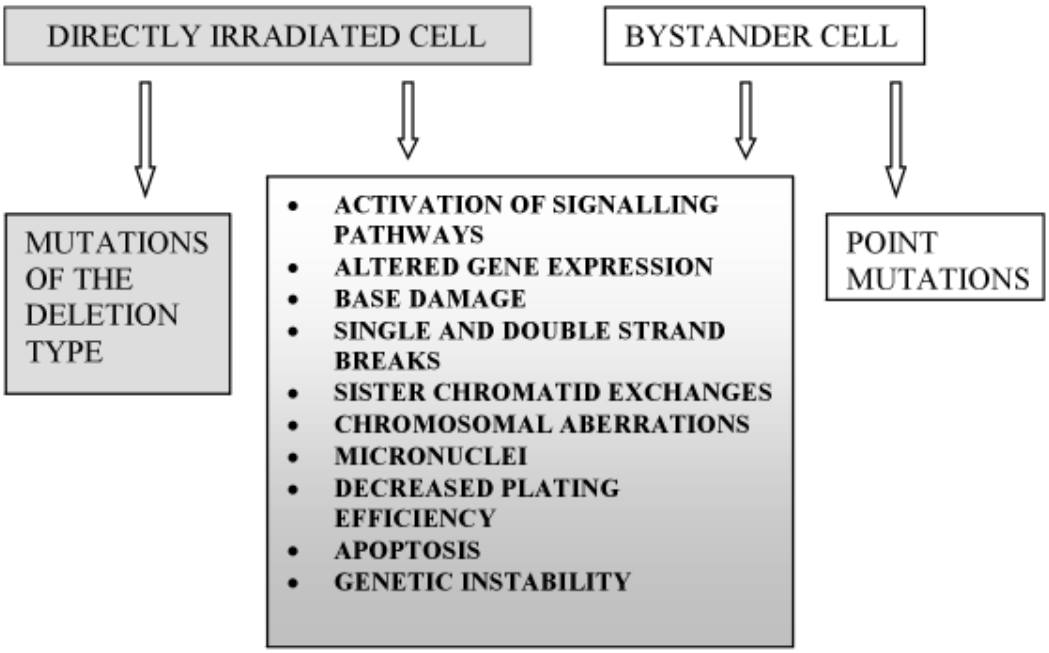

Fig. 1. Radiobiological effects common to directly targeted (irradiated) and bystander cells. Note, that point mutations in bystander cells do not prevail in cells with DSB repair defect [26].

concerning the bystander effect was published by Mothersill and Seymour in 2001 [23].

Examples of bystander effects

Some examples of bystander effects are given below. They were chosen to illustrate and support the subsequent discussion of the possible molecular and cellular mechanisms underlaying the phenomenon.

Reduced clonogenic survival

Discovery of the bystander effect was made in 1992, when Nagasawa and Little found that extremely low doses of alpha particles induce sister chromatid exchanges in more cells than actually hit [27]. Exact determination of the effect on survival was relatively late, as other end-points turned to be easier to establish experimentally.

In 2001, in alpha particle microbeam experiments it was observed that more C3H 3T3 cells were killed by radiation than expected for the particle fluence applied [33, 34]. The fact that non-hit cells could be killed by the sole vicinity of cells damaged by ionising radiation was subsequently documented in an ingenious experimental system [35]. Chinese hamster V79 cells were stably transfected with hygromycin- or neomycin-resistance genes. V79 cells transfected with a hygromycin resistance gene were then stained with a nuclear dye and plated together with unstained neomycin-resistant cells in proportion 1:9. The charged-particle microbeam was used to irradiate all the stained cells. The mixed cell populations were then replated and 24 h later placed in geneticin-supplemented medium for 14 days. Thus, the hit, hygromycin resistant cells were selectively eliminated by exposure to the antibiotic. Clones scored after the incubation period were formed by the bystander, neomycin-resistant V79 cells. Survival thus obtained was compared to that obtained when all cell nuclei on the microbeam dish were exposed to the same number of alpha particles. The results, shown in Fig. 2 indicate that there was a considerable lethal effect in bystander cells.

It should be noted that V79 cells were plated at a density allowing gap junction communication. In cells not communicating through gap junctions, forming low number of junctions or seeded at low densities the biological effect may be weaker. The experiments described by Zhou *et al.* [48] allowed to conclude that "*irradiated cells released certain cytotoxic factor(s) into the culture medium that killed the non-irradiated cells... However, different bystander end points may involve different mechanisms with different cell types*".

Seymour and Mothersill [37] made an attempt at establishing the relative contribution of bystander and targeted cell killing to the low-dose region of the radiation

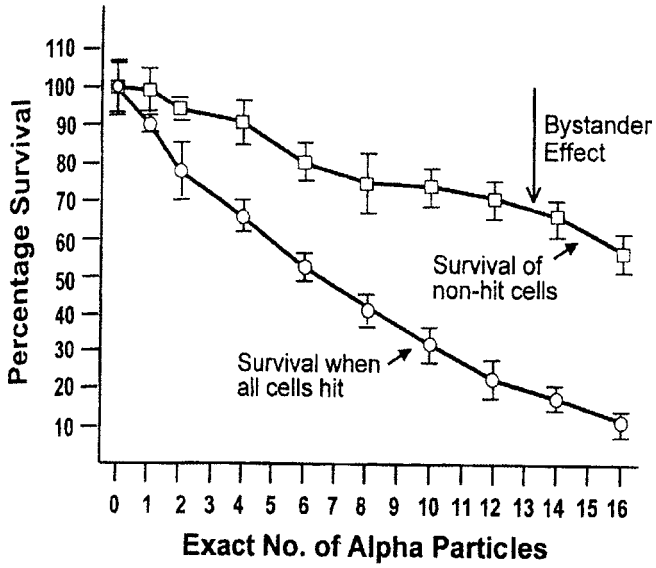

Fig. 2. The bystander effect for cell survival in V79 cells. Reproduced by permission of Radiation Research Society (with authors' approval) from the paper of Sawant *et al.* (2002) The radiation-induced bystander effect for clonogenic survival. Radiat Res 157:361–364 [35].

dose-response curve. They used human keratinocytes and determined survival by cloning. The cells showed a bystander effect when exposed to low doses of low-LET radiation. Seymour and Mothersill introduced a method of correcting the overall survival curve that enabled to estimate the relative contributions of the bystander effect and the effect of direct interaction of radiation with the target cells. Total cell death was estimated from cloning, bystander death – from cloning of unirradiated cells exposed to the medium from an irradiated cell culture. Direct radiation induced death was the survival difference between the two. It should be added that the medium was in contact with irradiated cells for 75 min, then filtered through a 0.22 μm pore filter to sterilise it and eliminate any cells and finally, added to the recipient unirradiated cells. Contact time between 30 min and 4 h did not significantly affect the extent of the subsequently observed bystander effect [21].

In these experiments, doses of 0.01–0.5 Gy killed about 40% cells due to the bystander effect alone. The effect was relatively constant, and apparently dose-independent in the range of 0.01–5 Gy. After irradiation with doses greater than 0.5 Gy, the survival curves were the result of a dose-dependent non-bystander effect and a dose-independent bystander effect. The results of the described experiments were contradictory to the current radiobiological dogma and indicated that directly targeted cells rapidly secreted factor(s) that were stable for at least 4 h and exerted an cytotoxic effect. The effect was apparently saturated at very low radiation dose.

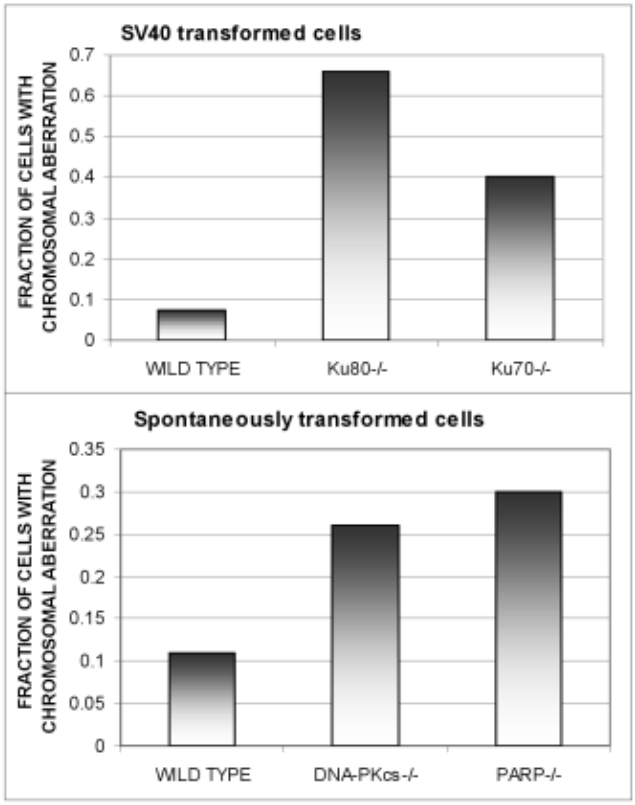

Fig. 3. Fractions of cells with chromosomal aberrations in wild type and knock-out mouse cells lacking DNA repair genes, as indicated. The fraction expected for the mean alpha particle dose applied, 0.5 cGy, was 0. 047 (data from Table 3, Little *et al.* [15]).

Cytogenetic damage

Studies on the bystander effect revealed sister chromatid exchanges and chromosomal aberrations [20, 25, 27, 29, 47] in bystander cells. These observations indicated that DSB must be a very important or perhaps critical lesion in bystander cells. This view has recently gained a strong support, as shown below.

A frequently used experimental system for studying bystander effect is a monolayer cell culture exposed to very low fluences of alpha particles [10, 25–27]. Such a system was used in studies of chromosomal aberrations [15] in mouse cells, wild type and knock-outs missing the DSB (double strand break) repair pathway, NHEJ (nonhomologous end-joining). The initial part of this repair pathway involves the DNA-dependent protein kinase, DNA-PK. The knock-out cell lines used in [15] were: xrcc5-/- (lack of Ku80), G22p1-/- (lack of Ku70), Prkdc-/- (lack of the DNA-PK catalytic subunit, DNA-PKcs). Additionally, mouse cells Adprt-/- (devoid of PARP-1 (poly (ADP-ribose) polymerase) activity) were examined. All cell cultures were irradiated in G1 phase and the frequency of chromosomal aberrations was scored in the first metaphase after irradiation.

All cell cultures were in G1 phase of the cell cycle and chromosomal aberrations were scored in the first metaphase after irradiation. Figure 3 shows part of the data taken from [15] and presented in a graphical form. They illustrate the bystander effect in wild type cells (compare the expected fraction of nuclei irradiated – 0.047 – and fractions of cells with chromosomal aberrations). This example also shows the impact of DSB repair defects on the examined bystander effect.

The authors of the quoted paper [15] analysed the relation between total numbers of chromosomal aberrations induced per track and the mean dose of alpha particle radiation, as well as percentage of cells with chromosomal aberrations as a function of dose. All the data, including the example given in Fig. 3, indicated the largest (very similar) bystander effect in cells lacking Ku80 or Ku70. In wild type cells, both SV40 and spontaneously transformed, the effect was comparable and much smaller than in knock-out cells.

Interestingly, an intermediate, very similar bystander effect was found in cells lacking either DNA-PKcs or PARP-1, although these proteins are expected to play different roles in repair: The catalytic subunit is engaged in the NHEJ pathway, whereas PARP-1 is participating in base excision repair [46]. Nevertheless, absence of poly(ADP-ribosyl)ation due to dominant negative PARP-1 expression induces a shift from rapid to slow DSB rejoining and thus, may increase the risk of misrepair [32]. The difference between the knock-out cells lacking Ku or DNA-PKcs may be explained by the observation of Hashimoto *et al.* [8] that the Ku70/80 complex (Ku heterodimer) has a role in the repair of clustered lesions in DNA such as an oxidised base close to an opposing SSB. On the other hand, both PARP-1 and Ku heterodimer may sequester the DSB formed during repair of such a complex lesion.

Mutation frequency

As shown in Fig. 1, bystander effects comprise practically all radiation damage effects. So far, one main difference has been noticed, namely, in mutation types, those in bystander cells, in contrast with the directly targeted cells, being predominantly of the point mutation type [10]. It should be noted, however, that point mutations in bystander cells do not prevail in cells with DSB repair defect, as recently found by Nagasawa *et al.* [26] for xrs5 cells which miss Ku80 and hence, do not have a functional NHEJ. On the contrary, mutations in xrs5 bystander cells are of deletion type, as in directly targeted wild type cells. This points to a considerable importance of DSB repair in mutagenesis involved in the bystander effect.

Mutation frequencies after exposure to very low doses of ionising radiation were in the past estimated by extrapolation of the dose-effect curve, assuming a non-threshold, linear dose-effect relationship. Technical advancement made it possible to measure mutation frequency in cells exposed to single alpha particles or to their precisely determined numbers. This brought an unexpected discovery [28] of a very high number of mutations induced by a particle fluence corresponding to traversal of one nucleus per 20 cells. In CHO cells it was 71 times higher than the background level. With the dose increasing from 1 cGy to about 10 cGy the mutation frequency per track dropped from 10^{-6} to 3×10^{-5} and it stayed at that level with the dose increasing up to 1 Gy. Additionally, other studies [9] have shown that one alpha particle traversing cytoplasm gave an only twofold increase over the background level.

The conclusion was that, apart from the targeted cells, also the bystander cells were mutated. In another study [49], it was found that irradiation of 10% of a confluent mammalian cell population with a single alpha particle per cell resulted in a mutant yield close to that observed when all of the cells in the population were targeted. This effect again was in contrast with the established radiobiological dogma which assumed that mutations take place in cells hit by particles or photons. Interestingly, in that particular study [49] the unusually high mutation frequency was almost completely suppressed in cells with impaired gap junction-mediated intercellular communication (cells pretreated with octanol, or transfected with a dominant negative connexin 43 vector).

Subsequent studies on mutagenesis in bystander cells indicated – as mentioned in Fig. 1 – that the mutation type in bystander cells was different from that in directly targeted cells [10]. Chinese hamster ovary (CHO) cells (DNA repair competent) were exposed to very low fluences of alpha particles and HPRT mutant colonies selected and examined by PCR (polymerase chain reaction). The mutated gene codes an enzyme called hypoxanthine: guanine phosphoribosyl transferase (HPRT).

After subtracting spontaneous mutations, it was found that 97% of the mutants induced by 0.5 cGy were bearing point mutations. In cell cultures exposed to the mean dose of 10 cGy 44% of the cells were directly targeted by one or more alpha particles and 11% – by 2 or more alpha particles; 62% of the mutants induced by 10 cGy were a result of partial or total deletions. The authors concluded that the mutations arise by different mechanisms and recalled experiments showing that both ROS and cytokines present in the medium can induce ROS production inside thus treated, unirradiated cells [5, 30]. Further studies confirmed this view, as discussed below.

Possible mechanisms of the bystander effects

Radiation-induces ROS generation has been long documented and ROS contribution to damage to various components of the irradiated cells is generally acknowledged. As mentioned above, recently, it was found that exposure to alpha particles leads to production of superoxide anion radicals and hydrogen peroxide [30]. Moreover, ROS was indirectly shown to be implicated in bystander effects, e.g. [5, 16, 18]. Apart from ROS, also nitric oxide, a stable radical, was implicated in mediating the bystander effect after exposure to alpha particles or heavy ions [19, 39].

In a more detailed study carried out by Azzam *et al.* [2], confluent human fibroblast monolayers were exposed to low doses of alpha particles, with the use of the methodological approach already mentioned above, where only a part of the cell population was directly targeted. When SOD or catalase were added to the cell culture medium 30 min before exposure, such treatment considerably decreased the percentage of micronucleated binucleate cells in the cytokinesis block/micronucleus test (see Table 1). As shown in the last column of the Table, an even more pronounced decrease could be obtained by DPI treatment. DPI inhibits NAD(P)H oxidases, a family of enzymes that produce ROS.

The most prominent member of the enzyme family is a plasma membrane-bound NAD(P)H oxidase. It can be activated by extracellular signals [3], hydrogen peroxide

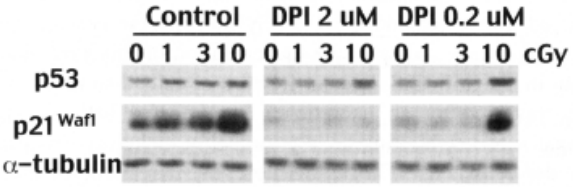

Fig. 4. Western blot analyses of p53 and p21(Waf1) in alpha particle irradiated control and DPI treated AG1522 fibroblasts cultures. Cultures were held at 37°C for 3 h before harvest. Reproduced by permission of the American Association for Cancer Research (with authors' approval) from the paper of Azzam *et al*. (2002) Oxidative metabolism modulates signal transduction and micronucleus formation in bystander cells from α-particle-irradiated normal human fibroblast cultures. Cancer Res 62:5436–5442 [2].

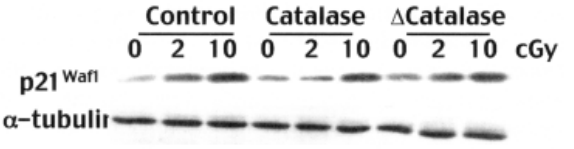

Fig. 5. Western blot analysis of p21(Waf1) expression in alpha particle irradiated AG1522 confluent fibroblast cultures in the presence of active or inactive (Δ) catalase (20 μg/ml). Reproduced by permission of the American Association for Cancer Research (with authors' approval) from the paper of Azzam *et al*. (2002) Oxidative metabolism modulates signal transduction and micronucleus formation in bystander cells from α-particle-irradiated normal human fibroblast cultures. Cancer Res 62:5436–5442 [2].

Table 1. Micronucleus formation in AG1522 cell cultures exposed to alpha-particles.
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Treatment	Percentage of micronucleated binucleate cells*			
(mean dose in cGy)	Sham-manipulated control	SOD-treated cultures	Catalase-treated cultures	DPI-treated cultures
0	3.1 ± 0.9	3.3 ± 0.9	6.0 ± 1.0	4.2 ± 0.9
1	11.9 ± 1.6	6.5 ± 1.3	7.1 ± 1.0	3.0 ± 0.8
2	10.0 ± 1.5	6.2 ± 1.2	7.2 ± 1.2	2.7 ± 0.8
10	15.3 ± 1.7	12.7 ± 1.8	13.6 ± 1.3	10.3 ± 1.4

*Percentage of binucleate cell containing micronuclei over total binucleate cells in sham-manipulated control, SOD (100 μg/ml)-, or DPI (0.2 μm)-treated cultures exposed to mean doses of 0, 1, 2 or 10 cGy, subcultured, and placed for micronucleus formation 3 h after irradiation.

[12], lipid hydroperoxides [13], and the activation is prevented by inhibitors of kinases p38 and MAPK.

DNA damage, activation of receptor tyrosine kinases and ROS from directly targeted cells, all can be the source of signalling that takes place in the bystander cell. Azzam *et al.* [2] found up-regulation of proteins p53 and p21(Waf1) (also known as CDKIN1A); both were affected by DPI (see Fig. 4) in cells irradiated with 1 or 3 cGy; at a higher mean dose (10 cGy) and lower DPI concentration there is a suppression of the effect. This indicates that in the case of irradiation with 10 cGy, most cells are directly traversed by alpha particles and the directly inflicted damage is the cause of p53 and p21(Waf1) up-regulation. Hence, the bystander effect becomes masked by the more pronounced effect of damage inflicted by direct targeting of DNA. Also catalase prevented p21(Waf1) up-regulation in cells irradiated with 2 cGy but not with 10 cGy (Fig. 5). The explanation of these observations is diagrammatically shown in Fig. 6 (see [40] for review concerning this signalling pathway).

Since p53 stabilisation/tetramerisation is directly related to the extent of ATM activation by DNA lesions and p21(Waf1) synthesis is proportional to the activity of p53 as transcriptional factor, p21 can be considered as a marker of DNA damage. Therefore, it is significant that – as found by Azzam *et al.* [2] in the experimental system described above – cells immunostained for p21 were in clusters, in numbers higher than those directly targeted according to the mean alpha particle dose applied. Therefore, it could be concluded that both directly targeted and bystander cells were stained. In SOD treated cell monolayers only isolated cells were immunostained; thus, SOD obviously prevented

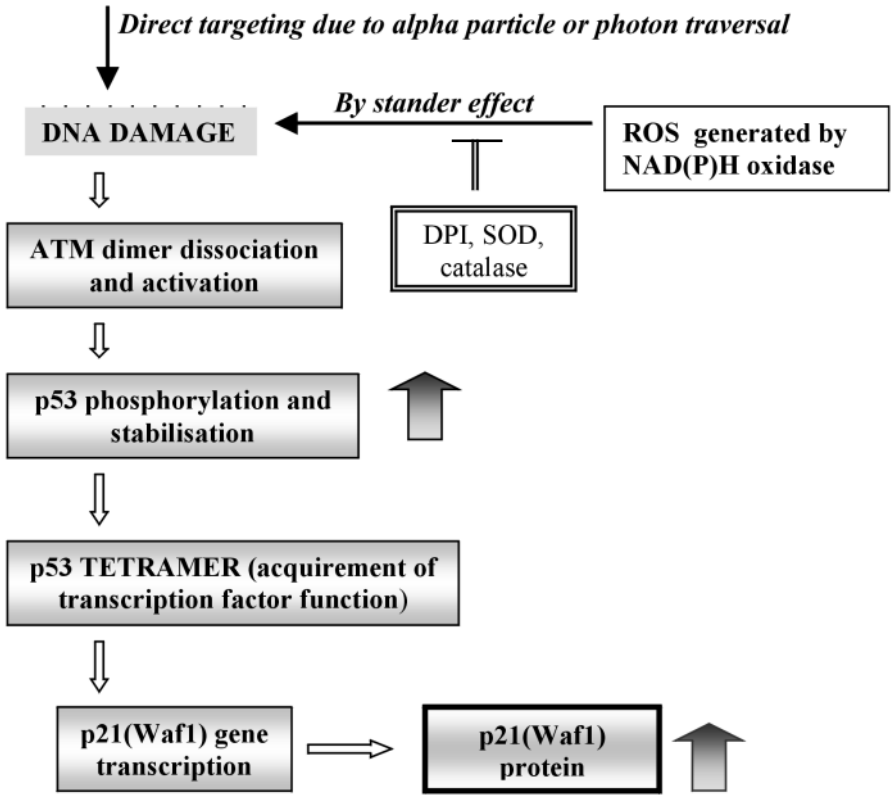

Fig. 6. Diagram explaining the cellular events underlying the results of Azzam *et al.* [2] presented in Figs. 6 and 7. Shaded arrows indicate increases in protein quantity detected by Western blotting.

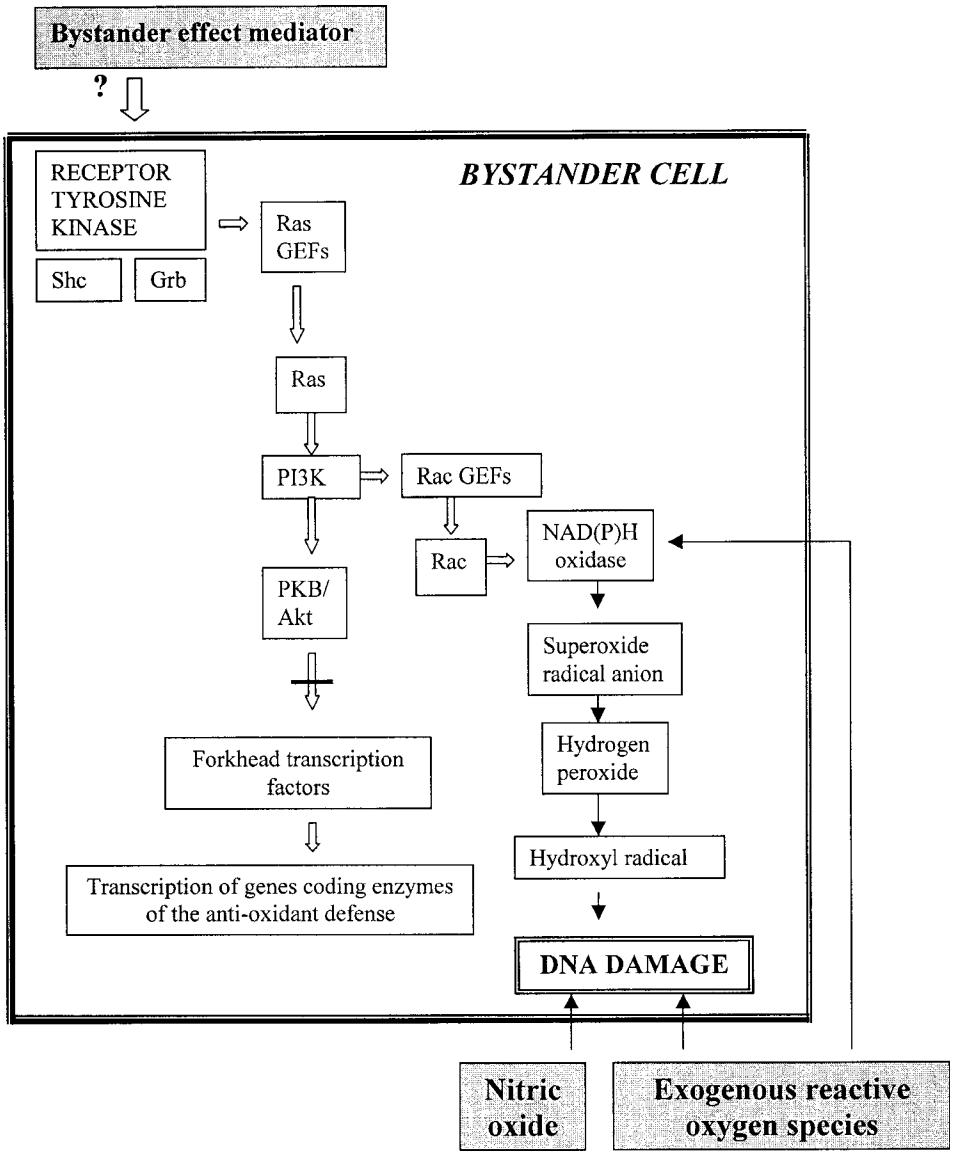

Fig. 7. Diagram presenting the possible pathways leading to activation of NAD(P)H oxidase and generation of ROS. Shc and Grb are adaptor proteins of the receptor complex. See list of abbreviations and text for other explanations.

the bystander cells to become damaged, as independently shown in experiments with micronuclei frequency, as endpoint examined (see Table 1).

Figure 7 shows a diagram that explains the possible events in a bystander cell. The contribution of a plasma membrane receptor is hypothetical, but supported by the work of Nagasawa *et al.* [25]. These authors stated the involvement of membrane signalling in the bystander effect. They used filipin, an agent that disrupts lipid rafts, and thus inhibits signalling initiated at the plasma membrane. Filipin treatment suppressed the induction of sister chromatid exchanges and HPRT mutations by very low fluences of alpha particles (mean doses 0.17–0.5 cGy). In contrast, after exposure to 10 cGy, when most mutations occurred in directly irradiated cells, no effect of filipin was observed.

The identity of bystander mediator remains to be discovered. It is not known whether there are several mediators or an universal one. Not every cell type may be able to produce the bystander signal and this is independent of the cell's ability to respond to bystander signals from another cell type [21]. So far, the only candidate for bystander mediator is TGF-B1 (transforming growth factor B1) [11]. On the one hand, exposure to alpha particles increased the extracellular TGF-B1 in a dose-independent way (at 3.6–19 cGy during 30 min – 8 h interval after exposure) that could be attributed to a posttranscriptional/posttranslational mechanism. On the other hand, this cytokine stimulated ROS production in unirradiated cells similarly to the medium from irradiated cells; moreover, anti-TGF-B1 antibody abolished the effect of that medium on the ROS-bystander effect. An identical effect as with the antibody could be obtained with DPI, in agreement with the earlier work on NADH oxidase activation by TGF-B1 [42–44]. To complete the consistent picture – NADH activation by TGF-B1 is tyrosine phosphorylation-dependent. Since the experimental data indicate that the latent TGF-B1 is activated by $^{\cdot}OH$ radical and activated TGF-B1 stimulates NADH oxidase [43], thus increasing ROS production, these events provide a feed-forward mechanism amplifying the original effect of irradiation on directly targeted cells. It seems plausible that also NAD(P)H oxidases-activating mitogenic growth factors [43] are involved as mediators in the bystander effect, especially that they also act as ligands for the receptor tyrosine kinases.

Whatever the origin of ROS (from directly targeted cells or endogenously generated by activated NAD(P)H oxidases), the chain of events eventually leads to production of $OH^{\cdot}$ radicals in the iron ions-involving Fenton reaction. Both $^{\cdot}O_2$ and hydrogen peroxide are stable enough to penetrate from one cell to another through gap junctions, as well as through the plasma membrane into the medium and from the medium into the bystander cell. Also the distance to the nucleus is short enough and the presence of iron in the nucleus [41] enables $OH^{\cdot}$ radical generation in the vicinity of DNA. Nitric oxide is another factor that mediates the effect but the exact mechanism remains unknown. This explains the origin of DNA lesions in bystander cells and all the biological effects that can subsequently be revealed with suitable cytogenetic or cytological methods.

Concluding remarks

Recent advances in studies on the effects of low doses of ionising radiation have demonstrated generation of transferable factor(s) that cause radiobiological effects in unexposed cells. Therefore, the effects of very low doses may be greater than predicted by conventional radiobiology. Hence, the question, how general is the phenomenon and how important for effects of very low dose irradiation. In one study [24], 13 cell lines were examined and these cell lines that had a radiation dose-response curve with a wide shoulder showed no bystander effect, notwithstanding p53 status (wild type or mutant). As the effect may, to some extent, depend on the activity of various signalling pathways that differ between cell types, comparison should be made between gene expression profiles, proteome phosphorylation profiles and the radiobiological characteristics of various cell types. Clearly, there is a need to continue the study, in order to reach understanding of the mechanisms of the cellular response to low doses of ionising radiation, since the long-standing radiobiological dogma, most rational in appearance, had to be revised after discovery of the bystander effect.

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Abbreviations

ATM – ataxia telangiectasia mutated kinase
DPI – diphenyleneiodonium
DSB – DNA double strand break
ERK – extracellular signal regulated kinase
GEF – GTP exchange factor
HPRT – hypoxanthine: guanine phosphoribosyl transferase
NAD(P)HOX – nicotinamide-adenine dinucleotide oxidase
NADH oxidase – nicotinamide-adenine dinucleotide:flavin:O_2 oxidoreductase
PARP-1 – poly(ADP-ribose)polymerase-1
PI3K – phosphatidylinositol-3 kinase
PKB – protein kinase B
ROS – reactive oxygen species
SCE – sister chromatid exchange
SOD – superoxide dismutase
SSB – DNA single strand break