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Radiation-Induced Bystander Effects in Malignant Trophoblast Cells Are Independent From Gap Junctional Communication

Ferya Banaz-Yaşar,[1] Klaus Lennartz,[2] Elke Winterhager,[1] and Alexandra Gellhaus[1]*

[1]Institute of Anatomy, University Hospital Essen, Hufelandstr. 55, 45122 Essen, Germany
[2]Institute of Cell Biology, University Hospital Essen, Hufelandstr. 55, 45122 Essen, Germany

Abstract It is controversially discussed that irradiation induces bystander effects via gap junction channels and/or diffusible cellular factors such as nitric oxide or cytokines excreted from the cells into the environment. But up to now the molecular mechanism leading to a bystander response is not well understood. To discriminate between both mechanisms of bystander response, (i) mediated by gap junctional communication and/or (ii) mediated by diffusible molecules, we used non-communicating Jeg3 malignant trophoblast cells transfected with inducible gap junction proteins, connexin43 and connexin26, respectively, based on the Tet-On system. We co-cultivated X-ray irradiated and non-irradiated bystander Jeg3 cells for 4 h, separated both cell populations by flow cytometry and evaluated the expression of activated p53 by Western blot analysis. The experimental design was proven with communicating versus non-communicating Jeg3 cells. Interestingly, our results revealed a bystander effect which was independent from gap junctional communication properties and the connexin isoform expressed. Therefore, it seems more likely that the bystander effect is not mediated via gap junction channels but rather by paracrine mechanisms via excreted molecules in Jeg3 cells. J. Cell. Biochem. 103: 149–161, 2007.

Key words: connexin43; connexin26; gap junctions; bystander effect; p53; p21; trophoblast; Jeg3; X-ray irradiation

Radiation-induced bystander effects are described as processes which occur in cells that are not directly irradiated but receive signals from neighboring irradiated cells [Nagasawa et al., 1992; Hickman et al., 1994]. Several types of bystander responses have been reported and include changes in gene expression [Iyer et al., 2000; Azzam et al., 2002], induction of genetic effects such as mutations [Zhou et al., 2000; Little et al., 2002, 2003; Nagasawa et al., 2003], DNA damage [Azzam et al., 2001; Little et al., 2003], cell killing [Lyng et al., 2002], and malignant transformation [Sawant et al., 2001]. There has been increasing recognition of the importance of bystander effects, especially when using low doses, as they may have an impact on our understanding of the radiation-induced biological response mechanisms and developing risk estimations [Little et al., 2003; Mothersill and Seymour, 2003]. Bystander effects were commonly measured by analysing cell survival, the induction of stress-inducible activated proteins p21 and p53, micronuclei formation and γ-H2AX foci. Though there is a growing interest in the role of bystander effects in the biological response of mammalian cells to ionizing radiation the molecular mechanisms of bystander effects are not well understood and they are likely to be involved in multiple pathways resulting at least in cell damage.

It is controversially discussed that the radiation-induced bystander effect is mediated directly via gap junctional intercellular communication (GJIC) and/or diffusible cellular factors excreted from irradiated cells [Azzam et al., 1998, 2001; Bishayee et al., 2001;

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*Correspondence to: Alexandra Gellhaus, Institute of Anatomy, University Hospital Essen, Hufelandstr. 55, 45122 Essen, Germany.
E-mail: alexandra.gellhaus@uni-essen.de

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Nagasawa et al., 2002; Shao et al., 2003; Mitchell et al., 2004; Zhou et al., 2005]. Evidence for the participation of GJIC in the transmission of damage signals from α-particle irradiated to non-irradiated confluent cultures of mammalian cells was obtained using GJIC-deficient cells as well as gap junction inhibitors [Azzam et al., 1998, 2001; Zhou et al., 2000, 2001].

Gap junctions are favored candidates for explaining bystander effects because they form clusters of intercellular membrane channels connecting the cytoplasm of two neighboring cells. Gap Junction channels consisting of twelve connexin (Cx) proteins may mediate bystander effects by allowing the direct intercellular exchange of small molecules such as cAMP, IP_3, and Ca^{2+} up to 1 kDa. To date the connexin gene family consists of 20 members in the murine and 21 members in the human genome [Sohl and Willecke, 2004] and the different connexin channels play a critical role in cellular and tissue function which has been proven by generating knockout mice [Wei et al., 2004]. The connexins reveal mostly unique channel function but experiments with double-knockout and functional replacement of a channel by another connexin (knockin) demonstrated that some of the gap junction channels display both unique and shared functions [Sohl and Willecke, 2004]. The phenomenon of the bystander effect mediated by GJIC derives originally from an observation in cancer gene therapy with ganciclovir as gap junctions mediate the transfer of gene products from transfected into non-transfected cells resulting in neighboring cell death [Mesnil and Yamasaki, 2000].

Recently, Azzam et al. [2001] demonstrated that GJIC is involved in transferring high linear energy transfer (LET) α-particle radiation-induced damage signals from irradiated to non-irradiated fibroblasts and epithelial cells using GJIC-competent and deficient confluent cultures. In these studies modulation of the expression of proteins involved in the p53/p21 stress-induced signaling pathway and induction of DNA damage in bystander cells were observed only in the communicating cell lines. So far, molecules which can pass the channels and could be responsible for a direct bystander effect on neighboring cells are not known. However, Udawatte and Ripps [2005] could give proof that at least cytochrome *c* was able to induce apoptosis in adjacent baby hamster kidney (BHK) cells mediated via ions and molecules such as Ca^{2+}, cAMP, and IP_3, but only when the cells were transfected with connexins.

As mediators of the paracrine effects free radicals (reactive oxygen species (ROS), nitric oxide (NO)) and soluble factors like cytokines such as tumor necrosis factor (TNF), interleukin 1, and 8 (IL-1, IL-8) and transforming growth factor-β1 (TGF-β1) are discussed [Lehnert et al., 1997; Narayanan et al., 1997; Iyer et al., 2000; Mothersill and Seymour, 2001; Shao et al., 2002, 2005]. It is known that co-culture of non-irradiated cells with conditioned medium from irradiated cultures lead to induction of biological effects. For the first time, Mothersill and Seymour [1997] could show that non-irradiated epithelial cell lines that received media from γ-irradiated cells significantly reduced the clonogenic survival. Recently, Zhou et al. [2005] provide evidence that cyclooxygenase-2 (COX-2) signaling cascade—which activates the mitogen-activated protein kinase (MAPK) pathways—plays an important role in mediating the radiation-induced bystander effect in human fibroblasts.

To discriminate between both mechanisms of bystander response, (i) mediated by GJIC and/or (ii) mediated by diffusible cellular factors, we used non-communicating Jeg3 malignant trophoblast cells stably transfected with the inducible gap junction protein Cx43 and Cx26 [Gellhaus et al., 2004]. After induction of the connexins with doxycycline the different cell lines demonstrate high amounts of connexin mRNA and protein which correlated with a strong coupling efficiency (GJIC) as measured by Calcein dye transfer. In previous reports, it has been shown that the level of Cx43 is changed after ionizing radiation which could in turn modify the bystander effect [Azzam et al., 2003a; Glover et al., 2003]. However, we could demonstrate that in contrast to other cell lines Cx43 expression in Jeg3 cells, transfected or non-transfected, is not induced or changed by irradiation itself [Banaz-Yasar et al., 2005]. Thus, these Cx-inducible Jeg3 cell lines represent an ideal system to study the response of neighboring cells upon radiation in the presence or absence of GJIC within the same cell clone.

In the present work, we investigated bystander effects in a long-term co-culture system with bystander cells co-cultured with irradiated cells. We found evidence for a bystander effect in both communicating and non-communicating Jeg3

cells independent from the connexin isoform expressed.

MATERIALS AND METHODS

Cell Line and Culture

The human choriocarcinoma cell line Jeg3 (ATCC HTB-36) was purchased from the American Type Culture Collection (Manassas). Jeg3 cells were grown in minimal essential medium (MEM, Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (certified tetracycline-free; Biochrome, Berlin, Germany).

Jeg3 Tet/Cx43 and Tet/Cx26 transfectants were cultivated in medium containing 500 μg/ml G418 sulfate (PAA laboratories, Cölbe, Germany) and 0.5 μg/ml puromycin (Sigma, Munich, Germany). The Cx43 and Cx26 expression in these cells was induced following 48 h treatment of the cells with 1 μg/ml doxycycline HCl (Dox) (Sigma).

Plasmid Construction and Transfection

The plasmid construction of the connexin expression plasmids based on the Tet-On system and the transfection procedure were described previously [Gellhaus et al., 2004]. For construction of the Cx26 plasmid based on the Tet-On system, the expression vector containing rat Cx26 (pPH9) was used [Hellmann et al., 1999]. The coding region for rat Cx26 was excised from the vector pPH9 by EcoRI digestion and subcloned into the EcoRI site of the rtTA-responsive plasmid pUHD10-3 [Gossen and Bujard, 1992] creating the plasmid pUHD/Cx26.

The transfected cell lines were routinely checked for induction of Cx43 and Cx26 expression respectively by immunocytochemistry following 48 h treatment with 1 μg/ml Dox (see above).

Cell Irradiation

Confluent cell cultures were irradiated at room temperature using a conventional Pantak X-ray machine (Pantak, East Haven, CT) operated at 310 kV, 10 mA, with a 2 mm AI filter (effective photon energy ~90 kV), at a distance of 75 cm and a dose rate from 2.7 Gy/min with 5 Gy ultrasoft X-rays. Dosimetry was performed with a Victoreen dosimeter that was used to calibrate an in-field ionizing monitor. Culture flasks were returned immediately to the incubator after irradiation.

Co-Culture Experiments and Cell Sorting by Flow Cytometry

For evaluating bystander effects Jeg3 cells were seeded in Petri dishes at a concentration of 1.5×10^6 cells. After 48 h, the cells were stained for 1 h at 37°C with 5 μM DiI dye (V-22885, Molecular Probes, San Francisco, CA), a permanent red membrane dye. After incubation time the cells were washed three times with fresh culture medium. Immediately after washing the DiI stained cells were exposed to 5 Gy X-rays. A further Petri dish remained unstained. After irradiation the DiI stained cells were trypsinized and mixed together with the unstained and non-irradiated cell population in a 2:1 ratio. The mixed cell cultures were incubated for 4 h at 37°C to establish the formation of cell–cell membrane contacts between the two cell populations before trypsinization and resuspension in culture medium. This coupling time is defined as the time leading to the strongest coupling efficiency [Gellhaus et al., 2004; Banaz-Yasar et al., 2005]. Separation of the two co-cultivated cell populations, stained, and unstained cells, by fluorescence activated cell sorting (FACS) was performed on a FACSDiVa cell-sorter (Becton Dickinson, San Jose, CA). DiI was excited with a 488 nm laser wavelength and fluorescence was measured through a 585/42 nm bandpass filter. For each experiment 200.000–330.000 cells were sorted by flow cytometry.

After the separation of the cells the purity of the separated cell populations was routinely reanalyzed again by flow cytometric analysis of 10.000 cells. The cellular protein was isolated from each separated cell population and the protein lysates were evaluated for p53 expression by Western blotting. The co-culture experiments were performed three times for parental Jeg3 and four times for Jeg3 Cx43 and Cx26 transfectants.

Controls

In each experiment (1) unstained and non-irradiated cells were cultivated with DiI stained, non-irradiated cell populations as a control for the co-culture experiments. In addition since cells respond with p53 activation to any stress, the following cells were used as further controls for each sort experiment, (2) unstained cells which stayed only in the incubator during the whole procedure,

(3) unstained cells which were carried to the radiation room but not irradiated, (4) DiI stained cells stayed in the incubator, and (5) DiI stained and irradiated cells. These control cells were separated by flow cytometry and the p53 expression was analyzed by Western blot analysis in the same way as for the co-cultivated cell populations.

Western Blot Analysis

Protein extracts were prepared with modified RIPA lysis buffer (50 mM Tris/HCl, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mM EDTA, 0.1% SDS) supplemented with EDTA free complete protease inhibitors (Roche, Penzberg, Germany).

Protein samples (150.000 cells for the co-cultivated cells and 100.000 cells for the control cells without any co-cultivation) were separated on a 15% polyacrylamide gel and electrophoretically transferred to polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ). Membranes were blocked with 5% non-fat dried milk in Tris-buffered saline (TBS) with 0.15% Tween-20 and incubated with the primary antibody. The following primary antibodies were used: rabbit anti-p53 (1:1000, Cell Signaling, Danvers, MA), mouse anti-p21 (1:2000, Cell Signaling, Danvers) and mouse anti-human GAPDH antibody (1:1000, Chemicon, Hampshire, UK) for normalization of protein expression. Primary antibody binding was detected using the following secondary antibodies: anti-rabbit and anti-mouse IgG antibody conjugated to horseradish peroxidase (1:2000, Santa Cruz Biotechnologies, Heidelberg, Germany). Detection was achieved with the ECL chemiluminescence kit (Amersham Biosciences) according to the protocol using Gel Imager (Intas, Göttingen, Germany).

Immunocytochemistry

Indirect immunocytochemistry on cells was performed as described previously [Winterhager et al., 1991]. The following primary antibodies were used: anti-Cx43 rabbit polyclonal antibody (1:100) [Traub et al., 1994] and anti-Cx26 rabbit polyclonal antibody (1:150, Zymed Laboratories, San Francisco, CA). Donkey anti-rabbit Alexa Fluor® 488 (1:300, MoBiTech, Göttingen, Germany) was used as a secondary antibody. Finally, cells were mounted with Mowiol (Sigma) to prevent photobleaching. Photomicrographs were obtained using a confocal laser scanning microscope (LSM 510, Zeiss, Oberkochen, Germany).

Statistics

Results are reported as the mean ± standard deviation of the mean (SD). Levels of significance were determined at the 0.05 level by the Student's *t*-test.

RESULTS

Analysis of p53 and p21 Expression Levels in Parental and Connexin-Inducible Jeg3 Cells After X-Ray Irradiation

Since both stress markers p53 and p21 have been used to identify bystander effects [Azzam et al., 2001], we analyzed the expression of both being better qualified for our experimental system. Cells were irradiated with 5 Gy X-rays, a clinically relevant irradiation dose for diagnosis and therapy [Dale and Carabe-Fernandez, 2005], which does not lead to obvious cell death in Jeg3 cells observed morphologically and confirmed by the lack of activated caspase 3 in Western blot analysis but to an arrest of the cells in G_2 phase of the cell cycle (unpublished work).

Moreover, this dose of low LET X-rays has been chosen for bystander experiments in numerous in vitro studies [Gerashchenko and Howell, 2003; Edwards et al., 2004; Mitchell et al., 2004; Yang et al., 2005; Facoetti et al., 2007].

The level of activated tumor suppressor protein p53 and cell cycle inhibitor p21 has been investigated after irradiation with 5 Gy X-rays in time course experiments in Jeg3 cells (Fig. 1).

For Western blot analysis the cellular proteins were harvested 15 min up to 24 h after irradiation. Already 15 min after irradiation parental Jeg3 cells revealed a significantly elevated expression of phosphorylated p53 which showed the highest amount after 30 min and remained at high levels for up to 24 h after irradiation compared to non-irradiated controls (Fig. 1A). Like the parental cells, Jeg3 transfectants without and with induction of connexin channels by doxycycline treatment showed a significant increase of p53 activated protein already 15 min after irradiation with highest expression 30 min after irradiation followed by a decline up to 24 h (Fig. 1C,E).

In contrast to p53, the expression of p21 in parental Jeg3 cells was not significantly upregulated before 2 h after irradiation but

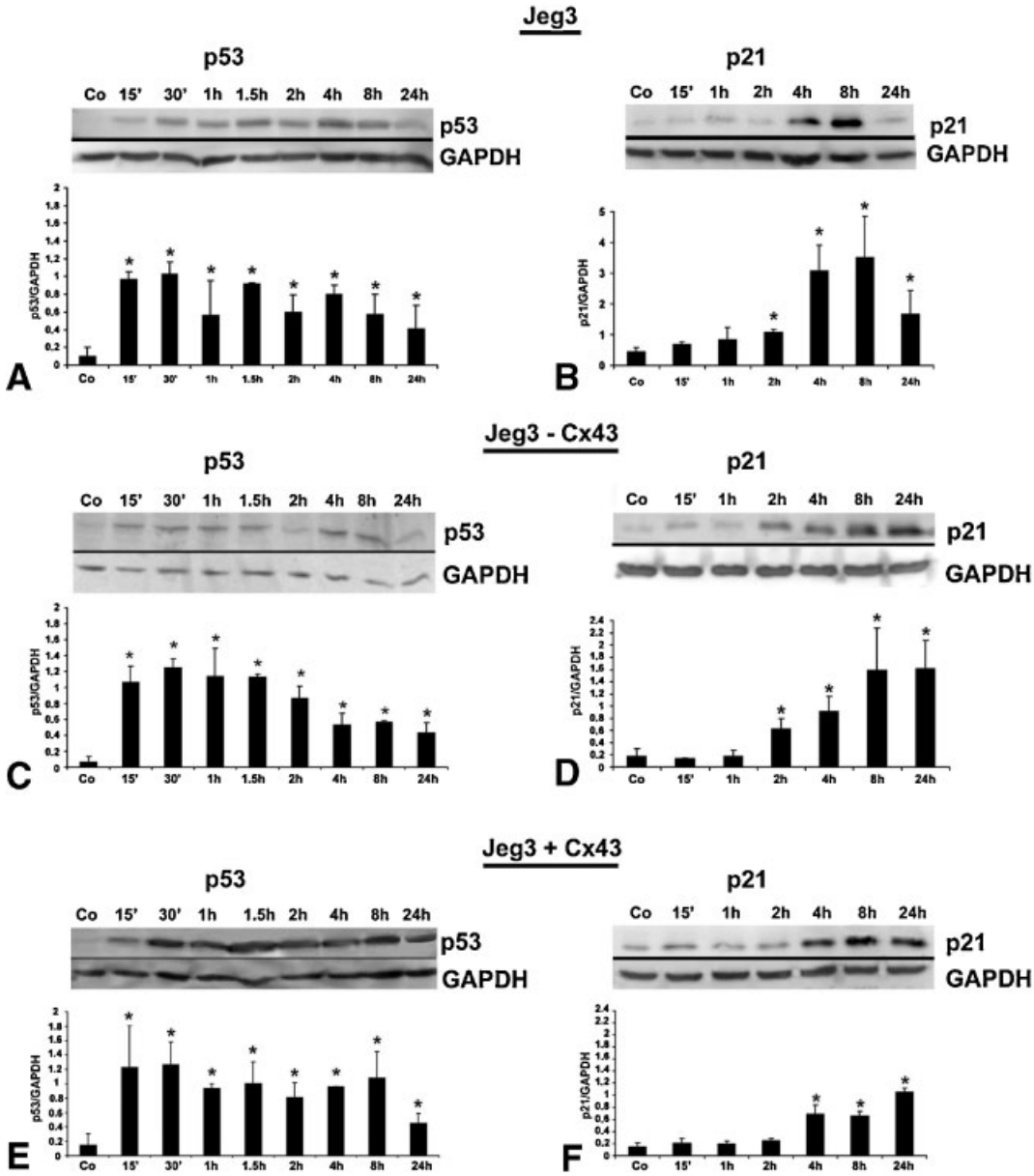

Fig. 1. Expression of p53 and p21 in parental Jeg3 and inducible Cx43 transfected Jeg3 cells after irradiation. Western blot and densitometric analysis of activated p53 (**A**,**C**,**E**) and p21 (**B**,**D**,**F**) expression in Jeg3 (A,B), Cx43 non-expressing (C,D), and Cx43 expressing Jeg3 cells (E,F) exposed to 5 Gy X-rays. Fifteen minutes after irradiation p53 was significantly upregulated in Jeg3 and Jeg3 Cx43 transfectants. In parental Jeg3 and Cx43 non-expressing Jeg3 cells p21 was significantly increased 2 h after irradiation whereas in Cx43 expressing cells p21 was significantly upregulated not until 4 h after irradiation. Data represent means ± SD (n = 3). $*P \leq 0.05$, significant increase in reference to the non-irradiated control (Co).

increased at later times reaching a maximum at 8 h. A decline to pre-irradiation levels was frequently observed at 24 h after irradiation (Fig. 1B). However, the Cx43 expressing cells exhibited an increase in p21 expression not before 4 h after irradiation (Fig. 1F), whereas the non-communicating transfectants showed a significant upregulation of p21 already after 2 h irradiation (Fig. 1D).

Taken together, the Cx43 expressing cells showed the same temporal pattern of p53 activation upon irradiation compared to the non-communicating cells but a later onset of the stress marker p21. In comparison to the rapid activation of p53 already 15 min after irradiation in Jeg3 cell lines, p21 revealed a decelerated induction of about 2–4 h upon irradiation.

Since for co-culture experiments 4 h are needed to reach strongest coupling efficiency between the cells, p21 was not the appropriate marker to indicate bystander effects under this experimental approach. For further investigations, we have chosen activated p53 as the marker protein to detect bystander effects.

Evaluation of Radiation-Induced Bystander Effects in Parental Jeg3 Cells After X-Ray Irradiation

For the co-culture bystander experiments, we cultivated an irradiated, DiI membrane stained cell population with an unstained, non-irradiated cell population. After 4 h incubation time, we have separated the two cell populations by FACS analysis, the proteins of each co-cultured cell population were isolated and the phosphorylated p53 protein level was evaluated by Western blotting. Preliminary experiments were conducted to establish that in the absence of radiation, DiI has no effect on p53 activation and that other required manipulations do not modify the endpoint under investigation. Therefore, for every experiment and investigated cell line several controls have been performed as described in Materials and Methods Section in detail. To show that DiI has no influence on activated p53 expression, we used DiI stained, non-irradiated cells (see Figs. 2B, 3D, and 4D). Furthermore, we analyzed unstained cells which were equally treated throughout the experiment to proof if p53 is not upregulated upon mechanical stress. In addition, a co-culture control was performed

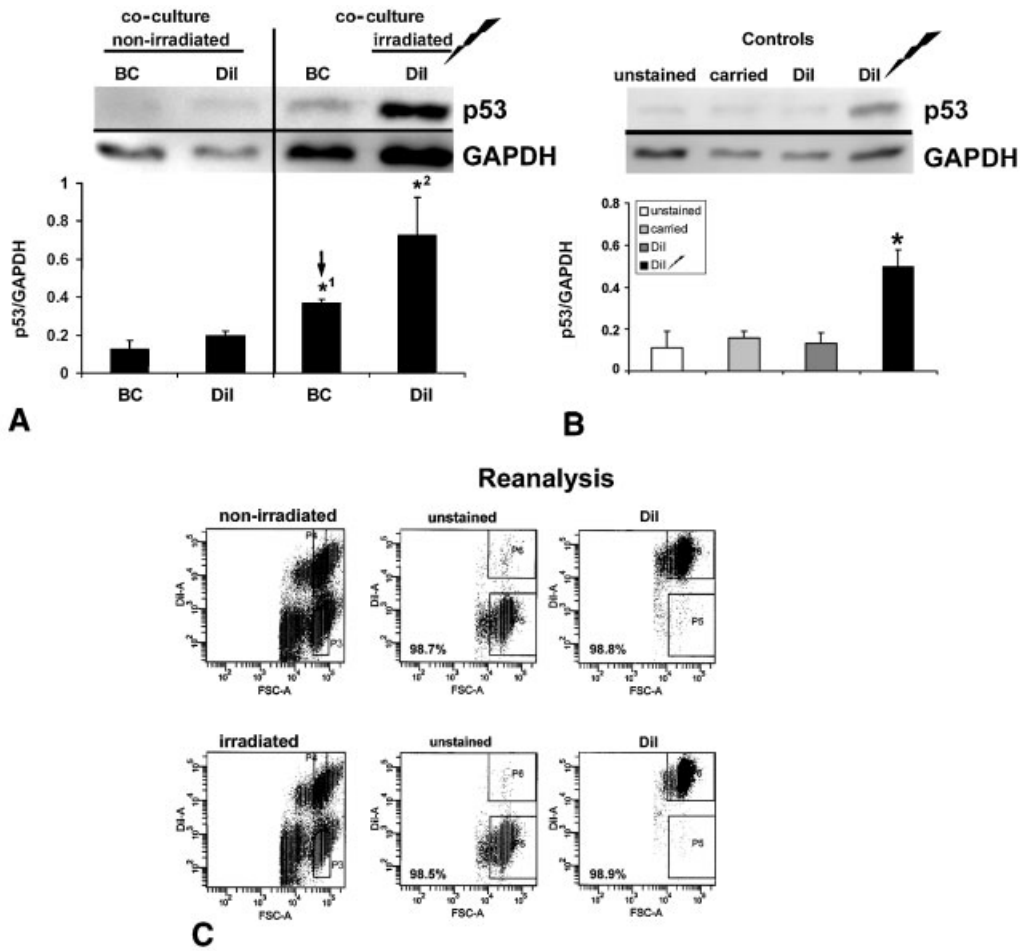

Fig. 2. Radiation-induced bystander effects in parental Jeg3 cells after X-ray irradiation. **A**: Western blot analysis of activated p53 in Jeg3 cells after 4 h of co-culture. The non-irradiated bystander cell population (bystander cells, BC) showed a significant upregulation of activated p53 after co-culturing with directly irradiated Jeg3 compared to the non-irradiated BC which were co-cultivated with non-irradiated Jeg3. **B**: p53 expression in control Jeg3 cells. **C**: Flow cytometric analysis of co-cultured cell populations with the reanalysis of the separated cell populations. X-axis, forward scatter characteristics (FSC); Y-axis, DiI fluorescence. Data represent means ± SD (n = 3), $^{*1,2}P \leq 0.05$, significant increase in reference to the non-irradiated control.

by cultivating DiI stained, non-irradiated cells with unstained, non-irradiated cells (see Figs. 2A, 3C, and 4C). All controls revealed only basic levels of p53 expression.

The results of the bystander experiments obtained for parental Jeg3 cells are shown in Figure 2. The parental malignant human trophoblast Jeg3 cell line is characterized as communication-deficient [Hellmann et al., 1996] which has been proven later on using the Calcein dye transfer assay analyzed by flow cytometry [Gellhaus et al., 2004]. Parental Jeg3 cells showed a weak p53 level in both unstained and DiI stained non-irradiated culture populations after co-culturing (Fig. 2A). The directly irradiated cell population revealed, as expected, a strong upregulation of p53 expression. However, after co-culturing non-irradiated, unstained Jeg3 cells with directly irradiated, DiI stained cells, the non-irradiated bystander cells exhibited a significant upregulation of p53 ($P \leq 0.05$) demonstrating a bystander effect in contrast to controls with unstained cells co-cultivated with non-irradiated Jeg3 bystander cells.

To exclude mixture of stained and unstained cell populations during the sorting process the purity of the separated cell populations was checked once more by FACS analysis (Fig. 2C). The reanalysis of the separated cell populations exhibited a mean purity of $98.7 \pm 0.17\%$.

Evaluation of Radiation-Induced Bystander Effects in Cx43 Inducible Jeg3 Cells After X-Ray Irradiation

Since the parental malignant human trophoblast Jeg3 cell line is characterized as communication-deficient, the cells have been stably transfected with exogenous Cx43, which can be induced by doxycycline treatment via a tetracycline-responsive inducible promoter system [Gellhaus et al., 2004].

Figure 3A,B shows the induction of Cx43 in these cells following 48 h treatment with Dox with a strong immunolabeling of Cx43 protein at the cell membranes of Jeg3 Cx43 transfected cells (Fig. 3B, arrow) compared to the uninduced cells (Fig. 3A). The coupling compatibility was confirmed by measuring the degree of gap junctional cell coupling by FACS analysis using the gap junction permeable dye Calcein [Gellhaus et al., 2004; Banaz-Yasar et al., 2005]. The co-culture experiments were proven with communicating via connexin channels as well as with non-communicating Jeg3 cells. The non-irradiated Cx43 expressing bystander cells revealed a significant upregulation of activated p53 expression ($P \leq 0.05$) compared to the bystander cells which were cultivated with the non-irradiated cell population (Fig. 3C). Interestingly, also the bystander cells which do not express Cx43 exhibited an upregulation of p53 compared to the non-irradiated bystander control cells.

After separation of the co-cultivated cell populations by cell sorting, the purity of each separated population was measured (Fig. 3E,F). The Cx43 non-expressing as well as Cx43 expressing Jeg3 cells showed a mean purity of $98.9 \pm 0.29\%$.

Thus, we observed a bystander effect upon irradiation with 5 Gy in parental Jeg3 cells as well as in Jeg3 Cx43 transfectants after co-culturing for 4 h independent from their GJIC properties.

Evaluation of Radiation-Induced Bystander Effects in Cx26 Inducible Jeg3 Cells After X-Ray Irradiation

To prove whether other connexin isoforms exhibit different properties in mediating bystander effects, we used Jeg3 cells which were transfected with inducible Cx26. We revealed a strong immunolabeling of Cx26 proteins at the cell membranes after 48 h treatment of the cells with Dox (Fig. 4B) compared to the uninduced cells (Fig. 4A).

For the analysis of bystander effects, the same co-culture experimental design was used as for the Cx43 transfected cells. The results are demonstrated in Figure 4C. Independent from Cx26 expression Jeg3 bystander cells revealed elevated levels of activated p53 after co-culturing with irradiated cells compared to non-irradiated controls. The p53 protein expression level was significantly upregulated ($P \leq 0.05$) compared to co-cultures with non-irradiated cells.

Reanalysis of the separated cell populations showed completely separated cell populations (Fig. 4E,F). The Cx26 non-expressing as well as the Cx26 expressing Jeg3 cells revealed a mean purity of $98.2 \pm 0.85\%$ after 4 h co-culturing and separation of the different cell populations.

Taken together, we observed a bystander effect in Jeg3 cells upon irradiation with 5 Gy X-rays after 4 h co-culture which was

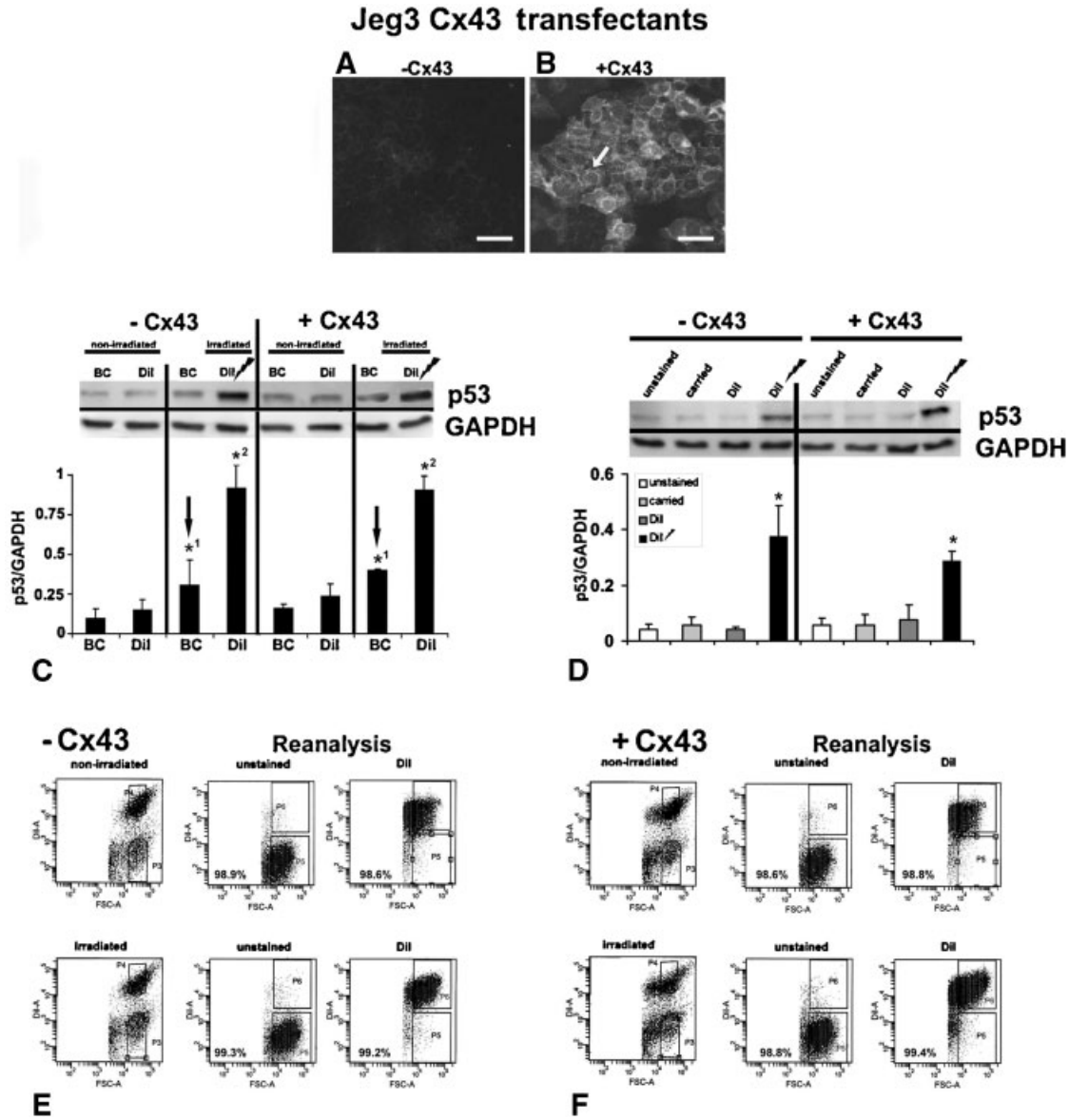

Fig. 3. Radiation-induced bystander effects in inducible Cx43 transfected Jeg3 cells after irradiation. Cx43 immunostaining of Cx43 transfected cells revealed strong immunoreactivity at the cell membranes in the Dox treated cells (**B**, arrow) in contrast to the very weak staining in the uninduced cells (**A**). **C**: Western blot analysis of activated p53 in non-irradiated and irradiated Cx43 non-expressing (−Cx43) and expressing (+Cx43) Jeg3 after 4 h co-culture. The non-irradiated bystander cells (BC) showed in the Cx43 non-expressing Jeg3 as well as in the Cx43 expressing Jeg3 a significant upregulation of p53 compared to the BC which were co-cultivated with non-irradiated cells. **D**: p53 expression in control Jeg3 Cx43 transfected cells. **E**,**F**: Flow cytometric analysis and reanalysis of co-cultured Jeg3 Cx43 non-expressing (E) and Cx43 expressing transfectants (F). X-axis, forward scatter characteristics (FSC); Y-axis, DiI fluorescence. Data represent means ± SD (n = 4), $^{*1,2}P \leq 0.05$, significant increase in reference to the non-irradiated control. Scale bar in (A,B): 40 μm.

independent from GJIC and the connexin isoform expressed.

DISCUSSION

It is well known that irradiated cells are capable of providing signals to neighboring unirradiated cells resulting in damage to the cells which are called bystander effect. In previous studies, it has been intensely debated whether GJIC is involved in mediating radiation-induced bystander effects and/or diffusible cellular factors such as ROS, NO, TGF-β1, IL-1, and IL-8 which are excreted from irradiated cells in the growth medium [Azzam et al., 1998, 2001; Bishayee et al., 2001; Nagasawa et al., 2002; Shao et al., 2003; Mitchell et al., 2004].

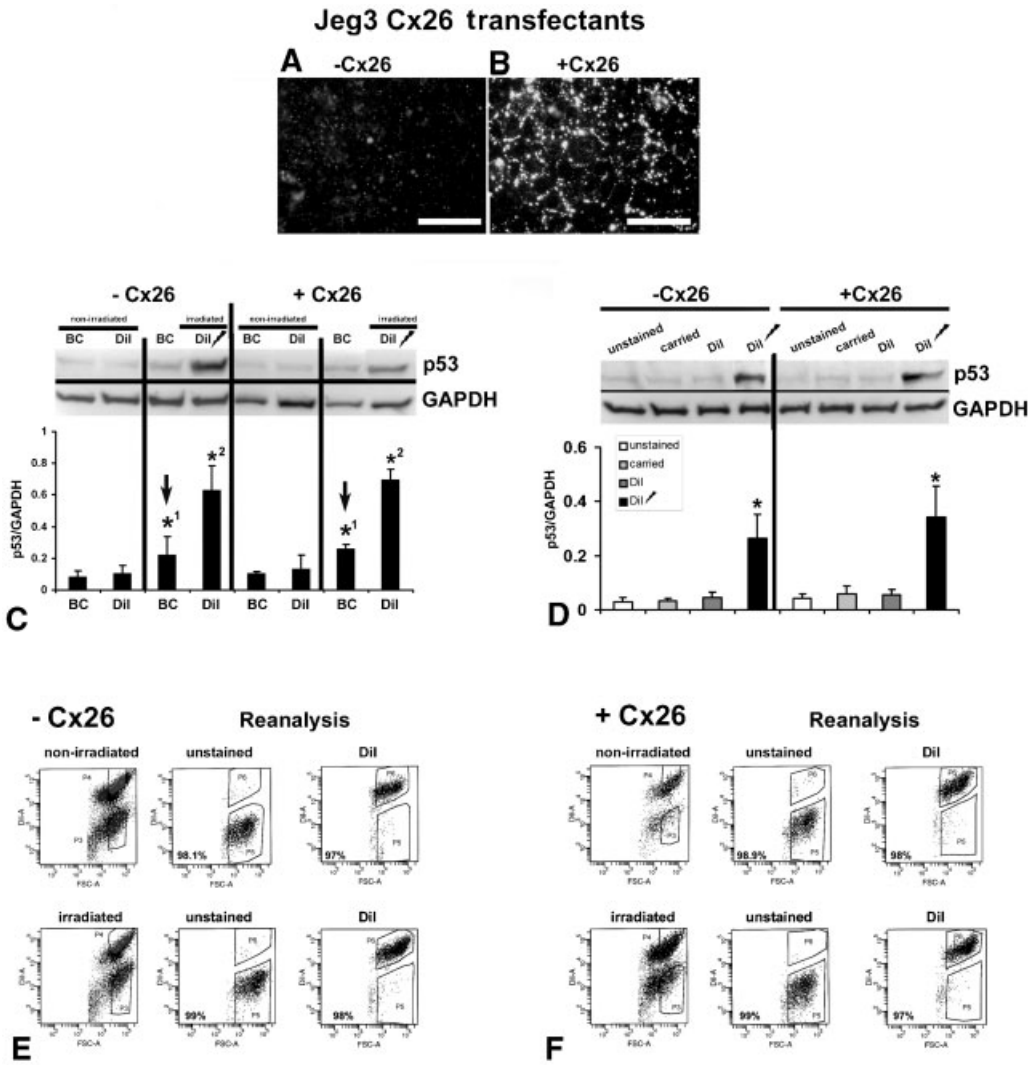

Fig. 4. Radiation-induced bystander effects in inducible Cx26 transfected Jeg3 cells after irradiation. Cx26 immunostaining showed strong immunoreactivity at the cell membranes in the Cx26 induced (**B**) in contrast to the uninduced cells (**A**). **C**: Activated p53 expression of non-irradiated and irradiated Jeg3 Cx26 expressing and non-expressing cells analyzed by Western blotting. Both cell lines revealed a significant upregulation of p53 in the non-irradiated bystander cells (BC) after 4 h co-culturing with directly irradiated cells. **D**: p53 expression in control Jeg3 Cx26 transfected cells. **E**,**F**: Reanalysis of the separated cell populations by flow cytometry. X-axis, forward scatter characteristics (FSC); Y-axis, DiI fluorescence. Data represent means ± SD (n = 4), $^{*1,2}P \leq 0.05$, significant increase in reference to the non-irradiated control. Scale bar in (A,B): 80 μm.

Many experiments have shown that incubation of non-irradiated cells with conditioned medium from irradiated cultures may lead to induction of biological effects [Azzam et al., 2003b]. Since the bystander effect is relevant to malignant transformation of healthy cells in the environment of irradiated tumors, it might have significant implications for risk estimation to radiation exposure. But up to now the nature of the bystander effect signal, how it impacts on the unirradiated cells and the signaling pathways involved in sustaining damage to these cells remain to be elucidated.

In our current study, we show for the first time a bystander effect in co-cultured malignant trophoblast cells Jeg3 after exposure to 5 Gy X-rays using the activation of the stress-inducible protein p53 as a marker for radiation-induced bystander effects with and without gap junctional communication. The results of the present study revealed that this bystander effect in Jeg3 cells was not mediated via gap junctional communication under this co-culture approach because the activation of p53 could be observed in both communicating and non-communicating Jeg3 cells and was even independent from

the connexin isoform expressed. Neither the Cx43 nor the Cx26 transfected Jeg3 cells revealed a difference in the degree of the bystander effect.

The upregulation of the activated p53 damage response pathway was a consequence of DNA damage because p53 was phosphorylated on serine 15 which could be confirmed by using an antibody specific for phosphorylated p53 in Western blot analysis. Only few studies used p53 as a damage sensor for the investigation of bystander effects [Azzam et al., 1998, 2001]. Most of the bystander analyses were performed analysing micronucleus formation, γH2AX foci, and apoptosis by immunocytochemistry [Azzam and Little, 2004]. However, p53 is an early and rapid-induced stress response protein activated by multiple endogenous and environmental insults and therefore, an appropriate key mediator of the DNA damage response cascade following cellular exposure to ionizing radiation [Cuddihy and Bristow, 2004].

In contrast, the analysis of micronucleus formation or γH2AX foci was not as rapid as p53 activation and shows mostly significant effects not before 24 h after irradiation [Sokolov et al., 2005]. Moreover, the micronuclei and γH2AX foci were only quantified by subjective immunocytochemical observation and not by quantitative Western blotting.

Because of this rapid activation of p53 in the Jeg3 cell line already 15 min following X-ray irradiation independent from gap junction communication, whereas p21 exhibited only an induction not until 2–4 h after irradiation, we preferred the quantification of phosphorylated p53 protein for our co-culture experiments. Our results are in accordance with observations of Fournier et al. [2004] who report about the same difference in time kinetics of p53 and p21 activation in human fibroblasts after exposure to X-rays. This could be partly explained by the fact that p21 is transcriptionally activated by p53 [Ewen and Miller, 1996]. Interestingly, the increase in p21 expression was delayed in those Jeg3 cells which have the possibility to communicate with one another suggesting a dampening of the checkpoint response. This again supports a favored theory that communicating cells are more protected against radiation when direct cell communication via gap junctions is enhanced [Knedlitschek et al., 1990; Hamada et al., 2003].

For our experiments, we performed a co-culture system of unstained, non-irradiated, and DiI-stained, irradiated cells combined with flow cytometry using a very strict gating strategy to obtain clearly separated cell populations which has only been partly described before by Gerashchenko and Howell [2003].

To address the question if the connexin channels are involved in mediating this bystander effect, we have used the doxycycline inducible gene expression system. With this system, we were able to discriminate between both mechanisms of bystander response, (i) mediated by GJIC and/or (ii) mediated by diffusible cellular factors by using non-communicating Jeg3 malignant trophoblast cells stably transfected with inducible gap junction proteins Cx43 and Cx26 within the same cell clone. Many studies performed bystander experiments using gap junction channel inhibitors such as carbenoxolone (CBX) or γ-isomer of hexa-chloro-cyclo-hexane (lindane). But the disadvantage of those inhibitors is the possible damage of the cell membrane as well as the change of membrane fluidity and the non-specific inhibition [Spray et al., 2002]. Therefore, we used in our experiments doxycycline inducible Jeg3 Cx transfected cells where the uninduced cells, Cx non-expressing cells, serve as an internal control. Previous reports revealed that the induction or increase in expression of Cx43 in human fibroblasts as well as in liver epithelial cells is modulated by ionizing radiation [Glover et al., 2003; Azzam et al., 2003a]. This inducible Cx43 transfected human epithelial Jeg3 cell line, however, demonstrated neither an induction of the endogenous Cx43 nor a modulation of the exogenous Cx43 expression upon irradiation as evidenced previously [Banaz-Yasar et al., 2005].

In contrast to our findings that the bystander effect is independent from the direct communication properties, Azzam et al. [1998, 2001] revealed that Cx43-mediated GJIC is involved in the bystander response observed in fibroblasts and epithelial cells exposed to low fluences of α-particles. Additional confirmation of the role of GJIC in mediating the α-particle-induced bystander response results from mutation studies using microbeam irradiation. Cells genetically deficient in Cx43 function or chemically inhibited with the gap junction blocker lindane demonstrated no bystander effect [Zhou

et al., 2000, 2001; Bishayee et al., 2001]. Also Hu et al. [2006] confirmed that a bystander effect in α-particle irradiated AG1522 human skin fibroblasts is mediated via gap junctions by treating the cells with lindane or DMSO.

However, there are other reports about a gap junction independent bystander mechanism. Human lung carcinoma cell lines as well as a rat tumor cell line for example exhibited a bystander effect that was not altered by gap junction inhibitors or enhancers [Imaizumi et al., 1998; Princen et al., 1999]. Furthermore, Yang et al. [2005] demonstrated a bystander effect in X-ray irradiated human fibroblasts which is independent from gap junctional communication using several markers including induction of p21 protein and γ-H2AX foci, formation of micronuclei and reduction of cloning efficiency of bystander cells. Moreover, Gerashchenko and Howell [2003] demonstrated that only cell proximity is a prerequisite for the proliferative bystander response of γ-irradiated cells but not gap junctional communication or soluble extracellular factors under the used experimental conditions.

All these studies give evidence that bystander effects occur in a variety of cell types of human and rodent origin and involve GJIC, oxidative metabolism, and secreted diffusible factors [Azzam and Little, 2004]. These various mechanisms converge to regulate at least GJIC and they possible contribute to radiation-induced bystander effects in concert. However, it is also possible that specific bystander effects are regulated by some mechanisms and not by others dependent on the cell type investigated, the growth state of the cell, the type of radiation, the delivered dose, and the biological endpoint being measured.

The role of ROSs released into the cell-culture medium for bystander effects has been shown applying antioxidants such as superoxide dismutase or inhibitors of superoxide and NO generators [Shao et al., 2002; Shao et al., 2005]. It has been reported that ROS and NO induced the inflammatory cascade by the activation of cytokines and prostaglandins [Geronikaki and Gavalas, 2006]. Recently, the involvement of prostaglandins in mediating the radiation-induced bystander effect in human lung fibroblasts has been revealed by Zhou et al. [2005]. Treatment of bystander cells with a COX-2 inhibitor reduced the bystander effect and suppressed the induction of MAPK pathways such as ERK, c-Jun N-terminal kinase, and p38 kinase. It is further known that NO regulates the expression of the cytokine IL-8 in human pancreatic cancer cell lines [Xiong et al., 2001]. Not only the ability to release cytokines but also the receptor profiles are likely to modulate the bystander responses and the final outcome. This could be demonstrated by Facoetti et al. [2007] analyzing the involvement of IL-8 and its receptor (CXCR1) in mediating bystander responses in glioblastoma cells.

Moreover, signaling amines such as serotonin and glycine have recently been found to modulate bystander effects when added to the cell culture medium [Poon et al., 2007]. Taken together, various mediators of different origin leading to different signaling cascades are involved in generating bystander effects in a probably tissue-specific manner [Little, 2006].

In conclusion, our data showed a bystander effect in co-cultured malignant trophoblast cells Jeg3 after exposure to X-rays. However, this bystander effect is independent from the possibility of a direct cell to cell communication via gap junction channels and independent from the connexin isoform, Cx43 or Cx26. Therefore, we suggest that it is more likely that the bystander effect in this trophoblast cell line using a co-culture system is mediated by paracrine mechanisms through excretion of diffusible signaling molecules.

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REFERENCES

Azzam EI, de Toledo SM, Gooding T, Little JB. 1998. Intercellular communication is involved in the bystander regulation of gene expression in human cells exposed to very low fluences of alpha particles. Radiat Res 150:497–504.

Azzam EI, de Toledo SM, Little JB. 2001. Direct evidence for the participation of gap junction-mediated intercellular communication in the transmission of damage signals from alpha -particle irradiated to nonirradiated cells. Proc Natl Acad Sci USA 98:473–478.

Azzam EI, De Toledo SM, Spitz DR, Little JB. 2002. Oxidative metabolism modulates signal transduction and micronucleus formation in bystander cells from alpha-particle-irradiated normal human fibroblast cultures. Cancer Res 62:5436–5442.

Azzam EI, de Toledo SM, Little JB. 2003a. Expression of CONNEXIN43 is highly sensitive to ionizing radiation and other environmental stresses. Cancer Res 63:7128–7135.

Azzam EI, de Toledo SM, Little JB. 2003b. Oxidative metabolism, gap junctions and the ionizing radiation-induced bystander effect. Oncogene 22:7050–7057.

Azzam EI, Little JB. 2004. The radiation-induced bystander effect: Evidence and significance. Hum Exp Toxicol 23:61–65.

Banaz-Yasar F, Tischka R, Iliakis G, Winterhager E, Gellhaus A. 2005. Cell line specific modulation of connexin43 expression after exposure to ionizing radiation. Cell Commun Adhes 12:249–259.

Bishayee A, Hill HZ, Stein D, Rao DV, Howell RW. 2001. Free radical-initiated and gap junction-mediated bystander effect due to nonuniform distribution of incorporated radioactivity in a three-dimensional tissue culture model. Radiat Res 155:335–344.

Cuddihy AR, Bristow RG. 2004. The p53 protein family and radiation sensitivity: Yes or no? Cancer Metastasis Rev 23:237–257.

Dale R, Carabe-Fernandez A. 2005. The radiobiology of conventional radiotherapy and its application to radionuclide therapy. Cancer Biother Radiopharm 20:47–51.

Edwards GO, Botchway SW, Hirst G, Wharton CW, Chipman JK, Meldrum RA. 2004. Gap junction communication dynamics and bystander effects from ultrasoft X-rays. Br J Cancer 90:1450–1456.

Ewen ME, Miller SJ. 1996. p53 and translational control. Biochim Biophys Acta 1242:181–184.

Facoetti A, Ballarini F, Cherubini R, Gerardi S, Nano R, Ottolenghi A, Prise KM, Trott KR, Zilio C. 2007. Gamma ray-induced bystander effect in tumour glioblastoma cells: A specific study on cell survival, cytokine release and cytokine receptors. Radiat Prot Dosimetry [Epub ahead of print].

Fournier C, Wiese C, Taucher-Scholz G. 2004. Accumulation of the cell cycle regulators TP53 and CDKN1A (p21) in human fibroblasts after exposure to low- and high-LET radiation. Radiat Res 161:675–684.

Gellhaus A, Dong X, Propson S, Maass K, Klein-Hitpass L, Kibschull M, Traub O, Willecke K, Perbal B, Lye SJ, Winterhager E. 2004. Connexin43 interacts with NOV: A possible mechanism for negative regulation of cell growth in choriocarcinoma cells. J Biol Chem 279:36931–36942.

Gerashchenko BI, Howell RW. 2003. Flow cytometry as a strategy to study radiation-induced bystander effects in co-culture systems. Cytometry A 54:1–7.

Geronikaki AA, Gavalas AM. 2006. Antioxidants and inflammatory disease: Synthetic and natural antioxidants with anti-inflammatory activity. Comb Chem High Throughput Screen 9(6):425–442.

Glover D, Little JB, Lavin MF, Gueven N. 2003. Low dose ionizing radiation-induced activation of connexin 43 expression. Int J Radiat Biol 79:955–964.

Gossen M, Bujard H. 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci USA 89:5547–5551.

Hamada N, Kodama S, Suzuki K, Watanabe M. 2003. Gap junctional intercellular communication and cellular response to heat stress. Carcinogenesis 24:1723–1728.

Hellmann P, Winterhager E, Spray DC. 1996. Properties of connexin40 gap junction channels endogenously expressed and exogenously overexpressed in human choriocarcinoma cell lines. Pflugers Arch 432:501–509.

Hellmann P, Grummer R, Schirrmacher K, Rook M, Traub O, Winterhager E. 1999. Transfection with different connexin genes alters growth and differentiation of human choriocarcinoma cells. Exp Cell Res 246:480–490.

Hickman AW, Jaramillo RJ, Lechner JF, Johnson NF. 1994. Alpha-particle-induced p53 protein expression in a rat lung epithelial cell strain. Cancer Res 54:5797–5800.

Hu B, Wu L, Han W, Zhang L, Chen S, Xu A, Hei TK, Yu Z. 2006. The time and spatial effects of bystander response in mammalian cells induced by low dose radiation. Carcinogenesis 27:245–251.

Imaizumi K, Hasegawa Y, Kawabe T, Emi N, Saito H, Naruse K, Shimokata K. 1998. Bystander tumoricidal effect and gap junctional communication in lung cancer cell lines. Am J Respir Cell Mol Biol 18:205–212.

Iyer R, Lehnert BE, Svensson R. 2000. Factors underlying the cell growth-related bystander responses to alpha particles. Cancer Res 60:1290–1298.

Knedlitschek G, Anderer U, Weibezahn KF, Dertinger H. 1990. Radioresistance of rat glioma cell lines cultured as multicellular spheroids. Correlation with electrical cell-to-cell-coupling. Strahlenther Onkol 166:164–167.

Lehnert BE, Goodwin EH, Deshpande A. 1997. Extracellular factor(s) following exposure to alpha particles can cause sister chromatid exchanges in normal human cells. Cancer Res 57:2164–2171.

Little JB, Azzam EI, de Toledo SM, Nagasawa H. 2002. Bystander effects: Intercellular transmission of radiation damage signals. Radiat Prot Dosimetry 99:159–162.

Little JB, Nagasawa H, Li GC, Chen DJ. 2003. Involvement of the nonhomologous end joining DNA repair pathway in the bystander effect for chromosomal aberrations. Radiat Res 159:262–267.

Little JB. 2006. Cellular radiation effects and the bystander response. Mutat Res 597(1–2):113–118.

Lyng FM, Seymour CB, Mothersill C. 2002. Early events in the apoptotic cascade initiated in cells treated with medium from the progeny of irradiated cells. Radiat Prot Dosimetry 99:169–172.

Mesnil M, Yamasaki H. 2000. Bystander effect in herpes simplex virus-thymidine kinase/ganciclovir cancer gene therapy: Role of gap-junctional intercellular communication. Cancer Res 60:3989–3999.

Mitchell SA, Marino SA, Brenner DJ, Hall EJ. 2004. Bystander effect and adaptive response in C3H 10T(1/2) cells. Int J Radiat Biol 80:465–472.

Mothersill C, Seymour C. 1997. Medium from irradiated human epithelial cells but not human fibroblasts reduces the clonogenic survival of unirradiated cells. Int J Radiat Biol 71:421–427.

Mothersill C, Seymour C. 2001. Radiation-induced bystander effects: Past history and future directions. Radiat Res 155:759–767.

Mothersill C, Seymour C. 2003. Low-dose radiation effects: Experimental hematology and the changing paradigm. Exp Hematol 31:437–445.

Nagasawa H, Little JB, Tsang NM, Saunders E, Tesmer J, Strniste GF. 1992. Effect of dose rate on the survival of irradiated human skin fibroblasts. Radiat Res 132:375–379.

Nagasawa H, Cremesti A, Kolesnick R, Fuks Z, Little JB. 2002. Involvement of membrane signaling in the bystander effect in irradiated cells. Cancer Res 62:2531–2534.

Nagasawa H, Huo L, Little JB. 2003. Increased bystander mutagenic effect in DNA double-strand break repair-deficient mammalian cells. Int J Radiat Biol 79:35–41.

Narayanan PK, Goodwin EH, Lehnert BE. 1997. Alpha particles initiate biological production of superoxide anions and hydrogen peroxide in human cells. Cancer Res 57:3963–3971.

Poon RC, Agnihotri N, Seymour C, Mothersill C. 2007. Bystander effects of ionizing radiation can be modulated by signaling amines. Environ Res [Epub ahead of print].

Princen F, Robe P, Lechanteur C, Mesnil M, Rigo JM, Gielen J, Merville MP, Bours V. 1999. A cell type-specific and gap junction-independent mechanism for the herpes simplex virus-1 thymidine kinase gene/ganciclovir-mediated bystander effect. Clin Cancer Res 5:3639–3644.

Sawant SG, Randers-Pehrson G, Geard CR, Brenner DJ, Hall EJ. 2001. The bystander effect in radiation oncogenesis: I. Transformation in C3H 10T1/2 cells in vitro can be initiated in the unirradiated neighbors of irradiated cells. Radiat Res 155:397–401.

Shao C, Furusawa Y, Aoki M, Matsumoto H, Ando K. 2002. Nitric oxide-mediated bystander effect induced by heavy-ions in human salivary gland tumour cells. Int J Radiat Biol 78:837–844.

Shao C, Furusawa Y, Aoki M, Ando K. 2003. Role of gap junctional intercellular communication in radiation-induced bystander effects in human fibroblasts. Radiat Res 160:318–323.

Shao C, Folkard M, Michael BD, Prise KM. 2005. Bystander signaling between glioma cells and fibroblasts targeted with counted particles. Int J Cancer 116:45–51.

Sohl G, Willecke K. 2004. Gap junctions and the connexin protein family. Cardiovasc Res 62:228–232.

Sokolov MV, Smilenov LB, Hall EJ, Panyutin IG, Bonner WM, Sedelnikova OA. 2005. Ionizing radiation induces DNA double-strand breaks in bystander primary human fibroblasts. Oncogene 24:7257–7265.

Spray DC, Rozental R, Srinivas M. 2002. Prospects for rational development of pharmacological gap junction channel blockers. Curr Drug Targets 3:455–464.

Traub O, Eckert R, Lichtenberg-Frate H, Elfgang C, Bastide B, Scheidtmann KH, Hulser DF, Willecke K. 1994. Immunochemical and electrophysiological characterization of murine connexin40 and -43 in mouse tissues and transfected human cells. Eur J Cell Biol 64:101–112.

Udawatte C, Ripps H. 2005. The spread of apoptosis through gap-junctional channels in BHK cells transfected with Cx32. Apoptosis 10:1019–1029.

Wei CJ, Xu X, Lo CW. 2004. Connexins and cell signaling in development and disease. Annu Rev Cell Dev Biol 20: 811–838.

Winterhager E, Stutenkemper R, Traub O, Beyer E, Willecke K. 1991. Expression of different connexin genes in rat uterus during decidualization and at term. Eur J Cell Biol 55:133–142.

Xiong Q, Shi Q, Le X, Wang B, Xie K. 2001. Regulation of interleukin-8 expression by nitric oxide in human pancreatic adenocarcinoma. J Interferon Cytokine Res 21(7):529–537.

Yang H, Asaad N, Held KD. 2005. Medium-mediated intercellular communication is involved in bystander responses of X-ray-irradiated normal human fibroblasts. Oncogene 24:2096–2103.

Zhou H, Randers-Pehrson G, Waldren CA, Vannais D, Hall EJ, Hei TK. 2000. Induction of a bystander mutagenic effect of alpha particles in mammalian cells. Proc Natl Acad Sci USA 97:2099–2104.

Zhou H, Suzuki M, Randers-Pehrson G, Vannais D, Chen G, Trosko JE, Waldren CA, Hei TK. 2001. Radiation risk to low fluences of alpha particles may be greater than we thought. Proc Natl Acad Sci USA 98: 14410–14415.

Zhou H, Ivanov VN, Gillespie J, Geard CR, Amundson SA, Brenner DJ, Yu Z, Lieberman HB, Hei TK. 2005. Mechanism of radiation-induced bystander effect: Role of the cyclooxygenase-2 signaling pathway. Proc Natl Acad Sci USA 102:14641–14646.