Cyto genetic detection of a trans-species bystander effect: induction of sister chromatid exchanges in murine 3T3 cells by ganciclovir metabolized in HSV thymidine kinase gene-transfected Chinese hamster ovary cells

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Due to the very limited transduction capacity of hitherto available vectors, the success of gene therapy of tumours by means of suicide genes has so far essentially depended on the transfer of toxic drug metabolites from transduced (metabolizing) cells to adjacent non-transduced cells via gap junctions (bystander effect). Most experimental systems for the detection of a bystander effect yield net data of cell losses and cannot differentiate between killed transduced versus killed bystander cells. Here we report on metabolic cooperation in vitro between CHO cells stably transfected with the thymidine kinase gene of herpes simplex virus type-1 (HSVtk) (metabolizing cells) and Swiss albino 3T3 cells (bystander cells). Both cell lines are readily distinguishable by single cell and colony morphology and by their chromosomal constitution. While 3T3 cells cultured alone were refractory to the antiviral drug ganciclovir (GCV), co-culture with CHO-HSVtk+ cells led to a dramatic reduction in plating efficiency as well as to a 4-fold increase in sister chromatid exchange rates induced by very low GCV concentrations in the 3T3 bystander cells. The modulator of gap junctional cooperation, all-trans retinoic acid, caused a strong augmentation of the bystander effect, while 18α-glycyrrhetinic acid, an inhibitor of gap junctional communication, drastically diminished the toxicity of GCV in the bystander cells. Whereas CHO-HSVtk+ cells showed a distinct immunoreactivity for connexin43 in the cell membranes, 3T3 cells were almost negative. The co-culture system described here allows unequivocal distinction between metabolizing and bystander cells. In this way, mechanistic aspects of the transfer of genotoxic/cytotoxic metabolites to cells, which per se are unable to form them, become accessible to investigation.

Introduction

Gene therapy of neoplastic diseases by means of ‘suicide gene’ transduction, as originally designed by Moolten (1986), has raised expectations and is still a focus of anticancer research. The principle of this novel therapy originates from antiviral chemotherapy and relies on the fact that herpesviruses encode their own nucleoside kinases which, owing to a much lower substrate specificity than their eukaryotic counterparts, are able to monophosphorylate certain nucleoside analogues whose triphosphates interfere with the function of herpesvirus DNA polymerases, thus causing an interruption of virus replication (for reviews see Darby, 1994; De Clercq, 1994). If such herpesvirus-encoded nucleoside kinase genes, in particular that of the thymidine kinase of herpes simplex virus type 1 (HSVtk), are transduced and expressed in eukaryotic cells and an antiviral drug, e.g. ganciclovir (GCV), is administered, metabolism to the triphosphate of the harmless nucleoside analogue and its subsequent incorporation into genomic DNA kills the host cells, mainly by apoptosis (Freeman et al., 1993; Hamel et al., 1996). Similar to many other novel anticancer strategies, the method worked well in in vitro systems and in transplant tumours in vivo, however, clinical trials in human patients were rather disappointing (Rainov, 2000; Rochlitz, 2001). The most important reason of this failure is the limited transduction capacity (<<10%) of hitherto available vectors. Although several possibilities exist to optimize this therapy regimen, e.g. by improving the vectors, transduction of tk mutants with higher monophosphorylating activity (Black et al., 2001) or by use of other enzyme/prodrug combinations (for reviews see Wildner, 1999; Greco and Dachs, 2001; Kirn et al., 2002), it has become obvious that success or failure of suicide gene cancer therapy, especially that of the HSVtk/GCV combination, crucially depends on the ‘bystander effect’. The term bystander effect in this case means the killing of cells that do not themselves express the critical transgene, due to metabolic cooperation with suicide gene-expressing adjacent cells. Although the bystander effect is a very complex phenomenon and appears to be based on different mechanisms, most important is transfer of the toxic GCV metabolite, GCV triphosphate (GCV-TP), from HSVtk-positive cells to their HSVtk-negative neighbours via gap junctions (Vrionis et al., 1997; Mesnil and Yamasaki, 2000; for a review see van Dillen et al., 2002). Gap junctions are cell–cell contacts consisting of connexins that permit the passage from one cell to the other of hydrophilic molecules smaller than 1 kDa, such as nucleoside phosphates, which otherwise cannot freely pass the cellular membrane (reviewed by Kumar and Gilula, 1996). In experimental systems, the bystander effect of the HSVtk/GCV combination is measurable in transplant tumours consisting of a known ratio of HSVtk-transfected and HSVtk-negative cells where, after GCV administration, tumour size or survival times of the animals serve as parameters (Freeman et al., 1993; Dilber et al., 1997; Berenstein et al., 1999; Namba et al., 2000). In vitro co-cultures containing a mixture of metabolizing and bystander cells are another alternative to investigate the bystander effect. In these systems, cell counting or biochemical assays were used to detect cytotoxicity (Freeman et al., 1993; Vrionis et al., 1997; Princen et al., 1999; Tanaka et al., 2001). With the exception of a few cases where sophisticated methods

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of cell sorting were applied or the bystander cells expressed a marker gene (Ishii-Morita et al., 1997; Boucher et al., 1998; Rubsam et al., 1999), however, these models yielded net data of cell losses and the unequivocal distinction between killed HSVtk+ cells and HSVtk+ bystander cells remained elusive.

In the present study, we used HSVtk gene-transfected Chinese hamster ovary cells for nucleoside analogue activation ('metabolizing cells') and mouse 3T3 cells as bystander cells for the detection of damage. Both cell types are readily distinguishable with respect to morphology and karyotype. Among the various antiviral nucleoside analogues proposed for suicide gene therapy, ganciclovir was chosen in this investigation because: (i) it is the most widely used drug in combination with suicide gene therapy; (ii) its bystander capacity has been proven in various systems in vitro and in vivo; (iii) GCV is a very potent cytogegetic genotoxin, which in extremely low concentrations induces sister chromatid exchanges (SCEs) in metabolically competent (HSVtk-transfected) cells (Thust et al., 2000a,b). The latter property makes GCV a very suitable candidate drug for the detection of chromosomal damage in cells which themselves are unable to activate the prodrug but are connected with HSVtk+ cells via gap junctions. Using the SCE assay, this is the first study that allows a direct comparison of genotoxic effects 'in situ', i.e. in metabolizing versus bystander cells.

Materials and methods

Chemicals

Ganciclovir [9-(1,3-diacyclo-2-propoxymethyl)-guanine; Cymevene®] was obtained from Syntex GmbH (Grenzach-Wyhlen, Germany) and dissolved in complete culture fluid. All-trans-retinoic acid (ATRA) and 18α-glycyretinic acid (AGA) were purchased from Sigma and dissolved in DMSO. The freshly prepared stock solutions were diluted in complete culture fluid giving a final concentration of 1 μM ATRA and 50 μM AGA, respectively. Maximum DMSO concentrations in the cultures did not exceed 0.2%.

Cells

Swiss albino 3T3 cells were used as bystander cells. They were purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and cultured in Ham's F12/Dulbecco's minimal essential medium (1:1) containing 10% inactivated foetal bovine serum and supplemented with 50 μg/ml gentamicin sulphate. CHO cells transfected with the thymidine kinase gene of herpes simplex virus type 1 (CHO-HSVtk+ cells) were used as the metabolizing system. Generation and properties of the transfectant clones has been described elsewhere (Thust et al., 2000a). They were grown in the same medium but containing 5% foetal bovine serum. The CHO clones were used as the metabolizing cells, whereas the bystander cell line (3T3) was obtained from Syntex GmbH (Grenzach-Wyhlen, Germany) and cultured in Ham's F12/Dulbecco's minimal essential medium (1:1) containing 10% inactivated foetal bovine serum and supplemented with 50 μg/ml gentamicin sulphate. GCV a very suitable candidate drug for the detection of damage. Both cell types are readily distinguishable with respect to morphology and karyotype. Among the various antiviral nucleoside analogues proposed for suicide gene therapy, ganciclovir was chosen in this investigation because: (i) it is the most widely used drug in combination with suicide gene therapy; (ii) its bystander capacity has been proven in various systems in vitro and in vivo; (iii) GCV is a very potent cytogegetic genotoxin, which in extremely low concentrations induces sister chromatid exchanges (SCEs) in metabolically competent (HSVtk-transfected) cells (Thust et al., 2000a). They were grown in the same medium but containing 5% foetal bovine serum. The CHO clones were used as the metabolizing system. Generation and properties of the transfectant clones has been described elsewhere (Thust et al., 2000a,b). The latter property makes GCV a very suitable candidate drug for the detection of chromosomal damage in cells which themselves are unable to activate the prodrug but are connected with HSVtk+ cells via gap junctions. Using the SCE assay, this is the first study that allows a direct comparison of genotoxic effects 'in situ', i.e. in metabolizing versus bystander cells.

Cytotoxicity assay

Experiments were started by seeding a total of 5 × 10^5 cells (3T3 and/or CHO-HSVtk+ cells in different ratios as given in Results) per 25 cm² flask. On the next day, GCV was added for 24 h. Thereafter, the cells were plated in 6-well multiplates at a density of 400 cells/well (surface area 9.6 cm²) with three wells per treatment condition. About 1 week later, the cultures were fixed with methanol and stained with crystal violet. Relative cell survival (plating efficiency) was calculated from the number of colonies per well in relation to those in untreated controls.

Sister chromatid exchange assay

Mixtures of metabolizing cells (CHO-HSVtk+) and bystander cells (3T3) as given in Results in a total amount of 5 × 10^5 cells were seeded per 25 cm² flask. About 8 h later the cultures were treated with GCV for the duration of one 3T3 cell cycle, i.e. ~16 h. Thereafter, they were carefully rinsed with fresh medium and cultured for another two cell cycles in the presence of 10 μg/ml 5-bromodeoxyuridine. For metaphase arrest, colcemid (0.05 μg/ml) was added 3 h prior to chromosome preparation. Chromosome preparations were made in the usual manner after treatment of the cultures with 0.25% trypsin, 0.02% EDTA. SCEs were visualized using a modified fluorescence plus Giemsa technique according to Wolff and Afzal (1996). A total of 30 metaphases per sample of the 3T3 cell stemline were enumerated. Statistical significance was checked using Student's t-test.

Figure 1. Relative cell survival (colony formation) of 3T3 cells alone or in co-culture with CHO-HSVtk+ cells (mix 1:1) exposed to ganciclovir without and with addition of all-trans-retinoic acid (ATRA) (1 μM) or 18α-glycyretinic acid (AGA) (50 μM).

Immunofluorescence microscopy

Cells cultured on glass coverslips were fixed for 10 min at 4°C with acetone and dried. After rehydration with phosphate-buffered saline, cells were incubated for 30 min with primary rabbit polyclonal antibody against connexin37 (Cx37) (2.5 μg/ml) (Zymed Laboratories, San Francisco, CA), which was followed by a 30 min incubation with Cy-3-conjugated goat polyclonal anti-rabbit IgG (affinity purified, 1:400) (Jackson Immunoresearch Laboratories, West Grove, PA). Nuclei were counterstained with 1 μg/ml 4',6-diamidino-2-phenylindole added to the second incubation medium. To test specificity, Cx37 antibody was blocked by pre-incubation for 10 min with the Cx37 inhibitory peptide (20 μg/ml) (Zymed Laboratories). Cells were observed with a confocal laser scanning microscope (Zeiss LSM 510; Jena, Germany).

Results

It is obvious that the number of intercellular contacts depends on cell density, i.e. a maximum of junctions occurs in confluent cultures. On the other hand, however, the mode of adverse action of GCV in eukaryotic cells depends on the incorporation of GCV triphosphate into the genomic DNA of the cells and its subsequent processing by repair enzymes (see Discussion). For this reason, we had to empirically find an initial cell density which would not only allow the formation of intercellular contacts but also an almost unrestricted replication of both cell types in co-culture during the whole assay period.

In order to detect cytotoxicity, the colony formation assay was used. An essential precondition for these experiments was that both cell types are clearly distinguishable with regard to single cell morphology in suspension as well as to the appearance of colonies. In suspension, 3T3 cells are larger than CHO cells and, as revealed by phase contrast microscopy,
show a rough cell surface with ‘spikes’, while the surface of the smaller CHO cells is completely smooth. 3T3 colonies consist of loosely grown, very flat cells; CHO colonies, on the other hand, are densely packed and distinctly delimited. Both cell lines, CHO-HSVtk+ and 3T3, have quite different absolute plating efficiencies. Whereas under control conditions >66% of the plated CHO cells are usually able to form colonies, the absolute plating efficiency of untreated 3T3 cells was <10% (data not shown). Therefore, in untreated co-cultures CHO-HSVtk+ colonies overgrew 3T3 colonies and prevented their detection. Thus, the relative plating efficiency of 3T3 cells in GCV-treated co-cultures had to be calculated in relation to the plating efficiency of 3T3 controls. In the concentration range up to 10 μM GCV, the plating efficiency of 3T3 cells cultured alone remained essentially unaltered, while co-culture with CHO-HSVtk+ cells almost abolished their colony forming ability. The second lowest GCV concentration, 0.37 μM, applied according to the treatment schedule as given in Materials and methods almost completely killed the CHO-HSVtk+ cells and dramatically diminished the plating efficiency of 3T3 bystander cells by ~50% (Fig. 1).

Both cell lines, 3T3 and CHO-HSVtk+, have an approximately equal cell cycle length of 14–16 h. This allows a concomitant demonstration of sister chromatid differential contrast in both cell types and a direct comparison of SCE rates induced by GCV in the metabolically competent CHO-HSVtk+ cells and in the 3T3 bystander cells, respectively (Fig. 2). In the range of concentrations tested in the SCE assay, 0.1–1 μM, 3T3 cells alone exposed to GCV for one cell cycle show SCE rates at the background level, i.e. these cells are unable to activate the prodrug. In co-culture with CHO-HSVtk+ cells a distinct bystander effect with regard to SCE induction in the 3T3 cells is demonstrated. Even the lowest GCV concentration of 0.1 μM induced a statistically significant increase in the SCE rate. Furthermore, the SCE rate depends on the ratio of CHO-HSVtk+ and 3T3 cells, i.e. the higher the proportion of metabolizing cells the higher is the SCE rate in the bystander cells (Fig. 3A). On the other hand, when the SCE rates of the CHO-HSVtk+ cells were at the limit of countability and the metaphases partially showed structural chromosome aberrations (Fig. 2B), the SCE rate of the bystander cells was maximally ~4 times the control and clastogenic effects were not detected in 3T3 cells (data not shown). Principally, after GCV exposure the SCE rates in co-cultured 3T3 cells showed an unusually wide dispersion of single values as reflected by the high standard deviations (Fig. 3A and B). This suggests a large variation in the frequency of intercellular contacts between metabolizing and bystander cells (Fig. 2B and C).

The above-mentioned findings prove metabolic cooperation between CHO-HSVtk+ and 3T3 cells but do not yield a clue to its mode of action. For this reason, the influence of a modulator of gap junctional intercellular communication, all-trans-retinoic acid, on the bystander parameters studied here was determined. The ATRA concentration chosen (1 μM) neither changed the plating efficiency nor the cell cycle progression (as revealed by 5-bromodeoxyuridine labelling) of 3T3 and CHO-HSVtk+ cells (data not shown), but it distinctly augmented the bystander toxicity (Fig. 1) as well as CHO-HSVtk+-mediated SCE induction by GCV in 3T3 cells (Fig. 3B). On the other hand, AGA, which at a concentration of 50 μM neither alters the morphology nor the plating efficiency of 3T3 cells (not shown), dramatically diminished GCV toxicity in the bystander cells (Fig. 1). To our surprise, in the presence of AGA with 0.12 μM GCV ~10% of the plated CHO-HSVtk+ cells

Figure 2. Sister chromatid exchanges in CHO-HSVtk+/3T3 co-cultures. Fluorescence plus Giemsa staining. (A) Untreated control metaphases showing complete sister chromatid differential contrast in both cell types. (B) Co-culture (mix ratio 1:1) treated with 0.11 μM ganciclovir. While the CHO-HSVtk+ cells have an almost innumerable SCE rate, that of 3T3 metaphase cells is in the background range, presumably due to a lack of cell contacts to CHO-HSVtk+ cells. (C) Co-culture (mix ratio 1:1) in the presence of 1 μM all-trans-retinoic acid and treated with 1 μM ganciclovir. Strongly increased SCE rate in a 3T3 mitosis.
were able to form colonies, while at the same GCV concentration in the normal cell mixture (without any supplement) or in the presence of ATRA the CHO-HSVtk\(^{+}\) cells completely lost their colony forming ability (data not shown; see Discussion).

The occurrence and localization of the gap junctional protein assumed to be predominantly involved in the bystander effect (Cx43) was investigated. Immunofluorescence revealed the presence of Cx43 in CHO-HSVtk\(^{+}\) cells as well as in the bystander 3T3 cells. While the 3T3 cells showed a very weak, partially diffuse, in part granular distribution of the labelling, in the CHO cells a distinct and punctate fluorescence mainly at the cell membrane, but also intracytoplasmically, was found. Fluorescence labels were completely abolished in both cell types upon addition of the Cx43 inhibitor peptide, i.e. labelling was specific for Cx43. Treatment with ATRA appears to enhance the Cx43 immunoreactivity of CHO cells (Fig. 4). Whether there is a true increase in connexin protein content or whether the increased bystander effect is due to connexin translocation and assembly within the cell membrane, however, must be checked by other methods.

**Discussion**

Among the antiviral nucleoside analogues so far investigated with respect to genotoxicity, ganciclovir is unique in its extraordinary chromosome damaging potency (for review see Wutzler and Thust, 2001). This property is presumably a consequence of the particular mode of action of this drug and it was an essential precondition of the studies presented here. Contrary to most publications on gene therapy with the HSVtk/ GCV combination, which assume chain termination upon incorporation of GCV-TP into the genomic DNA of the host cell as the cause of apoptosis induction, it has been proven repeatedly and unequivocally that GCV incorporation is extensive and internal and does not interrupt ongoing replication (St Clair et al., 1987; Rubsam et al., 1998; Thust et al., 2000a; Tomicic et al., 2002). Moreover, cytotoxicity, clastogenic damage and SCE induction do not become manifest immediately after GCV exposure but occur one or more cell cycles later, suggesting that adverse effects arise during the processing of incorporated GCV (Thust et al., 2000a,b; Tomicic et al., 2002). By using polymerase \(\beta\) knockout cells or a specific inhibitor of polymerase \(\beta\) we have shown previously that polymerase \(\beta\)-mediated short- and long-patch base excision repair play important roles in the protection of cells against GCV-induced genotoxic and cytotoxic damage (Tomicic et al., 2001). It warrants future investigations whether further repair pathways are also involved.

It is noteworthy that, while the applied GCV doses induced an almost innumerable number of SCEs in metabolizing cells, the SCE rates in 3T3 cells were maximally quadruplicated. This gives a coarse idea of how few of the ultimate GCV metabolites hit the bystander cells but nevertheless were sufficient to dramatically reduce their plating efficiency. This makes it very unlikely that GCV-induced damage detectable as SCEs alone is decisive in cell killing by this nucleoside analogue. Meanwhile, we have shown that other antiviral nucleoside analogues, penciclovir and (\(E\))-5-(2-bromovinyl)-2'-deoxyuridine, kill metabolically competent cells without being similarly genotoxic (Thust et al., 2000a; Tomicic et al., 2003). These drugs also provoke a bystander effect in our model system, but are less potent (Tomicic et al., in preparation).

The present findings on the influence of ATRA on bystander toxicity and SCE induction provide circumstantial evidence for the pivotal role of connexins in gap junctional intercellular communication. Expression of connexin proteins can be modulated by various chemicals, e.g. by c-AMP mimicking agents, corticoids, retinoids, carotenoids and flavonoids (Mesnil and Yamasaki, 2000). All these compounds, however, can cause very disparate alterations in cell physiology. All-trans-retinoic acid was chosen here because its influence on the bystander effect in suicide gene cancer therapy with the HSVtk/GCV combination is well documented (Park et al., 1997; Imaizumi et al., 1998; Mesnil and Yamasaki, 2000) and, furthermore, because pilot experiments had shown that ATRA in the applied dose (1 \(\mu\)M) did not change plating efficiency,
cell cycle progression or background SCE rates of our 3T3 and CHO cells (not shown). As has been demonstrated, this retinoid distinctly enhances the bystander cytotoxicity and SCE induction by ganciclovir in our co-culture system. 18α-

Figure 4. Connexin 43 immunoreactivity revealed by confocal laser scanning microscopy in CHO-HSVtk+ cells (A) and in 3T3 cells (B) as well as in mixtures of both cell lines under normal culture conditions (C) and cultured for 3 days in the presence of 1 μM all-trans retinoic acid (D). Inhibition of the immunoreactivity by the Cx43-specific peptide in CHO-HSVtk+ (E) and 3T3 cells (F).
Glycyrrhetinic acid belongs to the class of most specific inhibitors of gap junctional communication so far known. It binds to a single proteinaceous binding site in the plasma membrane and causes a conformational change in connexin structure that results in closure of the channel (Goldberg et al., 1996). The dramatic inhibition of GCV-induced bystander toxicity, as observed in the present study, once more underlines the decisive role of gap junctions in the CHO-HSV/tk-mediated killing of 3T3 cells by GCV. The experiments with AGA yielded another interesting phenomenon, i.e. the formation of CHO-HSV/tk colonies at the lowest GCV concentration which under similar assay conditions but without AGA were never observed. The numerous experiments with our CHO-HSV/tk clone did not give any indication that the transgene was lost or switched off in some cells. The most pertinent explanation for the effect of AGA on the survival of CHO-HSV/tk cells seems to assume a diversification of herpes simplex virus type 1 thymidine kinase activity as a consequence of partial methylation of the transgene (Kuriyama et al., 1998) that, in the absence of intercellular communication, could lead to survival of CHO-HSV/tk cells with decreased herpes simplex virus type 1 thymidine kinase activity. This, however, was checked by specific assays.

About 20 different connexin proteins have been described (Evans and Martin, 2002). The occurrence of Cx43 in 3T3 and CHO cells was studied here because it appears to be the most important connexin with regard to the bystander effect (Park et al., 1997; Mesnil and Yamasaki, 2000; Tanaka et al., 2001; Sanson et al., 2002). Unexpectedly, the Swiss albino 3T3 cells used in this study showed an extremely low immunoreactivity for Cx43 that was mainly localized in the cytoplasm and in the nuclei but not in the cell membranes, suggesting that these cells were unable to form functioning gap junctions. On the other hand, CHO-HSV/tk cells were distinctly Cx43-positive, with the connexin being predominantly localized in the ‘right’ position, i.e. in the cell membranes. These findings confirm previous observations of gap junctional communication between connexin-positive and connexin-negative cells (Tanaka et al., 2001). It should be considered, however, that the bystander effect observed here might be caused by connexin types other than Cx43 (heterotypic junctions).

It should be mentioned that gap junction-mediated transfer of GCV metabolites is not the only mode of bystander killing in vitro. While a previously assumed induction of apoptosis due to phagocytosis of apoptotic vesicles (Freeman et al., 1993) has been shown to be very unlikely because of the kinetics of bystander cell death (Vrioni et al., 1997), several research groups have independently demonstrated that various cell types, e.g. certain colon carcinoma and glioma cell lines, are able to release GCV metabolites without the involvement of any direct intercellular contact (Boucher et al., 1998; Bai et al., 1999; Princen et al., 1999; Drake et al., 2000). How the toxic metabolites leave the metabolizing cells and are taken up by bystander cells in these cases is not yet understood. The modulation of the bystander end-points studied here by ATRA, however, make such a direct transfer of GCV-TP highly unlikely in our cell lines. Metabolic cooperation via gap junctions is not restricted to isologous cell types but also occurs between different tissue types and even between cells of different species. A trans-specific bystander effect has been shown to occur, for example, between 3T3 cells and various human tumour cell types (Fick et al., 1995) as well as between different neoplastic human and murine cell lines (Ishii-Morita et al., 1997; Imaizumi et al., 1998), and the present findings are a further example.

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