A Tumor Suppressor Gene, Cx26, Also Mediates the Bystander Effect in HeLa Cells

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ABSTRACT

The connexin 26 (Cx26) gene suppresses the growth of HeLa cells in vitro and in vivo. We explored the possibility that the Cx26 gene not only suppresses growth but can also mediate the bystander effect that is observed in some gene therapy. In gene therapy mediated by the herpes simplex virus thymidine kinase, the toxicity of ganciclovir affects not only the cells transduced with the gene but also affects neighboring tumor cells; it has been suggested that gap junctional intercellular communication (GJIC) may play a role in such a bystander effect. HeLa cells expressing the Cx26 gene (Cx26* or not expressing the Cx26 gene were transfected with the herpes simplex virus thymidine kinase (tk) gene, producing Cx26*-tk-, Cx26*-tk*, Cx26*-tk*, and Cx26*-tk* cells. By making different kinds of cocultures of these cells, we observed a clear bystander killing effect, assessed by the neutral red toxicity test, in the coculture of Cx26*-tk*/Cx26*-tk* cells. The bystander effect was markedly prevented by a long-term inhibitor of GJIC, 18-α-glycyrrhetinic acid, demonstrating that a major part of the bystander effect seen occurred through Cx-mediated GJIC. These data suggest the possibility of using of Cxs as both tumor suppressor genes and as diffusers of ganciclovir toxicity in therapeutic approaches.

INTRODUCTION

In gene therapy mediated by the HSV-tk, the toxicity of ganciclovir is extended from HSV-tk-transduced cells to neighboring cells (1, 2). Such propagation of toxicity, called “bystander effect,” is highly significant for gene therapy, because it allows a limited number of positively transduced cells to promote the nearly complete killing of tumors. We have previously demonstrated that propagation of the killing effect can be mediated through GJIC (3), and recently, we and others have further demonstrated that GJIC can mediate the bystander effect of HSV-tk/ganciclovir treatment (4—7). However, the inability or diminished capacity of tumor cells to communicate through gap junctions could limit the effectiveness of the HSV-tk/ganciclovir strategy of gene therapy (8, 9). Cx gene transduction together with HSV-tk gene transduction in the same tumor cells may be an efficient gene therapy by enhancing the GJIC and thus the bystander effect, leading to optimal elimination of the tumor cell population during ganciclovir treatment. Moreover, increasing evidence suggests that the Cx genes themselves are tumor suppressor genes (10—14). For instance, Cx43 gene transfection did not modify the phenotype of HeLa cells, but Cx26 expression was correlated with inhibition of tumorigenicity, a slower growth rate, and a decreased cell density in vitro (15).

Therefore, we were interested to see whether Cx26, which is a tumor suppressor in HeLa cells, could also mediate a strong bystander effect. Our results suggest that in addition to its tumor suppressor role, which was described previously (15), Cx26 was able to induce a bystander effect. These results support the idea of using specific Cxs to achieve efficient cancer control by direct cell growth control and by exerting bystander effect on HSV-tk/ganciclovir gene therapy.

MATERIALS AND METHODS

Cell Lines. The parental cell lines we used in this study (HeLa cells transfected or not transfected with Cx26 or Cx43 genes) were obtained from the laboratory of Prof. K. Willecke (Institute of Genetics, University of Bonn, Bonn, Germany). Their phenotypes and culture conditions have been described previously (15, 16). The pUT649 expression vector containing a zeocin-resistance gene and carrying the HSV-tk gene under the control of human cytomegalovirus promoter was kindly provided by Prof. G. Tiraby (Cayla, Toulouse, France) and transfected into HeLa cells with or without the Cx26 gene by using Lipofectin (Life Technologies, Inc.) as reported previously for HeLa cells expressing Cx43.

Northern Blot Analysis. Total RNA of the clones used in this study was extracted by a single-step technique described previously (15). Twenty μg of total RNA from each sample were separated by electrophoresis in denaturing formaldehyde 1% agarose gels. Gels were capillary-blotted onto Hybond-N nylon membranes (Amersham). Hybridizations were carried out for 24 h under high-stringency conditions (50% formamide, 42°C) with [γ-32P]dCTP-radio-labeled cDNA probe for HSV-tk that was prepared by using the rapid multiprime DNA-labeling system (Amersham). After washing, the blots were exposed to hyperfilm-MP (Amersham).

In Vitro Dye Transfer Assay to Measure GJIC Capacity. To estimate the GJIC capacity of cultured cells, 5% (w/v) Lucifer yellow CH (Sigma) was transferred to a glass needle. Cells were impaled and injected with needles as described previously (15). After 10 min, the number of cells that became fluorescent (communicating cells) was estimated.

Estimation of the Bystander Effect. To examine the bystander effect, we performed the neutral red toxicity assay. Cells expressing HSV-tk (Cx26*-tk-, Cx43*-tk-, and Cx*-tk-) were mixed at different ratios (50 or 10%) with corresponding cells that do not express it (Cx26*-tk+, Cx43*-tk+, and Cx*-tk+). They were seeded in 96-well plates (total cell number, 104 cells/well). Plates were prepared in such a manner that all controls were present: untreated cultures; cells treated with the long-term inhibitor of GJIC (AGA; Sigma; 70 μM); Plates were prepared in such a manner that all controls were present: untreated cultures; cells treated with AGA; Sigma; 70 μM; Ref. 17) to test its toxicity; cells treated with ganciclovir (20 μM; Syntex, Puteaux, France) to test the bystander effect; and cells treated both with ganciclovir (20 μM) and AGA (70 μM) to test the extent of the bystander effect when GJIC is inhibited. For each experiment, two plates were stopped at different days after the beginning of the treatment.

The extent of ganciclovir toxicity was estimated by neutral red uptake. Cells expressing HSV-tk (Cx26*-tk-, Cx43*-tk-, and Cx*-tk-) were mixed at different ratios (50 or 10%) with corresponding cells that do not express it (Cx26*-tk+, Cx43*-tk+, and Cx*-tk+). They were seeded in 96-well plates (total cell number, 104 cells/well). Plates were prepared in such a manner that all controls were present: untreated cultures; cells treated with the long-term inhibitor of GJIC (AGA; Sigma; 70 μM; Ref. 17) to test its toxicity; cells treated with ganciclovir (20 μM; Syntex, Puteaux, France) to test the bystander effect; and cells treated both with ganciclovir (20 μM) and AGA (70 μM) to test the extent of the bystander effect when GJIC is inhibited. For each experiment, two plates were stopped at different days after the beginning of the treatment.

RESULTS

In HeLa cells, the bystander effect of a transfected Cx43 gene on the killing by ganciclovir of HSV-tk-transfected cells was very strong;
almost complete elimination of cells was seen, even when only 10% of them expressed the HSV-tk gene (4). To see whether a similar bystander effect could be obtained using a Cx that down-regulates the growth of HeLa cells, we transfected HeLa Cx26+ cells with the HSV-tk gene carried in the pUT649 expression vector driven by a human cytomegalovirus promoter. After zeocin selection, several clones were obtained. The clone (Cx26+-tk+) showing the highest sensitivity to ganciclovir treatment (40 or 20 μM) was kept and expanded for all subsequent studies. These Cx26+-tk+ cells expressed a similar amount of HSV-tk transcripts as the Cx43+-tk- cells (Fig. 1). Moreover, the expression of the HSV-tk gene did not significantly modify either the GJIC capacity of the cells or the amount of the Cx26 transcript (Fig. 1). We have previously shown that HeLa Cx26 cells grow more slowly and that their saturation density is lower than that of the parental HeLa cells (15). This has also been confirmed in the present study for Cx26+-tk+ cells (data not shown).

To test whether Cx26 can mediate a bystander effect, we mixed 50% of Cx26+-tk+ cells with Cx26+-tk- cells and treated them with 20 μM ganciclovir. After 2 weeks, the cell culture was completely eliminated. As expected, no such effect was observed in cocultures of noncommunicating HeLa cells (Cx-tk+/Cx-tk-), indicating that Cx26 was responsible for the bystander effect (Fig. 2).

To quantify the bystander effect, we performed similar cocultures (50% of Cx26+-tk+/Cx26+-tk-) cells in 96-well plates with or without 20 μM ganciclovir, and the extent of toxicity was estimated by the neutral red technique. For comparison, similar cocultures of Cx43 transfectants were prepared and treated with 20 μM ganciclovir for 2 weeks. As shown in Fig. 3, the extent of the bystander effect was similar for Cx43+-tk+ cells and Cx26+-tk+ cells after 2 weeks of treatment, with less than 20% of cells surviving.

As expected, we noted a slight bystander effect in the coculture of Cx+-tk+/Cx+-tk- cells (Fig. 3), indicating that there are: (a) other mechanisms than GJIC by which the toxicity of ganciclovir is transferred; or (b) HeLa cells have residual GJIC. To examine to what extent Cx-mediated GJIC is responsible for the bystander killing, we used a long-term inhibitor of GJIC, AGA (17). We observed that even if AGA did not completely inhibit the Cx26-mediated cell-cell communication (50% inhibition), as it did for Cx43 transfectants (data not shown), it inhibited the bystander effect mediated by Cx26 as well as that mediated by Cx43 (Fig. 3). This means that the partial inhibition of Cx26-mediated GJIC by AGA is sufficient to prevent the extent of the bystander effect.

The bystander effect was still observed when only 10% of Cx26+-tk+ cells were mixed with the Cx26+-tk- cells (Fig. 4). However, the bystander effect was not complete, and some cells started to grow 2 weeks after the beginning of the ganciclovir treatment.

DISCUSSION

The results presented here confirm and extend our previous findings that the expression of Cx genes mediates an extensive bystander effect in vitro (4). In this paper, we show that the presence of Cx26 enables the toxicity of ganciclovir from cells expressing HSV-tk to cells that do not express it. The involvement of GJIC in this process
is confirmed by the reduction or absence of the bystander effect in the presence of a long-term inhibitor of GJIC, AGA.

The fact that Cx26 is able to mediate a bystander effect may have important consequences for possible therapeutic approaches. First, Cx26 was shown to inhibit the tumorigenicity of HeLa cells in nude mice and to reduce their growth rate and their density in vitro (15). Despite its tumor suppressor role, Cx26 induces a bystander effect that is similar to that mediated by Cx43 in these cells, even if it is lower when only 10% of cells express HSV-tk. This means that in experimental anticancer gene therapy, Cx26 could provide both down-regulation of cell growth and an extensive bystander effect. Cx26 is thought to be a tumor suppressor of breast cancer and indeed down-regulates the growth of human breast cancer cells (18). Because the tumor suppressor role of Cxs may depend on the target cancer cells, we can imagine HSV-tk-mediated experimental therapies in which specific Cxs could be used as both a tumor suppressor and a diffuser of ganciclovir toxicity. This would be possible by transducing both the appropriate Cx gene and the HSV-tk gene into a tumor. Another possible advantage of Cx26 is that it is an unphosphorylated Cx. In contrast to other Cxs, such as Cx43 (19), Cx26 function cannot be inhibited by any particular phosphorylation of the molecule. Because many oncogenes have kinase activity, and because aberrant phosphorylation is commonly observed in cancer cells, Cx26 function might be less affected by particular cancer phenotypes and could be used as a standard bystander effector.

The results of this study suggest a possible dual effect of the Cx26 gene as a tumor suppressor and mediator of the bystander effect in HeLa cells. Tumor suppression by Cx genes does not imply cell killing but rather does imply growth reduction. Although the bystander effect in HSV-tk/ganciclovir therapy leads to rapid killing of cells, the effect would never be complete enough to kill a whole tumor cell population. Therefore, the growth control effect of Cx genes becomes important to control the growth of those tumor cells not killed by suicide gene (e.g., HSV-tk) therapy, so that they remain relatively inert until the next wave of cell killing by the gene. Thus, theoretically, it seems that the two actions of Cx genes may work synergistically to eliminate tumor cells efficiently.

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