

Increased Genetic Stability of HeLa Cells after Connexin 43 Gene Transfection¹

Weibin Zhu, Nikolai Mironov, and Hiroshi Yamasaki²

Unit of Multistage Carcinogenesis, International Agency for Research on Cancer, 150, cours Albert Thomas, 69372 Lyon Cedex 08, France

Abstract

To test the hypothesis that intact gap-junctional intercellular communication (GJIC) is necessary for genomic stability, we compared the spontaneous and chemically induced mutation frequencies in GJIC-proficient and -deficient HeLa cells. Thus, we determined microsatellite instability and mutation frequency in the *HPRT* gene in parental HeLa cells, which have no GJIC ability, and in HeLa cells in which GJIC was restored by transfection with the *connexin 43* (*Cx43*) gene. When HeLa cells with (*Cx43*⁺) or without *Cx43* gene (*Cx43*⁻) were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) or methylnitrosourea, the *Cx43*⁺ cells survived better than *Cx43*⁻ cells. The mutation frequency at CA repeats was measured with a shuttle vector; in the vector, the coding region of the β -galactosidase gene was rendered out of frame by insertion of CA repeats, and the frame could be restored by insertion or deletion mutations of the CA repeats. The mutation frequency at CA repeats was 2-fold lower in *Cx43*⁺ cells than in *Cx43*⁻, both before and after exposure to MNNG or methylnitrosourea ($P < 0.05$). The frequency of spontaneous *HPRT* gene mutations, selected by their resistance to 6-thioguanine, was 3-fold lower in *Cx43*⁺ cells than *Cx43*⁻ cells. Similarly, the frequency of MNNG-induced *HPRT* mutations was significantly higher in *Cx43*⁻ cells ($P < 0.001$). Similar results were obtained even when the mutant selection process was carried out in the presence of α -glycyrrhetic acid, a long-term inhibitor of GJIC, suggesting that the observed effect is not due to unwanted killing of cells by GJIC-mediated metabolic cooperation. Thus, our data demonstrate that HeLa cells transfected with the *Cx43* gene become more resistant to spontaneous as well as chemically induced genetic changes.

Introduction

Connexin genes code the proteins that are responsible for the formation of intercellular membrane channels, *i.e.*, gap junctions (1, 2). GJIC³ has been postulated to play an important role in cell homeostasis by making possible exchange between cells of cytoplasmic compounds of low molecular weight, such as metabolites, ions, and second messengers of signal transduction pathways. GJIC allows tissues to respond appropriately, to eliminate waste products, normalize catabolism, and decrease the effects of xenobiotics (3–5).

Accumulated evidence indicates that aberrant control of GJIC is involved at certain stages of carcinogenesis (reviewed in Refs. 6–8). Thus, many tumor-promoting agents inhibit GJIC, and most tumors and transformed cells show lower levels of GJIC. More recent studies have shown that growth of tumor cells can be suppressed by transfection of connexin genes, establishing connexins as a family of tumor suppressor genes. For example, after transfection of the *Cx43* gene into rat glioma C6 and into chemically transformed mouse 10T^{1/2} cells, their growth was inhibited (9, 10).

HeLa cells do not exhibit detectable GJIC, as measured by dye-

transfer assay (11), nor do they contain detectable levels of mRNA for various connexins, including *Cx26*, *Cx32*, *Cx40*, or *Cx43* (12). However, GJIC of HeLa cells can be restored by transfection of various connexin genes (12).

Genomic instability is considered to be induced during carcinogenesis and facilitates the accumulation of multiple genetic changes in a given cell. Thus, genomic instability is found in most human cancers (13). Because aberrant GJIC is also found in most tumors and because the essential role of GJIC is the maintenance of global homeostasis, it is conceivable that GJIC itself plays a role in maintaining genomic stability in multicellular organisms. In the present study, we have tested this hypothesis using HeLa cells with or without transfected *Cx43* gene. We found that the expression of *Cx43* and resulting high level of GJIC were accompanied by lower frequencies at CA repeats and *HPRT* gene following exposure to methylating carcinogenic agents.

Materials and Methods

Cell Culture. The preparation of GJIC-proficient HeLa cells has been described earlier (14). The transfection was performed with a DNA construct containing coding DNA of murine *Cx43* under control of the SV40 early promoter in the pBEHpac18 vector. Both *Cx43*⁻ and *Cx43*⁺ HeLa cells were cultured in DMEM (Life Technologies, Inc.), complemented with L-glutamine, penicillin-streptomycin (Life Technologies, Inc.), and 10% FCS (Organics, Ltd., Yavue, Israel). Cell cultures were maintained in a 37°C incubator under a humidified 5% CO₂ atmosphere and were routinely subcultured by trypsinization with a change of medium twice weekly.

Cell Survival. Exponentially growing cells were seeded in the appropriate growth medium in 60-mm Petri dishes with 1000 cells/dish. After 12 h, cells were treated for 40 min at 37°C with various concentrations of MNNG. The culture medium was then removed, and the cells were rinsed with Earles' solution and fed with the culture medium. After 10–14 days, surviving colonies were fixed with methanol, stained with Giemsa, and counted. Three dishes were counted for each concentration of MNNG.

Mutagenicity Assay at CA Repeat. The pCAR2 vector (15) containing two copies of a (CA)₁₄ insert within the coding sequences of β -galactosidase genes, was provided by Dr. B. Vogelstein (Oncology Center, Johns Hopkins University, Baltimore, MD). The vector contained the EBV origin of replication and a hygromycin-resistant element. Insertions or deletions in the CA repeat could result in restoration of the reading frame, restoring β -galactosidase activity. The plasmid was transfected into *Cx43*⁻ and *Cx43*⁺ HeLa cells using lipofectin, and after growth for 17–21 days in a medium with hygromycin, resistant cells were used for treatment with carcinogens.

For analysis of CA repeat mutations, 8×10^5 cells were seeded and cultured for 12 h in 60-mm Petri dishes. Treatment with MNNG or MNU was continued for 30 min. The cells were then briefly washed with Earles' solution and cultured for 3–4 days in DMEM as described above.

DNA was purified from Hirt supernatant and used to transform Epicurian Coli XL2-blue MRF⁺ (Stratagene) ultracompetent cells by using the Stratagene instructions. Transformed cells were spread onto L-agar containing isopropyl- β -D-thiogalactopyranoside (6 μ g/ml), 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (20 μ g/ml), and ampicillin (10 μ g/ml), and the numbers of blue colonies (resulting from α -complementation) and total colonies were counted. The difference between the data obtained for *Cx43*⁻ and *Cx43*⁺ HeLa cells was statistically examined by Poisson regression analysis.

Selection of *HPRT* Mutants Induced by MNNG. Briefly, one million cells were seeded in 100-mm Petri dishes. After 12–16 h, they were exposed

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² To whom requests for reprints should be addressed.

³ The abbreviations used are: GJIC, gap-junctional intercellular communication; Cx, connexin; 6-TG, 6-thioguanine; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MNU, methylnitrosourea.

to various concentrations of MNNG (0–0.1 μM) for 40 min. As negative controls, the same amount of DMSO used as a solvent was added instead of chemicals. After treatment, the medium containing chemicals was removed, and the cells were washed twice with Earles' solution and fed with DMEM. For the expression of mutations, the exposed cells were kept in culture for 8 days, with subculturing every 2–3 days. For mutant selection, each culture of exposed cells was plated on 100-mm Petri dishes (3×10^5 cells/dish) and fed with complete medium containing 5 $\mu\text{g/ml}$ freshly prepared 6-TG. Clone-forming ability was measured at the same time by plating 3000 cells in 100-mm Petri dishes with complete medium but no 6-TG. After 10 days of growth, the mutant colonies were fixed, stained, and counted. The mutation frequency was determined by dividing the number of resistant colonies by the total number of cells plated, corrected for the clone-forming ability.

Data on *HPRT* gene mutations were analyzed by ANOVA by using the Student-Newman-Keuls criterion.

Results

Survival of Cells. Curves of cell survival in relation to carcinogen concentration are shown in Fig. 1. Both MNNG and MNU are methylating agents that produce *O*⁶-methylguanine. Corresponding to the known lack of *O*⁶-alkylguanine-DNA alkyltransferase (*Mex*⁻ status) of HeLa cells, a quite low concentration of these carcinogens is necessary to produce a maximal toxic effect. The survival of both types of cells (*Cx43*⁻ and *Cx43*⁺) was the same at the beginning of the curves. At higher doses, however, the tolerance of *Cx43*⁺ cells was greater than that of *Cx43*⁻ cells, for both MNNG and MNU.

Frequency of Mutation of CA Repeats. The frequency of mutations at the CA microsatellite was determined by a shuttle vector that was exposed to carcinogens in HeLa cells and produced blue colonies in *Escherichia coli* in the case of alterations restoring the transcriptional frame of the β -galactosidase gene (15). Cell lines manifesting instability at (CA)_n and other simple repeats are considered to show replication error phenotypes (RER⁺), and such cell lines have often been found to be deficient in mismatch repair capacity (15). HeLa cells are known not to be mismatch repair deficient. Without treatment

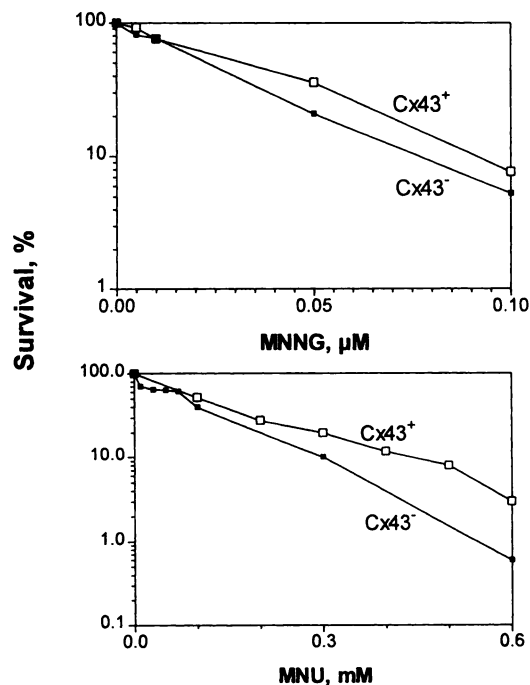


Fig. 1. Cytotoxicity of *Cx43*⁺ and *Cx43*⁻ HeLa cells in response to carcinogens. Cells were treated as described in "Materials and Methods" at the indicated concentrations of MNNG or MNU. The percentage of surviving cells was calculated based on the number of colonies formed.

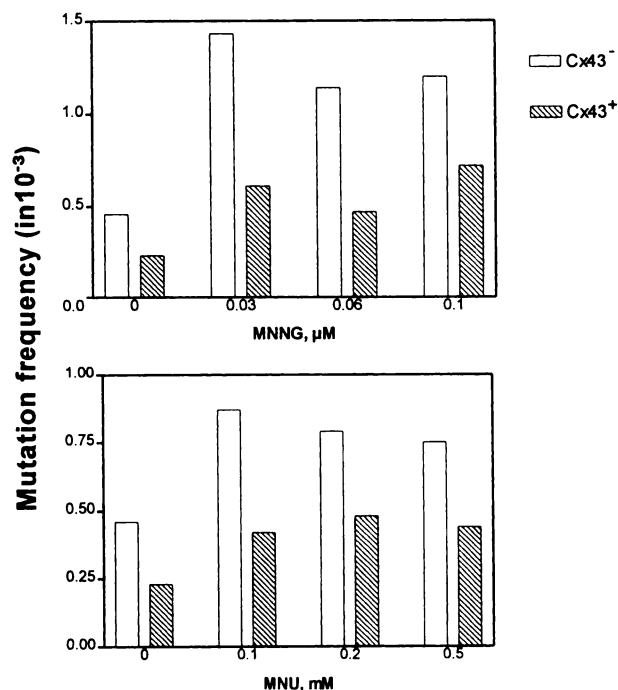


Fig. 2. Mutation frequency at CA repeats. Cells containing a shuttle vector with a CA repeat in the β -galactosidase gene were treated with carcinogens. Mutation frequency was calculated as frequency of blue colonies after transformation of *E. coli* as described in "Materials and Methods."

with carcinogens, the mutation frequency (ratio of blue colonies to total number) was 4.6×10^{-4} for *Cx43*⁻ HeLa cells and 2.3×10^{-4} for *Cx43*⁺ HeLa⁻ (Fig. 2). Treatment with 0.03 μM of MNNG increased the mutation frequencies to 14.3×10^{-4} and 6.1×10^{-4} for these cells, respectively. Treatment with two higher concentrations of MNNG did not greatly change these mutation frequencies of *Cx43*⁻ and *Cx43*⁺ HeLa cells (Fig. 2).

The results were very similar when cells were treated with MNU (Fig. 2). At a concentration of 0.1 mM, this carcinogen gave a mutation frequency of 8.7×10^{-4} for *Cx43*⁻ HeLa cells and 4.2×10^{-4} for *Cx43*⁺. Again, the ratio between these two types of cells did not greatly change with higher concentrations of MNU used.

The difference between *Cx43*⁻ and *Cx43*⁺ HeLa cells in their levels of alterations at CA repeats was statistically significant, either with or without exposure to mutagens ($P < 0.05$).

***HPRT* Mutation Frequency.** This was measured as the frequency of clones resistant to 6-TG after treatment of cells with MNNG, an agent that produces predominantly G \rightarrow A transitions due to formation of *O*⁶-methylguanine adducts (16). This kind of mutation might be quite distinct from alterations at CA repeats that are due to a slippage error of replication.

The frequency of spontaneous mutations was $(0.73 \pm 0.13) \times 10^{-6}$ for *Cx43*⁻ HeLa cells and $(0.39 \pm 0.06) \times 10^{-6}$ for *Cx43*⁺ HeLa cells (Fig. 3). After treatment with 0.05 μM MNNG, the mutation frequencies were increased to $(1.80 \pm 0.23) \times 10^{-6}$ and $(0.53 \pm 0.09) \times 10^{-6}$, respectively ($P < 0.001$). The difference in mutation frequency between the two types of cells was also statistically significant when the MNNG concentration was 0.1 μM . These results indicate that *Cx43*⁺ cells have a lower *HPRT* mutation frequency.

The selection of mutants in the presence of 6-TG is possible because wild-type *HPRT* phosphorylates it to form the toxic 6-TG monophosphate. In the presence of GJIC, 6-TG monophosphate might be transferred from one cell to another (metabolic cooperation), killing not only wild-type cells but also mutant cells. To examine whether the decreased *HPRT* mutation frequency observed in *Cx43*⁺ cells was

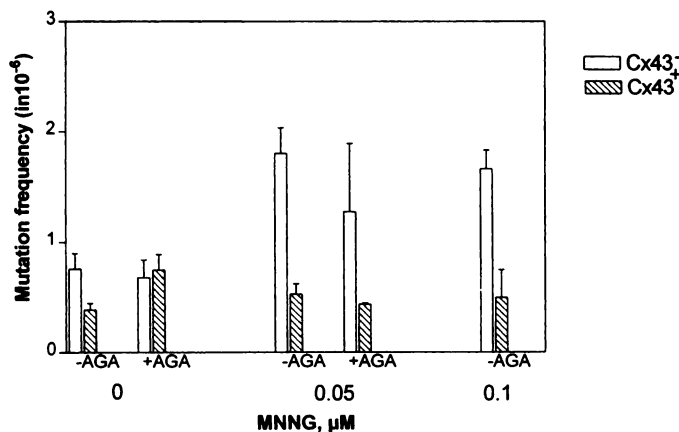


Fig. 3. Mutation frequency at the *HPRT* gene. Mutants were selected by their resistance to 6-TG in the absence or presence of 70 μM α-glycyrrhetic acid.

due to such an effect, we compared the mutation frequency at the *HPRT* gene in the presence of 70 μM α-glycyrrhetic acid, which inhibits GJIC (17). We found no statistically significant increase in the yield of 6-TG-resistant cells in the presence of α-glycyrrhetic (Fig. 3). Thus, the density of cells during the selection was appropriate to avoid exchange of toxic metabolites, and the difference in mutation frequencies is due to the presence of Cx43 itself.

Discussion

In the present study, we used GJIC-proficient and -deficient HeLa cells to examine whether GJIC plays a role in the maintenance of genetic stability. Our results provide strong evidence that increased GJIC may result in genomic instability; HeLa cells transfected with Cx43 showed lower microsatellite instability and a lower *HPRT* mutation frequency than wild-type HeLa cells, both in the absence and in the presence of exogenous mutagens. Although microsatellite instability is believed to be induced by a defect in mismatch repair enzyme system (15), *HPRT* mutations can be induced by various mechanisms. It would be interesting to further examine whether any types of mutations can universally be affected by this type of cell-cell communication.

Connexin genes seem to form a new family of tumor suppressor genes. Thus, transfection of connexin genes into various types of tumorigenic cells leads to suppression of their growth *in vitro* and/or *in vivo* (9, 10, 18, 19). Cx26 but not Cx32, Cx40, or Cx43, suppresses the growth of HeLa cells, presumably reflecting the fact that cervical tissue expresses Cx26 as a major connexin; the HeLa cell line was established from a cervical tumor (14). Thus, the Cx43⁺ HeLa cells used in this study represent GJIC-proficient but still tumorigenic cells. We avoided using growth-suppressed HeLa cells (*i.e.*, those containing the *Cx26* gene), because the difference in their growth rate compared with wild-type HeLa cells would make the interpretation of our comparative mutation studies difficult. We conclude, therefore, that the genomic stabilization we observed in Cx43⁺ HeLa cells is not related to a growth control effect of connexins but rather to GJIC *per se*.

Our results also show that Cx43⁺ HeLa cells are more resistant than Cx43⁻ HeLa cells to the cytotoxic effect exerted by MNNNG and MNU. In such toxicity assays, cells are sparsely seeded (*e.g.*, 1000 cells in 60-mm plates) so that they are not in contact and thus not able to form GJIC with each other. Therefore, the apparent protective effect of Cx43 against toxicity to HeLa cells must be due to an action of Cx43 that is not directly related to GJIC. It is not known whether connexins can exert biological effects directly without forming gap

junctions. However, this idea is compatible with the fact that Cx26-transfected HeLa cells grow much less in soft agar than the parental HeLa cells (14). Because single cells are seeded into soft agar, this effect must be due to a GJIC-independent effect of Cx26. It has been suggested recently that certain connexins may indeed operate directly in the process of signal transduction by binding to DNA (20).

GJIC has long been known to reduce the yield of mutants because of the phenomenon called "metabolic cooperation during the mutant selection process" (21). Two lines of evidence suggest that the decrease in mutation frequency induced by Cx43 is not due to such an effect: (a) we have seen the effect of Cx43 in reducing microsatellite instability, for which no metabolic cooperation can occur; and (b) when a potent and long-term inhibitor of GJIC, α-glycyrrhetic acid, was added during the mutant selection process, no increase of mutant yield was observed.

When cells are connected via GJIC, they are considered to be in homeostasis, keeping their neighboring cells in check. Thus, many of their cellular functions are under control. Our results on the protective effect of Cx43 on genetic stability are in line with this concept. However, how such homeostasis is maintained and whether and how it stabilizes genetic integrity await further studies.

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