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Bystander effect from cytosine deaminase and uracil phosphoribosyl transferase genes *in vitro*: A partial contribution of gap junctions

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ABSTRACT

Among gene therapy strategies elaborated to kill cancer cells, one uses the *CodA* gene, coding for cytosine deaminase (CD) that converts 5-fluorocytosine (5-FC) into toxic 5-fluorouracil (5-FU). To enhance 5-FC metabolic activation, we prepared a vector carrying *CodA* and *upp* (uracil phosphoribosyl transferase) genes which rendered HeLa cells sensitive to 5-FC and enhanced a bystander effect not mediated by gap junctions. However, 1% CD^+–UPP^+ cells were able to kill 40% of the cell population if the cells were communicating. This suggests that, at very low percentages of CD^+–UPP^+ cells, *CodA* and *upp* induce a bystander effect through gap junction-dependent mechanisms.

1. Introduction

Ways to suppress tumor cells selectively have been investigated with the goal of avoiding the systemic administration of chemotherapeutic treatments to cancer patients. One such approach is the suicide gene therapy in which a gene that can convert an inactive prodrug into a toxic drug is introduced into the tumor cells. One model uses the *CodA* gene that codes for cytosine deaminase (CD) capable of converting 5-fluorocytosine (5-FC) into the chemotherapeutic agent, 5-fluorouracil (5-FU). The transfer of the *CodA* gene was shown to induce lethal sensitivity to 5-fluorocytosine in mammalian cells [1], and this raised the possibility of using such a gene transfer strategy to kill tumor cells selectively *in vivo* [2]. Moreover, the killing of cells expressing the *CodA* gene (CD^+ cells) by 5-FC treatments has been reported to be accompanied by the elimination of neighboring tumor cells that did not express the gene [3–5]. This bystander effect led to a significant elimination of tumor cells even when no more than 10% of CD^+ cells were present [3,6]. From a therapeutic point of view, it is crucial to enhance this phenomenon for compensating the low level of gene transfer which prevents gene therapy to be efficient. Rendering the target cells more sensitive to the 5-FC treatment, in order to increase the bystander effect, by enhancing the metabolism of 5-FU can be accomplished by co-expressing the *CodA* and *upp* genes [7]. In the present study, we have investigated

Abbreviations: CD, cytosine deaminase; CodA, cytosine deaminase gene; Cx, connexin; 5-FC, 5-fluorocytosine; 5-FU, 5-fluorouracil; GJIC, gap junctional intercellular communication; upp, uracil phosphoribosyl transferase.

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the effect of the dual expression of *CodA* and *upp* genes on the sensitivity of HeLa cells to 5-FC treatment. Our principal goal was to estimate the extent of the induced bystander effect and determine whether it was related or not to the presence of gap junctions. To do so, HeLa cells, which are relatively resistant to the CD/5-FC combination, were transfected with a vector carrying both the *CodA* and *upp* genes. The cells expressing these two genes (CD^+–UPP^+) were then more sensitive to the 5-FC prodrug than cells expressing the *CodA* gene alone. There was also a more efficient bystander effect that led to a complete elimination of a whole cell population within 1 week of 5-FC treatment, even if 5% of the cells were CD^+–UPP^+. Moreover, the use of HeLa cells transfected or not with a cDNA coding for a gap junction protein (connexin43; Cx43) has shown that gap junctions did not appear to be involved in this bystander effect. However the role of gap junction became obvious when only 1% of the cells were CD^+–UPP^+. This result reinforces the idea that gap junctional intercellular communication (GJIC) may be a parameter to consider especially if very few cells do express the *CodA* and *upp* genes.

2. Materials and methods

2.1. Cell lines

The parental HeLa cells we used in this study (lacking or transfected with the cDNA coding for the gap junction protein Cx43) were obtained from K. Willecke (Bonn, Germany). Their culture conditions have been described previously [8,9]. Cells transfected with the *CodA* gene without (pUT816 expression vector; CAYLA, Toulouse, France) or with the *upp* gene (pZEO.SG7 expression vector, CAYLA) were obtained by selection with zeocin treatment (100 μg/ml). The transfection process has been described previously [10]. The function of the transfected *CodA* and *upp* genes was examined by assessing the sensitivity of the cells to 5-FC treatment (St Louis, USA). Only the clones that were most sensitive to 5-FC were kept and analysed for the bystander effect.

2.2. Cloning efficiency

Cells expressing the *CodA* gene with or without the *upp* gene or the parental HeLa cells (expressing or not Cx43) were seeded into 60-mm dishes (500 cells per dish). The cultures were treated, while cells were seeded, with various concentrations of 5-FC, in triplicate. Two weeks later, cells were fixed with methanol, stained with GIEMSA, and colonies were counted.

2.3. Estimation of the bystander effect

Cells expressing the *CodA* gene with or without the *upp* gene (CD^+–UPP^+ cells or CD^+ cells) were mixed in various ratios (1, 5, 10 or 50%) with their non-transfected counterparts (CD^-–UPP^- cells or CD^- cells). For communicating Cx43-expressing HeLa cells (Cx^+), mixtures were made between Cx^+/CD^+–UPP^+ and Cx^+/CD^-–UPP^- cells or between Cx^+/CD^+ and Cx^+/CD^- cells. The cells were seeded in 96-well plates (10^4 cells/well) and treated with 5-FC (250 μg/ml). For each experiment, two plates were stopped at different days.

The extent of 5-FC toxicity was estimated by neutral red uptake. The medium was replaced with culture medium containing neutral red (50 μg/ml; Sigma). Plates were incubated for 3 h at 37 °C. The absorbance of the neutral red was read at 550 nm on a Titertek Multiskan Plus MKII linked to a Victor V286C computer. The results are presented as the percentages of untreated cultures. Each point is the average of three independent experiments, and two plates were tested per experiment.

The importance of cell–cell contact on the 5-FC toxicity, was estimated by coculturing CD^-–UPP^- and CD^+–UPP^+ cells in the same dish and preventing contact between the two cell populations. 2×10^5 CD^-–UPP^- cells were seeded into an insert inside a dish containing the same number of CD^+–UPP^+ cells. One day after seeding, the cocultures were treated with 5-FC (250 μg/ml), and medium was changed twice a week. Two weeks later, cultures were fixed, stained, and photographed.

3. Results

3.1. Increased sensitivity to 5-FC due to co-expression of the CodA and upp genes in HeLa cells

In order to increase the intracellular metabolism of 5-FU, we transfected HeLa cells with a vector (pZEO.SG7) carrying both the *CodA* and *upp* cDNAs. Cloning efficiency indicated that the co-expression of *CodA* and *upp* genes made the cells ten times more sensitive to 5-FC (250 μg/ml) than HeLa cells expressing the *CodA* gene alone (pUT816 vector) (Fig. 1). The expression of *upp* gene enhanced the sensitivity to 5-FC, pre-

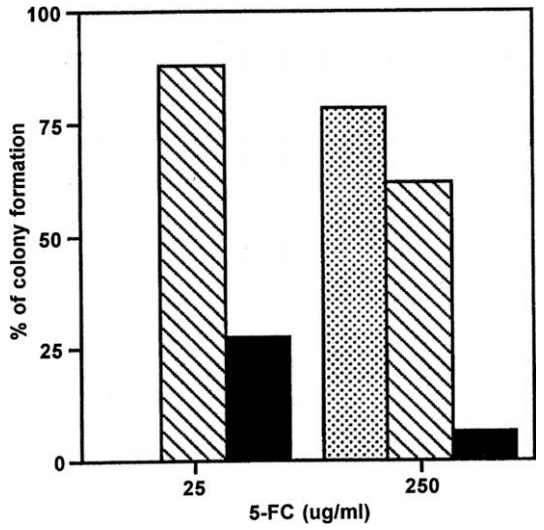

Fig. 1. Differential sensitivity to 5-FC of HeLa cells expressing CD alone or CD and UPP. Five hundred HeLa cells were seeded and treated immediately with 5-FC (25 or 250 μg/ml). Ten days later the cultures were fixed, stained and the number of colonies was estimated. The results are presented as the percentage of colony formation after treatment compared to untreated cultures. CD^-–UPP^- cells (▨); CD^+–UPP^- cells (▩); CD^+–UPP^+ cells (■). The treatment of CD^-–UPP^- cells with 25 μg/ml 5-FC had no effect on cloning efficiency (result not shown). All experiments were performed in triplicate. Standard errors were similar and too low to be shown.