Novel Method for Selective Killing of Transformed Rodent Cells through Intercellular Communication, with Possible Therapeutic Applications

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ABSTRACT

A novel method for killing transformed cells selectively, without affecting surrounding nontransformed cells, has been developed. The method is based on our finding that transformed cells form their own gap-junctional communication compartment which is independent of that of adjacent nontransformed cells; transformed cells and adjacent normal cells transfer molecules through gap junctions among their homologous cells, but there is no heterologous transfer. Thus, when Lucifer Yellow CH is microinjected into transformed cells, it spreads only among the transformed cells and not to surrounding nontransformed cells. Subsequent irradiation of cells with blue light (around 430 nm) kills only those cells containing Lucifer Yellow CH (i.e., transformed cells), and surrounding normal cells continue to grow after treatment. We succeeded in killing BALB/c 3T3 transformed foci induced in situ by a chemical carcinogen, and surrounding normal cells continue to grow after treatment. We succeeded in killing BALB/c 3T3 transformed foci induced in situ by a chemical carcinogen, and surrounding normal cells continue to grow after treatment. We succeeded in killing BALB/c 3T3 transformed foci induced in situ by a chemical carcinogen, and surrounding normal cells continue to grow after treatment.

INTRODUCTION

Gap-junctional communication is the only known means by which living cells freely and directly interchange substances of low molecular weight ($M_r < 1000$) from the interior of one cell to that of adjacent cells (1, 2). Thus, this form of communication is considered to play an important role in the control of both tissue homeostasis and cell proliferation and differentiation (1, 2). Since cancer cells show aberrant control of cell proliferation and differentiation in comparison to surrounding normal cells, it is likely that the intercellular communication control mechanism is altered during multistage carcinogenesis. Results from recent studies suggest that the block of gap-junctional communication may play an important role in the clonal expansion of potential tumor cells during tumor promotion (3, 4).

The comparison of tumor cells and normal cells in terms of their ability for gap-junctional communication has not resulted in a clear picture; some tumors show decreased gap-junctions and/or gap-juncti onal communication, while others retain a level similar to that of their normal counterparts (1, 5–9). Therefore, the original hypothesis that tumor cells may be characterized by decreased gap-junctio nal communication (1, 5) is not generally proven. Recently, we proposed an alternative hypothesis: the degree of communication among tumor cells may not be a primary determinant for maintenance of their transformed phenotypes, but, more important, tumor cells do not communicate with surrounding normal cells; i.e., there is selective cell-cell communication (10, 11).

We demonstrated previously that, when transformed foci of BALB/c 3T3 cells are induced in vitro by a carcinogen, the cells indeed show a selective communication capacity; transformed cells show a normal level of intercellular communication among themselves but do not communicate with surrounding normal cells (10). Such selective communication was seen with BALB/c 3T3 cells transformed by a variety of chemical carcinogens, by ultraviolet light, or by transfection of an activated human oncogene, pEJ-ras-H-1 (Ref. 12; Footnote 3). Selective communication was also observed with transformed and nontransformed rat liver epithelial cells in coculture. These results indicate that transformed cells have a gap-junctional communication compartment independent of that of surrounding normal cells. We proposed that due to this selective communication pattern, transformed cells can maintain their phenotypes since there would be no transfer of growth-regulatory factors from surrounding normal cells through gap junctions (10, 11).

These findings provided us the theoretical basis for supposing that, when a toxic substance—gap junction permeable but not cell membrane diffusible—is microinjected into a transformed cell, it would spread only among transformed cells but not to surrounding normal cells, resulting in selective killing of transformed cells. Here, we report selective killing of transformed cells by microinjection of Lucifer Yellow CH followed by blue-light irradiation, taking advantage of the finding that Lucifer Yellow CH can kill cells when activated by blue light at around 430 nm (13). We also report that the communication capacity of transformed cells can be increased by dibutyryl cAMP and they can thus be killed more efficiently. Possible extension of this finding to cancer therapy is suggested.

MATERIALS AND METHODS

Materials. Lucifer Yellow CH and MCA were purchased from Sigma Chemical Co. (St. Louis, MO). pEJ-ras (14) (a pBR322-derived plasmid containing a 6.6-kilobase human DNA fragment encompassing the entire activated human Ha-ras oncogene cloned into the vector's BamHI site) was a kind gift of Dr. R. Newbold. Chemical Transformation. BALB/c 3T3 A31-1-1-cell s, obtained from Dr. T. Kakunaga (15), were cultured in Eagle's minimal essential medium supplemented with 10% fetal calf serum. MCA (3 /µg/ml) was added to cultures seeded the previous day at 1 x 10⁴ cells per 60-mm Falcon plastic dish. After 72-h treatment, MCA was removed by replacement with fresh medium. The culture medium was replaced twice a week thereafter. Morphologically transformed cells (16) started to appear 2 to 3 wk after the end of MCA treatment.

DNA Transfection of Human H-ras Gene. Confluent BALB/c 3T3 cultures were transfected with pEJ-ras DNA by the calcium phosphate method, essentially as described by Graham and van der Eb (17). Cells were exposed to purified plasmid EJ-ras DNA (0.3 /µg/plate) and diluted in a transfection solution of phosphate buffer (280 mM NaCl, 1.5 mM Na₂HPO₄·25 mM HEPES, pH 7.12) and calcium chloride (1.25 mM CaCl₂·125 mM HEPES, pH 7.12) containing calf thymus carrier DNA (4 /µg/plate). After 6 h of this treatment, the cultures were rinsed twice with serum-free minimal essential medium and then placed in medium supplemented with 10% fetal calf serum. The medium was changed twice a week until transformed foci appeared (2 to 3 wk).

Rat Liver Epithelial Cell Lines. Epithelial cell lines used in this study, IAR 20 and IAR 6-1, were isolated from livers of BD IV rats (18, 19).

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The abbreviations used are: cAMP, cyclic AMP; MCA, 20-methylcholangan-threne; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

3 Unpublished results.
4 Unpublished observation.
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and IAR 6-1 cells were transformed in vitro by N-nitrosodimethylamine as described previously, while IAR 20 is a nontumorigenic cell line (18, 19). Cells were cultured in William’s E medium supplemented with 10% fetal calf serum and L-glutamine, penicillin, and streptomycin (100 units/ml), in a 5% CO2 incubator at 37°C.

Killing of Transformed Cells by Microinjection of Lucifer Yellow CH Solution and Subsequent Blue Light Irradiation. For selective killing of transformed cells, a solution of 10% Lucifer Yellow CH solution in 0.33 M lithium chloride was first microinjected into transformed cells, as described previously (20). Then, 10 to 30 individual cells in a BALB/c 3T3 transformed focus or in the area of transformed rat liver epithelial cells (IAR 6-1) were microinjected. The culture dish was returned to a CO2 incubator for 2 h. The Petri dish was then exposed to blue light under an Olympus IMT2 fluorescence inverted microscope through 4× objective lens for 10 min; the diameter of the light exposure field was 5 to 6 mm. We used a mercury lamp (OSRAM HBO 100 W/2) as the light source and a dichroic mirror unit (IMT-DMB) provided for standard B excitation by Olympus Optical Co., Ltd., Tokyo, Japan. This unit consists of BP490 (excitation), DM500 (dichroic), and AFCad 0515 (barrier) filters; the major spectral lines transmitted are at 435.8, 434.8, and 404.7 nm; and these three lines are within the absorption spectral band of Lucifer Yellow CH molecules; the maximum absorption occurs at around 430 nm. There are also a weak line at 433.9 nm and continuous spectral regions near 490 nm.

RESULTS

Selective Killing of Transformed BALB/c 3T3 Foci. We chose to use BALB/c 3T3 cells, since in this system transformed cells are produced in situ over normal monolayer cells, stimulating carcinogenesis in vivo (21). Fig. 1 shows MCA-transformed foci before and after killing by microinjection of Lucifer Yellow CH and blue-light irradiation. The combination of our filters allows transmission of blue light in the wavelength range of 400 to 440 nm. It is clear from the figure that only transformed cells were killed by blue-light irradiation; when surrounding normal cells were microinjected with Lucifer Yellow and exposed to blue light, only these and not adjacent transformed cells were killed (not shown). The irradiation time of 10 min was chosen, since the time course of selective killing showed that 72% and 90% of Lucifer Yellow-containing cells were killed after 5-min and 10-min irradiation, respectively, whereas those cells without Lucifer Yellow molecules were not killed by 10-min irradiation. Longer exposure, however, also killed the control cells: 34%, 69%, and 100% after 15-, 20-, and 30-min exposure, respectively.

Recent studies suggest that activation of cellular oncogenes is the genetic basis of carcinogenesis (22, 23). We considered it important to investigate whether oncogene-transformed cells could also be selectively killed by our method. Thus, we transfected BALB/c 3T3 cells with an activated oncogene isolated from a human bladder carcinoma, pEJ-ras-H. When Lucifer Yellow CH was microinjected into these transformed cells, they could again be selectively killed by blue-light irradiation (Fig. 2). When many transformed cells in a focus were killed, the central part of the focus disappeared, since the cells were no longer able to adhere to the culture dish (Fig. 2). Cell death was usually examined by trypan-blue staining, but was confirmed by microscopic observation to detect any residual proliferative capacity. Many dead cells were raised above the surface of the dish, although the foci peripheries remained attached.

Selective Killing of Transformed Epithelial Cells. In order to extend our method to epithelial cells, we used transformed and nontransformed rat liver epithelial cells from a line established by Montesano et al. (18, 19). Since there is no method for inducing transformed epithelial cell foci by carcinogens in situ, we artificially cocultured transformed and nontransformed rat liver epithelial cells so that the two types of cell were in contact. When transformed cells (IAR 6-1) were cocultured with nontransformed cells (IAR 20), the border between the two types was clearly visible, because of the difference in their morphological appearance (Fig. 3). Microinjected Lucifer Yellow CH again spread selectively, and, following exposure to blue light, the targeted cells were killed (Fig. 3).

Relationship between Intercellular Communication Capacity and Killing Efficiency. The efficiency of such selective killing is directly dependent on the communication capacity of the target cells. Thus, the greater the number of cells into which Lucifer

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Fig. 1. Selective elimination of a focus of BALB/c 3T3 cells transformed by MCA. Twenty to 30 cells in the focus presented here were microinjected with Lucifer Yellow CH solution followed by blue light irradiation, as described in “Materials and Methods.” A, phase-contrast micrograph of a transformed focus on the monolayer; B, trypan-blue staining of the same focus after Lucifer Yellow CH microinjection and blue-light irradiation. Note that only the transformed focus is stained; C and D, phase-contrast micrographs of the focus 24 and 48 h after incubation following B, respectively. Note that many cells in the transformed focus are dead and raised above the surface of the Petri dish, while surrounding nontransformed cells look healthy. Elimination of 4 other transformed foci was confirmed. Selective killing of the major part of large foci was accomplished with more than 10 independent foci.
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Yellow CH spread, the more cells that were killed by blue-light irradiation (Fig. 4). After microinjection of Lucifer Yellow CH into a single cell, BALB/c 3T3 cells typically show 25 to 35 fluorescent surrounding cells (20), whereas rat liver epithelial cells have a greater communication capacity (50 to 100 cells) (9), and the number of killed cells per injection is higher.

We also increased killing efficiency by stimulating gap-junctional communication with an exogenous compound, dibutyryl cAMP (Fig. 4). When we added dibutyryl cAMP (1 mM) to a culture dish in which transformed foci of BALB/c 3T3 cells were present, we observed stimulation of intercellular communication of cells in both transformed foci and the nontransformed monolayer, but the two types of cell still did not cross-communicate for at least 24 h. Selective killing of transformed cells was also more efficient in these dishes. It is also interesting to note that longer treatment with dibutyryl cAMP for more than 5 days resulted in elimination of selective intercellular communication, and transformed phenotypes started to disappear. 

DISCUSSION

We report here that transformed cells can be killed by microinjection of a cytotoxic or potentially cytotoxic substance; a single injection can kill many neighboring tumor cells, since the substance spreads through gap junctions. In this study, we successfully used Lucifer Yellow microinjection followed by irradiation with blue light. In this case, cell killing was probably due to strong heat locally generated as the result of activation of Lucifer Yellow molecules by irradiation (13). In theory, many chemicals could be used in this newly developed method of selective killing, provided that they can pass through gap junctions and that they do not diffuse through membranes. Membrane-diffusible compounds and compounds that are metabolized to membrane-diffusible species kill cells other than the targeted cell population, since they can diffuse freely into surrounding nontarget cells. Currently available cancer chemotherapeutic agents are designed to penetrate easily into cells by membrane diffusion; their side effects in cancer patients result from the fact that these agents penetrate both malignant and normal cells. The method described here uses only chemicals that do not penetrate cells through membrane diffusion, and selective blockage of gap-junctional communication between tumor cells and normal cells would ensure protection of the normal cells from toxic effects.

Our results have been obtained in vitro. A number of problems must be resolved before our method can be extended to the killing of tumorigenic cells in vivo. An important question is whether tumor cells show selective intercellular communication in vivo. Furthermore, since cells in certain solid tumor types probably have diminished communication capacity (1, 5), it would be important to devise treatments that increase junctional communication, such as use of cAMP as demonstrated by us in vitro, in order to amplify selective cell killing.

The method developed here requires microinjection of compounds into target tumor cells. Since many of the fatal human cancers occur in internal organs, the target cells may not be accessible to microinjection. It is important, however, to emphasize that gap-junctional communication has been measured using microelectrodes in tissue slices of rat liver (24) and mouse skin (25). It would also be possible to combine our method with the recently developed chemotherapeutic method of monoclonal antibody targeting of tumors (26–28). If a cytotoxic compound that is non-membrane diffusible is coupled to a specific antibody and brought to tumors by antibody recognition, possibly with the aid of liposomes (29) (thus avoiding the need for microinjection), the therapeutic effect may be enhanced greatly via specific spread throughout the tumor communication compartment. Although possible applications to chemotherapy are still at the hypothetical stage, the method is effective for killing tumor cells in vitro. We believe this to be an important new approach.
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REFERENCES

7. Fentiman, I. S., Hurst, J., Ceriani, R. L., and Taylor-Papadimitriou, J.
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