Characterization of a Human Teratocarcinoma Cell Assay for Inhibitors of Metabolic Cooperation

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ABSTRACT

Although a Chinese hamster V79 cell-based assay for inhibitors of metabolic cooperation is currently available, the development of a human cell-based assay is desirable in order to avoid inappropriate extrapolation from animal cells to human cells.

Cells derived from a human teratocarcinoma cell line (designated PA-1), which has a stable pseudodiploid karyotype and excellent in vitro growth properties, were used in a metabolic cooperation assay. The assay was based on the metabolic isolation of hypoxanthine-guanine phosphoribosyltransferase (HGPRT)-deficient variants in the presence of HGPRT-proficient cells and 6-thioguanine. Chemicals which inhibit the transfer of the lethal metabolite of 6-thioguanine from HGPRT-proficient to HGPRT-deficient cells will allow for recovery of the 6-thioguanine-resistant (HGPRT-deficient) cells.

Chemicals tested included 12-O-tetradecanoylphorbol-13-acetate and related analogues phorbol-12,13-didecanoate, mezerein, and 4-phorbol-12,13-didecanoate. Concurring with results previously obtained in V79 cells, 12-O-tetradecanoylphorbol-13-acetate and phorbol-12,13-didecanoate strongly inhibited metabolic cooperation, whereas mezerein was moderately inhibitory and 4α-phorbol-12,13-didecanoate was inactive. These cells thus hold promise as a human cell-based assay for inhibitors of metabolic cooperation.

INTRODUCTION

Gap junction mediated intercellular communication is an important regulatory process which has been implicated in cell growth and differentiation (1, 2), embryogenesis (3, 4), germ cell development (5-7), tissue repair processes (8, 9), and adult tissue homeostasis and function (10-12). Alterations in intercellular communication of this kind have been suggested to be involved in chemical carcinogenesis (specifically tumor promotion) (13-15), teratogenesis (16-18), reproductive dysfunction, and several other maladies (19).

The observations that the mouse skin tumor promoter TPA3 blocked metabolic cooperation in two distinct mammalian cell types (20, 21) led to the hypothesis that tumor promoters may act via their ability to isolate initiated cells from the physiological control provided by metabolic cooperation with normal cells and hence allow for the clonal expansion of an initiated cell to a larger target population in which additional mutagenic changes might take place (15).

Since the originally published observations in Chinese hamster V79 cells and mouse epidermal/3T3 cells, TPA has been shown to inhibit gap junctional communication in many other cell types, including human cells (22-29). These observations have recently been extended to include in vivo experiments in which a dose dependent decrease in the number of gap junctions in the epidermis of TPA treated mice was reported (30, 31). The inhibition of metabolic cooperation by many different classes of tumor promoters in a variety of cell types has now been well documented (32-48).

If indeed there is a relationship between inhibited cell-cell communication and various chemically induced disease states, the detection of these chemicals by a reliable easily conducted assay would be desirable. A Chinese hamster V79 cell-based assay for the detection of agents which inhibit metabolic cooperation has been developed for such a purpose (21, 43). This assay is based on the chemically induced isolation, recovery, and clonal growth of a few 6-TG resistant (HGPRT+) cells covaluated with many wild-type cells (HGPRT-+) in the presence of 6-TG. The number of recovered clones is inversely related to the amount of transfer of phosphorylated 6-TG (via gap junctions) from HGPRT+ to HGPRT- cells. Although in vitro-based assays may have the ability to detect potential tumor promoters, the effectiveness of these compounds in blocking cell-cell communication in vivo would of course depend upon many factors not easily controlled in tissue culture. Nonetheless, the use of these in vitro assays in screening for tumor promoters has its place in chemical risk assessment.

Since the effectiveness of many tumor promoters is species dependent, it would be desirable for purposes of human risk assessment to use human cells in an in vitro tumor promoter assay. However, most continuously growing human cell lines pose the problem of an unstable karyotype, which could possibly effect the physiological responsiveness of the cells.

We describe here the development of a human teratocarcinoma cell-based assay for inhibitors of metabolic cooperation and its responsiveness to TPA and three related analogues. The results show a good correlation between the activity of these compounds in vivo and their ability to inhibit metabolic cooperation in these human cells. Additionally, these cells undergo limited morphological differentiation in culture in response to some agents which are capable of inhibiting metabolic cooperation. Because of their excellent clonal growth properties, stable pseudodiploid karyotype, intercellular communication ability, responsiveness to known tumor promoters, and their in vitro differen-
tiation ability, these cells are an excellent candidate for a human cell-based assay for inhibitors of metabolic cooperation.

**MATERIALS AND METHODS**

**Chemicals.** 6-TG was obtained from Sigma Chemical Co., St. Louis, MO. TPA, MEZ, PDD, and 4α-PDD were obtained from Consolidated Midland Corp., Brewster, NY.

**Cells and Culture Methods.** The cells used in these studies (designated P3), were a gift from Dr. E. Huberman (Argonne National Laboratory) and were originally derived from PA-1 cells (49). These cells have a pseudodiploid karyotype [46,XX,t(15:20)] and grow without the aid of a feeder layer. A subclone of P3, designated HTP3-4, was derived from a single cell colony by the glass cylinder method of Ham and Puck (50). This clone had high colony forming ability and tight uniform colonies. Cells were routinely grown in modified Eagle's minimal medium with Earl's salts, with 50% increase in vitamin B and essential amino acids, except glutamine, 100% increase in nonessential amino acids, 1 mm sodium pyruvate, and 5% fetal bovine serum. Penicillin (E. I. Lilly, Indianapolis, IN) and streptomycin (Pfizer, Hoffman Estates, IL) were added to the medium at 100 units/ml and 100 μg/ml, respectively. The cells were incubated at 37°C in humidified air containing 5% CO2. Cells were checked for the presence of mycoplasma by the Hoechst 33258 method (51) and were free of contamination. Stocks of cells were stored frozen in 10% dimethyl sulfoxide in growth medium at -70°C and thawed as needed.

**Chromosome Analysis.** Karyotyping and chromosome analysis were kindly provided by the Michigan State University Cytogenetics Laboratory, under the direction of Dr. J. V. Higgins. Log-phase cultures of HTP3-4 cells were treated with Colcemid (0.5 μg/ml, final concentration) for 3 h. Cells were then trypsinized, treated with hypotonic (0.56%) KCl for 10 min, centrifuged, and the cells from the resulting pellet were resuspended in Carnoy's fixative. Cells were refrigerated in fixative overnight and prepared for Giemsa banding by the method of Seabright (52).

**Selection of 6-TG Resistant Variant.** HTP3-4 cells were irradiated in suspension with a 300-R dose using a Torex-150 X-ray machine operated at 150 kV and 5 mA. Cells were maintained in 75-cm² tissue culture flasks (Corning Glass Works, Corning, NY) in growth medium, and after a 10-day expression time (53), were replated into 100-mm dishes (Corning) containing 10 ml of 6-TG medium (10 μg/ml) at a cell density of approximately 10⁵ cells/cm². Ninety-six surviving colonies were screened for colony forming ability and colony morphology and six clones (designated HTXTG-1 through HTXTG-6) were selected from these for metabolic cooperation testing.

**Test of Metabolic Cooperation.** Two human 6-TG resistant cells from each of the six HTXTG lines were plated into separate 60-mm plates with either 0, 1, 2, 3, 4, or 5 × 10⁵ HTP3-4 cells. After a 4-h attachment period, 6-TG was added to the medium (10 μg/ml). Since the 6-TG resistant mutants are presumptive HGPRT−, they will only be killed by 6-TG if the lethal metabolite is transferred to them via gap junctions. The 6-TG resistant mutants are presumptive HGPRT−, they will only be killed by 6-TG, but not by HGPRT−. The background of HTXTG-1 cells escaping metabolic cooperation in the controls was of course higher with decreasing cell density, and thus the most effective cell density.

**RESULTS**

**Plating Efficiency and Chromosome Analysis.** The subclone HTP3-4 was found to have a plating efficiency of approximately 65%, and karyotype analysis (Fig. 1) revealed a chromosome pattern similar to the parental line, PA-1, from which these cells were derived (49).

**Test of Metabolic Cooperation between HTP3-4 and 6 HTXTG Variants.** Fig. 2 shows that the relative survival of all 6 HTXTG lines was progressively diminished as the number of wild-type cells was increased. HTXTG-1 was selected for further testing based on its cloning efficiency, colony morphology, and metabolic cooperation ability.

**Test of Metabolic Cooperation between HTXTG-1 and Other Human Cells.** The ability of HTXTG-1 teratocarcinoma cells to metabolically cooperate with normal human diploid fibroblasts (MSU-1), and a human epithelial bladder line (HCV-29) can be seen in Fig. 3. As is apparent from the figure, the teratocarcinoma cells communicate quite well with both cell types.

**Effect of TPA on Metabolic Cooperation between HTXTG-1 and HTP3-4.** TPA was effective in causing a dose dependent increase in the recovery of HTXTG-1 cells cocultivated with wild-type cells at a density of 3 × 10⁶ cells/60-mm plate in the presence of 6-TG (Fig. 4). This inhibition appeared to be maximal in the range of 0.75-1.0 ng/ml. Above these doses TPA began to be cytotoxic (data not shown). The morphology of surviving HTXTG-1 colonies was quite varied, with many colonies appearing whispy and faint, containing flattened cells with many processes. This was in contrast to the tight epithelial morphology of the few surviving control colonies (Fig. 5). This effect on cell morphology was not as apparent in the TPA treated cytotoxicity plates, suggesting that this was an effect related to cell density.

**Effect of TPA on Metabolic Cooperation between HTXTG-1 and HTP3-4.** One hundred HTXTG-1 and 3 × 10⁵ HTP3-4 cells were added to 60-mm plates in growth medium. After a 4-h attachment period, TPA was added at various concentrations in a vol of 10 μl. Two h after chemical treatment, 6-TG was added to the medium at 10 μg/ml. Three days later, treatment medium was replaced with fresh 6-TG-containing medium, and the cells were again refed on day 7. On day 10, colonies were rinsed, stained, and scored as above. Cytotoxicity plates containing 100 HTXTG-1 cells were treated in parallel with the metabolic cooperation plates. After 3 days in treatment medium, these plates were similarly given fresh selection medium and scored as above on day 10.

Cells were next tested for their responsiveness to TPA at different cell densities. Two hundred HTXTG-1 cells and either 0, 0.25, 1, and 3 × 10⁵ HTP3-4 cells were added per 60-mm plate, and after a 4-h attachment period TPA was added at various doses. After a 2-h exposure to TPA, 6-TG was added to the medium at 10 μg/ml. This treatment medium was replaced with selection medium on day 3 and the medium was changed again on day 7. Plates were scored on day 10. Concurrent cytotoxicity plates containing 100 HTXTG-1 cells/plate were treated in parallel in the fashion described above.

**Effect of TPA Analogues on Metabolic Cooperation.** As for the TPA experiment above, 100 HTXTG-1 and 3 × 10⁵ HTP3-4 cells were cocultivated in 60-mm dishes. The phorbol esters TPA, PDD, 4α-PDD, and the nonphorbol but structurally related analogue, MEZ, were tested at various concentrations for their ability to inhibit metabolic cooperation. Concurrent cytotoxicity plates containing 100 HTXTG-1 cells were also treated in parallel. Plates were stained and scored as indicated above.
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Fig. 1. Karyotype of the human teratocarcinoma cell line (designated PA-1). A–G refer to classes of chromosomes that are based on similar size and shape.

of the three densities tested for detecting inhibition of metabolic cooperation by TPA was with a wild-type cell density of $3 \times 10^5$ cells/60-mm dish, giving approximately a 9-fold increase in recovery over the control.

Effect of TPA Analogues on Metabolic Cooperation. Fig. 7 displays the effect of TPA, PDD, 4α-PDD, and MEZ on metabolic cooperation. PDD was as effective as TPA in inhibiting metabolic cooperation, whereas MEZ was intermediate in its effect and 4α-PDD was quite ineffective. However, all four compounds showed relatively little difference in cytotoxicity.

DISCUSSION

The PA-1 human teratocarcinoma cell derived lines used in these studies promise to be good candidates for a human cell-based assay for inhibitors of metabolic cooperation. These cells, in spite of many passages, have retained their originally described pseudodiploid karyotype [46,XX,t(15;20)]. Their high colony forming ability, tight colony morphology, and relatively short doubling time (approximately, 16 h) (53), and responsiveness to known tumor promoters make them an ideal candidate for a human cell-based assay for inhibitors of metabolic cooperation.

Huberman et al. (53), while designing a mutation assay with PA-1 derived P3 cells as target cells, demonstrated a cell density dependent effect on recovery of 6-TG resistant mutants, which was the initial indication that these cells were capable of metabolic cooperation. As shown in these studies (Figs. 2 and 3), they are capable of undergoing metabolic cooperation, not only with themselves, but equally as well with at least two other types of human cells.

As with the results found in studies of other animal and human cells (cited in the "Introduction"), TPA inhibited metabolic cooperation in a dose dependent manner in these teratocarcinoma cells. In addition PDD and MEZ were highly and moderately
HTXTG-2; D. HTXTG-3; A, HTXTG-4; •, HTXTG-5; A, HTXTG-6. on day 3. Plates were stained and colonies were scored on day 10. • HTXTG-1; tissue culture dishes in the presence of 6-TG. Fresh 6-TG medium was replaced were separately cocultured with increasing numbers of HTP3-4 cells in 60-mm and HTP3-4 cells. Two hundred cells from each of six cell lines resistant to 6-TG demonstrated in vitro activity would predict. Results similar to 4o-PDD was negative, as its in vivo and previously effective, respectively, in their ability to inhibit metabolic coop tion. 4o-PDD was negative, as its in vivo and previously demonstrated in vitro activity would predict. Results similar to these were also found with these and other phorbol ester ana logues in previously published reports (22, 26-28, 43).

One notable difference found between the human teratocarcinoma cells used here and the V79 cell metabolic cooperation assay is the increased cytotoxic response to TPA by the human cells (approximately $10^3$ times more sensitive than V79 cells). This phenomenon may be attributable to the fact that some of these cells are induced to “differentiate” (see Fig. 5) under the influence of TPA, and thus may have lost their ability to clone. This sensitivity may place some limitations on the utility of this assay for compounds that inhibit metabolic cooperation at concentrations that induce terminal differentiation. However, if both inhibition of metabolic cooperation and differentiation are mediated via the same mechanism, the differentiation phenomenon could act as a flag of potential cell-cell communication blocking activity in nondifferentiating cell types.

Although the mechanism of action of TPA induced reduction in metabolic cooperation is not known, several recent reports offer some clues. Yancey et al. (54) have shown the disappearance of gap junction from the surface of V79 cells treated with TPA. Kalimi and Sirsat (31) applied TPA and MEZ to the skin of mice and were able to show a dose dependent decrease in the number of gap junctions in the basal and suprabasal layers of interfollicular epidermis, as well as an increase in the intercellular spaces. These effects were apparent for MEZ only at high doses, agreeing in principle with the results obtained here and with the known relative weakness of this substance as stage 1 promoter (55). The observation that the intercellular spaces are increased in the basal layer of mouse epidermis by TPA (31, 56) suggests a change in normal cellular adhesion mechanisms. Kanno et al. (57) have shown the critical nature of Ca2+ dependent adhesion molecules in gap junction mediated intercellular communication. Antibodies raised to these molecules can inhibit metabolic co operation in mouse teratocarcinoma cells.

At the molecular level, recent advances in the understanding of TPA action have come from receptor binding studies (58-61), which show that the Ca2+ and phospholipid dependent protein kinase (protein kinase C) binding of TPA is correlated with its biological activity. This kinase is normally activated by diacylglycerol formed from phospholipase C mediated phosphatidylinositol turnover (62-64). The demonstration that diacylglycerol was also able to inhibit gap-junctional intercellular communication in mouse 3T3 cells also suggests a link between protein kinase C and tumor promotion (65, 66). Many molecular signals such as hormones, growth factors, and lectins are known to stimulate phosphatidylinositol metabolism via interaction of their respective receptors with phospholipase C (62, 64). This series of reactions is sensitive to the antagonistic effects of cAMP. Thus, many other molecular signals which increase cAMP can ultimately depress protein kinase C mediated phosphorylation while stimulating cAMP dependent protein kinase (protein kinase A) phosphorylation events. TPA, through its tight binding to protein kinase C, bypasses this regulatory feedback control mechanism and supports continuous protein kinase C catalyzed phosphorylation. It is known that cAMP can act to increase the junctional coupling between cells (67, 68). Enomoto et al. (22)

Fig. 2. Metabolic cooperation between six X-ray induced 6-TG resistant variants and HTP3-4 cells. Two hundred cells from each of six cell lines resistant to 6-TG were separately cocultured with increasing numbers of HTP3-4 cells in 60-mm tissue culture dishes in the presence of 6-TG. Fresh 6-TG medium was replaced on days 3 (and 7 for metabolic cooperation plates, bottom). Colonies were scored on day 10. I, HTXTG-1; O, HTXTG-2; □, HTXTG-3; △, HTXTG-4; ◆, HTXTG-5; ▲, HTXTG-6.

Fig. 3. Metabolic cooperation between HTXTG-1 cells and other human cells. Two hundred HTXTG-1 cells were cocultured with increasing numbers of either normal diploid fibroblasts (MSU-1) (△) or a human bladder cell line (HCV 29) (◆) in the presence of 6-TG.
have recently been able to demonstrate cAMP antagonism of the TPA induced reduction in gap junction permeability to dye transfer. Thus, it appears that competing phosphorylation reactions catalyzed by either of these two kinases may ultimately lead to increased or decreased gap junction mediated intercellular communication.

One possible consequence of the ensuing phosphorylation cascade known to occur subsequent to TPA binding is the ultimate modification of gap junction protein, or proteins associated with its assembly. Consistent with this model is the observation by Atkinson et al. (69) that temperature sensitive Rous sarcoma virus introduced into cells produced a temperature dependent reduction in metabolic cooperation. It is known that the transforming activity of this virus is associated with the Mr 60,000 phosphoprotein a tyrosine specific kinase (70). This oncogene product is known to locate proximal to gap junctions in the membrane (71), and thus may, as part of its transforming action reduce metabolic cooperation via phosphorylation of gap junction protein or associated protein such as vinculin. Recently, Chang et al. (72) and Azarnia and Loewenstein (73) have found that M, 60,000 phosphoprotein transformed NIH-3T3 cells have a reduced metabolic cooperation ability. Incidental to these observations is that one of the major breakdown products of phospholipase C catalyzed phosphatidylinositol breakdown is inositol triphosphate. This substance has recently been implicated in stimulating the release of intracellular Ca\textsuperscript{2+} stores (74). Increases in intracellular Ca\textsuperscript{2+} are known to reduce gap junction permeability (75-77), perhaps through the action of gap junction associated calmodulin (78). This may be one of the additional mechanisms by which molecular signals that stimulate phospholipase C (and hence protein kinase C) act to modulate gap junction permeability.
or 3 x 10^5 P) HTP3-4 cells/60-mm dish. TPA was added at various concentrations at 4 h postdating. 6-TG was added 2 h later. Fresh 6-TG medium was given on HTP3-4 cells. Two hundred HTXTG-1 cells were plated with 0 (D), 0.25 (O), 1 (•), on day 3 (and on day 7, bottom), and colonies were scored on day 10.

TPA is known to modify the nature of glycoconjugates at the cell junction mediated dye transfer between transformed and contact normal cells. The results thus give added weight to the concept that selective communication plays a role in altered differentiation and tumor development. The in vivo potency of the phorbols as tumor promoters and their capacity to affect differentiation are correlated.

The results obtained in these studies agree in general with metabolic cooperation studies that have been performed on other mammalian cells. TPA and its analogues affected metabolic cooperation and differentiation in a manner that was consistent with their previously known activity in vitro and in vivo. We have also conducted similar studies with a number of polybrominated biphenyl congeners in these cells, and the results of those studies agree with previously established activity of these compounds.

As with any carcinogenic cell line, these cells are not without some physiological abnormalities, but because of their excellent clonal growth properties, stable pseudodiploid karyotype, responsiveness to known inhibitors of metabolic cooperation, and their in vitro differentiation ability, these cells are a promising candidate for a human cell-based assay for inhibitors of metabolic cooperation.

REFERENCES


Transformation has been described as an altered differentiation process (80-81). The fact that these human teratocarcinoma cells are capable of limited differentiation (49, 82), and that compounds reported here which were able to block intercellular communication were also capable of inducing differentiation suggests a link between these two phenomena, at least as far as TPA-like compounds are concerned. TPA has been shown to induce differentiation in a number of other systems (reviewed in Ref. 83). Recently published results by Enomoto and Yamasaki (84) and Yamasaki et al. (85) have shown that in vitro transformation of BALB/c-3T3 cells is associated with a reduction in gap junction mediated dye transfer between transformed and contacting normal cells. The results thus give added weight to the concept that selective communication plays a role in altered differentiation and tumor development. The in vivo potency of the phorbols as tumor promoters and their capacity to affect differentiation are correlated (83).

Another possible cause of the decreased metabolic cooperation induced by TPA involves still other cell surface changes. TPA is known to modify the nature of glycoconjugates at the cell surface (79), potentially influencing cell recognition and adhesion...
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