Transformation of BALB/c-3T3 Cells: III. Development of a Co-Culture Clonal Survival Assay for Quantification of Chemical Cytotoxicity in High-Density Cell Cultures

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A co-culture clonal survival assay was developed to measure the cytotoxicity of test chemical treatments to BALB/c-3T3 cells because the standard clonal survival assay using 200 wild type (WT) cells frequently overestimates chemical cytotoxicity when compared with identical treatment doses in high-density cultures. The assay used co-cultures of 3.2×10^4 WT cells, the same seeding density used in the transformation assay, and 200 ouabain resistant (OUA^r) cells. After a 48-hr test chemical treatment, co-cultured cells were fed with culture medium containing 4 mM ouabain. The test chemical was cytotoxic to an equal percentage of WT and OUA^r cells. Ouabain treatments killed the remaining WT cells. Thus, OUA^r cells surviving the test chemical treatment measured the relative cloning efficiency (RCE) of all treated cells in the high-density cell co-culture. The co-culture assay succeeded because metabolic cooperation at the OUA^r locus was not detected in BALB/c-3T3 cells. Five chemicals induced comparable cytotoxic responses in both assays, including actinomycin D, 5-bromo-2'-deoxyuridine, N'-methyl-N-nitro-N'-nitrosoguanidine, dimethyl sulfoxide and sodium chloride. In contrast, chemical cytotoxic responses detected in the standard and co-culture assays differed by $\geq$ 10-fold for 11-aminoundecanoic acid, benzo[a]pyrene, cytosine arabinoside, and 3-methyl-cholanthrene and differed by >2-fold for 2-acetylaminofluorene and dimethylnitrosamine. Detection of 11-aminoundecanoic acid-induced transformation was shown to be dependent on selecting treatment doses from the co-culture assay data. Thus, this method permits more accurate assessment of both chemical-induced cytotoxicity and transformation.

Introduction

The selection of test chemical treatment doses for the BALB/c-3T3 cell transformation assay depends upon results obtained from assessment of chemical cytotoxicity (1–3). Three different procedures have been used to determine the cytotoxicity of chemical treatments: dye exclusion using trypan blue, a clonal survival assay using 200 wild-type (WT) cells (1,2), and a total cell count of chemical-treated versus control cell cultures in the transformation assay (4). The dye exclusion assay distinguishes viable and nonviable cells; however, the dye-exclusion assay does not measure the ability of a chemical-treated cell to replicate and form a colony of cells. Because cell replication is required for formation of a transformed focus, the dye exclusion test does not accurately predict

chemical cytotoxic responses to be used for the transformation assay. The standard clonal survival assay accurately predicts the ability of a cell to survive a chemical treatment and replicate; however, this assay uses only 200 WT cells. Because the BALB/c-3T3 cell transformation assay uses either 1×10^4 (1,5) or 3.2×10^4 cells/vessel (2,6), this assay does not measure the effect of the chemical on relative cloning efficiency (RCE) of cells in high density cell cultures (7). The third assay measures the total number of cells surviving the chemical treatment, but it does not measure the RCE of cells surviving the treatment. Thus, this procedure does not distinguish a chemical that kills most of the cells in a culture and permits a few cells to grow rapidly from a chemical that kills only a few cells and permits most of the cells in the culture to grow at a slower rate. Therefore, all of the standard assay methods used previously to measure the cytotoxicity of chemical treatments to BALB/c-3T3 cells have limitations and inaccurately estimate chemical cytotoxic responses.

Accurate detection of the cytotoxicity of chemical treatments is also important in many other *in vitro* assays. Many different types of chemical-induced genetic toxicity in cultured mammalian cells are detected optimally only

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when chemical treatments were moderately cytotoxic to the target cells, including point mutations (8) and chromosomal aberrations (9,10). Similarly, chemical enhancement of transforming activities in adenovirus-7 infected Syrian hamster embryo virus enhancement (SHE/SA7) transformation assay (11) has been optimally detected only when chemical treatments were moderately cytotoxic to the cells. Because genetic lesions are initiated in a single cell, the procedure used to assess cytotoxic damage must accurately reflect the survival and growth of a single altered cell within a population of many cells.

Malcolm et al. (12) have reported a co-culture clonal survival assay method for determining RCE in high-density cultures of chemical-treated Chinese hamster ovary (CHO) cells. The method uses co-cultures of 200 thioguanine-resistant (TG^r) cells and 1×10^5 WT cells. The TG^r cells chosen for the assay has two phenotypes that are different from the WT cells. They do not exhibit metabolic cooperation, and they are TG^r. After the chemical treatment is removed from the co-cultured cells, the co-cultures are fed with culture medium supplemented with thioguanine. The thioguanine kills all WT cells surviving the chemical treatment and leaves behind only the TG^r cells that survive the chemical treatment. Thus, this assay measurs the RCE of cells in a high-density culture of up to 1×10^5 cells. The co-culture method was developed because many chemical treatments determined to be highly cytotoxic in low-density CHO cell cultures were observed microscopically to be relatively noncytotoxic to high-density cell cultures. Thus, the standard clonal survival assay that uses low densities of cells does not accurately predict the cytotoxic effects of the chemical on a mass culture of cells.

This report describes the development of a co-culture clonal survival assay for use in the BALB/c-3T3 cell transformation assay. The OUA^r locus was chosen for these experiments because the spontaneous occurrence of OUA^r mutants has been reported to be about 1×10^{-7} in WT cells (13). Furthermore, it has been reported that there was no evidence for metabolic cooperation at the OUA^r locus in another immortal mouse cell line (14). Therefore, ouabain treatments of co-cultures of WT and OUA^r cells should only be lethal for the WT cells. A portion of this work has been presented in abstract form (13).

Materials and Methods

Cell Culture

The investigations in this report used the 1-13 clone of A31 BALB/c-3T3 cells (15,16). The materials and methods used to culture the cells have been previously reported in detail (2) and are summarized in part I of these investigations (17).

OUA^r BALB/c-3T3 Cells

The OUA^r cells used in this investigation were derived from a pool of five different OUA^r cell lines (13). Equal numbers of the five cell lines were combined to generate this pool to avoid the possibility that one cell line might have had a metabolic capacity and phenotypic properties that were significantly altered from WT cells. Each OUA^r cell line was isolated from an individual culture dish of WT cells that had been treated with 1.5 µg/mL N-methyl-N-nitro-N'-nitrosoguanidine (MNNG) (Sigma Chemical Co., St. Louis, MO). MNNG induced a high frequency of OUA^r variants of BALB/c-3T3 cells (13), and these variants were selected using three weekly treatments with culture medium containing 2 mM ouabain. WT cells were killed by ouabain treatments ≥ 0.3 mM. All five of these OUA^r cell lines were shown to be phenotypically stable and resistant to 10 mM ouabain treatments (13). In this study, a large pool of OUA^r cells was cryopreserved, and an ampule of cells was thawed to initiate a cell culture. Cultures were passaged biweekly and used between passage 4 (p4) and p30. To prevent phenotypic reversion of the OUA^r cells to the ouabain-sensitive (OUA^s) phenotype, the cultures were fed every other passage with medium supplemented with 4 mM ouabain.

Standard Clonal Survival Assay

The standard clonal survival assay was used to a) estimate the cytotoxic activity of a test chemical, b) select treatment doses for the preliminary co-culture clonal survival assay, c) assess the reproducibility of the chemical-induced cytotoxic responses, and d) determine the relative shift in test chemical cytotoxic responses between high- and low-density cell cultures. The standard clonal survival assay using low-density cultures of BALB/c-3T3 cells was conducted according to our modification (2,6) of the method described by Kakunaga (1). Briefly, 200 WT cells were seeded in either 60-mm culture dishes (or 25-cm^2 culture flasks), and chemical treatment doses were applied to triplicate cultures for 48-hr beginning 2 days after seeding. After a total culture period of 8 days, the vessels were washed, fixed in methanol, stained with Giemsa, and colonies of cells were hand tabulated according to the procedure described in part IV of these investigations (6).

Co-culture Clonal Survival Assay

This assay used a co-culture of 200 OUA^r cells and 3.2×10^4 WT cells (2,13). The two cell types were trypsinized from cell cultures and seeded into the same culture vessels on the same day. In addition, 200 WT, as well as 200 OUA^r cells, were seeded in triplicate culture vessels to determine the RCE of the two cell types. Two days after seeding, the culture vessels were treated 48 hr with chemicals, and the treatments were terminated as described above. Immediately after the last wash, the cultures were fed with medium supplemented with 4 mM ouabain. The ouabain-supplemented medium was added a second time 4 days later, and this medium remained on the cells for an additional 3 days. The prolonged selection period with ouabain was required because the cytotoxic effect of ouabain on high-density cultures of WT BALB/c-3T3 cells was relatively slow. After a total incubation period of 11 days, the culture vessels were fixed, stained, and OUA^r colonies of cells were hand tabulated. The LD_{50} cytotoxic

activity for individual chemicals was determined as described above.

Calculation of Cytotoxic Responses

The cytotoxic responses of test chemicals in the standard and the co-culture clonal survival assays were compared using the treatment dose concentration in millimoles that resulted in 50% clonal survival of chemical-treated cells relative to untreated control cultures. This LD_{50} treatment dose was extrapolated from graphs of dose-related changes in cytotoxic activity of each chemical.

Transformation Assay

Chemical-induced transformation of BALB/c-3T3 cells was evaluated in a standard transformation assay protocol that has been reported in detail (2) and summarized in these investigations in part IV (6). Briefly, each transformation assay contained three components: a standard clonal survival assay (2,6), a co-culture clonal survival assay (2,6,13), and a transformation assay (2,6). In each experiment, chemical-induced transformation was detected in the positive control that consisted of 20 vessels seeded with 3.2×10^4 cells/vessel. Benzo[a]pyrene BaP doses were applied to cell cultures for 48 hr, days 2–4, using standard procedures (2,6).

Evaluation of Transformed Foci

The method used to evaluate transformed foci of BALB/c-3T3 cells has been reported in detail (2) and is summarized in Part IV of this series (6). Briefly, the number of type I–III transformed foci of BALB/c-3T3 cells were identified microscopically using published criteria (1,5,18–20). Type III foci had three phenotypic properties: piling and overlapping cells, disorientation of cells at the periphery of the focus, and invasion of transformed cells into a contact-inhibited monolayer of WT cells. Type I and II foci also appeared in many different sizes, but they lacked one or more of the three phenotypic properties of the type III transformed focus.

Test Chemicals

11-Aminoundecanoic acid (CAS no. 2432-99-7) was provided by Radian Corporation (Houston, TX). 2-Acetylaminofluorene (53-96-3), actinomycin D (50-76-0), BaP (50-32-8), 5-bromo-2'-deoxyuridine (59-14-3), cytosine arabinoside (147-94-4), dimethylnitrosamine (62-75-9), MNNG (70-25-7), and sodium chloride (7647-14-5) were purchased from Sigma. Dimethyl sulfoxide (67-68-5) and 3-methylcholanthrene (56-49-5) were purchased from Aldrich Chemical Company (Milwaukee, WI) and Fisher Chemical Co. (Silver Spring, MD), respectively.

The procedures for handling test chemicals in this laboratory are described in detail in part IV of these investigations (6).

Statistical Methods

The method used to determine the distribution of spontaneous transformed foci of BALB/c-3T3 cells has been previously reported (2) and is described in detail in part I of these investigations (17). The statistical significance of a chemical-induced transformation response was determined using the analysis of variance (F-test) and modified Student's t-tests (6). The computations were performed using SAS software (19).

Results

Relative Cloning Efficiency of Co-cultured OUA^r and Wild-Type Cells

The RCE of WT and OUA^r cells, as well as co-cultures of WT and OUA^r cells, were determined in 36 experiments (Table 1). The cultures of WT cells used in all 36 experiments, as well as the cultures of OUA^r cells used in the first 12 experiments (group 1), were both fed with medium lacking ouabain. In contrast, the last 24 experiments (group 2) used cultures of OUA^r that were fed once a week with medium supplemented with 4 mM ouabain.

The RCE of 200 WT cells used in the clonal survival assay experiments in groups 1 and 2 were 41.4% and 39.7%, respectively (Table 1). Thus, the RCE of the WT cells in the two sets of experiments were nearly identical. In contrast, the RCE of the OUA^r cells in groups 1 and 2 were 18.7% and 51.6%, respectively (Table 1). Thus, the OUA^r cells in group 2 had a significantly higher ($p < 0.001$) RCE than the OUA^r cells in group 1. OUA^r cells in group 2 were maintained on medium supplemented with 4 mM ouabain; thus, any revertants from the OUA^r phenotype were eliminated from the cultures. In contrast, the OUA^r cells in the group 1 were maintained with culture medium lacking ouabain;

Table 1. Effect of different seeding densities of WT cells on the relative cloning efficiency of OUA^r BALB/c-3T3 cells.[a]

Seeding density, cells/vessel		% RCE, mean $\pm$ SD	
OUA^r cells	WT cells	OUA^r cells	WT cells
Cultures of OUA^r cells maintained without ouabain treatment (12 experiments)[b]			
200	31600	19.1 $\pm$ 9.0	—
200	10000	23.4 $\pm$ 12.9	—
200	3160	21.6 $\pm$ 10.7	—
200	Control	18.7 $\pm$ 14.1	—
Control	200	—	41.4 $\pm$ 5.6
Cultures of OUA^r cells maintained with ouabain treatment (24 experiments)[b]			
200	31600	66.3 $\pm$ 41.5	—
200	10000	61.7 $\pm$ 13.9	—
200	3160	54.0 $\pm$ 13.7	—
200	Control	51.6 $\pm$ 51.3	—

Abbreviations: OUR^r, ouabain resistant; WT, wild type; RCE, relative cloning efficiency.

[a]For methods, see text.

[b]The cultures of OUA^r cells in the first 12 experiments were maintained with minimal culture media. In contrast, the cultures of OUA^r cells in the second set of 24 experiments were maintained on culture media supplemented with 4 mM ouabain.

thus, revertants from the OUA^r phenotype were not eliminated from the cultures. The data demonstrate the necessity of treating cultures of OUA^r cells with ouabain in order to remove revertant or OUA^s cells.

The RCE of OUA^r cells was also examined in co-cultures of 200 OUA^r cells and different seeding densities of WT cells. In group 2 experiments, which used cultures of OUA^r cells maintained in the presence of ouabain, the RCE of OUA^r cells increased slightly from 54.0% to 66.3% as the number of WT cells increased from 0.32 × 10^4 to 3.2 × 10^4 cells/vessel. It is important to mention that the relative size of the OUA^r colonies in this assay depended on the number of WT cells that were co-cultured with the OUA^r cells. The size of the surviving OUA^r colony size was inversely proportional to the seeding density of the co-cultured WT cells. In addition, because the co-cultures of cells were not exposed to ouabain until 4 days after the cells were seeded, the first of two 4-mM ouabain treatments was added to a confluent monolayer in vessels seeded with 31,600 WT and 200 OUA^r cells. Taken together, these experiments show that there was no evidence of metabolic cooperation between the WT and OUA^r cells, because the cytotoxic effect of ouabain on WT cells did not have a cytotoxic effect on the co-cultured OUA^r cells.

Relative Cloning Efficiency of BaP-treated Cells

The data presented in Table 1 demonstrate that the RCE of 200 OUA^r cells can be determined when they are co-cultured in the presence of a large number of WT cells. Assuming chemical treatments have an equal opportunity to be cytotoxic to either the WT or the OUA^r cells, then co-

Table 2. Cytotoxic response of BaP detected in a standard and a co-culture clonal survival assay.

	Cytotoxic response, % RCE, mean ± SD		
	Standard assays[b]		Co-culture assay[c]
BaP, μg/mL[a]	WT	OUA^r	WT + OUA^r
Preliminary experiment			
0.633	1.3 ± 1.1	0 ± 0	11.2 ± 2.8
0.200	3.9 ± 1.0	6.7 ± 3.7	26.5 ± 7.4
0.0633	12.4 ± 1.3	12.0 ± 3.7	85.9 ± 12.6
0.0200	55.3 ± 11.3	32.1 ± 7.9	102.0 ± 13.0
Control	100	100	100
Mean responses from extended series of 36 experiments			
0.200	2.9 ± 3.6	ND	39.0 ± 16.6
0.0633	7.0 ± 5.3	ND	73.4 ± 12.9
Control	100	ND	100

Abbreviations: BaP, benzo[a]pyrene; ND, not determined; OUR^r, ouabain-resistant cells; % RCE, percent relative cloning efficiency; WT, wild-type cells.

[a]The BaP was dissolved in dimethylsulfoxide and diluted in medium to the final treatment dose concentrations.

[b]The standard clonal survival assay used culture vessels seeded with either 200 WT or 200 OUA^r cells/vessel. See Materials and Methods for details.

[c]The co-culture clonal survival assay used co-cultures of 3.3 × 10^4 WT cells and 200 OUA^r cells/vessel. See Materials and Methods for details.

cultures of OUA^r and WT cells could provide a method of determining clonal survival in chemical-treated high-density cell cultures. This hypothesis was tested by treating the co-cultures of WT and OUA^r cells with BaP, and the results of these experiments are presented in Table 2.

A preliminary experiment revealed that a BaP treatment of 0.063 μg/mL was cytotoxic to cultures of either 200 WT or 200 OUA^r cells, and it resulted in an RCE of 12.4% and 12.0%, respectively (Table 2). In contrast, the 0.063 μg/mL BaP dose resulted in a RCE of 85.9% and was essentially noncytotoxic to the co-cultures of 3.2 × 10^4 WT and 200 OUA^r cells (Table 2). This shift in cytotoxic response of BaP treatments detected in the standard assays using a low seeding density versus the co-culture assay using a high seeding density cell cultures was examined in an extended series of 36 experiments. These experiments confirmed that BaP treatments of 0.20 and 0.0633 μg/mL were far less cytotoxic in co-cultures of 3.2 × 10^4 WT and 200 OUA^r cells, versus 200 WT cells alone (Table 2).

Cytotoxic Responses of 11 Chemicals in Standard and Co-culture Clonal Survival Assays

The data presented in Table 2 demonstrate that BaP was far more cytotoxic to low-density cell cultures than it was to high-density cell cultures. It is possible that the difference in BaP cytotoxic responses detected in the two assays is specifically related to an effect of BaP on WT cells. Alternatively, many different test chemical treatments might exhibit different cytotoxic responses in low- and high-density cell cultures. To resolve this question, 10 test chemicals were selected that had a wide range of cytotoxic responses to BALB/c-3T3 cells.

The data presented in Table 3 demonstrate that 6/11 chemicals had > 2-fold difference in their cytotoxic response detected in the two clonal survival assays (2-acetylaminofluorene, AAF; 11-aminoundecanoic acid, AMI; BaP; cytosine arabinoside, ARAC; dimethylnitrosamine, DMN; and 3-methylcholanthrene, MCA; Table 3). The cytotoxic response shifts ranged from 2.24- to 152-fold. The most cytotoxic of these five chemicals was BaP, with an LD$_{50}$ treatment dose of 0.00050 mM detected in the co-culture clonal survival assay, and the least cytotoxic was DMN with an LD$_{50}$ of 310 mM. Thus, there was nearly a six-log difference in the cytotoxic responses of BaP and DMN. In addition, the second largest cytotoxic shift (44-fold) was obtained with MCA. The LD$_{50}$ treatment dose of MCA was 0.00093 mM in the standard assay and 0.0041 mM in the co-culture assay.

In contrast, 5/11 chemicals had < 2.0-fold difference in their cytotoxic responses detected in the two clonal survival assays (actinomycin D, ACTD; 5-bromo-2'-deoxyuridine, BUdR; dimethyl sulfoxide, DMSO; MNNG, and sodium chloride, NaCl; Table 3). The most cytotoxic of these five chemicals was ACTD, with an LD$_{50}$ of 0.0000067 mM detected in the co-culture clonal survival assay, and the least cytotoxic was DMSO, with an LD$_{50}$ of 400 mM. Thus, the cytotoxic responses of DMSO and ACTD were

Table 3. Comparison of the cytotoxic responses of 11 test chemicals detected in a standard and a co-culture clonal survival assay.

Chemical	Molecular weight	Cytotoxic response, LD_{50}, mM		Cytotoxic response shift LD_{50} CCA/LD_{50} SA[a]
		Standard assay	Co-culture assay	
Chemicals with cytotoxic response shifts > 2.0				
2-Acetylaminofluorene	223	0.076	0.17	2.24
11-Aminoundecanoic acid	201	0.101	15.4	152
Benzo[a]pyrene	252	0.000050	0.00050	10.0
Cytosine arabinoside	280	0.000064	0.00064	10.0
Dimethylnitrosamine	74	81	310	3.83
3-Methylcholanthrene	268	0.000093	0.0041	44.1
Chemicals with cytotoxic response shifts < 2.0				
Actinomycin D	1250	0.0000044	0.0000067	1.52
5-Bromo-2'-deoxyuridine	307	0.046	0.055	1.20
Dimethyl sulfoxide	78	400	400	1.00
N-Methyl-N'-nitro-N-nitrosoguanidine	147	0.014	0.018	1.29
Sodium chloride	58	100	147	1.47

Abbreviations: CCA, co-culture clonal survival assay; LD_{50}, lethal dose for 50% of the cells; SA, standard clonal survival assay.

[a]The difference in the cytotoxic responses of chemical treatments measured in the two clonal survival assays was designated a "cytotoxic response shift." The relative amount of this shift was calculated by dividing the LD_{50} treatment dose of a chemical determined in the co-culture clonal survival assay by the LD_{50} dose determined in the standard clonal survival assay. For example, the cytotoxic response shift for 2-acetylaminofluorene was 0.17/0.076 = 2.24.

nearly eight logs different. Only 6/11 test chemicals exhibited cytotoxic responses that were seeding density dependent. Furthermore, the presence or the absence of a test chemical seeding density-dependent cytotoxic responses was unrelated to the relative cytotoxicity of the test chemical to the WT cells.

Cytotoxic and Transformation Responses of 11-Aminoundecanoic Acid

Test chemical cytotoxic responses detected in a clonal survival assay are used to select treatment doses for the standard BALB/c-3T3 cell transformation assay (Materials and Methods). Thus, test chemicals that exhibit different cytotoxic response in a standard and a co-culture clonal survival assay predict two different sets of treatment doses for the transformation assay. In a preliminary series of cytotoxicity experiments, the test chemical AMI exhibited cytotoxic responses that were 152-fold different in the standard and the co-culture clonal survival assay (Table 3). Therefore, this chemical was chosen to be tested in transformation assays that selected four treatment doses based on the cytotoxic responses detected in either the standard or the co-culture clonal survival assays.

The AMI-induced cytotoxic and transformation responses detected in four experiments are presented in Table 4. In the first experiment (trial 1) the treatment doses for AMI were chosen based on the dose-related cytotoxic response data obtained from the standard clonal survival assay (Table 4). As predicted, 12.5, 25, 50, and 100 µg/mL of AMI induced highly significant cytotoxic responses in the standard clonal survival assay, including RCEs of 57.1, 9.3, 0, and 0%, respectively. In contrast, the same four treatment doses were noncytotoxic in the co-culture assay and induced RCEs of 98.0, 93.3, 91.3, and 89.3%, respectively. The same four treatment doses were inactive in the transformation assay, and resulted in type

III transformation responses of 0.49, 0.61, 0.37, and 0.58 foci/vessel compared to a spontaneous response of 0.33 foci/vessel. In a repeat experiment (trial 2) using a slightly higher range of treatment doses of 50, 100, 150 and 200 µg/mL, AMI was very cytotoxic to 200 BALB/c-3T3 cells in the standard clonal survival assay and noncytotoxic to 3.2×10^4 WT cells in the co-culture assay. Furthermore, AMI was inactive at all four treatment doses in the transformation assay.

In contrast to the results obtained in transformation assay trials 1 and 2, AMI was evaluated as active in trial 3 (Table 4). In trial 3, treatment doses of 2000 and 4000 µg/mL AMI induced significant type III focus transformation responses of 4.52 and 3.77 foci/vessel, respectively. Likewise, the 4000 µg/mL AMI treatment dose induced a significant cytotoxic response of 28.9% RCE in the co-culture clonal survival assay. Thus, AMI, as well as the positive control BaP, induced the highest transformation responses at a treatment dose that was determined to be cytotoxic in the co-culture clonal survival assay. In the repeat experiment (trial 4) using relatively high concentrations of AMI, both AMI and BaP induced significant transformation responses; however, the absolute magnitude of the transformation responses were both proportionately less than that observed in trial 3.

Taken together, it was imperative that the co-culture assay data be used to select treatment doses for the transformation assay. The standard clonal survival assay predicted that AMI treatments would be completely cytotoxic and result in 0% RCE over a dose range of 100–4000 µg/mL. In contrast, the co-culture assay predicted 11AMI treatment doses were essentially noncytotoxic to the high-density cells for treatment doses ≤ 2000 µg/mL. Because AMI-induced transformation was detected at treatment doses between 2000 and 4000 µg/mL, only cytotoxic response data from the co-culture assay permitted the correct AMI treatment doses to be selected for the transformation assay.

Table 4. Comparison of cytotoxic and transformation responses of 11-aminoundecanoic acid.

Trial no.	Chemical	Concentration, µg/mL	SA	CCA	I-III	III	(n)	Transformation response, type III foci/vessel[c]
1	BaP	0.200	0	47.1	249	94	(20)	4.43***
	BaP	0.0633	3.5	70.7	195	86	(20)	3.91***
	AMI	100	0	89.3	24	15	(20)	0.58*
	AMI	50.0	0	91.3	17	11	(20)	0.37
	AMI	25.0	9.3	93.3	54	17	(20)	0.61
	AMI	12.5	57.1	98.0	23	12	(20)	0.49
	AMI	Control	100	100	64	18	(40)	0.33
2	BaP	0.200	0	16.7	341	83	(20)	3.65***
	BaP	0.0633	5.7	62.5	211	86	(20)	3.73***
	AMI	200	0	93.3	26	4	(20)	0.13
	AMI	150	0	92.6	35	3	(20)	0.11
	AMI	100	0	94.6	65	7	(20)	0.27
	AMI	50.0	0	93.3	39	2	(20)	0.07
	AMI	Control	100	100	119	18	(40)	0.31
3	BaP	0.200	10.1	57.1	482	197	(19)	10.1***
	BaP	0.0633	6.4	85.5	297	115	(20)	5.46***
	AMI	4000	0	28.9	252	100	(20)	4.52***
	AMI	2000	0	83.2	201	87	(20)	3.77**
	AMI	1000	0	86.3	109	42	(20)	1.56
	AMI	500	0	81.9	124	53	(20)	1.79
	AMI	Control	100	100	219	91	(38)	1.97
4	BaP	0.200	5.8	34.3	90	48	(20)	2.07***
	BaP	0.0633	20.8	63.7	111	39	(20)	0.97**
	AMI	4000	0	71.8	69	17	(20)	0.64***
	AMI	2000	0	90.5	18	10	(20)	0.35*
	AMI	1000	0	86.9	16	10	(18)	0.39*
	AMI	500	0	87.6	14	9	(20)	0.30
	AMI	Control	100	100	11	5	(39)	0.09

Abbreviations: AMI, 11-aminoundecanoic acid; BaP, benzo[a]pyrene; CCA, co-culture clonal survival assay; n, number of culture vessels; % RCE, percent relative cloning efficiency; SA, standard clonal survival assay.

[a]The test chemical, 11-aminoundecanoic acid, was dissolved as a 5-fold concentrated stock in medium supplemented with 1.25% w/v pluronic F68 and was diluted 5-fold when it was added to cell cultures.

[b]The procedure used in the standard transformation assay, as well as the criteria used to evaluate transformed foci of BALB/c-3T3 cells, is furnished in Materials and Methods. The transforming activity is expressed as either the type I + II + III (I-III) foci or the type III foci alone that were scored in the number of vessels (n) in each treatment set.

[c]The transformation responses of BaP and AMI are expressed as type III foci/vessel, and the method used to calculate these responses is provided in Materials and Methods. The arithmetic value for the foci/vessel represents the antilog of the $\log_{10}$ mean transformation response minus one.

*Significant transformation response, $0.01 < p \leq 0.05$.

**Significant transformation response, $0.001 < p \leq 0.01$.

***Significant transformation response, $p \leq 0.001$.

Discussion

This investigation clearly demonstrates that certain test chemicals have cell density-dependent, cytotoxic responses in BALB/c-3T3 cells. Test chemicals with density-dependent cytotoxic responses were very cytotoxic to low-density cell cultures used in a standard clonal survival assay, and identical treatment doses were noncytotoxic in high-density cell cultures. The lack of chemical cytotoxicity could be observed microscopically using a phase contrast microscope (unpublished observations) or be measured quantitatively using the newly developed co-culture clonal survival assay (13). Whereas the standard clonal survival used only 200 WT cells (1,2), the co-culture clonal survival assay used co-cultures of 200

OUA[r] and 3.2×10^4 WT cells (13). The co-culture clonal survival assay accurately determined the cytotoxic responses of all test chemicals and thereby permitted the selection of treatment doses most likely to be active in the transformation assay.

The success of the co-culture clonal survival assay in measuring the RCE of chemical treatments in high-density cell cultures was directly dependent on the absence of metabolic cooperation at the OUA[r] locus in BALB/c-3T3 cells (Table 1). If metabolic cooperation for the sodium/potassium (Na/K) ATPase enzyme had been detected in these cells, then the cytotoxic effect of ouabain on WT BALB/c-3T3 cells would have cooperatively killed OUA[r] cells in high-density cell co-cultures. However, no

metabolic cooperation at the OUA^r locus was observed, and the RCE of OUA^r cells actually increased slightly in the presence of increasing seeding densities of co-cultured WT cells. Comparable observations and conclusions were made by Malcolm et al. (12) using mutant and WT CHO cells.

The mechanism by which some test chemicals induced different cytotoxic responses, or cytotoxic response shifts, in the standard and the co-culture clonal survival assays is unknown, and it was not the objective of this investigation. Nevertheless, the experimental data presented in this investigation demonstrate three important features that characterize the phenomenon. First, the cytotoxic shift was test chemical specific and unrelated to the relative cytotoxicity of the test chemical on WT BALB/c-3T3 cells. Only 6/11 test chemicals had cytotoxic shifts >2-fold, and the cytotoxic shifts varied from 2.24-fold for 2-acetyl-aminofluorene (Table 3) to 152-fold for 11-aminoundecanoic acid (Table 4). AAF, BaP, ARAC, and MCA were all very cytotoxic to the cells and had cytotoxic shifts $\geq$ 2.0-fold; however, ACTD, BUdR, and MNNG were equally cytotoxic and all had cytotoxic shifts < 2.0-fold (Table 3). Conversely, AMI and DMN were relatively noncytotoxic to the cells and had a shift >2.0-fold; however, the non-cytotoxic test chemicals DMSO and NaCl did not have a cytotoxic shift.

Second, the differences in cytotoxic responses detected in the two clonal survival assays was a highly reproducible biological property of individual test chemicals. Cytotoxic response shifts in the two assays were observed for BaP in 36 consecutive experiments (Table 2). Thus, the cytotoxic shift phenomenon was not obviously caused by an experimental parameter(s) that varied among experiments, such as the passage number of cultures or the ampule aliquot of cryopreserved cells used to initiate the cultures (20).

Third, the six test chemicals with cytotoxic response shifts in the two clonal survival assays that were $\geq$ 2.0-fold included five chemicals that have structural alerts (AAF, ARAC, BaP, DMN, and MCA) and one chemical (AMI) without an alert (22). In addition, three other chemicals that have structural alerts did not have a cytotoxic shift >2.0-fold, including ACTD, ARAC, and MNNG. Thus, the presence or absence of a cytotoxic response shift was not directly correlated with the presence of a known structural alert. Furthermore, the chemical with the largest cytotoxic shift of >152-fold, AMI (Table 4), has no obvious pathways for either metabolic activation or detoxification.

Two explanations for the cytotoxic shift can be hypothesized based on the data obtained in this investigation. First, WT A31-1-13 BALB/c-3T3 cells have been reported to metabolically cooperate with one another in sub-confluent cell cultures (23,24); thus, these cells are capable of forming gap junctions and passing low molecular weight chemicals between cells. While subconfluent low- and high-density cultures could form gap junctions, jap junction formation would be more efficient in cultures seeded with high (i.e., 3×10^4 cells/vessel) versus low (i.e., 200 cells/vessel) seeding densities. Therefore, parent test chemicals and related metabolites could be hypothesized to pass more efficiently between cell gap junctions in relatively

high-density cell cultures than in low-density cell cultures. Since the cytotoxic response of all the test chemicals was always less in the high- versus low-density cell cultures, then metabolic cooperation among chemical-treated BALB/c-3T3 cells could facilitate detoxification of test chemicals (25). The detoxified metabolite of a test chemical could be less cytotoxic than the parent test chemical; thus, the cytotoxic response of the test chemical would be less in the co-culture versus the standard clonal survival assay. If metabolic cooperation among those cells had favored activation of select test chemicals, then cytotoxic responses of test chemicals would have been predicted to increase in high-density cell cultures. In the latter situation, the parent chemical would have been presumably activated into an electrophilic form which bound to DNA and would have most likely been more cytotoxic than the parent molecule.

The second hypothesis is that the cytotoxic response shift in high- verses low-density cell cultures is caused by a chemical-specific, mass culture effect on the metabolism of WT cells. According to this hypothesis, certain test chemicals, when applied to high-density cell cultures, reduce the capacity of the entire cell culture to either transport the test chemical or metabolize the test chemical and thereby reduce the cytotoxicity of the chemical. The mass culture effect has to be chemical specific because only a portion of the test chemicals exhibited the seeding density-dependent cytotoxic response shift.

In conclusion, the standard and the co-culture clonal survival assay have been routinely used to investigate the cytotoxic responses of over 200 test chemicals. The data from these experiments have been used to select test chemical treatment doses for the standard BALB/c-3T3 cell transformation assay, and these results are being reported separately (6,26). For those test chemicals with cytotoxic shifts > 2-fold, the co-culture clonal survival assay has been important for selecting the treatment doses that would most likely induce significant transformation of the WT cells.

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