STPOST 06

Evaluation of cigarette smoke-induced cell transformation using three *in vitro* cell transformation assays

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ABSTRACT

Cell transformation assays (CTAs) detect phenotypic changes associated with neoplastic transformation. Experimental evidence suggests *in vitro* cell transformation may simulate early components of the multistage process of carcinogenesis. Moreover, CTAs may aid in identifying potentially non-genotoxic carcinogens not detected by conventional genotoxicity assays. We have tested whether total particulate matter (TPM) and cigarette smoke condensate (CSC) generated from Kentucky reference 3R4F cigarettes induce morphological transformation using three separate CTAs: the Syrian Hamster Embyro (SHE) cell transformation assay (24-hour and 7-day exposure protocols), the SHE assay (initiator/promoter protocols), and Bhas 42 initiator/promoter assays [Bhas 42 cells (*v-Ha-ras*-transfected Balb/c 3T3 cells)]. TPM and CSC were positive in the SHE 7-day exposure with 12.5- and 9-fold increases, respectively, in the number of morphologically transformed colonies as compared to solvent controls; CSC was positive (2.7-fold increase) while TPM was equivocal in the SHE 24-hour exposure assay. TPM and CSC induced positive responses in the SHE initiator and promoter assays with approximately 3-fold increases in the number of morphologically transformed colonies. In the Bhas 42 initiator assay, TPM produced a positive response (3.5-fold increase) while CSC was equivocal; however, in the Bhas 42 promoter assay both TPM and CSC were positive, inducing approximately a 12-fold increase in morphologically transformed foci. Among the CTA models investigated, the Bhas 42 promoter assay provided the most linear response coupled with a robust increase in transformed foci across the treatment range and may therefore be a useful technique to evaluate cigarette samples.

MATERIALS & METHODS

Cigarettes: Kentucky Reference 3R4F, University of Kentucky, Lexington, KY USA

Smoking Regimen: 35 mL puff, 2 second duration, 1 puff/minute

TPM Collection: TPM from mainstream smoke was collected onto Cambridge filter pads and extracted to produce a stock concentration of 30 mg "Tar"/mL in dimethyl sulfoxide (DMSO)

CSC Collection: Mainstream smoke was condensed during passage through a series of impingers seated in a cryogenic bath. The condensed smoke (condensate or CSC) was rinsed from impingers with acetone, followed by rotary evaporation to reduce acetone and water content to prepare CSC stock concentration of 35 mg "Tar"/mL in acetone

Tar: TPM minus nicotine and water

Vehicle controls: 0.2% DMSO and 0.2% Acetone

Positive controls:
Benzo(a)pyrene [B(a)P, initiator]
Phorbol-12-Myristate-13-Acetate [TPA, promoter]

Cell Transformation Assays: Conducted per standard protocols (See Tables 1-3 and references) at BioReliance Laboratories. Exposure concentrations for the SHE and Bhas assays were determined in preliminary cell growth assays; concentrations for the SHE initiator/promoter assay were sub-transforming and determined from the SHE assay.

Table 1: SHE Assay Methodology

Culture conditions: SHE cells cultured in DMEM-L with 20% Fetal Bovine Serum, 4 mM L-glutamine, pH 6.7, atop feeder layer of x-ray irradiated SHE cells.

Treatment protocols:	Concentration ranges:	
24-hour treatment with 6-day recovery	3R4F TPM:	0.94 – 30 µg Tar/ml
	3R4F CSC:	1.09 – 35 µg Tar/ml
7-day treatment without recovery	3R4F TPM:	0.50 – 16 µg Tar/ml
	3R4F CSC:	1.09 – 35 µg Tar/ml

Test article considered positive if:
- at least two concentrations show significant* increase in transformation frequency vs. vehicle control
- one concentration shows significant* increase in transformation frequency vs. vehicle control with a significant concentration-response trend

** p<0.05, one-sided Fisher's Exact Test*

Table 2: SHE Initiator/promoter Assay Methodology

Culture conditions: SHE cells cultured in DMEM-L with 20% Fetal Bovine Serum, 4 mM L-glutamine, pH 6.7, atop feeder layer of x-ray irradiated SHE cells.

Initiator/promoter treatment protocol:	Concentrations: are 1/10 of the Noel doses in the SHE 24 hour or 7 day assays	
Treat with culture media, test article or B(a)P for 24 hours; remove and treat with culture media, test article or TPA for 6 days. (See Figure 2)	3R4F TPM as initiator:	1.50 µg Tar/ml
	3R4F CSC as initiator:	0.14 µg Tar/ml
	B(a)P as initiator:	0.333 ng/ml
	3R4F TPM as promoter:	0.05 µg Tar/ml
	3R4F CSC as promoter:	0.05 µg Tar/ml
	TPA as promoter:	0.1 ng/ml

Test article considered positive if: Transformation frequency > vehicle control* either as:
- Initiating agent in conjunction with the known promoter, TPA
- Promoting agent in conjunction with the known initiator, B(a)P

**p<0.05, one-sided Fisher's Exact Test*

Table 3: Bhas 42 Assay Methodology

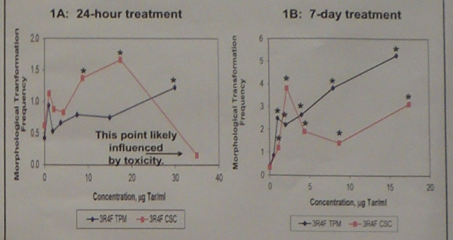
Culture conditions: Bhas 42 cells (v-Ha-ras-transfected Balb/c 3T3 clone A31-1-1 cells from Hatano Research Institute, Hatano, Japan) grown in MEM with 10% FBS; assays performed in DMEM/F12 with 5% Fetal Bovine Serum, 1% Penicillin/Streptomycin in 6-well plates

Initiator treatment protocol:	Concentration ranges:
Day 0: Seed 4000 cells/well Days 1-3: Treat with test article Days 4-21: Incubate with media only; re-feed with media on days 7, 10 and 14 Day 21: Fix and stain cells	3R4F TPM: 0.030 – 30 µg Tar/ml 3R4F CSC: 0.035 – 35 µg Tar/ml
Promoter treatment protocol:	**Concentration ranges:**
(Two repeat assays performed) Day 0: Seed 14,000 cells/well Days 1-3: Incubate with media only Days 4-13: Incubate with test article; re-feed with media containing test article on days 7 and10 Days 14-21: Incubate with media only Day 21: Fix and stain cells	3R4F TPM: 0.010 – 30 µg Tar/ml 3R4F CSC: 0.0117 – 35 µg Tar/ml

Test article considered positive if:
Two or more concentrations show significant increase* in number of transformed foci.

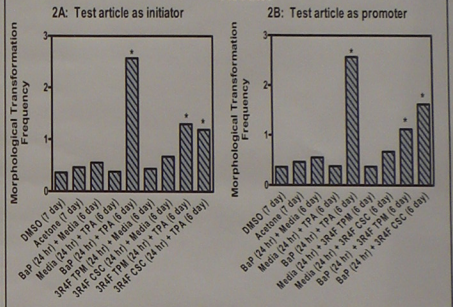
**p<0.05, one-sided ANOVA with Dunnett's correction*

Figure 1: SHE Results



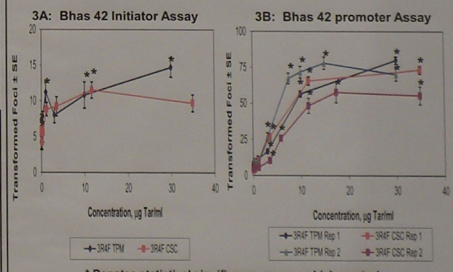
*** Denotes statistical significance versus vehicle control**
Note: scale of these two graphs differs

Figure 2: SHE Initiator/promoter Results



*** Denotes statistical significance versus vehicle control**

Figure 3: Bhas 42 Results



*** Denotes statistical significance versus vehicle control**
Note: scale of these two graphs differs

Table 4: Summary of responses in CTAs

CTA protocol	3R4F TPM	3R4F CSC
SHE 24-hour	Equivocal 2.9-Fold increase	**Positive** 2.7-Fold increase
SHE 7-day	**Positive** 12.5-Fold increase	**Positive** 9-Fold increase
SHE Initiator	**Positive** 3.6-Fold increase	**Positive** 2.6-Fold increase
SHE Promoter	**Positive** 3.1-Fold increase	**Positive** 3.5-Fold increase
Bhas 42 Initiator	**Positive** 3.5-Fold increase	Negative 1.9-Fold increase
Bhas 42 Promoter (average of two repeat assays)	**Positive** 12.7-Fold increase	**Positive** 14.8-Fold increase

SUMMARY & CONCLUSIONS

- 3R4F TPM and 3R4F CSC positive as both initiator and promoter
- SHE 7-day and Bhas 42 promoter assays gave robust responses
- Bhas 42 promoter assay gave the most linear response and was reproduced in a repeat experiment
- Of the CTA models investigated, the Bhas 42 promoter assay appears the most promising as a technique for evaluating cigarette samples

REFERENCES
Kerckaert, G.A., *et al.* (1996). A comprehensive protocol for conducting the Syrian hamster embryo cell transformation assay at pH 6.70. Mutat. Res. 356 (1996) 65-84.
Brahmy D, Zhang H, Massey ED. Application of a two-stage Syrian hamster embryo cell transformation assay to cigarette smoke particulate matter. Mutat. Res. 572 (2005) 45-57.
Ohmori, K. *et al.* An assay method for the prediction of tumor promoting potential of chemicals by the use of Bhas 42 cells. Mutat. Res. 557 (2004) 191-202.