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DEVELOPMENT OF AN IN VITRO CELL TRANSFORMATION MODEL IN SYRIAN HAMSTER ETBRYO CELLS FOR CARCINOGEN DETECTION AND MECHANISTIC STUDIES

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Summary

Results from studies demonstrating enhanced multi-stage neoplastic transformation of Syrian hamster embryo cells cultured at pH 6.70 compared to cells cultured in medium of higher pH are reviewed. These results are discussed in the context of the development of an in vitro cell transformation model for assessing the carcinogenic potential of chemicals.

Introduction

An ideal short-term assay for assessing carcinogenic potential should detect a wide spectrum of genetic changes in the cell induced via both direct and indirect mechanisms. The system should also allow for the assessment of whether the changes in the DNA will or will not result in the neoplastic phenotype. In theory, in vitro cell transformation models fit these criteria.

We have chosen to use early passage Syrian hamster embryo (SHE) cells for the development of a transformation model because they 1) have a finite life span in culture and rarely spontaneously transform to the neoplastic phenotype 2) are diploid and genetically stable thus facilitating studies designed to examine specific chromosome changes involved in neoplastic transformation, 3) possess a competent metabolic system for the activation of a wide spectrum of chemicals to their ultimate carcinogenic form, 4) the SHE assay reflects the multi-stage nature of neoplastic transformation and thus has proven to be a valuable experimental model for studying mechanisms of chemical carcinogenesis and 5) genetic endpoints can be measured concurrently with transformation endpoints to determine the implications of various types of genetic damage on the transformation process[1-3].

While theoretically an attractive model, several experimental difficulties with SHE cells have made their routine use for assessing carcinogenic potential problematic. These difficulties include 1) the variability in sensitivity of various isolates of SHE cells to chemical transformation, 2) the dependence of the transformation response on specific lots of fetal bovine serum, 3) the relative subjectivity of identifying morphologically transformed (MT) colonies, the endpoint most commonly used to assess whether a chemical has induced preneoplastic changes in these cells, 4) the relatively low frequency of MT induction following chemical treatment, thus requiring extremely large sample sizes for the conduct of appropriate statistical analysis or alternatively establishing arbitrary criteria for establishing a "positive" response and finally, 5) the difficulty in confirming that cells from chemically-induced MT colonies progress to the neoplastic phenotype with a higher frequency than cells from morphologically normal colonies[4].

Our approach has been to minimize or eliminate the variables of the SHE assay which have proven problematic in the past. Our initial studies were designed to optimize medium for the culture of SHE cells in which pH was the first parameter examined.

Methods

For these studies, 4×10^4 irradiated SHE

feeder cells were plated in 2 ml of a modified formulation of Dulbecco's modified Eagle's medium (DMEM) containing 20 % fetal bovine serum in a 60 mm culture dish followed by the plating of 60 - 80 early passage SHE cells (isolated from mid-gestation hamsters) in 2 ml of medium 24 hours later. Control medium or test chemical (for the transformation experments) was added to the cultures 24 hours after target cell plating in a volume of 4 ml of medium and the cultures were maintained at 37 ℃ in an atmosphere of 10 % CO_2 in air and 90 % relative humidity undisturbed for an additional 7 days. The cultures were then fixed, stained and evaluated for cloning efficiency (number of colonies/number of cells plated X 100) and the frequency of MT (number of MT colonies/total colonies scored X 100). Alternatively, colonies were identified by phase contrast microscopy, selectively detached using standard cloning techniques and further cultured to determine their potential to progress to the neoplastic phenotype. Detailed methods for these studies have been published previously[5, 6]. For the studies designed to determine the effect of pH on clonal proliferation and transformation, increasing amounts of $NaHCO_3$ ranging from 0.25 to 4.50 g / l were added to a bicarbonate - free formulation of Dulbecco's modified Eagle's medium and a pH series ranging from pH 6.50 to pH 7.45 was established[7].

Results and Discussion

Results from these studies indicated that the clonal proliferation of SHE cells was optimal at pH 6.65 - 6.75 in contrast to pH 7.35 used historically for the culture of these cells[7]. Subsequent studies indicated that the frequency of MT induced by several carcinogens was significantly higher undey pH 6.70 culture conditions compared to media of higher pH.[4]. It was also observed that the variability in different isolates of embryo cells and serum lots with respect to transformation induction was greatly reduced at pH 6.70 compared to media of higher pH. Thus, virtually all isolates of embryo cells are transformed at pH 6.70 and approximately 75 % of commercial lots of fetal bovine serum are adequate to support the transformation response. These results have been independently reproduced in a collaborative inter-laboratory study[5]. The increased frequency of MT also makes it possible to apply accepted statistical procedures (Fisher's Exact Test) for the analysis of the transformation response compared to controls[5].

Studies designed to determine the neoplastic potential of MT colonies generated at pH 6.70, clearly indicate that the MT phenotype is associated with an increased probability of cells progressing to an immortal line and thus represents a "preneoplassic" phenotype in the model[6]. Furthermore, it has also been shown that the cell lines generated from pH 6.70 colonies progress to the neoplastic phenotype with high frequency (95 %) with additional passaging in culture. This multi-step progression is associated with the acquisition of aneuploidy in most of the cell lines following escape from cellular sensecence[6].

Shown in Table 1 is a summary of chemicals examined to date by us in the pH 6.70 SHE assay. Several points are worth noting from this data base. The first is that good agreement exists between carcinogenic activity as defined in the rodent bioassay for chemicals from a variety of chemical classes[8] and results in the transformation assay. The second is that carcinogens which lack activity in the standard tests for genotoxic activity [reserpine, PBB's, diethylhexylphtalate (DEHP), phenobarbital] induced a significant increase in MT frequency compared to controls. This point is particularly significant because it is this class of carcinogens that the SHE assay must detect if it is going to be a useful addition to an in vitro genotoxicity battery for assessing carcinogenic potential.

The lack of agreement between bioassay and transformation results with nitrilotriacetic acid (NTA) may be explained on a mechanistic basis. Several lines of evidence indicate that it is the ability of NTA to deliver high levels of Zn to the kidney which is responsible for NTA-induced renal carcinogenesis and not a direct effect of

Table 1. Summary of transformation results for carcinogens and non-carcinogens examined in the pH 6.70 SHE cell transformation assay.

Chemical	Carcinogenicity	Transformation
2 - Acetylaminofluorene	+	+
Benzo [a] pyreme	+	+
Cadmium Chloride	+	+
Colcemid	?	+
Diethylhexylphthalate	+	+
Lead Acetate	+	+
3 - Methylcholanthrene	+	+
MNNG	+	+
Nitrilotriacetic acid (NTA)	+ ?	−
Zinc-NTA	+	+
Zinc Chloride	+	+
Phenobarbital	+	+
Polybrominated Biphenyls	+	+
Reserpine	+	+
4 - Acetylaminofluorene	−	−
Anilazine	−	−
Anthracene	−	−
Bisphenol A	E	−
Caprolactam	−	−
EDTA	−	−
Mannitol	−	−
3 - Nitropropionic Acid	E	−
Tetraethylthiuram Disulfide	−	+

NTA on the tissue[9]. A positive result with the Zn chelate of NTA but not NTA itself in the SHE assay supports this hypothesis. This hypothesis is supported further by the fact that Zn Cl_2 is effective at transforming SHE cells. The basis for the "false positive" result with TETD is unknown at this time but may be explained by chelation properties of TETD.

Recent unpublished results from our laboratory indicate that many effects of chelators (including mutagenesis) are dependent on whether the biological system is exposed to the chelator or the chelator-metal complex. Thus differences in the from of the chelator interacting with the biological system may explain differences in in vivo compared to in vitro results..

Based on data from our initial chemical screening studies, insight into a chemical's mechanism of induction of neoplastic transformation may be gained depending on the length of chemical exposure to the cells that is required to cause the transformation response. A specific example is phenobarbital. Phenobarbptal caused a significant, dose-dependent increase in MT frequency when it was left in the medium for the entire 7 day culture period (Fig. 1). The increase in MT frequency occurred in the absence of a significant decrease in relative plating efficiency compared to controls thus indicating that it is unlikely that the increase was a result of toxicity. In contrast, when the cells were exposed to phenobarbital for 24 hours and then refed with control medium for the subsequent colony development period, an increase in MT frequency was not observed at the end of the culture period (Fig. 1). In contrast, benzo [a] pyrene or N-methyl-N'-nitro-N-nitroso-guanidine (MNNG) caused a significant increase

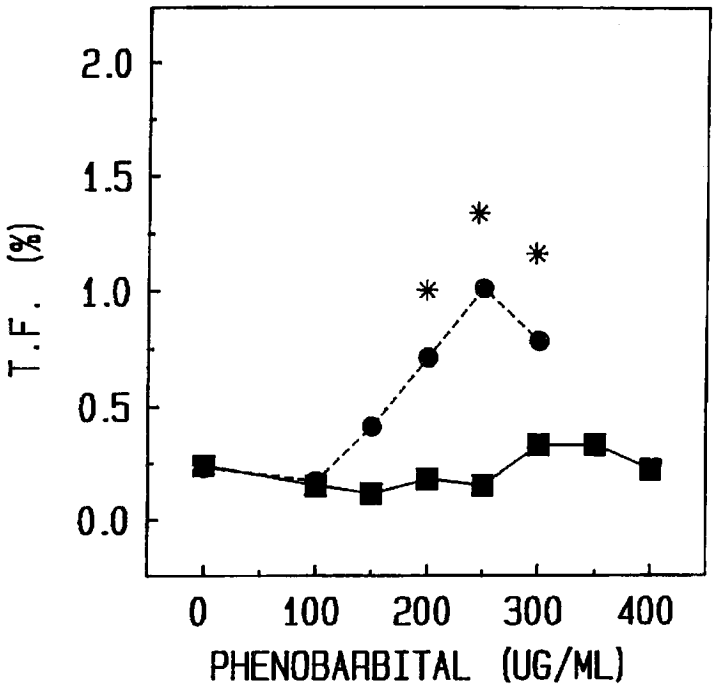

Fig. 1. The effect of exposure duration on phenobarbital-induced morphological transformation. Clonal SHE cell cultures were prepared as described in the Methods. Medium containing phenobarbital at various concentrations was added to the cultures 24 hours after SHE cell plating. Cells were then exposed to phenobarbital-containing medium for either 24 hours followed by culture in control medium for 6 days (■—■) or to phenobarbital-containing medium for the entire 7 day culture period (●···●) at which time the cultures were evaluated for MT frequency. Approximately 1000 to 1500 colonies were scored for each treatment group. T. F. = morphological transformation frequency; * indicates a statistically significant ($p<0.05$) increase in MT frequency compared to concurrent control as indicated by a one-sided Fisher's Exact Test[5].

in MT frequency with a 24 hour exposure to the chemical. A similar pattern of a significant increase in MT frequency induced with a 24 hour exposure to the chemical. A similar pattern of a significant increase in MT frequency induced with a 7 day exposure but no increase in MT with an exposure of shorter duration has also been reported to occur with the tumor promoter TPA[10]. Therefore it is plausible, that there exist s a qualitative difference between "non-genotoxic" and gonotoxic carcinogens in the SHE assay based on differences in the length of exposure required for the two classes of carcinogens to cause a significant increase in MT frequency. The duration of exposure required for transformation may ultimately be a valuable tool for helping to define the mechanistic basis for neoplastic transformation of chemicals shown to be carcinogenic *in vivo*.

In conclusion, pH 6.70 multi-stage transformation of SHE cells is promising model for assessing the carcinogenic potential of chemicals as well as understanding the mechanistic basis of chemically-induced neoplastic transformation. The impact of cell culture variables on the transformation response has been reduced at pH 6.70 and to date, a good agreement exists between bioassay and transformation results. Studies are curreutly in progress to extend the current data base as well as to define the molecular basis of

transformation in SHE cells.

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