The Use of Liver Epithelial Cultures for the Study of Chemical Carcinogenesis

Gary M. Williams, MD

Liver cultures offer several special advantages for the study of chemical carcinogenesis in cell culture; these include the sensitivity of the cells to procarcinogens requiring enzymatic activation, the epithelial nature of the cells which qualifies them as a model for epithelial carcinogenesis, and the opportunity to compare culture findings with the extensive information available on the effects of carcinogens on liver. The actions of chemical carcinogens have been studied in primary and long-term rat liver cell cultures. A variety of procarcinogens induced DNA repair in primary cultures, indicating the usefulness of this system for studying carcinogen metabolism, the interaction of carcinogens with DNA, and the repair of carcinogen-induced DNA damage. In addition, this system may provide a screen for chemical carcinogens in which metabolic activation occurs in the target cell. Carcinogen treatment of long-term cultures initiated from the primary cultures resulted in morphologic transformation accompanied by an increased growth in soft agar and an increased frequency of 8-azaguanine-resistant mutants. Cultures with a high fraction of cells in S phase were found to be most sensitive to the induction of 8-azaguanine-resistant mutants. (Am J Pathol 85:739–754, 1976)

THE STUDY OF CHEMICAL CARCINOGENESIS in cell culture offers the possibility of elucidating the mechanisms of malignant conversion in a simplified, easily manipulatable, readily observable system. Most investigations have utilized fibroblast cultures[1-6] because of their ease of cultivation, but attention has been directed to other cell types for a variety of reasons. The considerable interest in liver cell cultures appears to stem largely from the hope of maintaining the functions of the versatile hepatocyte in culture. As regards carcinogenesis, Katsuta and Takaoka[7] pointed out that, whereas sarcomas resulted from malignant conversion of fibroblasts, most human cancers were carcinomas and they, therefore, recommended the study of liver cell cultures. Williams *et al.*[8] noted that the fibroblast cultures had displayed sensitivity to limited classes of carcinogens and suggested that the liver, as the organ with the broadest capability of metabolic activation, should provide cultures with respon-

From the Naylor Dana Institute for Disease Prevention, American Health Foundation, Valhalla, New York.

Presented at the Sixtieth Annual Meeting of the Federation of American Societies for Experimental Biology, Anaheim, California, April 13, 1976.

Supported by Grant BC-133A from the Alexander Rolston Peacock Memorial Fund for Cancer Research of the American Cancer Society and by Contract NO1-CP-55705 from the National Cancer Institute.

Address reprint requests to Dr. Gary M. Williams, Naylor Dana Institute for Disease Prevention, One Dana Road, Valhalla, NY, 10595.

siveness to the widest variety of carcinogens. These two features continue to be the most frequently cited rationales for studying chemical carcinogenesis in liver cell cultures. In addition, another major consideration that has been noted [9] is that the extensive research on the action of carcinogens on liver provides great potential for comparison to cell culture findings.

This report reviews studies on the effects of chemical carcinogens on cultured liver cells with attention to the progress made in utilizing the described features which recommend liver cells for such studies.

Enzymatic Activation of Carcinogens

Long-Term Liver Cell Cultures

Long-term cultures have displayed toxic responses to a variety of procarcinogens,[10-14] implying that activation occurred. However, most descriptions of malignant conversion have dealt with direct-acting carcinogens which do not require enzymatic activation [7,15-17] Transformation with aflatoxin B_1 (AFB_1) has been reported,[18] but the neoplasms resulting from inoculation of the treated cells were fibrosarcomas. The first report of malignant conversion by a variety of procarcinogens was that of Williams *et al.*[8] Subsequently, Montesano *et al.*[19] also obtained transformation with dimethylnitrosamine (DMN) and with a direct-acting carcinogen. These studies thus suggest that cultured liver cells do retain activating capability.

The ability of liver cultures to activate procarcinogens may be studied by measuring the nature of metabolites or their interaction with cellular macromolecules. The interaction with DNA has been examined by studying the sedimentation pattern of DNA from carcinogen-treated cultures. Direct-acting carcinogens readily induced single strand DNA breaks,[9,20] but the procarcinogens, AFB_1, 7,12-dimethylbenz[a]anthracene (DMBA), DMN, and *N*-2-fluorenylacetamide (AAF) at 10^{-6} M all failed to do so.[9] The repair of DNA damage was active in these cultures,[9,20] and therefore, it was suspected that a low level of activation might produce so little interaction that it would be readily repaired without accumulating to a degree producing sufficient breakage to be detectable. To examine this possibility, the induction of breakage was studied in the presence of chloroquine, an agent shown to inhibit DNA repair.[20] With this addition, all four procarcinogens produced DNA breakage.[9] This could result from additive toxicity, since the addition of chloroquine, even after the removal of carcinogen and with an interval for production of maximal DNA damage, resulted in enhanced cell killing.[20] But even so, it would indicate carcinogen-induced toxicity. Thus, additional evidence was obtained for the ability of these liver cultures to metabolize procarcinogens.

Table 1—Agents Studied for Induction of DNA Repair in Primary Hepatocyte Cultures

Compounds	Carcinogenicity*	DNA repair†
Direct acting carcinogens		
Methyl methanesulfonate	++	+
Methylazoxymethanol acetate	++	+
Procarcinogens and analogs		
Aflatoxin B_1	++	+
Aflatoxin B_2	+	+
Aflatoxin G_1	++	+
Aflatoxin G_2	+	+
N-2-Fluorenylacetamide	++	+
N-4-Fluorenylacetamide	+	+
7,12-Dimethylbenz[a]anthracene	++	+
Benz[a]anthracene	+	+
Anthracene	+	+
Dimethylnitrosamine	++	+
Dimethylformamide	+	+
3'-Methyl-4-dimethylaminoazobenzene	++	+
4-Aminoazobenzene	+	+

* ++ = Definite, + = weak, + = noncarcinogenic.
† DNA repair was determined by the autoradiographic measurement of unscheduled DNA synthesis as in Williams.[23] + = positive, + = negative.

Primary Cultures

Because of the apparent low level of carcinogen-activating capability in long-term cultures, primary cultures were developed [21,22] for such studies. The activation of carcinogens was determined by their induction of DNA repair as evidence of interaction with DNA.[23] As *in vivo*, the level of replication in the adult hepatocytes was low, being about 0.05 to 0.1% at 24 to 48 hours in culture.[21,23] This low level of replicative DNA synthesis made it possible to study autoradiographic DNA repair which is otherwise obscured by replicative synthesis. Methyl methanesulfonate (MMS), AFB_1, and AFB_2 induced DNA repair.[23,24] Since most chemical carcinogens in their ultimate reactive form are electrophilic reactants [25] which interact with DNA [25-28] inducing repair synthesis,[29-33] this system with its metabolic capability was suggested to be a possible screen for chemical carcinogens.[23,24] Subsequent studies [34] have shown that a number of carcinogens, including representatives of several classes involving different pathways of metabolic activation, would induce DNA repair in the cultures (Table 1). In view of these results and since liver has the enzyme systems involved in the metabolism of all known procarcinogens,[35] these primary cultures have substantial potential as a screen for carcinogens. In

addition, the primary cultures may also be used to study several aspects of carcinogen metabolism and DNA damage and repair. Owens and Nebert [36] have noted differences between the induction of aryl hydrocarbon hydrozylase and cytochrome P_1-450 in primary fetal rat liver cultures and in adult liver, emphasizing the possibility that this might result in marked differences in metabolic products of carcinogens. Bissell and Guzelian [37] reported an 80% drop in cytochrome P-450 content of adult primary rat liver cultures during the first 24 hours, but provided no data on viability. The present system being derived from mature liver and remaining nonproliferative should, in contrast to fetal cultures, yield carcinogen metabolism more similar to that of the adult liver. In addition, the maintenance of at least 80% viability for the first 24 hours in culture [21] permits meaningful studies on carcinogen metabolism.

Liver Cultures as a Model for Epithelial Malignant Conversion

Carcinomas From Liver Cultures

The suggestion of Katsuta and Takaoka [7] that liver cultures would provide a model for epithelial carcinogenesis requires some consideration. The hepatocyte is a glandular epithelial cell, but it is not a true epithelial cell in the sense of those epithelia which cover external or internal surfaces. An important difference is that hepatocytes do not mature into permanently nondividing functional cells and, therefore, may not be an appropriate cell type for studies on the mechanisms by which carcinogens could interfere with maturation and cessation of replication. Nevertheless, similarly to typical epithelial cells, hepatocytes appear to pass through a series of stages in neoplastic development *in vivo* (see Williams [38]) and, therefore, may represent a suitable model for epithelial carcinogenesis. Liver cells in culture have behaved as epithelial-like cells,[15,39-43] displaying growth of closely adherent polygonal cells in mosaic-like sheets. Their epithelial nature is further supported by the presence of tight cell junctions and desmosomes.[39,44,45] Therefore, such cultures may be used to study neoplastic conversion of epithelial cells, but the applicability of the findings depends upon whether neoplastic conversion results in neoplastic cells capable of forming carcinomas upon inoculation into hosts. There are numerous suggestive reports of this, but some of the documentation is difficult to evaluate for a variety of reasons. Sato *et al.*[46] reported back-transplantation of spontaneously transformed rat liver cell lines that produced mainly carcinosarcomas and, in the case of one line, carcinomas. The latter unfortunately were not illustrated, except for a small metastatic focus. Oshiro *et al.*[47] also described a neoplastic liver line which formed

carcinomas but which was not ever documented to be nonneoplastic by inoculation into syngeneic hosts. Diamond *et al.*[39] have illustrated a poorly differentiated intrahepatic carcinoma in a rat which received an intraperitoneal injection of spontaneously transformed liver cells. Namba *et al.*[16] and Katsuta and Takaoka[7] reported carcinogen-induced malignant transformation of rat liver lines and claimed to have obtained carcinomas following inoculation of treated cells. Their illustrations of peritoneal implants, however, show connective tissue stalks lined by small undifferentiated cells but fail to demonstrate either definitive glandular formations or characteristic epithelial nests of cohesive, tightly apposed cells with substantial cytoplasms and prominent cell membranes. Ultrastructural study of one of these tumors[48] also revealed no specific features of epithelial cells. On the other hand, Williams *et al.*[8] found that inoculation of carcinogen-treated liver lines produced poorly differentiated carcinomas and papillary and glandular adenocarcinomas. Similar results were obtained by Montesano *et al.*[19,49] using the same culture procedure. Thus, there is definitive evidence that epithelial cell lines can be initiated from rat liver and that these lines can be converted to carcinomatous cells. Therefore, regardless of the hepatic epithelial cell of origin of such lines, they do provide a culture model for study of malignant conversion of epithelial cells.

The Relation of Cultured Liver Epithelial Cells to Hepatocytes

If the knowledge of the effects of carcinogens on hepatocytes is to be applied to cell culture and vice versa, then the cultured cells logically should be derived from hepatocytes. Cultured liver epithelial cells were found by Diamond *et al.*[39] to form bile canaliculi and by Williams *et al.*[44] to possess a cytoplasmic morphology more similar to that of hepatocytes than bile duct epithelia. In addition, individual hepatocytic functions such as glucose-6-phosphatase,[39] tyrosine aminotransferase,[9] albumin,[9,15] and α-2-globulin[39] secretion, and α-fetoglobulin synthesis[9] have been detected. Williams *et al.*[44] demonstrated that the histochemical profile of 13 enzymes in cultured liver cells was closer to that of hepatocytes than any other cell type in the liver. Thus, the evidence is highly suggestive that cells of this type are related to hepatocytes. Furthermore, no evidence has been obtained that such cells are derived from any other progenitor. The fact that, following neoplastic conversion of these cells, they have the ability to form adenocarcinomas upon transplantation does not necessarily indicate a bile duct origin.[8] The embryologic differentiation of hepatocytes into bile duct epithelium[50] accounts for the ability of neoplastic hepatocytes to assume a ductular architecture. In conclusion, the current

evidence favors hepatocytic origin of these liver cultures. However, it is clear they are not representative of mature hepatocytes. The reason for this may well lie in a dichotomy between continued proliferation in culture and functional maturation.[44] Hepatocytes are normally nondividing, and it may be that the requirement of continued proliferation results in phenotypic simplification or acts as a selective factor for a subpopulation capable of proliferation but limited in its functional capacity. It has been demonstrated that certain culture conditions will enhance the phenotypic expression of rat liver cells [44] and hepatocellular carcinoma cells.[51] Hopefully, such experiments will elucidate the factors involved in determining the level of functional expression of cultured liver cells.

Functional hepatocytes in culture will, of course, be of great value for the study of hepatocarcinogenesis and as a model for malignant conversion of epithelial cells in culture. However, they may not be appropriate models for studying the role of factors involved in carcinogenesis in other epithelial tissues. For example, it is unlikely that they will be useful for examining the hormone responses involved in neoplastic conversion in hormone-sensitive tissues. Thus, it will be necessary to develop epithelial cultures from organs of interest to examine the role of special factors.

Transformation of Cultured Rat Liver Cells

One of the difficulties encountered in carcinogenesis studies on rat liver cell cultures is the lack of criteria for transformation. Several investigators have indicated that there are no reliable morphologic features of transformation.[17,49] To the contrary, Williams *et al.*[8] observed definite morphologic changes in four of five carcinogen-treated sublines before tumorigenicity was detected. The acquisition of an irregular outline of islands in subconfluent cultures was emphasized, and piling up (although not prominent in sublines in continuous culture) was marked after reculture of tumors arising from inoculated sublines. Borek [52] and Borenfreund *et al.*[15] have also noted this piling-up. In order to use these epithelial lines to study the pathogenesis of malignant conversion, it is necessary to develop reliable quantitative essays of transformation. Therefore, we have been studying the quantification of a variety of phenotypic properties in adult rat liver lines during carcinogen treatment. The lines were initiated from the primary cultures,[22,42] and interestingly, their morphology (Figure 1) was identical to that of the newborn rat liver lines.[43] The adult lines were exposed continuously to concentrations of 10^{-6} M of the carcinogens used previously for malignant conversion and for the DNA breakage studies. All sublines were subcultured synchronously and seeded at a constant density. In a few cases, the carcinogens slowed the growth rates of

exposed sublines such that they could not be maintained synchronously with their controls. After treatment intervals of 4 and 8 weeks, the carcinogens were discontinued and the sublines were assessed for the presence of various transformed properties.

Morphologically altered colonies appeared as early as 2 weeks (Figures 1 and 2) and persisted following removal of the carcinogens. These altered colonies were composed of pleomorphic cells and displayed the same irregular peripheral contour noted previously.[8] The colony-forming efficiency in cell culture of treated sublines did not differ from that of controls, about 10%.

As with other neoplastic lines, neoplastic and transformed liver cells have been found to form colonies in soft agar [17,19,40,52] This has always been treated as a qualitative change usually with no precise criteria for the definition of a colony. To quantify growth in agar we have adopted the criterion of formation of eight cell colonies. This requirement necessitates at least three rounds of cell division during the 9-day assay period and, therefore, identifies cells capable of at least modest growth in agar. This criterion adequately distinguished between normal rat liver lines, which had little or no ability to grow in agar under our modified conditions (Table 2), and carcinoma lines, in which a majority of cells could grow

Table 2—Growth of Liver Cells in Soft Agar*

Line	Duration in culture (mons)	Times tested	Colony-forming efficiency† (%)	
			Average	Range
Normal				
ARL 6	40	4	0.8	0.1-1.7
ARL 12	12	1	0	
ARL 14	5	1	0	
ARL 15	5	1	0	
ARL 16	3	2	0	
ARL 17	4	2	4.7	3.6-5.7
Carcinoma				
HTC	>24	10	73.6	52.8-90.1
RH 35	>24	3	55.8	33.0-68.3
NDI-RHCI	12	1	58.4	

* Cells were seeded in an overlay composed of 0.36% agar and 20% FBS in WE on top of an underlay containing 0.5% agar and 20% FBS in WE. The number of clumps of colony size was immediately counted for subtraction in determining colony formation. The overlay was fed 0.5 ml serum-free WE on Days 3 and 6, and the colonies were counted on Day 9.

† An aggregate of eight cells or greater was considered to be a colony, since this number would result from a minimum of three rounds of cell division during the assay interval. Therefore, the colony-forming efficiency was (No. of colonies on Day 9 − No. of aggregates on Day 0)/No. of viable cells seeded × 100.

Table 3—Colony-Forming Efficiency in Soft Agar Following Carcinogen Treatment*

Treatment	Interval of treatment of ARL 6 line		Interval of treatment of ARL 14 line	
	4 wks	8 wks	4 wks	8 wks
Stock (untreated)	1.7	0.0	1.8	—
Control (DMSO)	1.5	0.1	0.0	0.0
AFB_1	3.5	2.6	3.8	4.1
DMBA	0.5	1.1	0.0	0.0
DMN	0.0	4.7	0.0	0.0
AAF	0.8	1.0	0.0	0.0
Positive control HTC	71.1	52.8	68.3	101.0

* Four or eight weeks at 10^{-6} M of carcinogens and 0.1% DMSO.

(Table 2). The growth potential in agar of these normal lines and experimentally treated cells was always tested in conjunction with a carcinoma line as a positive control. Treatment with AFB_1 consistantly produced an increase in colony-forming cells (Table 3), and DMN appeared to do so in one experiment.

Concanavalin A (con A) agglutination has been suggested to be a reliable marker for neoplastic hepatocytes *in vivo* [53] and in culture.[54,55] We have found that rat liver cells dissociated either with or without collagenase were agglutinable with con A [38] in agreement with Kapeller and Doljanski,[56] but at variance with the findings of Becker [53] on liver cells prepared in a different manner. Also, cultured liver cells were agglutinable (Table 4) as Borek *et al.*[54] reported. However, Katsuta and Ta-

Table 4—Concanavalin A Agglutination of Normal and Carcinoma Liver Lines*

Line	Times tested	Average percent agglutination†	
		Con A alone‡	Con A plus MMP‡
Normal			
ARL 6	3	70 ± 14	6
ARL 12	1	55	0
ARL 14	1	48	0
ARL 15	1	73	0
Carcinoma			
HTC	3	31 ± 10	6
RH35	3	47 ± 14	0
NDI-RHCI	2	61	13

* Cells were harvested with 0.5 mM EGTA in Ca^{2+} Mg^{2+}-free Hank's balanced salt solution. A 1 ml agglutination mixture containing 0 to 60 μg con A and 0.5 × 10^6 cells was gently agitated for 15 minutes. An aliquot was counted in a hemocytometer for the number of unagglutinated single cells and the degree of clumping (1 to 4+). The number of unagglutinated cells was used to calculate the percent agglutination.

† (No. single cells 0 μg per ml con A − No. single cells 60 μg per ml con A)/No. single cells 0 μg per ml con A × 100.

‡ Con A at 60 μg/ml was the highest concentration used. Methyl-α-D-mannopyranoside at 10^{-2} M was used as a specific inhibitor of con A-mediated agglutination.

kaoka [7] and Roth *et al.*[55] were unable to agglutinate normal lines. The basis for this difference in findings on the agglutinability of normal cells is unclear, but it has been established that liver cells have con A receptors [55,57] and are, therefore, potentially agglutinable. The demonstration of agglutination in the present cells may be due to the methods of maintaining and handling the cells. For example, Borek *et al.*[54] stated that vitamin A treatment would increase agglutinability. Vitamin A is present in the medium WE [51] that was used in these studies.

There was no increase in the con A agglutination of carcinogen-treated cells at 4 or 8 weeks. However, the agglutinability of hepatocellular carcinoma lines was generally lower than that of the control epithelial-like lines (Table 4). It has been repeatedly confirmed that the carcinoma lines which grew regularly in agar with efficiencies of 50 to 90% were less agglutinable than control lines which, at best, had a colony-forming efficiency in agar of 5.7% (Table 2). Therefore, high con A agglutinability was neither a feature of malignant hepatocytes nor a sensitive marker for transformation of liver epithelial cells. Although these results differ from other observations on liver cells systems,[54,55] they are similar to the finding of Glimelius *et al.*[58] that in a human lymphoid cell system there was no systematic correlation between the neoplastic state and sensitivity to con A.

Another change which was studied following carcinogen treatment was the frequency of 8-azaguanine–resistant mutants (AG^r) (Table 5). This mutation results in a deficiency of hypoxanthine-guanine phosphoribosyltransferase and thereby prevents mutant cells from incorporating the toxic purine analog. The rat liver epithelial cells were quite sensitive to 8-azaguanine, but interestingly, rat liver fibroblasts were completely resist-

Table 5—Frequency of 8-Azaguanine–Resistant Mutants in a Rat Liver Line Following Carcinogen Treatment*

Treatment	Number of AG^r cells/10^6 colony-forming cells†	
	ARL 6	ARL 14
None (stock ARL 6)	53	147
DMSO (Control)	108	0
AFB_1	148	1320
DMBA	75	0
DMN	153	0
AAF	268	—

* Eight weeks at 10^{-6}M of carcinogens and 0.1% DMSO.

† Cells were seeded at 10^4 cells/sq cm; 60 μg/ml 8-azaguanine in WE was started 1 day after inoculation and refed every 2 days. At 12 days, the cultures were fixed and stained for counting visible 8-azaguanine-resistant (AG^r) colonies. The percentage of colony-forming cells was measured by inoculating flasks at 20 cells/sq cm in WE containing 10% FBS.

ant.[59] In our epithelial lines, there were up to 150 AG^r cells/10^6 colony-forming cells. With carcinogen treatment, this level was increased by AFB_1 DMN, and AAF. Since there are always spontaneous mutants in these and other lines, such a result could be due to selection of pre-existing mutants or the induction of new mutants. Regardless, it clearly indicates an effect of the carcinogens which was not random, since it always involved an increase in AG^r cells by carcinogens. Therefore, it seems hopeful that this property may be used to study the effects of carcinogens on rat liver cells.

In summary: These studies have confirmed the induction of morphologic changes by carcinogens and have established an association with other phenotypic changes. But it remains to be demostrated that the quantity of morphologically altered cells corresponds to the level of other quantified changes and that the morphologically altered cells are, in fact, neoplastic. The testing of clonal isolates will be required to establish the latter correlation. Nevertheless, the inducibility of quantifiable phenotypic changes offers some opportunities for the study of mechanisms of carcinogenesis.

Relation of Carcinogen Effects to Cell Cycle Sensitivity

There is considerable evidence that carcinogens induce a greater oncogenic response in dividing cells than in nondividing cells.[60,61] In particular, liver cells stimulated to proliferate are more suspectible to malignant conversion than resting liver cells.[62-65] Whether this is due to a requirement of cell division to fix a mutation or reflects a sensitivity of certain phases of the cell cycle can only be resolved by cell cycle studies. Cell culture studies have yielded conflicting suggestions that maximal sensitivity to transformation may occur in the late G_1[66] or S[67] phases. The sensitivity of the liver epithelial cells to mutagenesis was studied using a procedure for minimizing and enriching cells in DNA synthesis which did not involve metabolic inhibition. Cultures rendered quiescent by 1% serum feeding contained only 20% of cells in DNA synthesis.[68] Stimulation of such cultures with medium containing 10% FBS resulted in an increase at 13 hours to over 50% of the cells in DNA synthesis. Treatment of paired quiescent controls in which cells were distributed in G_2 and early G_1 (not shown) and stimulated cultures with a 20-minute pulse of 3 mM MMS produced greater cell killing in the stimulated controls and a higher level of mutagenesis (Table 6). This suggests that during DNA synthesis in the S phase there is both greater sensitivity to the toxic action of carcinogens and increased susceptibility to their mutagenic effects. This finding complements that of Marquardt [67] of enhanced transformation in

Table 6—8-Azaguanine-Resistant Liver Cells Following Carcinogen Treatment* of Cultures With High and Low Levels of DNA Synthesis

Culture	Percent cells in S phase during treatment	Colony-forming efficiency following treatment	AG^r cells/10^6 treated colony-forming cells†
Quiescent	18	9.5%	32
Stimulated	53	4.4%	330

* Methyl methanesulfonate at 3 mM for 20 minutes.
† Determined as described in Table 5.

the S phase. However, Bertram and Heidelberger [66] have reported that late G_1 was the phase most susceptible to oncogenic effects of carcinogens. The ultimate goal of identifying cell cycle differences is to relate the observed differences to the induction of a molecular event. It still remains to be determined to what molecular event (e.g., the degree or type of damage inflicted or the lack of opportunity to repair it) the observed results were due. If this can be determined, apparent discrepancies will be resolved.

Conclusions

Results from a number of laboratories indicate that rat liver epithelial cultures offer special properties for the study of chemical carcinogenesis and that the study of neoplastic conversion in these cultures will have relevance to the problem of epithelial carcinogenesis.

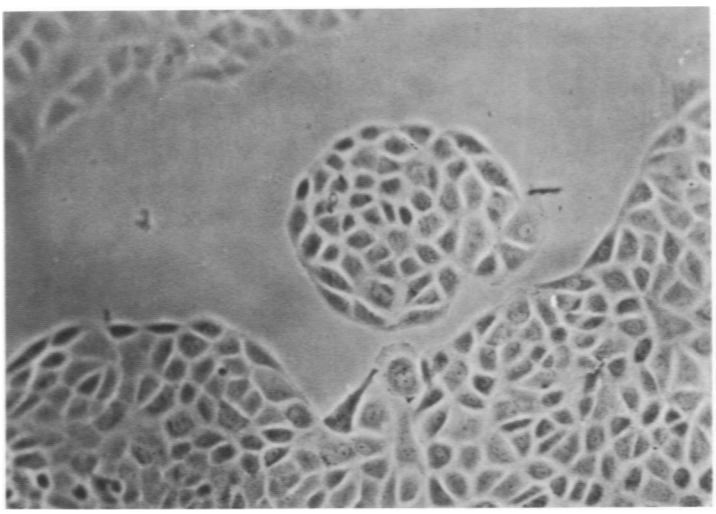
References

1. Berwald Y, Sachs L: In vitro transformation of normal cells to tumor cells by carcinogenic hydrocarbons. J Natl Cancer Inst 35:641–661, 1965
2. Borenfreund E, Krim M, Sanders FK, Sternberg SS, Bendich A: Malignant conversion of cells in vitro by carcinogens and viruses. Proc Natl Acad Sci USA 56:672–679, 1966
3. DiPaolo JA, Donovan PJ: Properties of Syrian hamster cells transformed in the presence of carcinogenic hydrocarbons. Exp Cell Res 48:361–377, 1967
4. Heidelberger C, Iype PT: Malignant transformation in vitro by carcinogenic hydrocarbons. Science 155:214–217, 1967
5. Sato H, Kuroki T: Malignization *in vitro* of hamster embryonic cells by chemical carcinogens. Proc Jap Acad 42:1211–1216, 1966
6. Sivak A, Van Duuren BL: Studies with carcinogens and tumor-promoting agents in cell culture. Exp Cell Res 49:572–583, 1968
7. Katsuta H, Takaoka T: Carcinogenesis in tissue culture. XIV. Malignant transformation of rat liver parenchymal cells treated with 4-nitroquinoline-1-oxide in tissue culture. J Natl Cancer Inst 49:1563–1576, 1972
8. Williams GM, Elliott JM, Weisburger JH: Carcinoma after malignant conversion in vitro of epithelial-like cells from rat liver following exposure to chemical carcinogens. Cancer Res 33:606–612, 1973
9. Williams GM: The study of chemical carcinogenesis using cultured rat liver cells. Gene Expression and Carcinogenesis in Cultured Liver. Edited by LE Gerschenson, EB Thompson. New York, Academic Press, Inc., 1975, pp 480–487

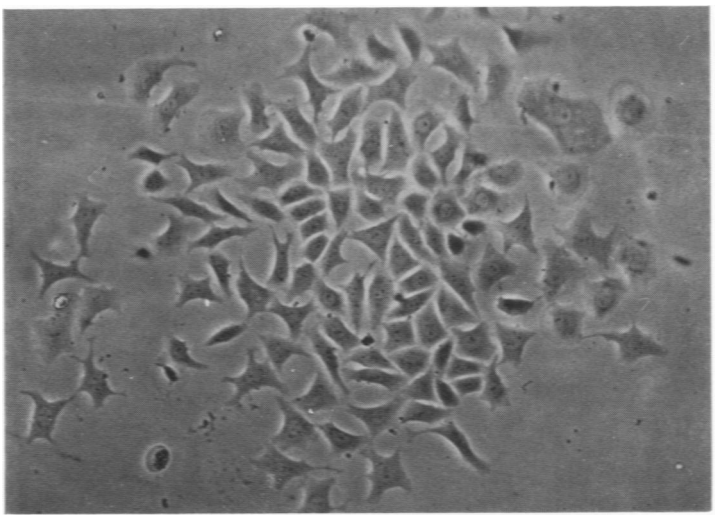
10. Bausher J, Schaeffer WI: A diploid rat liver cell culture. I. Characterization and sensitivity to aflatoxin B_1. In Vitro 9:286–293, 1974
11. Sato J, Yabe T: Carcinogenesis in tissue culture. V. Effects of long-term addition of 4-dimethyl-aminoazobenzene and 3'-methyl-4-dimethyl-aminoazobenzene on liver cells in culture. Jap J Exp Med 35:445–462, 1965
12. Schwartz AG: The protective effect of benzoflavone and estrogen against 7,12-dimethylbenz(a)anthracene and aflatoxin-induced cytotoxicity in cultured liver cells. Cancer Res 34:10–15, 1974
13. Sullman SF, Armstrong SJ, Zuckerman AJ, Rees KR: Further studies on the toxicity of the aflatoxins on human cell cultures. Br J Exp Pathol 51:314 316, 1970
14. Umeda M, Saito M: Carcinogenesis in tissue culture. XI. Cytotoxic effects of 4-dimethylaminoazobenzene and its derivatives on HeLa Cells and on rat liver and kidney cells in culture. Jap J Exp Med 39:601-613, 1969
15. Borenfreund E, Higgins PJ, Steinglass M, Bendich A: Properties and malignant transformation of established rat liver parenchymal cells in culture. J Natl Cancer Inst 55:375–384, 1975
16. Namba M, Masuji J, Sato J: Carcinogenesis in tissue culture. IX. Malignant transformation of cultured rat cells treated with 4-nitroquinoline-1-oxide. Jap J Exp Med 39:253–265, 1969
17. Weinstein IB, Yamaguchi N, Gebert R: Use of epithelial cell cultures for studies on the mechanism of transformation by chemical carcinogens. In Vitro 11:130–141, 1975
18. Toyoshima K, Hiasa Y, Ito N, Tsubura Y: In vitro malignant transformation of cells derived from rat liver by means of aflatoxin B_1. Gann 61:557–561, 1970
19. Montesano R, Saint Vincent L, Tomatis L: Malignant transformation in vitro of rat liver cells by dimethylnitrosamine and *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine. Br J Cancer 28:215–220, 1973
20. Michael RO, Williams GM: Chloroquine inhibition of repair of DNA damage induced in mammalian cells by methyl methanesulfonate. Mutat Res 25:391–396, 1974
21. Laishes BA, Williams GM: Conditions affecting primary cultures of functional adult rat hepatocytes. I. The effect of insulin. In Vitro 12:521–532, 1976
22. Williams GM: Primary and long-term culture of adult rat liver epithelial cells. Methods Cell Biol 14:357–364, 1976
23. Williams GM: Carcinogen-induced DNA repair in primary rat liver cell cultures: A possible screen for chemical carcinogens. Cancer Letters 1:231–236, 1976
24. Williams GM: Carcinogen-induced DNA repair in primary rat liver cell cultures: A possible screen for chemical carcinogens. National Cancer Institute Carcinogenesis Program, Fourth Annual Collaborative Conference. Washington, DC, US Department of Health, Education and Welfare, 1976, p 173 (Abstr)
25. Miller JA, Miller EC: Chemical carcinogenesis: Mechanisms and approaches to its control. J Natl Cancer Inst 47:5–14, 1971
26. Brookes P: On the interaction of carcinogens with DNA. Biochem Pharmacol 20:999-1003, 1971
27. Irving CC: Interaction of chemical carcinogens with DNA. Methods Cancer Res 7:907–920, 1973
28. Sarma DSR, Radjalakshmi S, Farber E: Chemical carcinogenesis: Interactions of carcinogens with nucleic acids. Cancer, A Comprehensive Treatise, Vol 1. Edited by FF Becker. New York, Plenum Press, 1975, pp. 235–287
29. Laishes BA, Stich HF: Repair synthesis and sedimentation analysis of DNA of human cells exposed to dimethylnitrosamine and activated dimethylnitrosamine. Biochem Biosphys Res Commun 52:827–833, 1973
30. Lieberman MW, Baney RN, Lee RE, Sell S, Farber E: Studies on DNA repair in

human lymphocytes treated with proximate carcinogens and alkylating agents. Cancer Res 31:1297–1306, 1971
31. Roberts JJ, Pascoe JM, Plant JE, Sturrock JE, Crathorn AR: Quantitative aspects of the repair of alkylated DNA in cultured mammalian cells. I. The effect of HeLa and Chinese hamster cell survival on alkylation of cellular macromolecules. Chem Biol Interact 3:29–47, 1971
32. Setlow RB, Regan JD: Defective repair of N-acetoxy-2-acetylaminofluorene–induced lesions in the DNA of xeroderma pigmentosum cells. Biochem Biophys Res Comm 46:1019–1024, 1972
33. Stich HF, San RHC: DNA repair and chromatid anomalies in mammalian cells exposed to 4-nitroquinoline-1-oxide. Mutat Res 10:389–404, 1970
34. Williams GM: The detection of chemical carcinogens by induction of DNA repair in primary liver cell cultures. (Unpublished observations)
35. Weisberger JH, Williams GM. Metabolism of chemical carcinogens.[28] pp 185–234
36. Owens IS, Nebert DW: Aryl hydrocarbon hydroxylase induction in mammalian liver-derived cell cultures: Stimulation of "cytochrome P_1 450 associated" enzyme activty by many inducing compounds. Mol Pharmacol 11:94–104, 1975
37. Bissell DM, Guzelian PS: Microsomal functions and phenotypic change in adult rat hepatocytes in primary monolayer culture.[9] pp 119–136
38. Williams GM: Functional markers and growth behavior of preneoplastic hepatocytes. Cancer Res 36:2540–2543, 1976
39. Diamond L, McFall R, Tashiro Y, Sabatini D: the WIRL-3 rat liver cell lines and their transformed derivatives. Cancer Res 33:2627-2636, 1973
40. Iype PT: Cultures from adult rat liver cells. I. Establishment of monolayer cell-cultures for normal liver. J Cell Physiol 78:281–288, 1971
41. Weinstein IB, Orenstein JM, Gebert R, Kaighn ME, Stadler UC: Growth and structural properties of epithelial cell cultures established from normal rat liver and chemically induced hepatomas. Cancer Res 35:253–263, 1975
42. Williams GM, Gunn JM: Long-term cell culture of adult rat liver epithelial cells. Exp Cell Res 89:139–142, 1974
43. Williams GM, Weisburger EK, Weisburger JH: Isolation and long-term cell culture of epithelial-like cells from rat liver. Exp Cell Res 69:106–112, 1971
44. Williams GM, Stromberg K, Kroes R: Cytochemical and ultrastructural alterations associated with confluent growth in cell cultures of epithelial-like cells from rat liver. Lab Invest 29:293–303, 1973
45. Iype PT: Studies on chemical carcinogenesis in vitro using adult rat liver cells. Chemical Carcinogenesis Essays. Edited by R. Montesano, L Tomatis. Lyon, International Agency for Research in Cancer, 1974, pp 119–132
46. Sato J, Namba M, Usui K, Nagano D: Carcinogenesis in tissue culture. VIII. Spontaneous malignant transformation of rat liver cells in long-term culture. Jap J Exp Med 38:105–118, 1968
47. Oshiro Y, Gerschenson LE, DiPaolo JA: Carcinomas from rat liver cells transformed spontaneously in culture. Cancer Res 32:877–879, 1972
48. Koshiba K, Namba M, Oda T: Electron microscopic studies on cultured rat liver cells transformed by 4-nitroquinoline-1-oxide. Gann 61:233–238, 1970
49. Montesano R, Saint Vincent L, Drevon C, Tomatis L: Production of epithelial and mesenchymal tumors with rat liver cells transformed in vitro. Int J Cancer 16:550-558, 1975
50. Elias H: Origin and early development of the liver in various vertebrates. Acta Hepatologica 3:1–56, 1955
51. Gunn JM, Shinozulka H, Williams GM: Enhancement of phenotypic expression in cultured malignant liver epithelial cells by a complex medium. J Cell Physiol 87:79–87, 1976
52. Borek C: Neoplastic transformation in vitro of a clone of adult liver epithelial cells

into differentiated hepatoma-like cells under conditions of nutritional stress. Proc Natl Acad Sci USA 69:956–959, 1972
53. Becker FF: Differential lectin agglutination of fetal, dividing postnatal, and malignant hepatocytes. Proc Natl Acad Sci USA 71:4307–4311, 1974
54. Borek C, Grob M, Burger MM: Surface alterations in transformed epithelial and fibroblastic cells in culture: A disturbance of membrane degradation versus biosynthesis? Exp Cell Res 77:207–215, 1973
55. Roth J, Neupert G, Bolck F: Concanavalin A receptors in the plasma membrane of rat liver cells: Comparative electron microscopic studies on normal cells and on cells in vivo transformed by diethylnitrosamine. Exp Pathol 10:143–155, 1975
56. Kapeller M, Doljanski F: Agglutination of normal and Rous sarcoma virus-transformed chick embryo cells by concanavalin A and wheat germ agglutinin. Nature [New Biol] 235:184–185, 1971
57. Berzins K, Blomberg F: Identification of concanavalin A-binding plasma membrane antigens of rat liver. FEBS Letters 54:139–143, 1975
58. Glimelius B, Nilsson K, Ponten J: Lectin agglutinability of non-neoplastic and neoplastic human lymphoid cells in vitro. Int J Cancer 15: 888–896, 1975
59. Berman JJ, Williams GM: Unpublished data
60. Berenblum I: A speculative review: The probable nature of promoting action and its significance in the understanding of the mechanisms of carcinogenesis. Cancer Res 14:471–477, 1954
61. Frei JV, Harsono T: Increased susceptibility to low doses of a carcinogen of epidermal cells in stimulated DNA synthesis. Cancer Res 27:1482–1484, 1967
62. Craddock VM: Liver carcinomas induced in rats by single administration of dimethylnitrosamine after partial hepatectomy. J Natl Cancer Inst 47:899–907, 1971
63. Marquardt H, Sternberg SS, Philips FS: 7,12-Dimethylbenz(a)anthracene and hepatic neoplasia in regenerating rat liver. Chem Biol Interact 2:401–403, 1970
64. Pound AW: Carcinogenesis and cell proliferation. NZ Med J 67:88-99, 1968
65. Warwick GP: Effect of the cell cycle on carcinogenesis. Fed Proc 30:1760–1765, 1971
66. Betram JS, Heidelberger C: Cell cycle dependency of oncogenic transformation induced by N-methyl-N'-nitro-N-nitrosoguanidine in culture. Cancer Res 34:526–537, 1974
67. Marquardt H: Cell cycle dependence of chemically induced malignant transformation in vitro. Cancer Res 34:1612–1615, 1974
68. Berman JJ, Williams GM: Enhanced susceptibility of cultured rat liver epithelial cells to mutagenesis during DNA synthesis. Proc Am Assoc Cancer Res 17:158, 1976



1



2

Figure 1—Control subline (ARL 14) after 2 weeks of exposure to 0.1% DMSO. The epithelial cells are tightly adherent in islands with smooth outlines. (Phase contrast, × 100) **Figure 2**—AFB, subline (ARL 14) after 2 weeks of exposure to AFB_1, at 10^{-6} M. The cells are pleomorphic and nonadherent and islands no longer have a smooth contour. (Phase contrast, × 100)

[*End of Article*]