

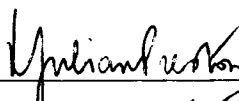
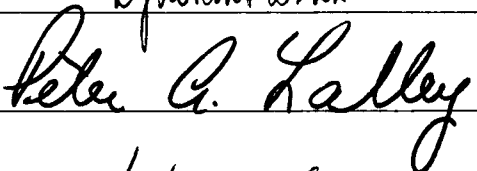
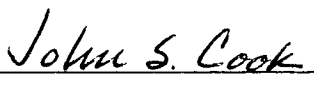
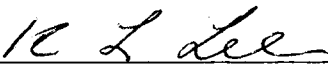
To the Graduate Council:

I am submitting herewith a dissertation written by William J. Wasilenko entitled "An Analysis of Metabolic Properties of Normal and Carcinogen-Altered Rat Tracheal Epithelial Cells." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.



Ann C. Marchok, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:



The Graduate School

AN ANALYSIS OF METABOLIC PROPERTIES OF
NORMAL AND CARCINOGEN-ALTERED RAT
TRACHEAL EPITHELIAL CELLS

A Dissertation
Presented for the
Doctor of Philosophy
Degree
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William J. Wasilenko

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ABSTRACT

Normal primary cell cultures (NPC) of rat tracheal epithelial cells require exogenous pyruvate for growth in vitro, whereas carcinogen-altered tracheal cells do not. The aim of this dissertation was to identify metabolic differences between these two cell types which may be related to their dissimilar growth requirements for exogenous pyruvate. The loss of requirement for exogenous pyruvate by the carcinogen-altered cells was used to selectively grow the altered cells, and these selected primary cell cultures (SPC) include cells from early stages in the progression of neoplasia.

A study of the effects of exogenous pyruvate on the growth and differentiation of NPC indicated that normal cells have a stringent and concentration dependent growth requirement for pyruvate. In pyruvate deficient medium, the degree of cell exfoliation and terminal differentiation (keratinization) gradually increased in NPC.

Distinct metabolic differences were found between NPC and SPC in the utilization of several key metabolites. Compared to SPC, the NPC have higher levels of hexose uptake, glycolysis, glucose flux in the pentose cycle, and exogenous pyruvate metabolism. Glutamine and alanine utilization are comparable in NPC and SPC, and similar levels of glutaminolysis are also present in both cell types. However, mitochondrial containing particulate fractions from SPC and two types of tumor-derived cell cultures have significantly higher levels of malic enzyme activity than particulate fractions of NPC.

The results of this study suggest that SPC do not achieve a growth advantage by: 1) increased rates of glycolysis; 2) a more efficient utilization of glucose; 3) major shifts in the utilization of glucose, alanine, or glutamine; and 4) increased levels of glutaminolysis.

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LIST OF ABBREVIATIONS

CS	- Citrate synthase
dGlc	- 2-deoxyglucose
DMBA	- 7,12-Dimethylbenz(a) anthracene
FBS	- Fetal bovine serum
{ ³ H}-Thd	- Tritiated thymidine
HBSS	- Hanks balanced salt solution
Hepes	- 4-(2-hydroxyethyl)-1 piperazine-ethane-sulfonic acid
IU	- International unit
MDH	- Malate dehydrogenase
NPC	- Normal primary cell culture
PBS	- Phosphate buffered saline
PFK	- Phosphofructokinase
TCA	- Trichloroacetic acid
TPC	- Tumor primary cell culture
REE	- Reestablished epithelia
SPC	- Selected primary cell culture

CHAPTER I

INTRODUCTION

The development of cancer is presumed to be a multistep process in which putative initiated cells gradually progress through a series of stages that culminate in frank neoplasia (Foulds, 1969). A preponderant amount of research has been focused on late manifestations of this disease, such as tumors or neoplastically transformed cell cultures. If a better understanding of the entire neoplastic process is to be realized, then scrutiny must be given to the properties of cells in earlier stages of the progression of neoplasia.

The concept of a stepwise progression of neoplasia has been supported by numerous morphological studies of cancer development in humans and laboratory animal models (Farber & Cameron, 1980). An early indication of neoplastic progression was found in the initiation-promotion studies with skin (Boutwell, 1964; Berenblum, 1954), which showed that papillomas sometimes occurred after initiation as an intermediate stage before the appearance of cancer. Putative precancerous lesions have subsequently been observed during the evolution of neoplasia in many other tissues, such as cervix (Burghart, 1973), urinary bladder (Friedell et al., 1977), respiratory tract (Sacomanno et al., 1974; Griesemar et al., 1976; Nettesheim & Marchok, 1983), and particularly liver (Pitot et al., 1978; Farber & Cameron, 1980).

The morphological description of putative precancerous lesions varies depending on the type of tissue studied. These descriptions range from: metaplasias with varying degrees of atypia, dysplasias and carcinoma in situ (cervix, urinary bladder and parts of the respiratory tract); alveolar hyperplasias, ductal hyperplasias and atypical lobules (mammary gland); and hyperplastic nodules, hyperplastic areas and hyperbasophilic foci (liver) (Farber & Cameron, 1980). Each of these lesions share the common property of harboring morphologically abnormal cells, believed to be preneoplastic, which may undergo further progression and ultimately form tumors (Farber & Cameron, 1980).

The evidence that tumors do indeed arise from presumptive preneoplastic lesions is largely circumstantial. Although many putative precursor lesions frequently regress, persistent lesions in their most advanced stages commonly show properties similar to tumor cells, such as aberrant differentiation and altered growth control (Farber & Cameron, 1980). The destruction and fixation of tissue in morphological studies precludes the attainment of unequivocal evidence showing that presumptive preneoplastic lesions are indeed the source of cells which constitute the progenitors of tumors. Direct proof of a precursor-progeny relationship between these lesions and neoplasia can be made only by repeatedly monitoring the fate of individual lesions as they progress to neoplasia (Farber & Cameron, 1980; Nettesheim & Marchok, 1983).

The progressive nature of cancer development has also been demonstrated in several in vitro studies conducted with a variety of chemically transformed cells. A progression from the preneoplastic to neoplastic state has been observed in studies with carcinogen-altered epithelial cell cultures from rat trachea (Marchok et al., 1978), mouse epidermis (Yuspa & Morgan, 1981) and human esophagus (Stoner et al., 1982). In most cases, cell culture studies support the generalization that altered growth control is a hallmark of neoplastic cells. This altered growth control is generally demonstrated by seemingly indefinite (immortalized) proliferative potentials for neoplastically transformed cells (Hayflick & Moorhead, 1961; Martin et al., 1970), in contrast to rather finite in vitro lifespans for normal cells. Recently, Newbold et al. (1982) observed that the progeny of immortal variants of carcinogen-altered Syrian hamster fibroblasts gradually acquired anchorage independence and tumorigenicity after extended periods of culture. These findings indicate the immortality occurs as an early event in the progression of neoplasia in vitro (Newbold et al., 1982), and strongly suggest that alterations in growth control also occur at early stages.

The altered growth control that typifies neoplastic cells has received extensive investigation. Current theory suggests that malignantly transformed cells have a reduced requirement for key growth regulatory substances, such as peptide mitogens (Holley, 1975; Scher et al., 1978; McKeehan, 1982b), nutrients (McKeehan, 1982b) or ions (Lechner, 1979; McKeehan, 1982b). In contrast, the

growth of normal cells in vitro is highly dependent upon these substances (Holley, 1975). Neoplastic cells may reduce their dependency for extracellular growth requirements by producing and secreting endogenous growth factors (Todaro et al., 1981), increasing the uptake of nutrients (Holley, 1972; Weber, 1972), or by altering metabolic requirements for key intracellular nutrients (McKeehan, 1980).

The hypothesis of altered growth control through reduced-growth-requirements is supported by transformation studies conducted mainly with fibroblastic cell culture systems (Holley, 1975). With epithelial cells which can often terminally differentiate, the role of cell differentiation in growth control must also be considered (Lechner et al., 1983; Peehl & Standbridge, 1981). It is becoming increasingly evident that the growth of normal epithelial cells is regulated by extracellular differentiation-inducing factors. Several examples of factors that induce terminal differentiation in normal cells are: a) high Ca^{++} in mouse epidermis (Yuspa & Morgan, 1981), and b) platelet factors in bronchial cells (Lechner, 1983). Tumor cells are believed to be nonresponsive to differentiation-inducing factors and thus able to escape the constraints of normal growth control in their presence (Yuspa & Morgan, 1981; Stanbridge et al., 1982). These findings have led to an alternate hypothesis to explain altered growth control in epithelial cells, i.e., aberrant differentiation control in malignantly transformed epithelial cells favors proliferation and prevents terminal differentiation (Lechner et al., 1983; Yuspa &

Morgan, 1981). This aberrant control of differentiation seems to be positively correlated with carcinogenesis (Lechner et al., 1983).

The metabolic properties of neoplastically transformed cells have received much scrutiny. Many metabolic investigations were fostered by the early observations of Warburg (1930), who demonstrated higher glycolytic rates in tumors. A great diversity of metabolic changes are noted in malignantly transformed cells. Some of the major changes include: higher rates of glucose uptake and aerobic glycolysis (Bissell et al., 1973; Lang & Weber, 1978; Eigenbrodt & Glossman, 1980); alterations in the regulation and activity of key enzymes (Weber, 1983); shifts to fetal isozyme programs (Weber, 1983; Weinhouse, 1982); new patterns of substrate utilization (Fenselau et al., 1977; Sauer et al., 1980), and a decreased regulation of lipid synthesis (Coleman & Lavietes, 1981). However, for several reasons it has been difficult to determine which of these properties is indicative of an early and fundamental metabolic change that is intrinsic to the neoplastic process itself. First of all, tumors are heterogeneous, and not all tumors express the same metabolic aberrations (Weinhouse, 1982). Secondly, many of the above studies have used cells which best represent a late stage in neoplastic progression (tumors and transformed cell lines), thus making it difficult to discern causal events from those that are merely incidental to tumorigenicity. For these reasons, it is necessary to examine the cellular and metabolic properties of cells from earlier stages of neoplastic progression, where fundamental events are likely to be expressed. Such studies

have been hampered in the past by difficulties in growing preneoplastic cells.

Major advances in the study of neoplastic progression in vitro have recently been reported by Marchok et al. (1977, 1978, 1984). Using a unique in vivo-in vitro model of chemical carcinogenesis in rat tracheas, Marchok et al. (1977, 1978, 1984), have been able to selectively grow primary cultures of carcinogen-altered rat tracheal epithelial cells and study their progression from the preneoplastic to neoplastic state. An additional advantage of their technique is that normal cells can also be grown and easily compared to altered cells. In the in vivo phase of the model, rat tracheal implants are grafted into isogenic hosts and exposed for varying lengths of time to beeswax pellets containing DMBA. These implants are then removed, cut into small pieces and planted onto tissue culture dishes in medium. The in vitro phase involves generating outgrowths of epithelial cells from the preexposed tracheal explants (Marchok, et al., 1977; Marchok, in press). Explants are then removed and the outgrowths expanded to yield primary cultures for future analyses.

In early studies with the tracheal implant-cell culture system described above, Marchok et al. (1977) demonstrated that carcinogen-altered tracheal epithelial cells had markedly enhanced in vitro growth capacities. Carcinogen-altered tracheal cells readily grew in a Waymouth MB 752/1 medium supplemented with insulin, hydrocortisone and serum, whereas normal cells derived from nonexposed explants did not. The sustained growth of normal

cells occurred only when this basic medium was further enriched with amino acids, fatty acids, putrescine and sodium pyruvate (Marchok et al., 1977). As an added indication of a greater in vitro growth capacity in the DMBA-altered cells, Marchok et al. (1977, 1978) observed that many carcinogen-altered primary cell cultures were subculturable. In contrast, normal cells were not subculturable (Marchok et al., 1977).

The enhanced in vitro growth capacity of carcinogen-altered tracheal cells appears to be an early event in the progression of neoplasia. Support for this view comes from several findings. First, an enhanced growth capacity is observed in cell cultures established from carcinogen-exposed tracheas long before tumors develop, and in some cases as early as two weeks after carcinogen treatment (Marchok et al., 1977; Marchok, in press). Secondly, immortalized tracheal cell lines can continue the progression of neoplasia in vitro regardless of whether progression is initiated in tracheal implants (Marchok et al., 1978) or in organ cultures (Steele et al., 1977). Finally, in an innovative study using exfoliative cell cytology to localize specific morphological lesions on DMBA-preexposed tracheal explants, Marchok et al. (1982) have recently demonstrated that lesions ranging from mild atypias to more severe atypias all yield cultures with an increased in vitro growth capacity. Such lesions are suspected of being preneoplastic in the respiratory tract of humans (Saccomano et al., 1974) as well as rodents (Schreiber et al., 1974; Topping et al., 1979). Taken together, these results indicate that primary cultures of

preneoplastic tracheal cells can indeed be isolated and grown using the tracheal implant-cell culture system.

Recently, Rice and Marchok (1980) demonstrated that removal of pyruvate from the enriched medium caused the death of normal tracheal cells, and that removal of both pyruvate and insulin accelerated their demise. In contrast, carcinogen-altered cells continue to grow when either pyruvate, or pyruvate and insulin are removed from the medium (Marchok et al., 1983, 1984). These observations suggest that pyruvate metabolism is very important for normal cell proliferation, and that a change occurs in carcinogen-altered cells that enables them to metabolically compensate for the absence of exogenous pyruvate. Since the loss of requirement for exogenous pyruvate is found in both preneoplastic and neoplastic tracheal cells, the metabolic basis for this phenomenon is likely to reflect a fundamental change from the metabolic pattern of normal cells that occurs early in the progression of neoplasia. Identifying this change could help to elucidate the basis for the growth advantage found in carcinogen-altered tracheal cells, and further our understanding of mechanisms underlying altered growth control during the progression of neoplasia. It is important, therefore, to identify metabolic differences between normal and carcinogen-altered rat tracheal epithelial cells.

The purpose of the following experiments was to identify major metabolic differences between normal and carcinogen-altered rat tracheal epithelial cells which may be related to their dissimilar growth requirements for pyruvate. In this dissertation, the loss

of requirement for exogenous pyruvate by carcinogen-altered cells was used to select out primary cell cultures of altered cells from earlier and late stages in the progression of neoplasia. Selected primary cell cultures (SPC), which include cells from earlier stages of neoplasia (Marchok, 1984), were established as outgrowths from pieces of DMBA-preexposed tracheal implants well before the appearance of recognizable tumors. Tumor primary cell cultures (TPC), derived as outgrowths from pieces of DMBA induced carcinomas, represented cells from late stages of neoplasia. Normal primary cell cultures (NPC) were generated as outgrowths from explants of normal rat tracheas.

Several avenues of investigation were undertaken. In an effort to gain insight into the nature of the pyruvate requirement in normal cells, the specificity of the pyruvate requirement and the effects of pyruvate on the total cell population dynamics of normal tracheal cell cultures was investigated. Glucose uptake in normal and carcinogen-altered cells was examined in detail, since glucose is a ready precursor of pyruvate in most cells. Precedent to this study are numerous investigations which have noted significant elevations in hexose uptake and glucose utilization in malignant cells (Eigenbrodt & Glossman, 1980). Subsequent to the study of hexose uptake, the utilization of several key metabolites was investigated in normal and selected cells in an attempt to identify major shifts or increases in the utilization of metabolites by carcinogen-altered cells. The final experiments focused on comparing the compartmentation and activities of malic enzyme and

malate dehydrogenase in normal, selected and tumor primary cell cultures, since the activities of these two competing enzymes can determine the capacity of mitochondria to oxidize malate into pyruvate. Increases in this capacity have recently been linked to neoplastic progression in hepatomas (Sauer et al., 1980), and to the maintenance of cell proliferation in general (McKeehan, 1982a).

CHAPTER II

PYRUVATE REGULATION OF GROWTH AND DIFFERENTIATION IN PRIMARY CULTURES OF RAT TRACHEAL EPITHELIAL CELLS

Introduction

Supplements of pyruvate to tissue culture medium enhance the proliferation of certain types of mammalian cells in culture, especially when cell densities are low (Neuman & McCoy, 1958; Moser, 1960; Ham, 1962). Originally, this need for pyruvate, a key anaplerotic metabolite, was ascribed to the ability of exogenous pyruvate to limit the loss of intracellular pyruvate pools into the surrounding culture medium (Eagle & Piez, 1962). In support of this early theory are observations of a reduced requirement for external pyruvate in dense cell cultures (Neuman & McCoy, 1958; Herzenberg & Roosa, 1960), presumably due to a conditioning of the medium by large cell numbers that adequately maintain the intracellular pyruvate pools. However, recent studies suggest a more complex role for 2-oxocarboxylates, such as pyruvate, in the control of cell proliferation. Depending on the type of cell studied, other 2-oxocarboxylates besides pyruvate (Herzenberg & Roosa, 1960; McKeehan & McKeehan, 1979), or a broad spectrum of intermediary metabolites (McGowan & Bucher, 1983) can stimulate cell proliferation in place of pyruvate. In addition, in some types of cell cultures an inverse relationship between levels of serum-derived growth factors and the enhancement of cell proliferation by 2-oxocarboxylates has been demonstrated (McKeehan & McKeehan, 1979;

Ellem & Kay, 1983; McGowan & Bucher, 1983), a phenomenon which McKeegan and McKeegan (1979, 1980) suggest may relate the metabolism of 2-oxocarboxylates to the activity of pyridine nucleotide-linked oxidoreductases and growth control by serum factors.

Although it is a widely held view that the proliferation of mammalian cells in vitro is dependent on a carefully coordinated interplay between serum-derived stimulatory factors, ions and nutrients (Ham, 1981; McKeegan, 1982b), little has been elucidated about the role of pyruvate metabolism in the regulation of cell proliferation. The significance of understanding this role is underscored by observations that many malignantly transformed cells have altered growth factor and/or nutrient requirements in culture (Dulbecco, 1970; McKeegan, 1982b).

Since recent observations suggest that pyruvate is required for the growth of normal rat tracheal epithelial cells (Rice & Marchok, 1980; Marchok, in press), a detailed characterization of the pyruvate growth requirement in normal cells was necessary. In this chapter, the effects of pyruvate on the maintenance, expansion and differentiation of normal rat tracheal epithelial cells is investigated. An understanding of these effects should help to further elucidate the metabolic dissimilarities between normal and carcinogen-altered tracheal cells.

Materials And Methods

Materials

Weymouth's MB 752/1 and sodium pyruvate were obtained from Grand Island Biological Co., Grand Island, New York. Insulin was obtained

from Calbiochem-Behring Corp., La Jolla, California. Fetal bovine serum was purchased from Microbiological Associates, Walkersville, Maryland. Diaminobenzoic acid and {methyl-³H}-thymidine (63 Ci/mmol) were obtained from Aldrich Chemical Co., Milwaukee, Wisconsin and ICN, Irvine, California, respectively. All other reagents and chemicals were acquired from Sigma, St. Louis, Missouri.

Culture Conditions and Establishment of Primary Cultures

All cell cultures were routinely maintained as described previously (Marchok et al., 1976) in growth medium consisting of Waymouth's MC 752/1 supplemented with pyruvate (2 mM), amino acids, putrescine, hydrocortisone (0.1 µg/ml) and insulin (10 µg/ml) plus 10% fetal bovine serum. Components tested for growth promoting effects in the absence of pyruvate were added directly to fresh medium, lacking pyruvate, before each feeding. All cultures were incubated under humidified 5% CO₂/95% air at 37°C, and media were changed twice weekly.

Primary epithelial cell cultures were generated from outgrowths of normal rat tracheal explants (Marchok et al., 1976). Briefly, tracheas obtained from specific pathogen-free female Fischer 344 rats were cleared of loose connective tissue and cut into 2 x 3 mm pieces. These pieces were planted on the bottom of 35 mm plastic tissue culture dishes (Falcon, Oxnard, California). The explants remained on the dish for seven to ten days to permit the outgrowth of sheets of epithelial cells. Three to four outgrowths were generated from each explant and fibroblasts, when present, were scraped away as described earlier (Marchok et al., 1976). The outgrowths

were allowed to expand in growth medium plus pyruvate for an additional three to six weeks, after which they were used in the experiments described below.

Determination of Cell Number and Cornified Envelopes

The number of cells attached to the culture dish was determined nondestructively by tracing from the bottom of each culture dish the perimeter of the cell culture onto clear Mylar plastic. This perimeter was then retraced on a graphics tablet interfaced with a PDP-11 computer, which calculated the total area. A mean cell density was determined by counting cells on phase-contrast photographs taken at intervals along the horizontal and vertical axis through the culture. Mean cell density x area gave total cell number. This method of determining attached cell numbers per culture gave values comparable to those obtained by counting trypsin-dissociated cultures in a hemocytometer (Marchok et al., in press).

Exfoliated cells in the culture medium were determined by collecting the old medium from cultures during each new medium change, and pelleting the cells at 400 x g for 10 minutes. The cells were resuspended in a small volume of HBSS and counted in a hemocytometer. Cornified envelopes in the exfoliated cell fraction were identified by procedures similar to those described elsewhere (Sun & Green, 1976). Exfoliated cells, collected in the above manner, were pelleted and resuspended in the PBS containing 1% SDS plus 1% mercaptoethanol, and heated for 5 minutes at 100° C. Aliquots of this suspension were removed and the SDS-heat resistant cornified envelopes quantitated by counting in a hemocytometer using phase contrast optics.

DNA Determination and $\{^3\text{H}\}$ -thymidine Incorporation

The DNA content in the cultures was determined in the following manner: cultures were scraped in 5% TCA (2-5°C) and centrifuged at 2,000 x g for 10 minutes. The subsequent cold TCA precipitates were then extracted sequentially with K^+ acetate + 95% ethanol (5°C) and chloroform:ethanol (1:3) (60°C). The residue was then extracted twice with hot 5% TCA (90°C). The hot TCA extracts were assayed fluorometrically to determine DNA concentrations using diaminobenzoic acid in a modification of the technique of Kissane and Robbins (1958). Calf thymus DNA was used as a standard.

The incorporation of $\{^3\text{H}\}$ -thymidine into DNA was followed in cell cultures by labeling cultures for 30 minutes at 37°C with one ml of fresh medium containing 4 μCi $\{^3\text{H}\}$ -thymidine. The cells were then rinsed with cold HBSS containing 80 μg unlabeled thymidine per ml, scraped, and the DNA extracted by the protocol given above. Aliquots of the hot TCA extract were counted for $\{^3\text{H}\}$ -thymidine incorporation, and aliquots from the same extract assayed for DNA.

Results

Effect of Pyruvate on Total Cell Number and Differentiation

In an effort to determine in more detail how pyruvate influences growth in primary cultures of rat tracheal epithelial cells, the effect of deleting pyruvate as a medium supplement on the total production of cells in culture was examined over a period of several weeks. All other medium components and conditions were unchanged. These measurements of growth were possible due to a nondestructive

method of counting attached cells permitting changes in the size of the same cell population to be repeatedly determined throughout culture. It is also important to note that in rat tracheal epithelial cell populations, which may turn over rapidly, the total number of cells produced is best assessed by determining the number of cells exfoliated into the medium as well as the number of attached cells on the culture dish. Under certain conditions the exfoliated cell compartment can contribute greatly to the total cell population, as was found in a tracheal epithelial cell line cultured in the presence of retinoic acid (Klann & Marchok, 1983).

Figure 1A shows that when the total number of cells was measured in the manner above, a steady increase in the production of cells occurred in cell populations cultured for 2 weeks in pyruvate supplemented medium. This was largely a result of a continuous increase in the number of attached cells. Exfoliated cells which accumulated in the medium from each culture were also counted on the days given in Figure 1. As shown in Figure 1A, exfoliation was low and a fairly constant amount of exfoliated cells was added throughout culture in the presence of pyruvate, such that by day 14 the cumulative exfoliated cell number represented only 22% of the total cells produced.

An increase in total cell production was also found in cell cultures during the first week, particularly the first 3 days, in medium lacking a pyruvate supplement (Figure 1B). This initial increase was similar to that found in cultures containing pyruvate (Figure 1A). However, after 7 days of culture the total number of cells (attached and exfoliated) began to decrease in the absence of

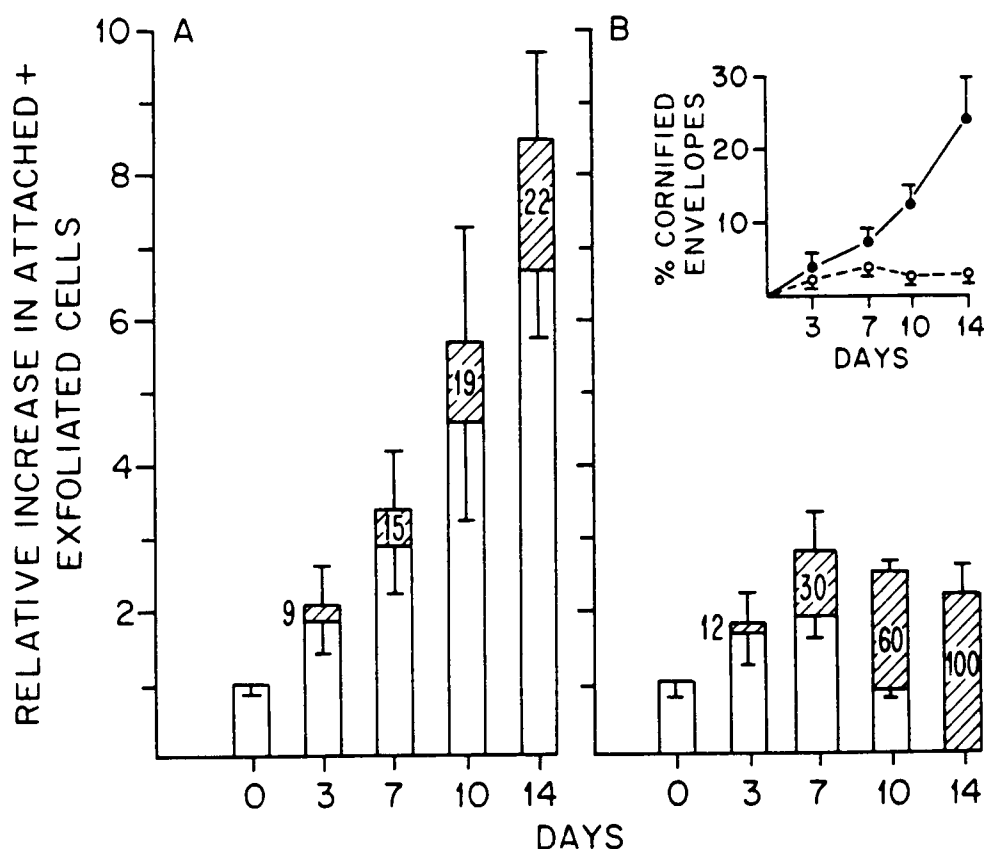


Figure 1. Effect of pyruvate on total cell population and keratinization in primary cultures of normal rat tracheal epithelial cells. Three to six week old primary cultures established in growth medium containing pyruvate were cultured in, (A) growth medium supplemented with 2 mM pyruvate, or (B) growth medium lacking a pyruvate supplement, and the change in the number of attached cells, relative to that at the start of the experiment, was determined (see Materials and Methods) in the same culture throughout 2 weeks of culture. Exfoliated cells were also collected and counted with each medium change. Open bars, relative attached cell number; hatched bars, relative change in size of cumulative exfoliated cell compartment. Numbers within hatched bars indicate percentage of total cells represented as cumulative exfoliated cells. Inset: Percentage of exfoliated cells that had cornified envelopes; (o---o), cornified envelopes from pyruvate-supplemented cultures (A); (●---●), cornified envelopes from pyruvate-depleted cultures (B). Results are the mean values from at least three individual cell cultures per treatment. Top S.E. bars and bottom S.E. bars represent error in determinations of relative total cell production and relative attached cell number, respectively.

pyruvate. This was largely a result of a marked reduction in the number of cells attached to the culture dish. An increase in exfoliation was observed in the absence of pyruvate as early as day 7, and this exfoliation continued to increase until all cells were lost from the culture dish (Figure 1B). These results clearly demonstrate that supplements of pyruvate in the medium are required for long term maintenance and growth of primary cultures of normal rat tracheal epithelial cells under these conditions.

To determine why a lag in the cessation of growth was observed after the deletion of pyruvate, a study of possible alterations in one pathway of cell differentiation, namely keratinization, was undertaken. The degree of keratinization in rat tracheal epithelial cells can vary (Marchok et al., 1981), and one way this can be measured is by quantitating the amount of cornified envelopes that are shed from keratinizing cell populations (Sun & Green, 1976). When this was done using the exfoliated cells collected from each sampling shown in Figure 1, a low and constant production of cornified envelopes in cultures containing pyruvate was observed (Figure 1, inset). In the absence of pyruvate, the level of cornified envelopes was initially low, but after 3 days of culture these levels began to increase. This increase in keratinization continued until approximately 24% of all cells collected after 2 weeks were actually cornified envelopes. Thus, differentiation towards keratinization is apparently induced in these cells when pyruvate is absent.

Effect of Pyruvate on DNA Synthesis

Since differentiation in these cells was affected by pyruvate, changes in the overall amount of DNA synthesis were studied in relation to the alterations in cell production and the increase in terminal differentiation. This was done by measuring, in the presence and absence of pyruvate, the incorporation of $\{^3\text{H}\}$ -thymidine into DNA. In contrast to the study illustrated in Figure 1, this experiment required different cultures at each day of assay, which contributes to the broad variation seen in Figure 2. As shown in Figure 2, control cultures were characterized by a fairly constant level of $\{^3\text{H}\}$ -thymidine incorporation per μg DNA (Figure 2A), although the size of the cultures (as indicated by total DNA content) increased greatly with time (Figure 2B). In the cultures lacking pyruvate, $\{^3\text{H}\}$ -thymidine incorporation into DNA was also initially high (day 1-6) and not significantly different from controls (Figure 2A). In addition, the size of these cultures increased during the first six days (Figure 2B). Thereafter (days 9-12), the level of DNA synthesis as well as the size of the cultures was greatly reduced to values well below those observed in control cultures. However, a low level of DNA synthesis was still present in the few residual cells left on the dish. By day 14, all cultures lacking external pyruvate had totally sloughed from the dish. Thus, in the absence of pyruvate there was not an immediate or complete inhibition of DNA synthesis in the primary cell cultures.

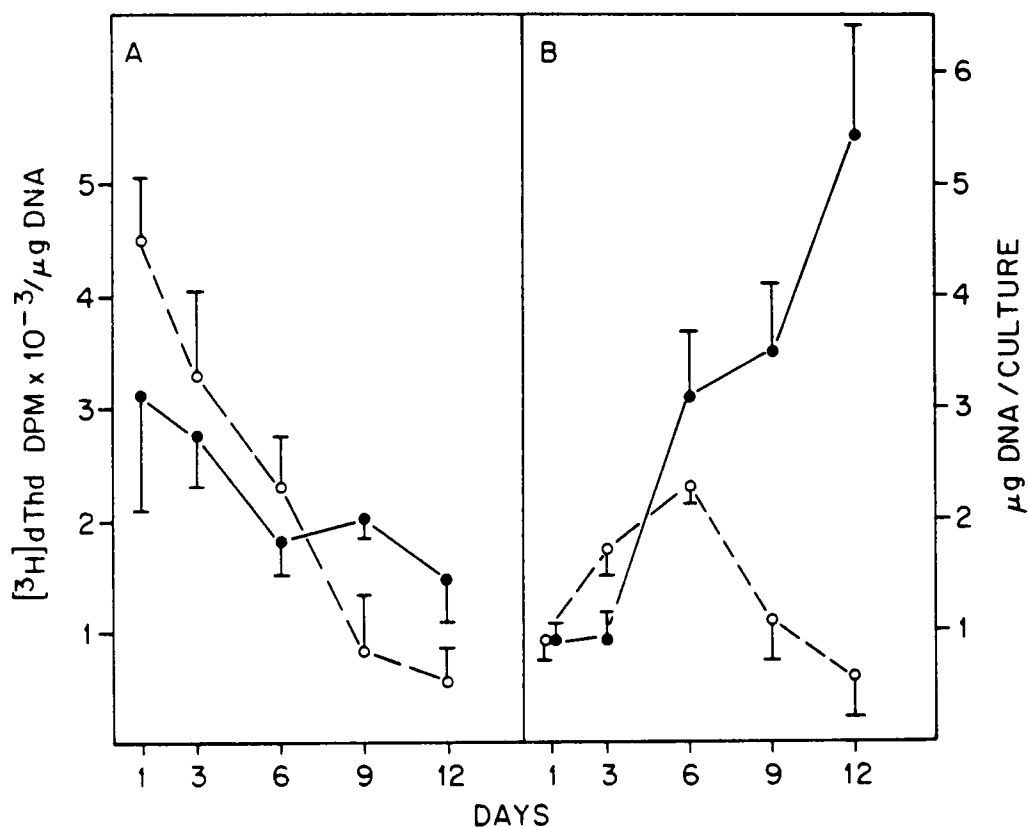


Figure 2. Effect of pyruvate on incorporation of $[^3\text{H}]$ -thymidine into DNA and culture size in primary tracheal cell cultures. Primary cell populations generated in growth medium containing pyruvate were cultured in growth medium with (●—●) or without (o---o) a supplement of 2 mM pyruvate for the length of time indicated. On the day of each assay, four different cultures were removed, pulsed with $[^3\text{H}]$ -thymidine and both the incorporation of label into DNA (A) and DNA content (B) were measured in each culture as described in Materials and Methods. Points represent mean values $\pm$ S.E.

Pyruvate Concentration-Dependency of Growth Response

A wide range of pyruvate concentrations were examined for their effects on the survival and growth of primary tracheal cell cultures. As shown in Figure 3, growth occurred during the initial week of culture with all concentrations tested except 80 mM, which was clearly toxic. Thereafter, growth began to decline in all cultures containing pyruvate supplements of 0.2 mM or less. By the end of 2 weeks, cultures supplemented with these lower pyruvate levels began to recede, and most sloughed off the dish by the end of 3 weeks (data not shown). In contrast, growth continued with supplements of 2.0 mM and 20 mM pyruvate, as routinely found.

Specificity of the Pyruvate Requirement

Since a broad range of substances, including other 2-oxocarboxylates (McKeehan & McKeehan, 1979), intermediary metabolites (McGowan & Bucher, 1983) and electron acceptors (Ellem & Kay, 1983), can effectively replace pyruvate in enhancing the growth of other types of cells, the effect of several of these on the growth of tracheal cells in the absence of pyruvate was studied (Table 1). This was assessed by noting changes in the sizes of cultures from tracings of the perimeter of each individual culture.

The growth response to most of the compounds tested was very similar to what was observed earlier in cultures lacking pyruvate (Figures 1B and 2B), that is, cultures expanded during the initial week and then stopped growing. Thereafter, the cultures began to recede and ultimately lifted off the dish. This was observed with the 2-oxocarboxylic acids, glyoxylic acid (750 μ M), 2-oxoglutaric

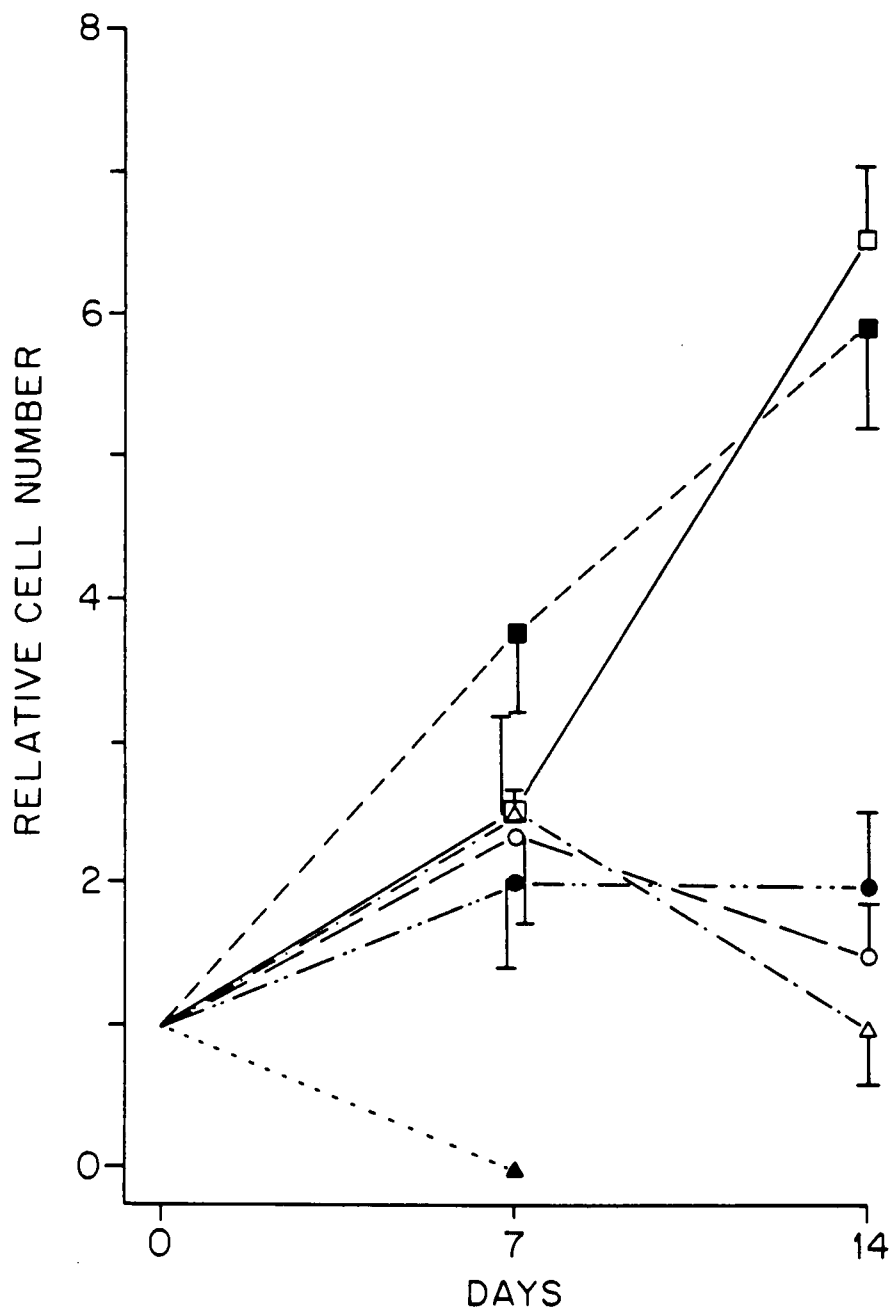


Figure 3. Effect of different pyruvate concentrations on the growth of tracheal cell cultures. Three to four week old primary cell cultures, generated in growth medium, were cultured for several weeks in medium supplemented with 0 (●); 0.02 mM (△); 0.2 mM (○); 2 mM (■); 20 mM (□) and 80 mM (▲) pyruvate, and the change in the number of attached cells in the same culture, relative to the initial number, was determined as described in Materials and Methods. Points represent average of three cultures, $\pm$ S.E.

Table 1
Effect of 2-oxocarboxylic Acids and Other Compounds
on the Growth of Tracheal Cell Cultures in the
Absence of Added Pyruvate^a

Component Tested	mM	Growth Response			
		Week 1	Week 2	Week 3	Week 4
Glyoxylic Acid	0.25	+	D		
	0.75	+	NC	D	
	5.00	D			
	20.00	D			
2-Oxoglutaric Acid	0.25	+	D		
	0.75	+	NC	D	
	5.00	+	NC	NC	D
	20.00	+	NC	D	
Oxalacetic Acid	0.05	+	D		
	0.75	+	R	D	
	5.00	+	R	D	
	20.00	+	+	+	+
Alanine	2.00	+	D		
	20.00	+	D		
Citric Acid	0.05	+	D		
	0.75	+	R	D	
	5.00	+	D		
Malic Acid	0.25	+	D		
	5.00	+	NC	R	D
	20.00	+	NC	D	
Succinic Acid	0.25	+	D		
	0.75	+	NC	D	
	5.00	+	NC	R	D
	20.00	D			
Aspartic Acid	20.00	+	D		
Lactic Acid	2.00	+	NC	R	D
	20.00	+	R	D	
Potassium Ferricyanide	1×10^{-4}	+	D		
	1×10^{-2}	+	D		
	5×10^{-2}	D			

^aCell cultures were established in the complete growth medium and then the pyruvate supplement was removed and replaced with the test compounds given above. The growth response of the cultures was assessed by determining changes in the perimeter of the same culture, relative to its size at the beginning of assay, as described in Materials and Methods. Growth response: (+), increase in culture size; (NC), no change in size; (R), reduction in size; (D) dead. Two to four cultures were used for each assay.

acid (750 μ M, 5 mM, 20 mM) and oxaloacetic acid (50 μ M, 750 μ M and 5 mM) as well as with malic acid (5 mM), succinic acid (750 μ M and 5 mM) and lactic acid (2 mM). Exceptions were seen with 5 mM glyoxylic acid, 20 mM succinic acid and 50 μ M ferricyanide, which were toxic. The electron acceptor, ferricyanide, was also ineffective after 2 weeks at either 10^{-4} mM or 10^{-2} mM. In addition, compounds which are potential metabolic precursors of pyruvate such as alanine, malic acid and lactic acid, were ineffective even at 20 mM levels. Of all the substances tested, the only one with growth promoting properties similar to pyruvate was oxalacetic acid at a concentration of 20 mM. It is likely however that this is caused by pyruvate formed from the degradation of oxaloacetic acid, since it easily decarboxylates into pyruvate in solution (Krebs, 1942). Taken together, the above results indicate that the control of growth by pyruvate in these cells is quite stringent.

Discussion

In the present study the nature of the growth requirement for pyruvate in primary cultures of normal rat tracheal epithelial cells was explored in greater detail. By following the growth of the same culture over a period of several weeks, it was shown here that pyruvate is stringently required for the long-term survival and growth of these cells. When pyruvate was added to the medium, these cultures maintained high levels of cell proliferation with a minimal degree of terminal differentiation. The latter was indicated by the low and constant rate of cell exfoliation and cornified envelope

formation found in the presence of added pyruvate. However, quite different results were observed when the total cell population kinetics of the cultures were examined in the absence of pyruvate. Rather than immediately dying and lifting off the dish as one might expect in response to a toxic nutritional deficiency, these cultures continued to expand for a considerable length of time before growth ceased and the integrity of the attached sheets of cells was lost. Accompanying this behavior was an increase in keratinization. Thus, it appears that pyruvate influences, in some as yet unknown way, the differentiation program expressed in these cell cultures.

The keratinocyte-like behavior (Green, 1977) of rat tracheal epithelial cells observed in vitro is not surprising since injured or vitamin A-deficient rat tracheal epithelium typically undergoes squamous metaplasia in vivo (Marchok et al., 1975). In fact, Phillips and Rice (1983) have recently demonstrated that epithelial cells derived from many different rat tissues, including trachea, are capable of exhibiting characteristics of keratinizing cells in vitro. It is surprising that an increase in terminal differentiation was triggered in the tracheal cell culture system by the deletion of pyruvate from an enriched medium containing high levels of glucose, serum and other nutrients. There have been few demonstrations of carbon sources, like pyruvate, affecting the differentiation of cells in culture. In one study with mouse teratocarcinoma cells, the pattern of differentiation was profoundly affected by the type of carbon source added to the medium (Avner et al., 1977). With these mouse cells, a complete program of differentiation was

dependent on either glucose or mannose, whereas differentiation was restricted with only pyruvate (Avner et al., 1977). It is not known at this time whether the deletion of other carbon sources or medium components, besides pyruvate, would also affect the differentiation program of tracheal epithelial cells.

In many respects, the squamous differentiation that was observed in tracheal cell cultures in the absence of pyruvate resembles the in vitro terminal differentiation that is induced by (a) platelet factors in normal human bronchial epithelial cells (Lechner et al., 1983), (b) elevations in Ca^{++} levels in murine epidermal cells (Yuspa & Morgan, 1981), or (c) tumor promoters in normal human keratinocytes (Hawley-Nelson et al., 1982). It is interesting to note that carcinogen-altered rat tracheal cells derived in this laboratory survive and proliferate in medium lacking pyruvate (Marchok, in press; Marchok et al., in press). Such cells may be unresponsive to signals that induce terminal differentiation, unlike normal tracheal cells, and thus are able to proliferate in the absence of pyruvate. A resistance to terminal differentiation is observed in several in vitro models of carcinogenesis (Yuspa & Morgan, 1981; Lechner et al., 1983) and this is likely to be related to the growth advantage displayed by many transformed cells (Peele & Standbridge, 1981; Yuspa & Morgan, 1981; Wille et al., 1982), including carcinogen-altered tracheal cell cultures (Marchok, in press; Marchok et al., in press).

The effects of deleting pyruvate supplements on the growth of the normal tracheal cells in culture were not immediately apparent. The considerable lag in the reduction of growth might have been

caused by the utilization of large intracellular pyruvate pools. It is also possible that these cultures maintain, even after the initial withdrawal of pyruvate, a large proliferative pool of cells that are still capable of dividing a limited number of times. In support of this latter view is the finding that DNA synthesis remains high during the first week of culture in medium lacking pyruvate. Cells in this proliferative pool may terminally differentiate only after several rounds of cell division. When overall DNA synthesis was eventually reduced, it was largely a result of more and more cells being lost from the proliferative pool, since exfoliation and cornified envelope production markedly increased during later periods of culture in pyruvate-deprived medium.

The actual concentration of pyruvate required for maintaining growth is difficult to determine from the results reported here since dialyzed serum was not used, and because pyruvate degrades somewhat during incubation in the tissue culture media (Wasilenko, unpublished results). However, the level of exogenous pyruvate found necessary for the growth of tracheal cells is considerably greater than levels previously reported as growth stimulatory in other types of cells (McKeehan & McKeehan, 1979; Ellem & Kay, 1983). The pyruvate requirement in this tracheal cell system also differs from that of other culture systems in several respects. First of all, pyruvate is required for the survival of normal tracheal cells, and not just for an enhancement of growth as it is in other types of cell cultures (Neuman & McCoy, 1958; McKeehan & McKeehan, 1979; Ellem & Kay, 1983). Secondly, an inverse relationship between levels of serum-derived

factors and growth promotion by pyruvate is not present in these tracheal cell cultures, since pyruvate is required for growth even in the presence of high levels of serum. This is in contrast to recent findings from studies conducted with human diploid fibroblasts (McKeehan & McKeehan, 1979) and melanoma cells (Ellem & Kay, 1983), which indicate that external serum-derived factors can modify the nutritional growth requirements of cells in culture. Thirdly, the pyruvate requirement in these tracheal epithelial cell cultures is quite stringent. Other types of medium supplements, including several 2-oxocarboxylates that effectively replace pyruvate as an enhancer of growth in some mammalian fibroblastic cells (McKeehan & McKeehan, 1979), were ineffective with rat tracheal epithelial cells. Although the bases for the above differences between tracheal cells and other cell types are currently unknown, such differences indicate that the regulation of cell proliferation in primary cultures of normal rat tracheal epithelial cells may be distinct from that found in other types of cells in culture.

At this time one can only speculate about the mechanism underlying growth control by pyruvate in tracheal cells. Preliminary evidence indicates that the requirement for external pyruvate is not caused by an impaired biosynthesis of pyruvate from other metabolic precursors, including glucose or amino acids (Wasilenko & Marchok, 1984; see Chapter IV). Some investigators have suggested the metabolism of 2-oxocarboxylates results in a redox state favorable for growth (McKeehan & McKeehan, 1979; Ellem & Kay, 1983), whereas recent work by Sens et al. (1982) using hamster embryo fibroblasts suggests that

pyruvate may affect the utilization of other key metabolic substrates, such as glucose and glutamine. Since the pyruvate requirement in rat tracheal cells is quite stringent, it is likely that pyruvate or some product of pyruvate metabolism is involved in a series of metabolic processes that inhibit terminal differentiation and maintain a large part of the cell population in a replicative cycle. Additional research is clearly needed to establish the mechanism for these effects.

CHAPTER III

HEXOSE UPTAKE IN 7,12-DIMETHYLBENZ(a) ANTHRACENE

PREEXPOSED RAT TRACHEAL EPITHELIAL CELLS

DURING THE PROGRESSION OF NEOPLASIA

Introduction

Since the pioneering work of Warburg several decades ago (1930), many studies have supported the generalization that malignant cells exhibit high rates of nutrient uptake and aerobic glycolysis (Holley, 1974; Barghava, 1977; Eigenbrodt & Glossman, 1980). Increases in hexose uptake and lactate production are often observed in cells from tumors of widely diverse origins (Hume & Weidemann, 1979). These two properties, in fact, are often correlated with neoplastic transformation in many in vitro cell studies with cell lines principally of mesenchymal origin (Isselbacher, 1972; Bissell et al., 1973; Lang & Weber, 1978). Most human tumors, however, are of epithelial origin. These tumors are believed to develop after initiated cells progress through a series of sequential changes that culminate in frank neoplasia (Foulds, 1975; Nettesheim & Marchok, 1983). It is difficult to distinguish from previous studies whether the increases in hexose uptake are causal or incidental events in the development of neoplasia since the cells generally represented the later stages of neoplastic progression (tumors and transformed cell lines). Assaying these parameters with epithelial cells in the early stages of neoplasia should greatly aid our understanding of the involvement of these events in carcinogenesis. This has not

been done widely in the past because of the difficulty of isolating and growing preneoplastic epithelial cell populations.

The tracheal implant-cell culture system described in Chapter II and utilized by Marchok et al. (1977, 1984), enables carcinogen-altered cells from various stages (early to late) of the progression of neoplasia to be selectively grown, and their cellular and biochemical properties analyzed in detail. Since the uptake of glucose, a potential precursor of pyruvate, is high in some types of neoplastic cells, it was hypothesized that elevations in hexose uptake might be related to the loss of requirement for exogenous pyruvate in carcinogen-altered tracheal cells. To test this hypothesis, and also to examine if progression-linked changes in hexose uptake occurred in carcinogen-altered tracheal cells, the uptake of hexose was measured in primary cultures of normal, carcinogen-altered and tumor-derived rat tracheal epithelial cells as well as in tumorigenic and nontumorigenic cell lines. These hexose uptake measurements are made with 2-deoxyglucose, a glucose analog that serves as a substrate for the facilitated transport of glucose across the plasma membrane, and it is not significantly metabolized past the initial deoxyglucose-6-phosphate. The uptake is a reflection of both the rate of transport and hexokinase activity (Renner et al., 1972).

The approach taken in this study is unique in that it allows one to determine if changes in hexose uptake occur early or late in the progression of neoplasia of an epithelial cell system. Since the tracheal implant cell culture system yields primary cultures

as continuous sheets of cells, these properties are assayed in cell cultures that are free of the rigors of proteolytic dissociation as well as any alterations that are likely to occur in cell cultures as they become immortalized cell lines.

Materials and Methods

Materials

Waymouth's MB 752/1 and sodium pyruvate were obtained from Grand Island Biological Co., Grand Island, New York. Insulin was obtained from Calbiochem-Behring Corp., La Jolla, California. Fetal bovine serum was purchased from Microbiological Associates, Walkersville, Maryland. Diaminobenzoic acid and fluorescamine were obtained from Aldrich Chemical Co., Milwaukee, Wisconsin, and Sigma Chemical Co., St. Louis, Missouri, respectively. 2-Deoxy-D-{1-³H}glucose (15 Ci/mmol) was obtained from Amersham/Searle Corp., Arlington Heights, Illinois. L-{1-³H}glucose was purchased from New England Nuclear, Boston, Massachusetts, and {methyl-³H}-thymidine (63 Ci/mmol) was obtained from ICN, Irvine, California. All other reagents and chemicals were acquired from Sigma, St. Louis, Missouri.

Culture Medium

All cell cultures were routinely maintained in growth medium consisting of Waymouth's MB 752/1 supplemented with pyruvate (2 mM), amino acids, putrescine, hydrocortisone (0.1 µg/ml) and insulin (10 µg/ml) plus 10% fetal bovine serum. Selection medium, used for the selection of carcinogen-altered cells, discussed below, consisted

of growth medium minus pyruvate and insulin. All media were changed twice weekly.

Cell Lines and Reestablished Epithelia (REE) of Each Line

Cloned cell lines, 1000-WT and T-8, were derived from a DMBA-induced squamous cell carcinoma and a N-methyl-N'-nitro-N-nitrosoguanidine-induced adenocarcinoma, respectively (Marchok et al., 1981). The nontumorigenic cell line, C-18, was subcultured and cloned by D. Knudson in Dr. Ann Marchok's laboratory from a long-term primary culture of rat tracheal epithelial cells generated as an outgrowth from a normal, nonexposed tracheal explant.

Tissue-like cell associations were reconstructed with each cell line using a protocol similar to that reported by Terzaghi et al. (1978). Briefly, dissociated cells of each cell line were inoculated into isolated tracheas denuded of their epithelium by freeze-thawing. These inoculated tracheas were then surgically grafted into the subscapular region of nude mice. After two weeks in the host mice, the repopulated tracheas were removed and cut into 2 x 3 mm pieces. These pieces were then planted on the bottom of 35 mm plastic tissue culture dishes to initiate the outgrowth of continuous sheets of epithelial cells. During the two weeks in the tracheas, each inoculum of dissociated cells formed tissue-like cell associations as described in earlier studies (Terzaghi et al., 1978), and these are referred to here as the reestablished epithelium (REE) of each cell line.

DMBA Treatment and Establishment of Primary Cultures

Rat tracheal implants were exposed to DMBA by methods described earlier (Griesemer et al., 1974; Kendrick et al., 1974). Briefly, tracheas obtained from specific pathogen-free male Fischer 344 rats were established subcutaneously and dorsal to the shoulders of isogenic recipient rats. Beeswax pellets containing 200 μ g DMBA were then inserted into the lumens of the implanted tracheas. After one month of exposure, the DMBA-beeswax pellets were removed. The implants were then left in the host animals for an additional four to eight months. At these times, they were removed and cut into 2 x 3 mm pieces and planted on the bottom of 35 mm or 60 mm plastic tissue culture dishes (Falcon, Oxnard, California). The explants remained on the dish in growth medium for seven to ten days to permit the outgrowth of sheets of epithelial cells. Three to four outgrowths were generated from each explant and fibroblasts, when present, were scraped away as described earlier (Marchok et al., 1977). After an initial two weeks of culture in growth medium, epithelial outgrowths from the DMBA-treated explants were placed in selection medium for an additional two weeks in order to select for carcinogen-altered tracheal cells that survive in medium lacking insulin and pyruvate supplements. Following this selection, the four week old cultures were refed with growth medium and used in the assays described below. These selected primary cell cultures (SPC) can be derived from tracheas long before the development of tumors, and thus represent carcinogen-altered cells from earlier

stages in the progression of neoplasia (Marchok & Huang, 1982; Marchok et al., 1983; Marchok, in press).

Tumor-derived primary cell cultures (TPC) were obtained from outgrowths of explants of carcinomas induced with DMBA using the tracheal implant model described above, or from pieces of carcinomas which formed in nude mice after inoculation of subcultured cells derived from DMBA-preexposed implants (Marchok et al., 1983). Outgrowths from explants of nonexposed tracheas maintained in growth medium were used to generate "normal" primary cell cultures (NPC). These normal cultures degenerate in selection medium (Rice & Marchok, 1980).

Primary cell cultures used in all assays were five to eight weeks old. None of the parameters assayed in this study was affected by this age variation. All assays made with cultures from later outgrowths of explants yielded values that were similar with those of cultures derived from earlier outgrowths. For experiments involving equal halves of individual primary cell populations, the cell cultures were established on plastic film-liners in 60 mm dishes (Falcon, Oxnard, California), and the liners were cut with scissors immediately before the assay.

Deoxyglucose Uptake and Lactic Acid Measurements

Hexose uptake was assayed by measuring the incorporation of 2-dGlc into the cells. The medium was removed from the culture dishes and the cells were washed with glucose-free Hanks balanced salt solution (HBSS) containing 10 mM Hepes (37°C). One ml of radioactive medium (HBSS + 10 mM Hepes + 10 μ M 2-dGlc, 1 μ Ci/ml)

was then added and the cultures incubated at 37°C for the designated time. Uptake was terminated by aspirating the radioactive medium and rinsing the cultures four times in cold HBSS (2-5°C). Ice cold 5% TCA (1 ml) was then added and the monolayers were scraped and centrifuged (2,000 X g for 10 min). The acid-soluble supernatant was collected and counted for radioactivity in Aquasol (New England Nuclear, Boston, Massachusetts). Cold TCA precipitates were further extracted (see below) for protein and DNA determinations. Nonspecific uptake and extracellular trapping of deoxyglucose were estimated by using [³H]-L-glucose in the above assay. This background was generally <10% of the uptake levels obtained with 2-dGlc and was subtracted from all experimental values.

Lactic acid was enzymatically assayed in aliquots of culture medium with lactate dehydrogenase, using the reagents and protocol given with a kit obtained from Sigma Chemical Co., St. Louis, Missouri.

DNA and Protein Determinations

DNA was extracted and measured as described in the Materials and Methods section of Chapter 2. Protein in the residue remaining after hot TCA extraction was solubilized with 0.5N NaOH and determined fluorometrically by the reaction of fluorescamine with primary amines by techniques similar to those reported (Bohlen et al., 1973). Bovine serum albumin (Fraction V) was used as standard. Preliminary studies examining the use of this assay as a measurement for protein in rat tracheal epithelial cells revealed

that protein values were generally 10%-50% greater than those obtained by the method of Lowry et al. (1951).

{³H}-Thd Incorporation and Determination of Cell Number

These techniques were similar to those described in the Materials and Methods section of Chapter 2.

Results

Hexose Uptake in Rat Tracheal Epithelial Cell Lines

Since most studies in the past which showed increases in hexose uptake with tumorigenicity were carried out in cell lines (Hume & Weidemann, 1979; Eigenbrodt & Glassman, 1980), this possibility was examined in cell lines established from rat tracheal epithelia. Table 2 shows the uptake of 2-dGlc in a nontumorigenic (C-18) and two tumorigenic (1000-WT, T-8) cell lines under conditions of increasing levels of cell association. At subconfluency, when cells covered about 1/3 of the dishes, no distinct differences were apparent between the cell lines when 2-dGlc uptake was expressed per mg DNA. A slightly lower level of hexose uptake was found in the C-18 cells if the uptake is given in pmols per mg protein. This is due to the higher protein:DNA ratio in these cells as compared to that found in the other cell lines at subconfluency. Hexose uptake was lowered in confluent cultures of all the cell lines with the nontumorigenic cell line clearly exhibiting the greatest reduction in 2-dGlc uptake (>3-fold). In contrast, uptake was reduced only 1.3-fold in T-8 cells. Cell line 1000-WT showed a 1.6-fold reduction when hexose uptake was expressed per mg DNA and

Table 2

2-dGlc Uptake in Cultures of Nontumorigenic (C-18) and
Tumorigenic (T-8; 1000-WT) Cell Lines at
Different Levels of Cell Association^a

Cell Line	N	P/DNA ^b	Subconfluent			Confluent			REE			
			pmoles 2-dGlc/min per:			pmoles 2-dGlc/min per:			pmoles 2-dGlc/min per:			
			μg DNA	mg protein	N	P/DNA	μg DNA	mg protein	N	P/DNA	μg DNA	mg protein
C-18	4	39	6.3 ± 1.3 ^c	167 ± 25	5	33	1.7 ± 0.6	54 ± 13	4	64	1.6 ± 0.5	32 ± 12
1000-WT	3	18	4.1 ± 1.5	233 ± 63	6	29	2.6 ± 0.4	94 ± 9 ^d	6	40	0.6 ± 0.1 ^d	22 ± 4
T-8	4	25	6.2 ± 1.1	231 ± 13 ^d	9	30	4.6 ± 0.4 ^d	171 ± 9 ^d	4	27	2.9 ± 0.6	115 ± 14 ^d

^a Cells were seeded at 5×10^4 in 35 mm culture dishes and assayed 2 days (subconfluent) or 5 days (confluent) later for 2 min of 10 μM 2-dGlc uptake as described in "Materials and Methods." Continuous sheets of cells generated from explants of denuded tracheas bearing reestablished epithelia (REE) of each cell line were also assayed for 2-dGlc uptake.

^b Ratio of mg protein/mg DNA per culture; S.E. was ≤ 15% of the mean.

^c Mean ± S.E.

^d Significant differences compared to C-18; $p \leq 0.05$ (Student's t-Test).

2.5-fold when expressed per mg protein. This latter result reflects the increase in the protein:DNA ratio in confluent cultures of 1000-WT cells.

Hexose uptake was next measured in sheets of epithelial cells generated as contiguous outgrowths from pieces of denuded tracheal implants bearing epithelia reestablished from the cell lines. These cultures should mimic more closely the primary cultures obtained directly from tracheas and tracheal implants in the studies discussed below. Under these conditions the nontumorigenic cell line C-18 only showed an additional lowering of 2-dGlc uptake when expressed per mg protein, which resulted from a further increase in protein to DNA. Hexose uptake was reduced somewhat in T-8 cells. The protein:DNA ratio remained essentially the same in these cells under all forms of culture growth, hence the 2-dGlc uptake reflects the activity of T-8 cells under the different conditions of cell association. The cells of line 1000-WT were particularly affected by reestablishment into sheets of cells. Uptake was reduced over 4-fold as compared to confluent cultures and 4.7- to 6-fold as compared to subconfluent cultures depending on whether the data were expressed per mg DNA or mg protein, respectively.

Under these assay conditions, deoxyglucose uptake was linear for at least 10 mins with an apparent K_m in C-18 cells and T-8 cells of 5 mM (data not given). The effects of inhibitors of facilitated glucose transport were also measured with 1000-WT cells to determine if deoxyglucose uptake occurred mainly by a facilitated transport mechanism in rat tracheal epithelial cells. Cytochalasin B and

and phloretin, two inhibitors of Na-independent hexose transport (Stein, 1967; Bloch, 1973), caused a dramatic and concentration dependent decrease in uptake (Table 3). Phloridizin, an inhibitor of Na-dependent hexose transport systems (Stein, 1967), did not inhibit deoxyglucose uptake at the concentration tested.

Taken together the above data indicate that under certain conditions (subconfluent and confluent cultures of dissociated cells), tumorigenic epithelial cell lines can have higher levels of hexose uptake than nontumorigenic cell lines. However, when the cells are cultured under conditions which should give cell associations closer to those found in intact epithelium (outgrowths from REE), strikingly different results may be obtained. This is not surprising since epithelial cell lines as well as primary epithelial cell cultures (see below) have markedly different growth properties. In this respect, 1000-WT is a heavily keratinizing cell line (D. Berry, personal communication; Marchok et al., 1981) in which the basal layer grows to high cell densities as tightly packed polygonal cells. This is especially so in cultures established from REE (Figure 4A). Even under subconfluent conditions, these cells grow closely apposed and display little random overlap. In contrast, T-8 cells show little keratinization (Marchok et al., 1981) and freely overlap neighboring cells (Figure 4B). At confluency as well as in outgrowths from REE, loose cell associations are maintained, and these preclude T-8 cells from attaining the high cell densities found in 1000-WT cultures. The morphology of nontumorigenic cell line C-18 is very similar to that found in NPC

Table 3

Effect of Inhibitors of Hexose Transport on
2-dGlc Uptake in 1000-WT Cells^a

Treatment	Relative 2-dGlc uptake ^b
None	1.00
+ Cytochalasin B	
2 μ M	0.58
20 μ M	0.09
+ Phloretin	
20 μ M	0.39
200 μ M	0.13
+ Phloridizin	
10 μ M	1.12

^aCells were seeded at 5×10^4 in 35 mm cultures dishes. After 48 hrs, medium was removed and the cultures were preincubated in HBSS for 15 min and assayed for 10 μ M 2-dGlc uptake after 5 min in the presence of inhibitors.

^bMean 2-dGlc uptake for control, non-treated cells was 20 pmols/min/ 10^5 cells. Cell counts were performed on parallel cultures. Variation between duplicate cultures was generally $\leq 10\%$ of mean values.

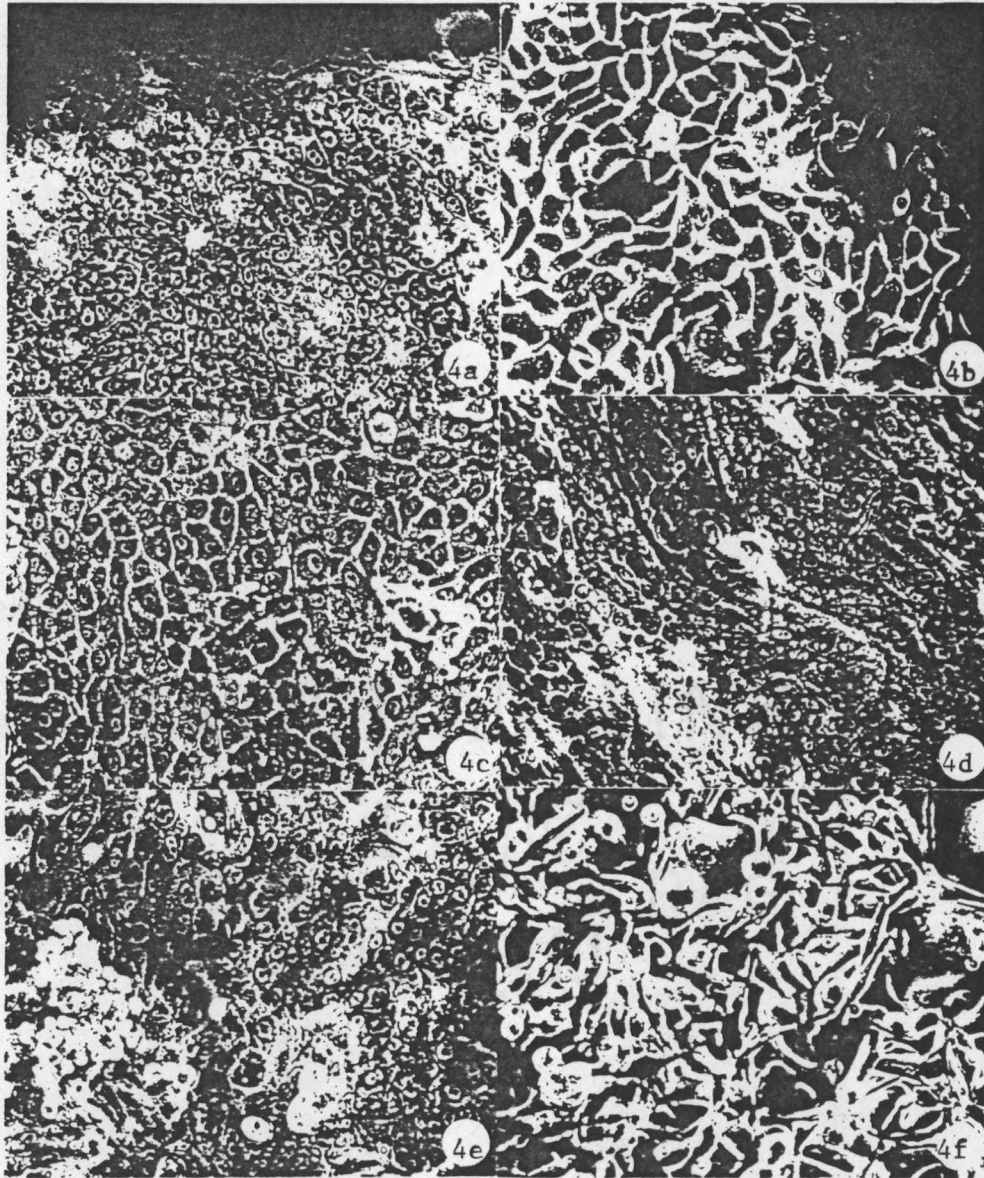


Figure 4. Phase contrast photomicrographs of rat tracheal epithelial cell cultures. a) Outgrowth from a REE of cell line 1000-WT. Note the tightly-packed, small cells with a high nuclear to cytoplasmic ratio. b) Outgrowth from a REE of cell line T-8. The cells are large, angular and more loosely associated. Dark regions in (a) and (b) denote edges of pieces of denuded tracheas bearing REE of each cell line. c) A NPC showing the characteristic polygonal cells with broad intercellular spaces found in cultures of normal epithelium. d) A SPC exhibiting small, closely associated irregularly-shaped cells. e) A TPC from Group 1 showing mostly polygonal cells of varying size. f) A TPC from Group 2 showing extensive overlap of elongated cells growing in a random pattern, X475.

(discussed below). It is interesting to note that the two tumorigenic cell lines, T-8 and 1000-WT, each display a high level of anchorage-independent growth (A. Marchok, personal communication) even though their in vitro growth characteristics are quite dissimilar.

2-dGlc Uptake by Primary Cultures During the Progression of Neoplasia

The uptake of 2-dGlc was measured next in cell populations at two stages in the development of neoplasia and compared to control cell populations. The latter were primary cultures of normal cells (NPC) generated from outgrowths of unexposed tracheas. A typical primary culture from a nonexposed trachea (NPC) is shown in Figure 4C. These cell cultures contain numerous polygonal-shaped cells separated by broad intercellular spaces. Cell populations exhibiting at least the first stage of neoplasia, i.e. altered growth control, were selected primary cell cultures (SPC) obtained by culturing outgrowths from DMBA-preexposed tracheal implants in the pyruvate and insulin deficient medium. These cultures (Figure 4D) were derived from exposed tracheas well before the appearance of recognizable tumors. Primary cell cultures from the last stage of neoplasia, i.e., frank tumors, were obtained from DMBA-preexposed tracheal implants which bore palpable tumors. Only the tumor areas were used. Small pieces of the tumors, fixed and stained for histology and/or in vitro exfoliative cytology from the culture pieces were used to positively identify the carcinomas (Marchok et al., 1978; Marchok, in press). Cell cultures were also generated from carcinomas which developed in nude mice after inoculating

tracheal epithelial cell lines derived from SPC (Marchok et al., 1984). The outgrowths from the tumors were also taken through the selection medium. These were designated tumor primary cell cultures (TPC).

Table 4 shows that the SPC have only half the 2-dGlc uptake of that found in NPC when the uptake is expressed per mg DNA. The difference is not as significant when expressed per mg protein because of the higher protein:DNA ratio in the normal cell cultures. Since some tumors were large, several pieces and subsequently several primary cultures could be obtained from a single tumor. Therefore, the 2-dGlc uptake in cultures from each of the tumors is given separately. Two different levels of hexose uptake were observed in the tumor-derived cell populations. The TPC from the four tumors in Group 1 had 2-dGlc uptake/mg DNA similar to that found in SPC and therefore lower than normal cell cultures. The in vitro morphology of these cultures was similar to that of SPC (Figure 4E). The protein:DNA ratios of the TPC were variable which made the uptake values expressed/mg protein in some cases higher and in others lower than that obtained from NPC. The uptake of 2-dGlc by TPC in Group 2 was distinctly higher than the controls. These epithelial cultures were distinguished by their loosely attached, markedly overlapping growth pattern (Figure 4F). TPC from this group came from rapidly growing, mostly undifferentiated carcinomas. Tracheal epithelial cell lines with these growth characteristics always show

Table 4

2-dGlc Uptake by Primary Cultures of Tracheal
Epithelial Cells in Various Stages of
the Progression of Neoplasia^a

Primary Culture	N	P/DNA ^b	pmoles 2-dGlc/min/per:	
			μg DNA	mg Protein
NPC	32	95	2.3 ± 0.2	30.0 ± 2.6
SPC	26	73	1.2 ± 0.1 ^c	23.5 ± 3.1
TPC				
<u>Group 1</u>				
T-1	9	77	1.4 ± 0.3 ^c	21.5 ± 5.8
T-2	3	76	1.4 ± 0.3 ^c	14.8 ± 5.1
T-3	8	31	1.7 ± 0.5	48.3 ± 7.2
T-4	3	22	1.1 ± 0.2 ^c	21.8 ± 6.7
<u>Group 2</u>				
T-5	3	58	5.2 ± 0.8 ^c	92.0 ± 15.0 ^c
T-6	2	35	3.7 ± 0.1 ^c	118.4 ± 32.7 ^c

^aCell cultures were established as outgrowths from tracheal explants as described in "Materials and Methods." SPC and TPC were selected by growing these cultures in selection medium (minus pyruvate and insulin) for 2 weeks. All cultures were returned to growth medium (plus pyruvate and insulin) for at least one week prior to assay. The 2 min uptake of 10 μM 2-dGlc was measured as described in "Materials and Methods."

^bRatio of mg protein/mg DNA per culture. S.E. was ≤ 15% of the mean.

^cSignificant differences compared to NPC; $p \leq 0.05$ (Student's t-Test).

high levels of anchorage independent growth (Marchok, unpublished results).

Substrate Accessibility and Influence of Cell Proliferation

Since the primary cultures in this system are differentiating sheets of cells, it was possible that substrate was not fully accessible to the cells. Therefore accessibility of the 2-dGlc to these primary cell cultures was examined by comparing uptake of 2-dGlc obtained after 2 min of incubation with that observed after 10 min (Table 5). Sister halves of individual primary cultures were used for the two time points. In each cell population tested, 2-dGlc uptake was linear for at least 10 min, and the differences observed between NPC and SPC populations after a two minute assay were equivalent to those obtained following 10 minutes of incubation (2.4 and 2.1 fold, respectively). This suggests that 2-dGlc is readily accessible to the cells under our assay conditions. Similar results were observed with several tumor-derived cultures tested in the same manner (data not shown).

The uptake of nutrients by cells in culture can also be influenced by cell proliferation (Koren, 1980). Therefore, proliferation effects on hexose uptake were assessed by measuring the incorporation of $\{^3\text{H}\}$ -Thd into DNA in one-half of a primary cell culture and by assaying the sister half for 2-dGlc uptake. By using this approach, it is clearly seen in Table 6 that there is no correlation between $\{^3\text{H}\}$ -Thd uptake and hexose uptake. The SPC incorporated 4.6 times more $\{^3\text{H}\}$ -Thd per mg DNA than did normal primary cultures. This suggests that SPC have much higher levels

Table 5

Effect of Incubation Time on 2-dGlc Uptake in
Normal (NPC) and Carcinogen-Altered (SPC)
Primary Cultures^a

Primary Culture	pmoles 2-dGlc/ μ gDNA per:	
	2 min	10 min
NPC	3.1 ± 0.4^b	15.4 ± 0.8
SPC	1.3 ± 0.2	7.4 ± 1.3

^aIndividual NPC and sister-halves of SPC grown on film-lined culture dishes were assayed for the uptake of 10 μ M 2-dGlc as described in "Materials and Methods."

^bMean $\pm$ S.E. of triplicate determinations.

Table 6

Cell Proliferation and 2-dGlc Uptake in Normal (NPC)
and Carcinogen-Altered (SPC)
Primary Cell Cultures^a

Primary Culture	N	dpm { ³ H}Thd x 10 ³ /μgDNA	pmoles 2-dGlc/min/μgDNA
Growth medium			
NPC	6	1.4 ± 0.2 ^c	2.9 ± 0.5
SPC	6	6.5 ± 1.5 ^d	1.6 ± 0.4 ^d
Selection medium ^b			
SPC	8	3.4 ± 0.9 ^d	1.3 ± 0.3 ^d

^aNPC and SPC, grown on film-liners, were cut into two equal-sized sections. One section was used for determining 2 min uptake of 10 μM 2-dGlc, while the second piece was assayed for the incorporation of {³H}Thd into DNA as described in "Materials and Methods."

^bSPC were grown for 5 days in medium lacking supplements of pyruvate and insulin prior to assaying for {³H}Thd incorporation and 2-dGlc uptake.

^cMean ± S.E.

^dSignificant differences as compared to NPC; $p \leq 0.05$ (Student's t-Test).

of DNA synthesis per culture than normal primary cell cultures. As found earlier (Table 4), 2-dGlc uptake in NPC was higher than in SPC cultures.

To further substantiate a lack of correlation between cell proliferation and hexose uptake, $\{^3\text{H}\}$ -Thd incorporation and 2-dGlc uptake were measured in sister halves of SPC cultures maintained for 5 days in the selection medium. Under these culture conditions the NPC begin to degenerate and ultimately die. Although $\{^3\text{H}\}$ -Thd incorporation was reduced in the SPC cultured in the selection medium, it was still more than twice that found in NPC maintained in the growth medium. Uptake of 2-dGlc by SPC cultured in selection medium was similar to that found for SPC maintained in growth medium. These data not only demonstrate the lack of correlation between cell proliferation and hexose uptake, but also show that 2-dGlc uptake into SPC cultures is not influenced by the presence or absence of insulin and pyruvate in the medium.

2-dGlc Uptake per Cell

The amount of protein per culture can vary markedly depending on the size of the cells, the amounts of specialized proteins accumulated, etc. In the same fashion, DNA content per culture can be markedly influenced by changes, for example, in ploidy. Therefore, 2-dGlc uptake was assayed in cultures in which the total cell number was first determined by a nondestructive method (see Materials and Methods). When 2-dGlc uptake was given on a per cell basis, the levels of activity found in the normal, carcinogen-altered and tumor cell populations (Table 7) were comparable to

Table 7

2-dGlc Uptake in NPC, SPC and TPC
Expressed Per Cell Number^a

Primary Culture	pmoles 2-dGlc/min/10 ⁵ cells
NPC	10.4 ± 1.8 ^c
SPC	3.1 ± 1.0
T-3 ^b	7.1 ± 1.6

^aPrimary cultures were first traced and photographed to determine cell number in a nondestructive manner as described in "Materials and Methods" and then assayed for 2 min uptake of 10 μ M 2-dGlc.

^bOutgrowths from tumor T-3 used in assays given in Table 4.

^cMean ± S.E. of at least 4 determinations.

that obtained when the uptake was expressed per mg DNA (Table 4, p. 45). That is, the NPC had the highest level of 2-dGlc uptake, tumor, T-3, an intermediate level and the SPC the lowest level. These data support the contention that it is suitable to normalize 2-dGlc uptake against DNA content in these cells on a routine basis.

Glucose Deprivation

Routine measurements of hexose uptake do not necessarily reveal all of the differences in metabolic requirements for hexose between NPC and SPC. Such differences may become apparent when glucose concentrations are limiting. Under conditions of glucose deprivation, many cells respond by elevating their hexose uptake (Martineau et al., 1972; Ullrey et al., 1975). Therefore, hexose uptake was measured in glucose-deprived NPC and SPC to determine if cells in neoplastic progression respond differently than normal cells to the absence of glucose. After 24 hours of glucose-starvation, a marked increase in 2-dGlc uptake was observed in NPC and SPC (Table 8). Hexose uptake increased 3-fold in NPC and 5-fold in SPC, thereby stimulating the uptake to an equivalent level of 14 pmol/ μ g DNA/min. Thus, SPC and NPC are capable of responding to glucose deprivation, and each type of culture can reach high levels of uptake when the cells demand it.

Lactic Acid Production

High rates of aerobic glycolysis are observed in many transformed cells (Warburg, 1930; Racker, 1976; Hume & Weidemann, 1979; Eigenbrodt

Table 8

Effect of Glucose Deprivation on
2-dGlc Uptake in NPC and SPC^a

Preincubation Medium	pmoles 2-dGlc/min/ μ gDNA	
	NPC	SPC
Growth medium		
+ glucose	3.9 ^b	2.6
- glucose	13.6	14.4

^aPrimary cultures were preincubated in growth medium with or without glucose plus 10% dialyzed serum for 24 hrs and then assayed for 2 min uptake of 10 μ M 2-dGlc as described in "Materials and Methods."

^bThe mean of 2 or 3 cultures.

& Glossman, 1980). This is generally assessed in vitro by measuring lactic acid, a byproduct of aerobic glycolysis, in cell culture medium. When this was done in NPC and SPC, the levels of lactic acid in the growth medium were found to continually increase with time in both types of cultures (Figure 5). Lactic acid production was 2-fold higher in NPC as compared to SPC. This is similar to the nearly 2-fold higher uptake of 2-dGlc observed earlier in NPC as compared to SPC (Table 3, p. 41). When glucose concentrations were reduced from 27.7 mM to 0.2 mM for a week prior to assay, a significant reduction (greater than 4-fold) in lactic acid formation occurred in both types of cultures. The NPC still retained a higher level than SPC although the difference was not as great as that found at the higher concentration of glucose. These data clearly show that carcinogen-altered cells do not achieve a growth advantage in vitro by acquiring a higher rate of aerobic glycolysis.

Discussion

In this study it is shown that hexose uptake actually decreases with respect to controls in cells from earlier stages of neoplasia while at later stages this uptake either remains low or greatly increases. This was investigated by using an in vivo-in vitro model of chemical carcinogenesis in rat tracheas that enables primary cultures of DMBA-altered cells from early (SPC) and late (TPC) stages of carcinogenesis to be isolated from neighboring normal cells. This "selection" was accomplished by growing carcinogen-altered cells in medium lacking pyruvate and insulin, a culture condition which

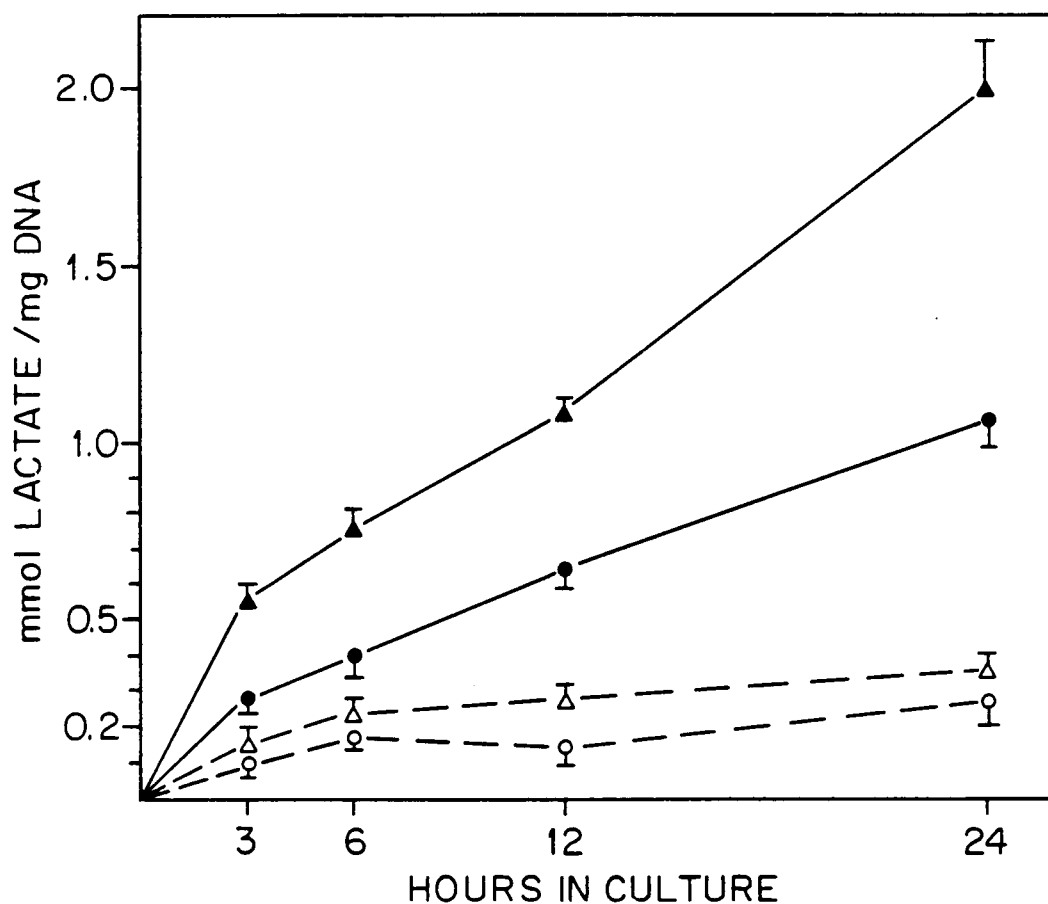


Figure 5. Lactic acid formation in NPC ($\blacktriangle, \triangle$) and SPC ($\bullet, \circ$). NPC and SPC were cultured in growth medium supplemented with 27.7 mM glucose ($\blacktriangle, \bullet$) or 0.2 mM glucose ($\triangle, \circ$) for one week. Prior to assay, the media were changed to growth medium with the same glucose concentrations but with 10% dialyzed serum, and aliquots of the culture medium were removed from each dish over a 24 hr. period and assayed for lactic acid as described in "Materials and Methods". Mean values of 3 dishes; bars, S.E.

causes normal cells to degenerate (Rice & Marchok, 1980; Marchok, in press). However, long-term cultures of nonexposed tracheal cells could be maintained in the presence of pyruvate and insulin, thereby allowing us to study directly the differences between normal and carcinogen-altered cells. The growth advantage displayed by carcinogen-altered tracheal epithelial cells in the absence of pyruvate and insulin is an event that occurs early in the progression of neoplasia (Marchok & Huang, 1982; Marchok et al., 1983; Marchok, in press). Its metabolic basis is unknown; therefore, the likelihood that increased hexose uptake was in some way related to this phenomenon was explored. This apparently does not play a role since hexose uptake in SPC and many TPC was less than that of normal cells. These results were observed when either protein or DNA was used as a reference for 2-dGlc uptake.

These findings are somewhat contradictory with most other investigations regarding hexose uptake and neoplasia (Warburg, 1930; Racker, 1976, Hume & Weidemann, 1979; Eigenbrodt & Glossman, 1980). In numerous studies, close correlations between increases in hexose uptake and neoplastic transformation have been found, although in recent years several exceptions have been noted (San et al., 1979; Pouyssegur et al., 1980; Peterkofsky & Prather, 1982). The apparent disparity between this and previous studies may be due to differences in the types of cultures used. Cell cultures of enzyme dissociated embryonic fibroblasts or specially selected cell lines are generally used to show transformation-related increases in hexose uptake (Isselbacher, 1972; Bissell et al., 1973; Siddiqi & Iype, 1975;

Lang & Weber, 1978; Hume & Weidemann, 1979). These types of cultures may lose many of the cell associations present in intact tissue. This could have an effect on several cellular events, particularly in epithelia. This method of generating primary cell cultures as contiguous sheets of cells is less likely to result in the loss of cell associations. In this regard, it is interesting to note that when elevations in uptake were observed in TPC (group 2, Table 4, p.45), these cultures grew in a loose irregular pattern with overlapping of the cells. This is similar to the growth pattern found in many transformed cultures derived from dissociated cell lines, including one of our tumorigenic tracheal epithelial cell lines (T-8).

That cell associations do indeed affect hexose uptake was clearly demonstrated in this study by using tumorigenic and nontumorigenic rat tracheal epithelial cell lines. As expected, in these cell lines the highest level of uptake was found in subconfluent cultures, with the nontumorigenic cell line having the lowest uptake when expressed on a protein basis. Growing the culture to confluency reduced hexose uptake to some extent in all the cell lines with the nontumorigenic line showing the greatest decrease. However, quite different results were obtained if the cell lines were grown in such a way as to mimic primary cultures from an intact epithelium. This was done by generating outgrowths from reestablished epithelia (REE) of each cell line. Under these conditions, hexose uptake was markedly reduced in one of the tumorigenic cell lines (1000-WT) to a level far below that found in the nontumorigenic

cell line (C-18), while the second tumorigenic cell line (T-8) retained the highest level of 2-dGlc uptake. These latter results are similar to those found in TPC, i.e., some had higher 2-dGlc uptake while others had lower uptake than NPC. Findings such as these indicate that cell associations may have a profound effect on hexose uptake, and generalizations concerning hexose uptake and tumorigenicity cannot be made.

Several other factors that may have influenced hexose uptake in primary cultures of tracheal cells were examined and found to have little effect. One possibility was that incomplete access of the substrate to all of the cells could restrict hexose uptake in continuous sheets of cells, especially if stratification occurred. This apparently was not a limitation, since uptake was linear and the results were unchanged throughout extended periods of assay.

In these studies, the uptake of 2-dGlc was expressed on a protein and DNA basis for several reasons. It has been suggested that transport as well as uptake measurements should be expressed as the amount of substrate accumulated per unit cell volume (Parnes & Isselbacher, 1978). However, cell volumes most likely vary extensively in epithelia, since heterogeneity in cell size is often observed in primary cultures (Heckman et al., 1978). Along with this, the protein content of epithelial cells may vary as well as DNA content because of changes in ploidy (Hsu, 1961; Braslawsky, 1982; Erlinger & Saier, 1982). Even so, comparable results were generally observed when hexose uptake was expressed on either a DNA or protein basis. In a few cases an apparent change in the

protein content per culture resulted in different data being obtained. This was reflected by a change in the protein:DNA ratio for that culture. Marked differences in the protein content of individual rat tracheal epithelial cell cultures are not surprising since the in vitro differentiative capacity, such as the degree of keratinization in these cells, does vary (Marchok et al., 1981). A greater degree of keratinization may have occurred in two of the rat tracheal epithelial cell lines (C-18, T-8), since elevations in their protein:DNA ratio were found with increasing culture density and cell association.

Another parameter which may influence hexose uptake is cell proliferation. Numerous studies in the past have shown close correlations between growth rate and nutrient uptake (Koren, 1980). However, recent investigations suggest that the rate of glucose uptake is not a prime regulator of cell proliferation (Peterkofsky, 1982; Romano & Connell, 1982). In mouse 3T3 cells, Romano et al. (1982) demonstrated little change in growth rate when glucose-derived metabolic pools were drastically reduced with 6-deoxy-D-glucose, a metabolically inert glucose analog. In HeLa cells, significant levels of proliferation occur in the complete absence of glucose in the medium (Wice et al., 1981). It was observed in this study that carcinogen-altered tracheal epithelial cells maintain high levels of $\{^3\text{H}\}$ -Thd incorporation into DNA without corresponding increases in hexose uptake. This suggests that hexose uptake and cell proliferation are not directly proportional in primary cell cultures of rat tracheal epithelial cells as well. The high levels

of $\{^3\text{H}\}$ -Thd incorporation in the SPC most likely reflect the increased in vitro growth capacity of these carcinogen-altered cells, an event that is associated with an early stage of neoplastic progression (Marchok et al., 1977; Nettesheim & Marchok, 1983).

Parallel rates of hexose uptake and aerobic glycolysis are often observed in cells (Pouyssegur et al., 1980; Eigenbrodt & Glossman, 1980; Peterkofsky & Prather, 1982). Each is frequently higher in neoplastic cells, although lower values, unrelated to tumorigenicity, have recently been reported (Pouyssegur et al., 1980; Peterkofsky & Prather, 1982). These data also support a close correlation between hexose uptake and aerobic glycolysis since each was lower in SPC and higher in NPC. This strengthens the findings regarding hexose uptake between NPC and SPC and also indicates that, like hexose uptake, increases in aerobic glycolysis do not play a role in the growth advantage exhibited by these cells in the early stages of carcinogenesis.

Finally, one may speculate that the reduced levels of hexose uptake and decreased in vitro growth requirements for pyruvate and insulin in these DMBA-altered cells are interrelated events. Each is likely to be a result of an overall metabolic strategy linked to the growth advantage these cells exhibit early in the progression of neoplasia. Lower levels of hexose uptake in the SPC may have been caused by a reduced activity of hexokinase, fewer transporters or alterations in the kinetic properties of transporters. Additional experimentation involving the assay of hexokinase activity and transport per se are needed to determine the rate limiting step

for hexose uptake in these cells. It is possible that the reduced level of hexose uptake in the SPC is a result of a more efficient utilization of hexose or a reduced requirement for glucose as an energy and/or anabolic substrate. The latter, however, appears unlikely since glucose deprivation in NPC and SPC resulted in an enhancement of hexose uptake in both cultures. This phenomenon has been observed in other cells (Martineau et al., 1972; Ullrey et al., 1975), and most likely reflects the metabolic importance of glucose in these cultures. Recent studies have shown that glucose is used less as an energy source by cells in vitro and more as a substrate for key anabolic pathways, such as the pentose phosphate pathway (Reitzer et al., 1979; McKeehan, 1982a). Many of these pathways are regulated by hormones as well as by metabolic intermediates. In SPC, these pathways may operate efficiently at lower intracellular glucose levels and/or be regulated less by medium components. The invariant levels of hexose uptake found in SPC maintained in selection medium suggest that the latter may indeed occur. However, it is also quite possible that alternate energy sources are utilized in these carcinogen-altered cells. In recent years, the importance of glutamine as a major energy substrate by cells in vitro has been suggested (Reitzer et al., 1979; McKeehan, 1982a). Elevations in glutamine utilization as well as in several key enzymes involved in glutamine metabolism have been reported in neoplastic cells (Knox et al., 1970; Sauer et al., 1980). McKeehan (1982a) has recently proposed a sequence of reactions, termed glutaminolysis, that utilize glutamine as an important source of energy

for proliferating cells in culture. An end product of these reactions is pyruvate. It would be interesting to know if glutaminolysis is a determinant of cell proliferation in rat tracheal epithelial cells. If so, then an increase in these reactions may convey a selective growth advantage on carcinogen-altered rat tracheal cells when extracellular pyruvate is limiting. This possibility warrants additional investigation.

CHAPTER IV

UTILIZATION OF KEY METABOLITES BY NORMAL AND CARCINOGEN-ALTERED RAT TRACHEAL EPITHELIAL CELLS

Introduction

Numerous metabolic changes have been documented in malignantly transformed cells. These range from alterations in the regulation and activity of key enzymes (Weber, 1983), isozyme shifts (Weinhouse, 1980), new patterns of metabolite utilization (Fenselau et al., 1976; Cederbaum & Rubin, 1976), and decreases in the regulation of lipid synthesis (Coleman & Lavietes, 1981). It is difficult to recognize, however, which of these varied metabolic changes is essential rather than incidental to the development of cancer, since most have been observed in cells that best represent late stages in neoplastic progression (tumors and transformed cell lines). Very little has been elucidated about the fundamental metabolic changes that occur in cells at earlier stages of neoplasia. For this reason, the data in Chapter III are quite significant. Not only do the results of Chapter III strongly suggest that increases in glucose uptake are not the mechanism by which SPC acquire a growth advantage over normal cells, but the findings also provide the first indication that there are indeed distinct metabolic differences between normal tracheal cells and tracheal cells from earlier stages of neoplastic progression. Elucidating these metabolic differences should help to unravel the mechanism(s)

underlying the reduced growth requirement for pyruvate in these carcinogen-altered cells.

In this chapter, the utilization of several key metabolites is examined in normal and carcinogen-altered tracheal cells in an attempt to identify metabolic differences between these cells which may be related to their dissimilar growth requirement for exogenous pyruvate. The research includes a detailed examination of glucose metabolism in these cells to determine if the lower uptake of hexose in SPC (Chapter III) is due to a more efficient utilization of glucose in pathways such as the pentose cycle or citric acid cycle. Also investigated is the incorporation of pyruvate, glucose, alanine and glutamine into the subcellular fractions and components of NPC and SPC. This was undertaken to assess if major shifts in the utilization of these substrates are related to the decreased requirement for exogenous pyruvate in SPC.

Materials and Methods

Materials

Most reagents and chemicals were obtained from the commercial sources described in Chapters II and III. D- $\{^{14}\text{C}(\text{U})\}$ glucose (240 mCi/mmol) was obtained from ICN Pharmaceuticals, Inc., Irvine, California. $\{1\text{-}^{14}\text{C}\}$ pyruvic acid (25.9 mCi/mmol) came from Amersham, Chicago Heights, Illinois. All other radiolabeled metabolic substrates were purchased from New England Nuclear, Boston, Massachusetts. These included: D- $\{1\text{-}^{14}\text{C}\}$ glucose (57.1 mCi/mmol);

D-{6-¹⁴C} glucose (56.1 mCi/mmol); {2-¹⁴C} pyruvic acid (15 mCi/mmol; L-{¹⁴C(U)} glutamine (281 mCi/mmol) and L-{¹⁴(U)} alanine (176.0 mCi/mmol).

Culture Conditions and Establishment of Primary Cell Cultures

These procedures were performed essentially as described in Chapters II and III.

Incorporation of ¹⁴C-Metabolites into Cell Fractions and Medium Lactate

Prior to most assays, cell cultures were preincubated for 24 hrs. in the appropriate test medium containing the indicated levels of the metabolite to be tested as well as 10% dialyzed fetal bovine serum.

Analysis of the incorporation of ¹⁴C-metabolites into various fractions was performed as follows. The medium used to pulse-label the cells was collected and stored at 4°C for later determinations of lactate. Cultures were then immediately acidified with 0.5 ml of cold (4°C) 0.5 N perchloric acid, scraped from the dish and centrifuged at 2,000 x g for 10 minutes. The supernatant was collected and stored (4°C) until further analysis (see below). The pellet was then extracted sequentially with K⁺ acetate + 95% ethanol (5°C) and chloroform + ethanol (1:3) at 60°C. These extracts were pooled and represent the lipid fraction of the cells. A nucleic acid fraction was then obtained by extracting the remaining residue with hot 5% trichloroacetic acid at 90°C (2X). These hot TCA extracts were assayed fluorometrically to determine

DNA content using diaminobenzoic acid in a modification of the technique of Kissane and Robbins (1958). Calf thymus DNA was used as a standard. The final residue remaining after hot TCA extraction was solubilized with 0.5 N NaOH and contained the cellular acid-insoluble protein fraction.

The perchloric acid-soluble cell fraction was neutralized with 5 N KOH (4°C) and the precipitated salt was separated from soluble metabolites by centrifugation at 2,000 x g (10 min). The soluble components were then concentrated by lyophilization (12 hr). Lyophilized extracts were resolubilized in 3X distilled water, and aliquots were counted for total ^{14}C -incorporated and analyzed for labeled metabolites by 2-dimensional paper chromatography.

Paper chromatography of lyophilized and reconstituted acid-soluble cell extracts was performed on Whatman 3M paper in 2-dimensions (8-10 hr each) using solvents similar to those reported elsewhere (Bissell et al., 1973). Solvents for the first dimension were phenol:H₂O:acetic acid (84:16:1). Solvents for the second dimension were butanol:H₂O:propionic acid (50:28:22). Lactic acid in the medium was also resolved by chromatographing medium samples in one dimension (5-6 hr) on 3M paper in ethyl acetate:acetic acid:H₂O (3:1:1) (Renner et al., 1972). ^{14}C -metabolites were visualized by fluorography using Enhance spray (New England Nuclear). Exposure was for 24-36 hr at -70°C. The homogeneity and identity of individual spots was established by eluting the unknowns in water at 37°C (15 min), and rechromatographing them alongside

freshly prepared standards (25 mg/ml in H₂O). Organic acids and amino acid standards were visualized with bromocresol green and ninhydrin, respectively. Recovery of label from chromatograms was generally > 90%.

Substrate Oxidation

For ¹⁴CO₂ collection, 35 mm culture dishes containing cells in the appropriate ¹⁴C-labeling medium were placed in airtight glass specimen jars and incubated at 37°C in a Dubnoff metabolic-shaking water bath. The jars were briefly gassed with 5% CO₂:95% air to equilibrate the pH of the medium. Lids on these jars were fitted with a rubber stopper through which a glass boat (Kontes, Vineland, New Jersey) was inserted. After incubation, 200 µl of NCS (Amersham) was added to the boats using a syringe inserted through the stopper. Perchloric acid (2.9 N; 150 µl) was then added by syringe to the culture medium to release ¹⁴CO₂. The collection jars were placed on ice, and ¹⁴CO₂ was collected for 1 hr (4°C). After CO₂ collection, the boats were removed and counted in Aquasol. Cultures were then processed for DNA extraction as described above. Efficiency of ¹⁴CO₂ trapping exceeded 90%. ¹⁴CO₂ collected from control jars containing labeling medium, but no cells, was subtracted from all determinations. This background value was generally < 10% of that obtained from experimental cultures.

Glucose and Lactate Measurements

Levels of glucose and lactic acid in culture media were determined using glucose oxidase-peroxidase and lactate dehydrogenase, respectively, in established procedures provided in a kit obtained from Sigma (St. Louis, Missouri).

Enzyme Assays

Crude homogenates of cell cultures were obtained for PFK and LDH assay as follows: Cultures were rinsed with phosphate-buffered saline (4°C) and immediately scraped from the dish into an ice-cold buffered solution containing 250 mM sucrose, 2 mM EDTA and 20 mM 4-(2-hydroxyethyl)-1 piperazine-ethane-sulfonic acid)-NaOH (pH 7.2). An aliquot of digitonin (15 mg per ml) prepared in the above buffer was then added to the scraped cells to adjust the final cell suspension to 1 mg digitonin per ml (McKeehan et al., 1982). Cells were mixed and centrifuged for 2 min. in an Eppendorf microcentrifuge. The supernatant was retained for immediate assay of PFK, and frozen at -70°C for later LDH assay.

PFK activity was assayed according to Kemp (1975) at 25°C in 1 ml of reaction mixture. A unit of PFK activity is defined as that amount of enzyme which catalyzes the formation of 1 μ mole NDH per min. LDH activity was measured using a sensitive radio-metric assay recently described by McKeehan et al. (1982a,b). This assay measured the LDH catalyzed conversion of H_2O_2 - acid labile $\{1-^{14}C\}$ pyruvate into stable $\{^{14}C\}$ lactate. All assays were conducted in a 4 ml polypropylene scintillation vials

(Wheaton Omni-vial) at 37°C in a reaction mixture consisting of 100 mM Tris-HCL (pH 7.6), 1 mM EDTA, 0.2 mM NADH, 53 M {1-¹⁴C} pyruvic acid (0.4 µCi/ml) plus cell extract. Assay volume was 100 µl and incubation was for 30 min. Reactions were terminated by placing the vials in a 90°C water bath for 10 min. Unreacted {1-¹⁴C} pyruvic acid was decarboxylated by the addition of a solution containing 2 M H₂O₂ + 1 M HCl (100 µl). The vials were heated for an additional 10 min and then cooled. Aquasol was added to the vials, and the retained {1-¹⁴C} lactate counted. One activity unit of LDH is defined as that amount of enzyme which stabilizes one pmol {1-¹⁴C} pyruvic acid per minute.

Determination of Cell Number

Cell number per culture was determined as described previously in Chapter II.

Results

Initial efforts to identify metabolic differences between normal and carcinogen-altered tracheal cells began with an investigation of glucose consumption, since glucose is a major precursor of pyruvate in most cells. The production of lactic acid was also examined to indirectly assess the level of glycolysis. These parameters were tested in both types of cultures under selecting conditions, since major metabolic differences between these cell types are most likely to be expressed when pyruvate is absent.

As shown in Figure 6, distinct differences between NPC and SPC were observed in both glucose consumption and lactic acid production after only 24 hours of culture in pyruvate-deficient medium. Glucose consumption was 2-fold greater in normal cells as compared to carcinogen-altered cells (Figure 6A). This was accompanied by a nearly 2-fold greater production of lactic acid in normal cells (Figure 6B). The above data suggest that carcinogen-altered cells may require less glucose-derived pyruvate, which would be consistent with their lack of requirement for exogenous pyruvate. In contrast, NPC appear to raise their levels of glucose consumption in the absence of pyruvate, perhaps in an attempt to form more pyruvate.

Oxidation of {1- ^{14}C } Glucose vs. {6- ^{14}C } Glucose

To further identify differences between NPC and SPC in glucose metabolism, the utilization of glucose in the citrate cycle as well as in the oxidative arm of the pentose cycle was measured in each type of culture. This was done by comparing the oxidation of {1- ^{14}C } glucose to {6- ^{14}C } glucose; the latter is oxidized primarily in the citric acid cycle. It should be noted that valid objections to precise measurements of pentose cycle activity by this approach have been made because of possible randomization of carbon atoms (Katz & Wood, 1960). However, the studies here are concerned with only assessing major shifts in the activity of these pathways between cultures, and incubation

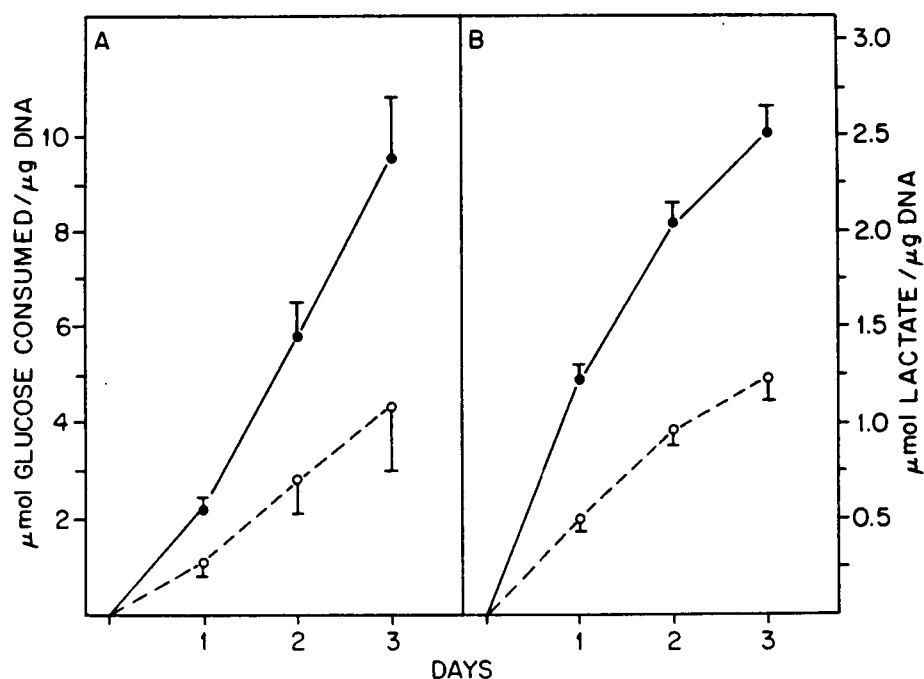


Figure 6. Consumption of glucose and production of lactic acid by NPC and SPC in the absence of pyruvate. NPC and SPC (3 each) were incubated in growth medium lacking pyruvate but containing 10% dialyzed FBS + 27.7 mM glucose. Aliquots of medium were removed on each day of culture over a 3 day period and analyzed enzymatically for glucose consumed (A) and lactic acid levels (B), as described in Materials and Methods. The cells were scraped off the dish and processed for DNA determination on day 3, and all data were normalized against these DNA values. NPC, ●—●: SPC, o---o.

times have been kept relatively short (1 hour) to minimize any recycling or randomization.

As Table 9 illustrates, {1-¹⁴C} glucose was oxidized to a considerably greater extent than {6-¹⁴C} glucose in both types of cultures, indicating that glucose utilization of energy via the citrate cycle represents only a minor fraction of the total glucose utilized in both NPC and SPC. The oxidation of {6-¹⁴C} glucose was similar in NPC and SPC, and not affected by exogenous pyruvate. The data in Table 9 also indicate that normal cells utilized nearly 1.7-fold more {1-¹⁴C} glucose along the oxidative arm of the pentose cycle than altered cells, regardless of whether pyruvate is present or absent. This finding highlights an additional difference in glucose metabolism between NPC and SPC. Interestingly, the oxidation of {1-¹⁴C} glucose was increased about 1.8-fold in both cell types when pyruvate was removed from the culture medium.

Enzyme Activities

Measurements of PFK activity were performed in NPC and SPC to determine if the differences in glucose utilization, noted above, were related to different levels of this key regulatory enzyme. LDH was also assayed, since increases in its activity have accompanied elevations in aerobic glycolysis in several types of neoplastically transformed cells (Bissell et al., 1971; Singh et al., 1975).

PFK activities from NPC and SPC were similar and not influenced by the removal of pyruvate from the medium

Table 9

Oxidation of {1-¹⁴C} Glucose and {6-¹⁴C} Glucose
by NPC and SPC in the Presence and Absence
of Added Pyruvate

Culture ^b	Substrate Oxidized ^a		C1/C6 ^c
	{1- ¹⁴ C} Glucose	{6- ¹⁴ C} Glucose	
NPC + Py	640 ± 210	70 ± 6	9.1
NPC - Py	1190 ± 320	70 ± 10	17.0
SPC + Py	380 ± 150	56 ± 9	6.8
SPC - Py	680 ± 20	60 ± 10	11.3

^aValues are expressed as pmol of substrate oxidized to ¹⁴CO₂ per hr per µg culture DNA. Each value represents the mean ± S.E. of 3-4 determinations.

^bFour to six week old cell cultures, maintained in complete growth medium, were preincubated 24 hr in growth medium containing 0.5 mM glucose + 10% dialyzed serum and supplemented with or without 2 mM pyruvate. Cultures were then pulsed for 1 hr in 1 ml of fresh preincubation medium containing the ¹⁴C-glucose noted above (2 µCi/ml, 0.5 mM).

^cRatio of ¹⁴CO₂ oxidized from {1-¹⁴C} glucose to {6-¹⁴C} glucose.

(Table 10). LDH activity was greater in NPC than SPC under each culture condition, and the activities of this enzyme were little affected by the removal of pyruvate. Only with respect to a higher level of LDH in the NPC as compared to SPC was there a direct correlation with higher lactate production in normal cells (Figure 6B).

Incorporation of Metabolic Substrates into Cellular Fractions

The incorporation of several key metabolites into subcellular fractions as well as specific cellular and medium components was next examined in the NPC and SPC under selecting conditions. This was undertaken to determine if the utilization of particular substrates differed between NPC and SPC when extracellular pyruvate was limiting. These data are presented in Table 11.

A marked difference between NPC and SPC was observed in the degree to which {2-¹⁴C} pyruvate was incorporated into all of the subcellular fractions and components. A 2.7 to 3.5-fold higher incorporation of ¹⁴C from pyruvate was found in several specific metabolites (lactate, glutamate, alanine) isolated from the acid-soluble fraction of the normal cells. This was paralleled by higher radioactivity (2.7 to 3.5 fold) in the macromolecular fractions from these cells. The normal cells also released four times more {¹⁴C} lactate into the medium. This increased utilization of {2-¹⁴C} pyruvate in NPC was accompanied by an 8-fold greater consumption (18.2 nmols/hr/ugDNA) of pyruvate from the medium compared to SPC (2.2 nmols/hr/ugDNA), as determined enzymatically using lactate dehydrogenase (not shown in table). Also, preliminary

Table 10

Activities of Phosphofructokinase and Lactate
Dehydrogenase from NPC and SPC Cultured
in the Presence and Absence
of Pyruvate

Culture ^a	PFK (milliunits/10 ⁶ cells)	LDH ^b (units/10 ⁶ cells) (x10 ³)
NPC + Py	28.9	398.7
NPC - Py	23.7	342.4
SPC + Py	33.9	195.7
SPC - Py	32.8	250.5

^aNPC and SPC (3 each) were preincubated for 24 hr in growth medium supplemented with or without pyruvate (2 mM) and 10% dialyzed FBS. Cells were scraped off and combined into one extract, which was assayed for PFK and LDH as described in Materials and Methods.

^bThe data represent the results obtained from a typical experiment.

Table 11

Incorporation of Pyruvate, Glucose, Glutamine and Alanine
Into Various Cellular Fractions
in the Absence of Pyruvate

Fraction	Substrate Utilized ^a					
	{2- ¹⁴ C} Pyruvate		{ ¹⁴ C(U)} Glucose		{ ¹⁴ C(U)} Glutamine	
	NPC	SPC	NPC	SPC	NPC	SPC
Total Acid Soluble	4.60	1.50	21.70	8.90	19.30	12.70
Lactate	2.24	0.76	2.20	0.60		
Glutamate	0.37	0.08			1.40	1.30
Aspartate			0.50	0.50		
Alanine	0.41	0.08				
Macromolecules						
Lipids	0.28	0.08	5.00	4.00	0.25	0.50
Protein	0.75	0.28	10.40	5.80	1.10	2.15
Nucleic Acids	0.08	0.03	2.60	2.30	0.10	0.20
Medium Lactate	31.60	7.80	194.40	31.20	11.50	9.20
					1.98	2.58

^aData are given as nmol ¹⁴C incorporated per 3 hr per μ g DNA. For each substrate examined, NPC and SPC (3 each) were preincubated for 24 hr in growth medium minus pyruvate but containing 10% dialyzed FBS and 0.5 mM levels of the substrate to be tested. These cultures were then pulsed for 3 hr in fresh preincubation medium containing the ¹⁴C-substrate (2-4 μ Ci/ml, 0.5 mM). After labeling, cell cultures were scraped off, combined, and the fractions isolated as described in Materials and Methods.

studies showed that pyruvate was incorporated at higher levels (2-fold) in the acid-soluble and protein fractions of NPC even in the standard pyruvate-supplemented medium.

Significant differences between NPC and SPC were also observed when the incorporation of three other metabolites was examined (Table 11). $\{^{14}\text{C}(\text{U})\}$ glucose was incorporated more than 2-fold higher in the acid-soluble and cellular lactate fractions of NPC. A 6-fold greater level of medium $\{^{14}\text{C}\}$ lactate was produced from $\{^{14}\text{C}(\text{U})\}$ glucose in NPC as compared to SPC, indicating that normal tracheal cells have a great capacity for glycolysis and the endogenous production of pyruvate. Similar levels of $\{^{14}\text{C}(\text{U})\}$ glucose were incorporated into most of the macromolecular fractions of normal and altered cells except for protein, which had a 1.8-fold higher level in normal cells.

The major patterns of glutamine and alanine utilization appeared to be similar in both types of cultures. In normal cells, the incorporation of ^{14}C from each amino acid into the acid-soluble fraction was 1.5-fold higher than that found in SPC. Glutamate was the only detectable component in the acid-soluble fraction that was labelled by $\{^{14}\text{C}(\text{U})\}$ glutamine, and this labeling was similar in both NPC and SPC. No detectable acid-soluble components were labelled by ^{14}C -alanine in either type of culture. Table 11 also indicates that the incorporation of $\{^{14}\text{C}(\text{U})\}$ glutamine and $\{^{14}\text{C}(\text{U})\}$ alanine into medium lactate was comparable in both cultures, suggesting that normal and carcinogen-altered tracheal cells may have similar capabilities of producing pyruvate

from each of these amino acids. However, the incorporation of each amino acid into macromolecules was nearly 2-fold higher in SPC.

Of the four metabolites studied in Table 11, glucose was utilized to the greatest extent for macromolecular synthesis. This suggests that glucose is a major anabolic substrate in both normal and carcinogen-altered tracheal cells.

Oxidation of Pyruvate and Glutamine

Since the above data demonstrate that NPC and SPC readily metabolize glutamine and pyruvate, the possibility that these two cell types might differ in the oxidation of either metabolite for energy was examined. {2- ^{14}C } pyruvate and { $^{14}\text{C(U)}$ } glutamine are oxidized to $^{14}\text{CO}_2$ for energy via the citric acid cycle. When the oxidation of {2- ^{14}C } pyruvate by NPC and SPC was measured, comparable levels of $^{14}\text{CO}_2$ were found in each type of cell culture (Table 12). These levels of CO_2 represent only a minor fraction of the total pyruvate utilized by both cultures, as assessed from the amount of pyruvate incorporated into other components (Table 11). This suggests that the requirement for exogenous pyruvate in normal cell cultures is not due to a greater reliance on pyruvate for energy via the citric acid cycle.

The oxidation of glutamine was also equivalent in NPC and SPC. However, when glucose in the medium was reduced, a marked increase (10-fold) in glutamine oxidation occurred in both cell types (Table 12). Although the details are not given here, this increased glutamine oxidation was accompanied by a 3 to 5-fold

Table 12

Oxidation of Glutamine and Pyruvate by NPC
and SPC in the Absence of Pyruvate^a

Substrate	Culture	
	NPC	SPC
{2- ¹⁴ C} Pyruvate ^b	0.44 (± 0.21)	0.29 (± 0.04)
{ ¹⁴ C(U)} Glutamine	1.05 (± 0.50)	1.00 (± 0.30)
{ ¹⁴ C(U)} Glutamine + Low Glucose ^c	10.16 (± 2.10)	11.83 (± 2.47)

^aData are expressed as nmol ¹⁴C incorporated into ¹⁴CO₂ per 3 hr. per µg DNA.

^bNPC and SPC (3-4 cultures each) were preincubated for 24 hr in growth medium lacking pyruvate but containing 10% dialyzed FBS and 0.5 mM substrate. The cultures were then placed in collection jars and pulsed for 3 hr in 1 ml of fresh preincubation medium containing 0.5 mM ¹⁴C-substrate. After labelling, ¹⁴CO₂ was collected and the cultures were further processed as described in Materials and Methods.

^cSame as (b) above, except that glucose levels were reduced from 27.7 mM to 0.2 mM during preincubation and the 3 hr incorporation.

increase in the incorporation of glutamine carbon into the lipid, protein and nucleic acid fractions of NPC and SPC. Thus, normal and carcinogen-altered tracheal cells appear to have similar capacities to utilize glutamine as an energy and anabolic substrate.

Discussion

The results reported here clearly demonstrate that normal rat tracheal epithelial cells metabolize significantly greater levels of exogenous pyruvate than carcinogen-altered tracheal cells. This major metabolic difference supports our earlier studies which indicate that normal cells require pyruvate for survival and growth, whereas carcinogen-altered cells do not (Rice & Marchok, 1980). Exogenous pyruvate was incorporated into the same components in NPC and SPC, although generally to a much greater extent in all the fractions of normal cells. The one exception was in the oxidation of pyruvate, which was similar in NPC and SPC. These findings suggest that normal cells do not preferentially rely on exogenous pyruvate as a major energy substrate via the citric acid cycle. The data in Chapter II showed that pyruvate is stringently required for normal cell growth. Additional metabolites including other 2-oxocarboxylic acids or citric acid cycle intermediates did not substitute for pyruvate in maintaining viable cultures of normal cells. Since exogenous pyruvate was so readily incorporated into many sub-cellular fractions and components of tracheal cells, it may

function as a key anaplerotic substrate that is utilized in major anabolic pathways.

NPC consumed more glucose, produced more lactate and incorporated greater levels of glucose into lactate than SPC, thus suggesting that normal cells are more glycolytic than carcinogen-altered cells from earlier stages in neoplastic progression. The basis for this dissimilarity in glucose metabolism was unrelated to PFK levels, since NPC and SPC had comparable amounts of this enzyme. Of particular interest, however, is the fact that normal cells still require exogenous pyruvate for survival and growth regardless of their great capacity for deriving pyruvate from glucose.

Neither type of tracheal cell generated much oxidative energy from glucose via the citric acid cycle, a finding that has been observed in other types of tissue culture cells (Reitzer et al., 1980; McKeehan, 1982). Instead, glucose functioned as a major anabolic substrate in both NPC and SPC. Compared to SPC, a greater reliance on glucose as a carbon source for protein synthesis and as a substrate in the oxidative segment of the pentose cycle was observed in normal cells. The flux of glucose in the oxidative arm of the pentose cycle was actually enhanced in both NPC and SPC upon the removal of pyruvate. This may have been an adaptive response by tracheal cells to maintain a critical level of pentose precursors. Such a response would imply that pyruvate serves as a regulator of, or precursor for, some segment of this cycle. There have been several reports of pyruvate modulating pentose

cycle activity, although the effects have largely been stimulatory and partially attributed to pyruvate acting as an electron-sink for the oxidation of NADPH (Wenner, 1959; Gumaa & Mclean, 1969). The nature of the pyruvate effect on the pentose cycle noted in tracheal cells warrants further study.

Other metabolites examined in this study were glutamine and alanine. Both of these amino acids are potential precursors of pyruvate and their utilization for the biosynthesis of endogenous pyruvate could have been substantially increased in SPC under selecting conditions. However, the utilization of glutamine and alanine was generally quite similar in NPC and SPC, except that both amino acids were incorporated to a somewhat higher extent into the macromolecular fractions of SPC. This may have been due to an increased synthesis of macromolecules that is likely to occur in the more actively proliferating SPC (see Chapter III, Table 6, p.48). Alanine is readily converted into pyruvate by transamination, whereas glutamine can be metabolized into pyruvate and other glycolytic intermediates through a linear oxidative pathway that utilizes part of the citric acid cycle (Sauer et al., 1980; McKeehan, 1982a). This pathway of glutamine metabolism, referred to as glutaminolysis (McKeehan, 1982a), is quite common in tissue culture cells which rely on glutamine as an anabolic and energy substrate (McKeehan, 1982a). Since much of the alanine and glutamine utilized by NPC and SPC were incorporated to a similar extent into lactate, it appears that transamination and glutaminolysis are quite common in primary cultures of normal and carcinogen-

altered rat tracheal epithelial cells as well. These results strongly suggest that NPC and SPC have similar capacities for deriving pyruvate from glutamine and alanine.

It is very significant that comparable levels of glutamine were oxidized in NPC and SPC. This suggests that the utilization of glutamine for energy is similar in both types of cell populations. Glutamine is a major energy substrate in several types of tissue culture cells, particularly when glucose levels are low (Zielke et al., 1978; McKeehan, 1982a). Under these conditions, marked increases in the utilization of glutamine were also observed in both NPC and SPC.

A most interesting finding in this study was that SPC had lower levels of glycolysis than NPC. Since increases in glycolysis are often seen in tumors and malignantly transformed cell lines (Eigenbrodt & Glossman, 1980), the lower glycolysis observed in the preneoplastic SPC indicates that marked increases in glycolysis are not a fundamental and early metabolic event in the development of neoplasia in rat tracheal epithelial cells. In support of this view are data in Chapter III which demonstrate that some rat tracheal tumor cells, mostly from fast growing, undifferentiated tumors, have significantly greater levels of hexose uptake than normal cells, whereas cells from earlier stages of neoplasia and some tumors have less uptake (see Chapter III, Table 6, p. 48). Therefore, it appears that marked elevations in glucose utilization may occur in some carcinogen-altered tracheal cells only at late stages in the progression of neoplasia.

In light of the apparent ability of NPC to make pyruvate from at least three potential pyruvate precursors (glucose, alanine, and glutamine), it is important to ask the question: Why do normal cells so stringently require exogenous pyruvate for growth? One possible explanation is that exogenous pyruvate limits the loss of intracellular pyruvate pools. This was originally suggested by Eagle and Piez (1962) as the mechanism by which exogenous pyruvate enhanced the growth of low density cell cultures. However, preliminary experiments in this laboratory showed no difference in levels of effluxed pyruvate in NPC and SPC. A second explanation might involve differences in the compartmentation of exogenous pyruvate and metabolically-derived pyruvate. Utilization of the latter may be restricted to reactions that are unessential for growth and in the immediate vicinity of the site of pyruvate production. This would be even more pronounced if the neighboring enzymes for these reactions had high affinities for pyruvate as substrate. In contrast, exogenous pyruvate may have greater access to reactions that are rate-limiting for cell proliferation, especially if those reactions are intramitochondrial. In the present study, exogenous pyruvate labelled several acid-soluble components that were not labelled to a detectable degree by pyruvate derived from the other three substrates. This implies that the metabolic fate of exogenous pyruvate may differ from that of metabolically-derived pyruvate, perhaps due to differences in compartmentation.

The results of this study allow several metabolic events to be dismissed as possible mechanisms responsible for the growth

advantage that is acquired by carcinogen-altered tracheal cells early in the progression of neoplasia. First, SPC do not achieve a growth advantage over NPC through elevations in aerobic glycolysis, or by a more efficient utilization of glucose in either the pentose cycle or citric acid cycle. These parameters were actually lower in SPC as compared to NPC under selecting conditions. Second, shifts in the major pattern of pyruvate, glucose, glutamine or alanine utilization appear to be unrelated to the altered growth control of SPC. This is suggested by the observation that each of these substrates was incorporated into the same detectable fractions and components in NPC and SPC. The possibility cannot be excluded, however, that an undetectable difference between NPC and SPC occurs in some minor pathway of metabolism for either of these substrates. Third, a greater degree of glutaminolysis in SPC can be dismissed as a mechanism since comparable levels of glutamine oxidation were observed in both types of cells, even under restricted glucose conditions.

Additional research is clearly needed to identify the metabolic change underlying the reduced requirement for exogenous pyruvate in carcinogen-altered tracheal cells. Several possibilities could be examined. 1) An increased utilization by SPC of other substrates, such as fatty acids or glycogen, should be studied since these could be pyruvate precursors whose metabolism is altered in transformed cells. 2) Differences in the capacity of NPC and SPC to maintain a redox state favorable for growth should be considered since McKeehan and McKeehan (1979, 1980) have suggested that the

metabolism of 2-oxocarboxylic acids, such as pyruvic acid, could affect NAD:NADH ratios through a mechanism involving pyridine nucleotide-linked oxidoreductases and serum factors. 3) Differences may occur between NPC and SPC in the compartmentation of pyruvate or of pyruvate-metabolizing enzymes that are essential for growth. Suggestive evidence for such differences is found in a recent study by Paradies et al. (1983), which showed that mitochondria from several Morris hepatomas and Ehrlich ascites tumor cells have lower capacities for transporting pyruvate than normal rat liver mitochondria. This suggests that malignant cell mitochondria may be less dependent upon cytoplasmically-derived pyruvate than normal cell mitochondria, perhaps due to an increased utilization of alternate metabolites or to an enhanced biosynthesis of intramitochondrial pyruvate. These possibilities may be relevant to the decreased requirement for exogenous pyruvate in carcinogen-altered tracheal cells, and should be investigated in the future.

CHAPTER V

MALIC ENZYME AND MALATE DEHYDROGENASE ACTIVITIES

IN RAT TRACHEAL EPITHELIAL CELLS DURING

THE PROGRESSION OF NEOPLASIA

Introduction

In recent years, an enhanced mitochondrial capacity to produce pyruvate from malate has been demonstrated in several types of proliferating normal and neoplastically transformed cells (Nagel et al., 1980; Sauer et al., 1980). The activity of a unique NAD(P)-dependent isozyme of malic enzyme appears to be associated with this production of pyruvate. Unlike the strictly NADP-dependent isozymes that are known to occur in the cytoplasm and mitochondria (Sauer & Dauchy, 1978), the NAD(P)-linked enzyme catalyzes the irreversible decarboxylation of malate to pyruvate using either NAD^+ or NADP^+ as coenzyme (Sauer & Dauchy, 1978).

The metabolic significance of the NAD(P)-linked malic enzyme activity in proliferating cells is largely unknown. Several studies have provided evidence which suggests that the NAD(P)-dependent activity may be associated with the efficient utilization of glutamine as an energy substrate (Sauer et al., 1980; McKeehan & McKeehan, 1982). One recent study has also shown that the activities of mitochondrial malic enzymes are progression-linked in Morris hepatomas (Sauer et al., 1980).

In Chapter IV, the utilization of several key metabolites, including pyruvate, was found to be qualitatively similar in normal

and carcinogen-altered cells. For this reason, it was speculated that these two cell types might differ in the compartmentation of pyruvate or pyruvate-metabolizing enzymes. Since mitochondrial malic enzyme activity is associated with pyruvate formation and may be progression-linked, differences in the level and compartmentation of this enzyme could be related to the dissimilar requirements for exogenous pyruvate between normal and carcinogen-altered tracheal cells. To examine this possibility, NAD and NADP-dependent malic enzyme activities were measured in normal, selected and tumor-derived tracheal cell cultures maintained with or without exogenous pyruvate. Malate dehydrogenase, a known competitor of malic enzyme for malate, was also assayed, since recent studies suggest that MDH activity might affect the net oxidation of malate to pyruvate in mitochondria (McKeehan & McKeehan, 1982). The activity of MDH is also crucial to the transport of reducing equivalents between the cytoplasm and mitochondria via the malate-aspartate shuttle (Pedersen, 1978). A new and sensitive radiometric technique (McKeehan & McKeehan, 1982) was used to assay these enzyme activities in the small-sized NPC and SPC.

Materials and Methods

Materials

L-{4-¹⁴C} Aspartic acid (55 mCi/mmol) and L-{1-¹⁴C} alanine (52 mCi/mmol) were from Amersham, Arlington Heights, Illinois. Sephadex G-25 was from Pharmacia, Piscataway, New Jersey. All

substrates, coenzymes and enzymes were obtained from Sigma, St. Louis, Missouri.

Culture Conditions and Establishment of Primary Cell Cultures

These procedures were performed essentially as described in Chapters II and III. TPC-1 and TPC-2 were generated from pieces of two different carcinomas which formed in nude mice after inoculation of subcultured cells derived from DMBA-preexposed implants (Marchok et al., 1983).

Digitonin Fractionation of Cell Cultures

Several primary cell cultures (3-5) were scraped from the dish, combined and fractionated with digitonin into particulate and soluble fractions according to methods described in Chapter III for PFK and LDH assay. All fractions were immediately stored at -70°C until assay. Digitonin is a detergent that interacts specifically with β -hydroxysterols, and it permeabilizes the plasma and outer mitochondrial membranes while leaving the inner mitochondrial membrane intact (Mackall et al., 1979). Treatment with digitonin is a convenient and rapid method for releasing cytoplasmic enzymes from cells (Mackall et al., 1979). Digitonin was purified according to Janski and Cornell (1980) to increase its solubility.

For the determination of malic enzyme activity, crude particulate and soluble cell fractions were passed through a G-25 column equilibrated with a buffer containing 20 mM Hepes (pH 7.2), 2 mM EDTA, 0.2 mM dithiothreitol, 50 mM KCl and 0.02% NaN_3 . This was

necessary to remove endogenous substrates which may interfere with the enzyme assays described below (pyruvate, α -ketoglutarate, etc.).

LDH and CS were radiometrically assayed as markers for the soluble and mitochondria in the particulate fractions, respectively. Details of these assays are given below. In typical experiments, > 95% of the total LDH activity was localized in the soluble fraction and > 85% of the total citrate synthase activity was in the particulate fraction. The distribution of the other enzyme activities between soluble and particulate fractions was calculated as described by Janski and Cornell (1980) using the distribution of LDH and CS.

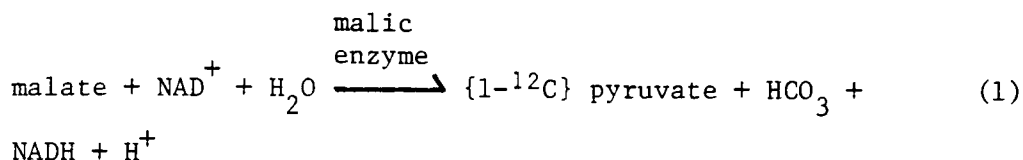
Protein in the fractions was determined using Coomassie Blue with a Bio-Rad protein assay kit (Bio-Rad, Richmond, California). Details for the determination of cell numbers per culture are given in the Methods section of Chapters II and III.

Radiometric Enzyme Assays

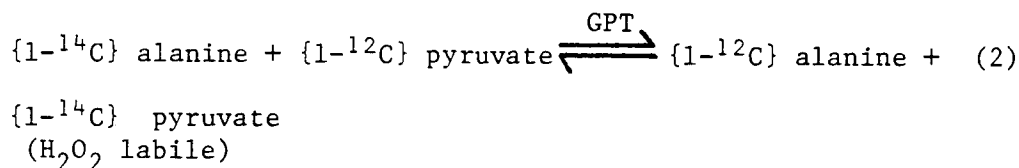
All cell cultures were preincubated for 48 hrs. before enzyme assay in growth medium + 10% dialyzed FBS with or without pyruvate (2 mM). Media were changed daily. The theory and details of the enzyme assays used in this study have been recently described by McKeehan and McKeehan (1981, 1982). All assays were carried out in uncapped 4 ml polypropylene scintillation vials (Wheaton Omni Vials) at 37°C in a fume hood. Maximum reaction volumes were 100 μ l.

The radiometric NAD and NADP-dependent malic enzyme assay is based on the following set of reactions (adapted from McKeehan and

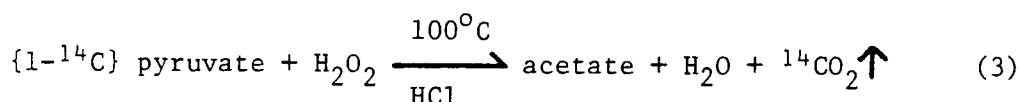
McKeehan, 1982). In reaction (1), malic enzyme catalyzes the formation of pyruvate from malate:



The $\{1\text{-}^{12}\text{C}\}$ pyruvate then undergoes an alanine aminotransferase catalyzed isotope exchange with $\{1\text{-}^{14}\text{C}\}$ alanine. $\{1\text{-}^{14}\text{C}\}$ alanine is H_2O_2 stable (Nisonoff et al., 1954).



The $\{1\text{-}^{14}\text{C}\}$ pyruvate formed from the exchange with $\{1\text{-}^{14}\text{C}\}$ alanine is decarboxylated by H_2O_2 .

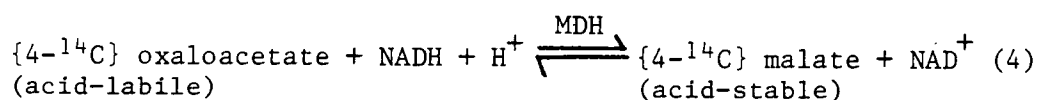


Thus, the quantity of $\{1\text{-}^{12}\text{C}\}$ pyruvate formed from malate via malic enzyme can be easily calculated from the amount of $\{1\text{-}^{14}\text{C}\}$ alanine that is lost from the vials as a consequence of its conversion to $\{1\text{-}^{14}\text{C}\}$ pyruvate and subsequent decarboxylation at ${}^{14}\text{CO}_2$.

Assay mixtures (100 μl) for malic enzyme activity contained 100 mM tris·HCl (pH 7.6), 5 mM MnCl_2 , 0.2 mM dithiothreitol, 20 mM malate, 2 IU per ml of alanine aminotransferase (GPT) Sigma (G9880),

50 μM $\{1\text{-}^{14}\text{C}\}$ alanine (0.4 μCi per ml), 1 mM NAD^+ or NADP^+ and the cell extract. After the allotted time of incubation, reactions were terminated by placing the vials in a boiling water bath for 10 min. A solution (100 μl) of freshly prepared 2 M H_2O_2 and 1 M HCl was then added, and the vials were boiled for an additional 10 min to decarboxylate $\{1\text{-}^{14}\text{C}\}$ pyruvate. The vials were then cooled, filled with Aquasol and counted for residual $\{1\text{-}^{14}\text{C}\}$ alanine left in the vial. Background activity in the absence of MnCl_2 was subtracted from the complete assay system. The amount of $\{1\text{-}^{14}\text{C}\}$ alanine that exchanged to $\{1\text{-}^{14}\text{C}\}$ pyruvate was converted to pmoles $\{^{12}\text{C}\}$ pyruvate produced from malate using a standard curve of pmoles alanine exchanged versus pmoles pyruvate added to the system (McKeehan & McKeehan, 1982). In this assay system, the conversion of $\{1\text{-}^{14}\text{C}\}$ alanine to $\{1\text{-}^{14}\text{C}\}$ pyruvate was proportional to the amount of $\{1\text{-}^{12}\text{C}\}$ pyruvate up to 1000 pmoles.

For determination of malate dehydrogenase activity, the conversion of acid-labile $\{4\text{-}^{14}\text{C}\}$ oxaloacetate to acid stable $\{4\text{-}^{14}\text{C}\}$ malate was calculated.



Commercially prepared $\{4\text{-}^{14}\text{C}\}$ oxaloacetate is not available, therefore, $\{4\text{-}^{14}\text{C}\}$ oxaloacetate was generated from $\{4\text{-}^{14}\text{C}\}$ aspartate in an incubation mixture (50 μl) consisting of 200 mM Tris-HCl (pH 7.6), 2 mM EDTA, 4 mM 2-oxoglutarate, 4 IU per ml aspartate aminotransferase Sigma (G2751) (GOT) and 20 mM $\{4\text{-}^{14}\text{C}\}$ aspartate (0.4 μCi per

ml). Incubation time was 15 min. After incubation, cell fractions and 0.2 mM NADH were added to a final volume of 100 μ l, and incubation was extended for 15 min. Reactions were terminated by adding 100 μ l of 2N HCl to each vial. Vials were then placed in a boiling water bath for 30 min. to decarboxylate unreacted {4- ^{14} C} oxaloacetate. After cooling, Aquasol was added and the vials were counted for {4- ^{14} C} malate remaining after acid treatment. Blank vials consisting of the reaction mixture minus NADH were subtracted from all values.

Details of the LDH assay are given in Chapter III (Methods). CS was assayed by substitution of 0.33 mM acetyl CoA for the NADH in the above MDH assay mixture, and the {5- ^{14} C} citrate formed from {4- ^{14} C} oxaloacetate was measured in the same manner as {4- ^{14} C} malate (McKeehan & McKeehan, 1982).

Concentrations of cell extracts were chosen to maintain a linear relationship between reaction rate and cell extract concentration. A unit of malic enzyme activity is defined as that amount of enzyme which catalyzes the production of 1 pmol pyruvate per minute. A unit of MDH is defined as that amount of enzyme which stabilizes 1 pmol oxalacetate per minute. Enzyme activity units per 10^6 cells (specific activity) were obtained from the slope of the linear response curve of activity units versus amount of cell fraction. Enzyme activities at 3 concentrations of cell fraction protein were generally used to determine the slope.

Results

NADP-Linked Malic Enzyme Activity

The NADP-linked catalyzation of pyruvate formation by the particulate and soluble fractions from normal, selected and tumor cells cultured in the presence and absence of pyruvate is shown in Figure 7. It can be seen that pyruvate formation was linear with time in all the fractions. The most prominent difference between NPC and all the carcinogen-altered cells cultured with or without pyruvate, was the low NADP-linked activity in the particulate fraction of the normal cells. Very high activity was found in the particulate fraction from SPC, and it was reduced somewhat by the removal of pyruvate from the medium. The two tumor cell cultures (TPC-1, TPC-2) also had higher particulate activity than NPC, and in TPC-1, this activity was markedly increased by the withdrawal of pyruvate. The NADP-linked malic enzyme activities in the soluble fractions from cells in all stages of carcinogenesis were much higher compared to the particulate activities. As with the particulate fraction, the activity in the soluble fraction of SPC was reduced by pyruvate withdrawal.

Comparison of NADP- and NAD-Dependent Malic Enzyme Activity

The units of malic enzyme activity using either NAD or NADP as coenzyme were assayed in these cells after 30 min of incubation to compare the levels of NADP- and NAD-dependent malic enzyme activity in the normal, selected and tumor-derived tracheal cells. As shown in Table 13, the results again demonstrate a very low

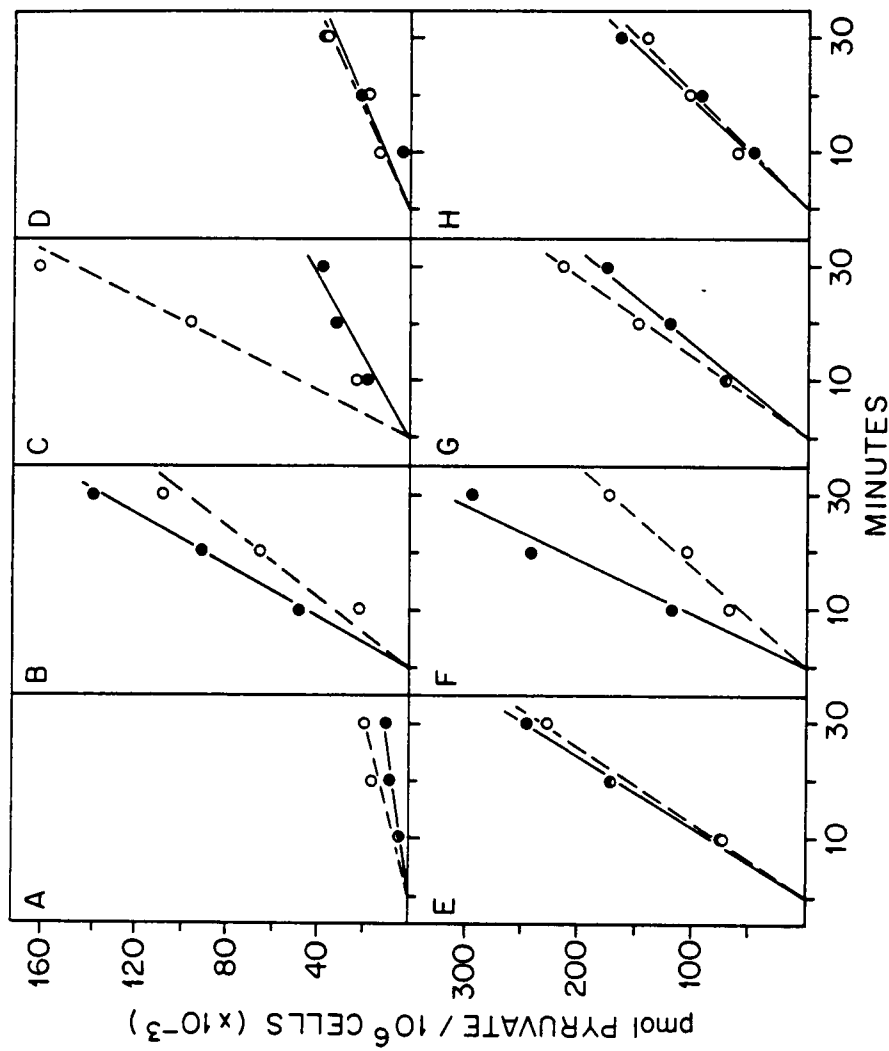


Figure 7. NADP-dependent malic enzyme activity in particulate and soluble fractions of normal, selected and tumor-derived rat tracheal epithelial cells. Primary cultures of normal (A,E), selected (B,F), TPC-1 (C and G) and TPC-2 (D and H) cells were cultured for 48 hr in growth medium supplemented with (o---o)pyruvate. Cultures were then fractionated into particulate (A-D) and soluble fractions (E-H). Malic enzyme was assayed in these fractions by isotope exchange as described in Materials and Methods. 2.5 μ g of protein from each fraction was used in the assay.

Table 13

Comparison of NADP- and NAD-Dependent Malic
Enzyme Activity in Normal, Selected
and Tumor-Derived Tracheal Cells

Cell Culture ^a	Medium Pyruvate	Malic Enzyme (Units per 10 ⁶ cells X 10 ²)			
		NADP			NAD
		Pt ^b	Sol ^b	% Total in Pt	Pt ^c
NPC	(+)	7.6	78.0	8.8	24.8
SPC		54.2	72.4	42.2	26.6
TPC-1		13.1	40.7	24.3	28.8
TPC-2		13.8	42.5	24.5	42.5
NPC	(-)	4.7	76.9	5.7	16.2
SPC		32.8	45.0	42.2	42.3
TPC-1		45.4	51.6	46.8	29.2
TPC-2		12.0	60.1	16.6	39.7

^aCell cultures were preincubated for 48 hr in growth medium with or without added pyruvate (2 mM) + 10% dialyzed FBS. were then scraped and combined into a single extract which was fractionated and assayed as described in Materials and Methods. Reaction time was 30 min and specific activity was determined as described (Materials and Methods).

^bPt, particulate fraction; Sol, soluble fraction.

^cNo soluble activity.

NADP-dependent activity in the particulate fraction of normal cells. SPC cultured with or without pyruvate had a 7-fold higher particulate NADP-dependent activity than NPC, despite the reduction in this activity that was again observed in both fractions upon the withdrawal of pyruvate. Particulate fractions from both tumor cell cultures were almost 2X higher in NADP-dependent activity than NPC. Again, this activity was markedly stimulated in TPC-1 when pyruvate was withdrawn. No distinct trend towards a higher soluble activity in one type of cell compared to the others was observed. Compared to normal cells, a greater percentage of total cellular NADP-dependent activity was localized in the particulate fractions of selected and tumor-derived cells.

NAD-dependent malic enzyme activity was detected exclusively in the particulate fractions of all the cells (Table 13). Distinctly different levels of NAD-dependent activity were only observed in the presence of pyruvate in TPC-2, which had an approximately 2-fold greater activity than all the other cell types. SPC again responded to the withdrawal of pyruvate, in this case with a 1.6-fold increase in NAD-dependent activity. Removal of pyruvate led to a 1.5-fold reduction of NAD-dependent activity in NPC, whereas activity remained high in TPC-2.

Malate Dehydrogenase Activity

Malate dehydrogenase (MDH), a known competitor of malic enzyme for malate, was also assayed to determine if its activity was progression-linked in carcinogen-altered tracheal cells. Compared

to malic enzyme, the activity of MDH in the particulate and soluble fractions of all cell types was markedly higher (Table 14). Both tumor cultures had distinctly lower levels of particulate MDH as compared to NPC or SPC: the latter two cell types had similar levels of activity. When pyruvate was withdrawn, particulate levels of MDH in SPC and TPC-1 were increased. In contrast, soluble levels of MDH were generally similar among all cell types, regardless of whether pyruvate was present or absent. The one exception was TPC-1, which had a 2-fold increase in soluble activity after the withdrawal of pyruvate.

Discussion

The major finding in this study is that selected and tumor-derived rat tracheal epithelial cells have markedly higher levels of particulate NADP-dependent malic enzyme activity than normal tracheal cells. This was observed with or without pyruvate in the medium. In contrast, soluble NADP-dependent activity was high in all cell cultures, and appeared to be unrelated to neoplastic progression. Other studies have shown that NAD(P)-dependent malic enzyme activity may be progression-linked in certain tumors. Since many SPC are preneoplastic (Marchok, 1984; Marchok et al., 1984), this study is the first to suggest that an induction of mitochondrial malic enzyme may be a fundamental metabolic event that occurs at an early stage in the progression of neoplasia.

High mitochondrial levels of NAD(P)-dependent malic enzyme activity have been associated with active rates of cell proliferation in both normal and tumor cell populations (Sauer et al., 1980;

Table 14

Malate Dehydrogenase Activity in Normal,
Selected and Tumor-Derived
Tracheal Cells

Cell Culture ^a	Medium Pyruvate	MDH (Units per 10 ⁶ Cells X 10 ³)	
		Pt ^b	Sol ^b
NPC	(+)	389	220
SPC		325	179
TPC-1		98	153
TPC-2		92	175
NPC	(-)	316	269
SPC		600	184
TPC-1		270	309
TPC-2		69	199

^a Aliquots from cultures used in Table 13 were assayed for MDH as described in Materials and Methods. Reaction time was 15 min.

^b Pt, particulate fraction; Sol, soluble fraction.

Nagel et al., 1980). Nagel et al. (1980) have observed significant levels of NAD(P) and NADP-dependent malic enzyme in rat lung tissue, a finding which was attributed to high rates of cell renewal in the lung. This conclusion was based on early morphological studies which overestimated the mitotic activity of respiratory tract tissue (Bertalanffy & LeBlond, 1953). However, recent studies clearly demonstrate that nontraumatized respiratory tract tissues have low mitotic activities (Kauffman, 1980). In support of this view are studies which show that normal rat tracheal epithelial cells have lower in vitro growth capacities (Marchok et al., 1977) and levels of DNA synthesis (see Chapter III, Table 6, p. 48) than carcinogen-altered tracheal cells. These findings, considered along with the lower particulate NADP-dependent activity observed in NPC, suggest that an association between cell proliferation and mitochondrial malic enzyme activity exists in tracheal epithelial cells. Thus, the differences between normal and carcinogen-altered tracheal cells in the activity of particulate malic enzyme could be due to dissimilar proliferation rates rather than the progression of tumorigenicity.

A most interesting finding in this study was that activity of MDH in the particulate fractions of both types of tumor cells was distinctly lower than that of NPC or SPC. This suggests that a decrease in mitochondrial MDH may be a progression-related change occurring at a late stage in neoplasia. However, Balinsky et al. (1984) recently reported MDH activity that was higher than normal

levels in the mitochondria of human lung tumors. The basis for these apparently contradictory findings is unknown, although they may be due to differences between species. McKeehan and McKeehan (1982) have recently compared malic enzyme and MDH activities in stationary and proliferating human fibroblasts. They suggest that a decrease in MDH activity relative to malic enzyme activity favors the production of pyruvate from malate in the proliferating fibroblasts (McKeehan & McKeehan, 1982). It is possible that coordinate changes such as these might occur in carcinogen-altered tracheal cells, and thus reduce their requirement for exogenous pyruvate. However, in this study markedly higher activities of MDH compared to malic enzyme were found in both fractions of all the cells, regardless of the presence or absence of exogenous pyruvate. In fact, MDH levels actually increased in SPC and TPC-1 upon the withdrawal of pyruvate. It appears, therefore, that coordinate shifts in the activity of MDH relative to malic enzyme do not occur in carcinogen-altered rat tracheal epithelial cells when exogenous pyruvate is absent.

It is significant to note that malic enzyme activity was detectable in the particulate fractions of these cells using either NADP or NAD as coenzyme. This suggests that an NAD(P)-dependent isozyme is present in the mitochondria of both normal and carcinogen-altered tracheal cells. It cannot be distinguished, however, whether the observed particulate NADP-dependent activities were due to a single NAD(P)-dependent isozyme acting alone or in concert with a strictly NADP-dependent enzyme. Also of particular interest was the apparent ability of exogenous pyruvate to modulate

the activity of NADP-dependent malic enzyme and MDH in SPC and TPC-1. Such modulations may represent adaptive responses by these carcinogen-altered cells to compensate for the lack of exogenous pyruvate. In contrast, the adaptive capacity of normal cells may not be as great, since the activities of these two enzymes in NPC were hardly affected by exogenous pyruvate.

The relationship between elevated levels of particulate NADP-dependent malic enzyme activity and the growth advantage by carcinogen-altered tracheal cells is unclear at the present time. An increased capacity to oxidize mitochondrial malate to pyruvate and a reduced requirement for exogenous pyruvate are likely to be more than a mere coincidence in these carcinogen-altered tracheal cells. Several studies suggest that NAD(P)-dependent malic enzyme activity is associated with the efficient metabolism of glutamine for energy (glutaminolysis) (McKeehan et al., 1982; Sauer et al., 1982). However, the data in Chapter IV clearly indicate that glutaminolysis is similar in normal and carcinogen-altered tracheal cells. These results strongly suggest that the high intramitochondrial NADP-dependent malic enzyme activity in carcinogen-altered tracheal cells may be involved in other important metabolic events besides glutamine metabolism. Several possibilities that could be examined include: 1) a generation of NADPH for anabolic reactions; 2) the production of intramitochondrial pyruvate for the maintenance of an active citric acid cycle during the oxidation of alternate energy substrates, such as glutamate or cytoplasmically-generated citric acid cycle intermediates.

The latter possibility was suggested by Sauer and Dauchy (1978) as a mechanism whereby tumor cells may acquire a metabolic advantage over normal cells for energy production. Such a role for mitochondrial malic enzyme may be relevant to the growth advantage by carcinogen-altered rat tracheal cells. In the preceding chapter, the lower utilization of pyruvate and glucose observed in SPC as compared to NPC, suggests that alternate substrates indeed might be utilized in carcinogen-altered tracheal cells. The metabolism of alternate substrates, such as amino acids, could traverse part of the citric acid cycle. A consequence of this might be a drawing off of critical citric acid cycle intermediates. Alternatively, an active and sustained level of cell proliferation may require the continual influx and efflux of citric acid cycle intermediates, especially if these intermediates are utilized for biosynthetic purposes. High levels of mitochondrial malic enzyme could facilitate these necessary exchanges of intermediates, and help to augment the level of intermediates when cellular demands for citric acid cycle activity are high. In fact, the reactions catalyzed by NADP-dependent malic enzyme are well recognized for their anaplerotic functions (Zubay, 1983). Clearly, additional research is needed to elucidate the role of high NADP-dependent malic enzyme activity in the progression of neoplasia in rat tracheal epithelial cells.

CHAPTER VI

SUMMARY

The purpose of this dissertation was to identify metabolic differences between normal and carcinogen-altered tracheal cells which may be related to their dissimilar in vitro growth requirements for exogenous pyruvate. The loss of requirement for pyruvate in carcinogen-altered tracheal cells was used to select primary cell cultures of altered cells from earlier stages in the progression of neoplasia. These were referred to as selected primary cell cultures. Tumor-derived primary cell cultures represented cell populations from late stages of neoplastic progression.

The effects of exogenous pyruvate on the growth and differentiation of normal tracheal cells were examined. Normal cells stringently require exogenous pyruvate in a concentration-dependent manner. This requirement could not be replaced by other 2-oxocarboxylates or citric acid cycle intermediates. Pyruvate does not directly stimulate DNA synthesis in normal cells. Instead, pyruvate indirectly promotes cell proliferation by inhibiting terminal differentiation.

The uptake of 2-deoxyglucose was measured in normal, selected and tumor-derived cells to determine if the growth advantage by carcinogen-altered cells was related to increases in hexose uptake. Generalizations cannot be made regarding tumorigenicity and hexose uptake in tracheal epithelial cells. This is partly due to the profound effects that cell associations have on hexose uptake

in tracheal cells. Carcinogen-altered tracheal cells from earlier stages of neoplasia do not achieve a growth advantage over normal cells by acquiring high rates of hexose uptake. Compared to normal cells, hexose uptake is lower in selected primary cultures as well as in some types of tumor cells. Other types of tumor cells have higher levels of hexose uptake than normal cells. These findings indicate that an increased rate of hexose uptake is an event that can occur in some types of carcinogen-altered cells primarily at a late stage in the progression of neoplasia.

A comparison of pyruvate, glucose, glutamine and alanine metabolism in normal and selected cells revealed several distinct metabolic differences between normal tracheal cells and carcinogen-altered tracheal cells from earlier stages of neoplasia. Most notable is a markedly higher metabolism of exogenous pyruvate by normal tracheal cells. This is consistent with their in vitro growth requirement for pyruvate. Pyruvate appears to be a major anaplerotic metabolite that is utilized extensively by normal cells for most anabolic purposes. The consumption of glucose and glycolytic rate in normal cells is also higher than that of selected cells. In both cell types, glucose is primarily utilized as a major anabolic substrate, and very little oxidative energy is derived from glucose metabolism. These findings indicate that exogenous pyruvate and glucose are very important to the basic metabolism of normal tracheal cells, and less so in carcinogen-altered cells.

The lower metabolism of glucose and exogenous pyruvate by selected cells strongly suggests that carcinogen-altered cells

rely on an increased utilization of alternate metabolic substrates. Since the general utilization of glutamine and alanine is similar in normal and selected cell cultures, these amino acids can be dismissed as alternate substrates whose metabolism is increased by selected cells. The oxidative metabolism of glutamine (glutaminolysis) was also similar in both cell types. A most interesting finding was that significant amounts of glutamine and alanine are metabolized to lactate in normal cells. Considered with the high glycolytic rate of normal cells, this finding indicates that the need for exogenous pyruvate in normal tracheal cells is not based upon an inability of normal cells to make endogenous pyruvate from potential precursors of pyruvate, such as glucose, alanine and glutamine.

Another major difference between normal and carcinogen-altered tracheal cells is that carcinogen-altered cells have distinctly greater intramitochondrial levels of malic enzyme. This was observed in selected and tumor-derived cells, suggesting that an induction of mitochondrial malic enzyme may be an early event in the development of neoplasia in tracheal cells. The increased levels of malic enzyme could be related to the growth advantage by carcinogen-altered cells, since this enzyme activity may facilitate the efficient utilization of alternate metabolic substrates, or catalyze key anaplerotic reactions. In contrast to malic enzymes, major changes in the level of MDH do not occur at early stages of neoplasia, suggesting that

activity may be unrelated to the decreased requirement for pyruvate in carcinogen-altered cells.

It can be concluded from this study that carcinogen-altered tracheal cells from earlier stages of neoplasia do not achieve a growth advantage over normal cells by acquiring: 1) increased rates of glycolysis; 2) a more efficient utilization of glucose for pentose precursor synthesis, or for oxidative energy; 3) major shifts or increases in the utilization of glucose, alanine, or glutamine; and 4) increased levels of glutaminolysis.

Other possibilities for future research could include:

a) measurements of the utilization of fatty acids, glycogen or other amino acids in carcinogen-altered cells to determine if these substances are utilized to a greater extent as alternate metabolic substrates; and b) measurements of the levels of citric acid cycle intermediates in both cell types in the presence and absence of exogenous pyruvate to determine if normal and carcinogen-altered cells differ in their utilization of pyruvate for the maintenance of an active citric acid cycle.

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