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INHIBITION OF *N*-DIETHYLNITROSAMINE METABOLISM BY HUMAN LUNG CANCER CELL LINES WITH FEATURES OF WELL DIFFERENTIATED PULMONARY ENDOCRINE CELLS

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Abstract—Cell lines derived from a human pulmonary carcinoid tumor (NCI-H727) and from a human pulmonary large cell carcinoma (NCI-H460) were investigated by transmission electron microscopy. Both cell lines, at early *in vitro* passage, demonstrated ultrastructural features of well differentiated pulmonary endocrine cells. Line NCI-H727 had more endoplasmic reticulum than line NCI-H460 and demonstrated L-dopa decarboxylase activity as well as production of calcitonin and bombesin. Because of their ultrastructural resemblance with normal pulmonary endocrine cells, these cell lines were used to test the theory derived from experiments in hamsters that human pulmonary endocrine cells can metabolize *N*-nitrosodiethylamine (DEN). The cells were incubated *in vitro* with [^{14}C]DEN. Metabolism was assessed by $^{14}CO_2$ production. Both cell lines metabolized DEN to a much greater extent than previously investigated human lung cancer cell lines of Clara cell and alveolar type II cell morphology. In keeping with its abundant endoplasmic reticulum, line NCI-H727 yielded $^{14}CO_2$ in the 300 nM range, whereas NCI-H460 was less active. Metabolism was time dependent. Preincubation with various enzyme inhibitors yielded a highly significant inhibition of DEN metabolism with the two inhibitors of the fatty acid cyclooxygenase component of prostaglandin endoperoxide synthetase, aspirin and indomethacin. Inhibitors of cytochrome P-450 (CO, piperoxylbutoxide) did not inhibit DEN metabolism. Preincubation with sinigrin yielded similar negative results as CO and piperonylbutoxide. Our data are in support of the theory that human pulmonary endocrine cells can metabolize nitrosamines. Moreover, the experiments with enzyme inhibitors suggest that in this cell type such metabolism is largely dependent on prostaglandin endoperoxide synthetase.

We have recently introduced well-differentiated human lung cancer cell lines as a unique system for investigations into cell type specific nitrosamine metabolism [1]. Such cell lines, which are characterized by electron microscopy for identification of cell type as well as degree of differentiation, consist of a uniform population of only one cell type per cell line. Generally, only those cell lines with ultrastructures closely resembling their normal non-cancerous cells of origin are selected for carcinogen metabolism studies. Since the ultrastructure of cells is nothing else but the morphological expression of biochemical function, it is only logical that, in fact, the major xenobiotic-metabolizing enzyme systems known to exist in normal Clara cells and alveolar type II cells were functional in cell lines featuring the structure of these cell types [1, 2]. In contrast, two small cell cancer lines failed to demonstrate any detectable levels of such enzymes and did not metabolize diethylnitrosamine [2]. Small-cell cancer, which is believed to be derived from pulmonary endocrine cells [3, 4], generally demonstrates a very poor cytoplasmic differentiation as compared with its putative cell of origin. In keeping with this, the investigated small-cell cancer cell lines had virtually no endoplasmic reticulum, very few mitochondria, and only scanty clusters of neuroendocrine granules [2]. We therefore felt that their lack of xenobiotic-metabolizing enzymes could not be extrapolated to their putative cells of origin, pulmonary endocrine cells, and was merely a result of advanced neoplastic transformation.

In the meantime, primary cultures of cell lines NCI-H727, derived from a lung carcinoid, and NCI-H460, derived from a large cell carcinoma, were established (NCI-Navy, Clinical Oncology Branch). Both cell lines at early passages exhibit features of well differentiated pulmonary endocrine cells by electron microscopy. Therefore, they were selected for studies on metabolic competence of human pulmonary endocrine cells for nitrosamines.

MATERIALS AND METHODS

Both cell lines (NCI-H727 passage 8 and NCI-H460 passage 11) which grow as monolayers were maintained in RPMI 1640 medium supplemented

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with L-glutamine (2 mM), fetal bovine serum (10%, v/v) and gentamycin (50 μg/ml) at 37° in an atmosphere of 95% air–5% CO_2. They were subcultured at a density of approximately 1.2×10^3/cm^2, and the spent medium was replaced with fresh medium every 3 days. NCI-H727 reached 75% confluency after 3 weeks. The cells were then washed with phosphate buffer solution, 0.1 M, pH 7.4, trypsinized, and resuspended in serum-free RPMI 1640 in 50-ml sterile centrifuge tubes. Incubations were carried out in tightly capped liquid scintillation vials in a gyrating water bath at 37°. The incubates contained 4–5 $\times 10^6$ cells/ml in a final volume of 2 ml cell suspension. Incubation with [^{14}C]DEN (39.3 mCi/mmol, 1.5 μCi in 0.25 ml water/vial) was for 10 min except when otherwise indicated (time course). Preincubation with enzyme inhibitors was for 10 min. Carbon monoxide/oxygen (80:20) was bubbled through the cells suspended in RPMI medium for 10 min prior to addition of [^{14}C]DEN. Aspirin (0.1 mM) was dissolved directly in the medium, whereas indomethacin (0.1 mM) was added as solution in DMSO (0.33%, v/v), and piperonylbutoxide (0.1 mM) as solution in 100% ethanol (0.33%, v/v). In DMSO or ethanol no cell damage was observed at the concentrations used, as assessed by viability counts with trypan blue. $^{14}CO_2$ was trapped by circular Whatman filter paper inserted in the vial caps (the diameter of which exactly matched the caps of liquid scintillation vials) soaked with 0.1 ml hyamine hydroxide. The reaction was started by adding the [^{14}C]DEN and was stopped by 5% phenol and removal of the caps and filters. The trapping filters were removed from each cap and put into prelabeled liquid scintillation vials with 10 ml PCS scintillation mixture (Amersham, Arlington Heights, IL). Samples (three for each group) were counted with a Tri-Carb liquid scintillation counter with automatic external standardization. Protein was determined by the method of Bradford [5] with slight modification. The cell suspension was sonicated twice for 15 sec. An 0.2-ml aliquot was removed and combined with 3.0 ml of Coomassie blue dye reagent (Bio-Rad Laboratory, Richmond, VA) and read at 595 nm.

For assessment of cell type and differentiation of the two cell lines, two samples representative of untreated control cells were processed for transmission electron microscopy. The cells were fixed in petri dishes with 2% cacodylate-buffered glutaraldehyde, postfixed with 1% cacodylate-buffered

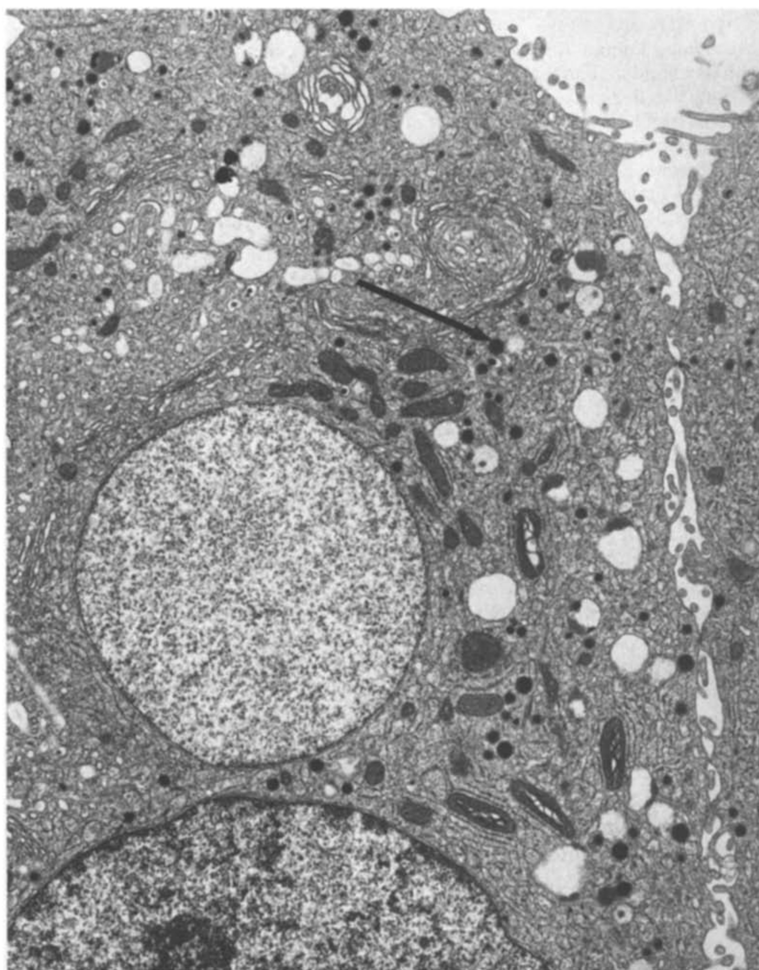

Fig. 1. Electron micrograph illustrating ultrastructure of the human lung carcinoid-derived cell line NCI-H727. Note abundant dense-cored granules (arrowed), well developed smooth and rough endoplasmic reticulum, and multiple Golgi-apparatuses. Magnification: ×10,000.

OSO_4, dehydrated, and embedded in Epon 812. The petri dishes were pulled off the embedded samples during polymerization in the oven. The large disc-shaped (size of a petri dish) samples were then divided with a jeweler's saw into smaller blocks for sectioning with an LKB Ultrotome V Ultramicrotome. Thin sections were mounted on copper grids, stained with uranyl acetate and lead citrate, and examined with a Philips 201 transmission electron microscope operated at 60 kV.

RESULTS

Both cell lines demonstrated ultrastructural features of pulmonary endocrine cells. However, cytoplasmic organelles were generally better differentiated in line NCI-H727 than in line NCI-H460. Cell line NCI-H727 was virtually indistinguishable from normal pulmonary endocrine cells by ultrastructural criteria. The cells contained abundant dense-cored endocrine granules (Fig. 1) that are the morphological hallmark of pulmonary endocrine cells. The granules occurred in clusters and as individual organelles. Vesicles and short tubules of smooth endoplasmic reticulum occupied the majority of the cytoplasm, while short tubules of rough endoplasmic reticulum were less numerous. Prominent multiple Golgi-apparatuses were found in many cells and were usually associated with small progranules of the dense-cored type [1] (Fig. 1). Cell line NCI-H460 (Fig. 2) demonstrated considerably fewer dense-cored granules than the carcinoid-derived cell line. Ribosomes and polyribosomes predominated in the cytoplasm while relatively little endoplasmic reticulum was noticeable. Well developed Golgi-apparatuses were occasionally present but were not multiple as in the other cell line.

Line NCI-H727 exhibits L-dopa decarboxylase activity and produces calcitonin and bombesin [6], whereas NCI-H460 was negative in these assays (A. F. Gazdar, unpublished observation). Both cell lines metabolized [^{14}C]DEN as assessed by measurement of $^{14}CO_2$ evolution. However, in keeping with their differences in cytoplasmic differentiation, the extent of such metabolism was different in the two cell lines. Line NCI-H727 which due to its abundant endoplasmic reticulum is more likely to possess significant levels of enzymes linked with this

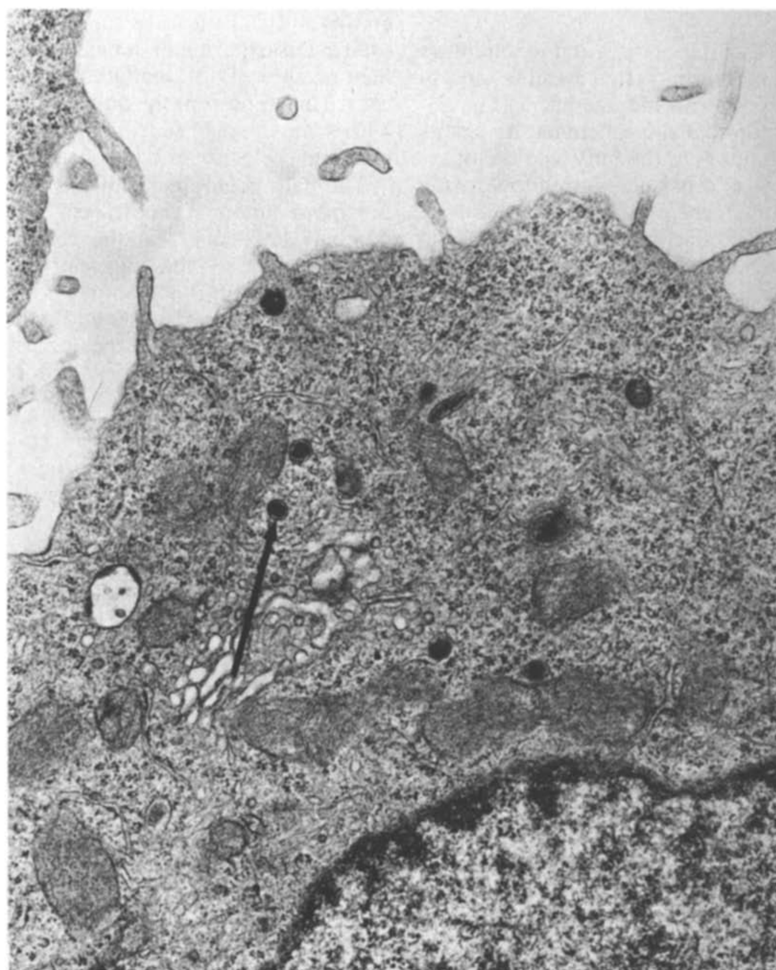

Fig. 2. Electron micrograph of human pulmonary large cell carcinoma-derived cell line NCI-H460. Note dense-cored granules (arrowed) but less endoplasmic reticulum than in NCI-H727. Magnification: ×25,000.

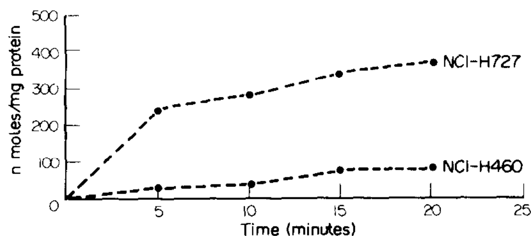

Fig. 3. Time-dependent metabolism of DEN as assessed by evolution of $^{14}CO_2$. The cells were incubated with [^{14}C]DEN in RPMI 1640 medium without serum (39.3 mCi/mmol, 1.5 μCi in 0.25 ml water/vial).

organelle (e.g. cytochrome P-450, prostaglandin H-synthetase) yielded $^{14}CO_2$ levels in the 300 nM range per mg protein per 10 min (Fig. 3). The less well differentiated cell line NCI-H460 was considerably less active in metabolizing the nitrosamine (Fig. 3). However, the yield of $^{14}CO_2$ (3–4 nmol per mg protein per 10 min) was still substantially above the levels obtained under identical conditions with adenocarcinoma derived cell lines composed of well differentiated Clara cells and alveolar type II cells respectively [2]. Metabolism of [^{14}C]DEN was time dependent.

Preincubation with a variety of enzyme inhibitors showed a marked inhibition of nitrosamine metabolism only with aspirin and indomethacin (Fig. 4). Both of these nonsteroidal anti-inflammatory agents are well known inhibitors of the fatty acid cyclooxygenase component of prostaglandin endoperoxide synthetase. In contrast, the established inhibitors of cytochrome P-450, CO and piperonylbutoxide, did not inhibit [^{14}C]DEN metabolism in this system (Fig. 4). Sinigrin, a natural constituent of cruciferous vegetables [7] which was shown recently to inhibit metabolism of nicotine-derived nitrosamine [7] was similarly ineffective.

DISCUSSION

Previous experiments have shown that several nitrosamines which are lung carcinogens induce a striking hyperplasia of pulmonary endocrine cells in hamsters [8, 9]. It was assumed that one reason for such a selective effect on this cell type—which has not been documented with any other class of chemicals—may be a high metabolic competence of pulmonary endocrine cells for nitrosamines. However, due to the scarcity of endocrine cells in the lungs of healthy adult mammals including the hamster [10], no *in vivo* or *in vitro* system was available to test this theory. Efforts to use cell lines derived from human small cell lung cancers for this approach failed [2]. Although pulmonary small cell cancer is believed to be derived from endocrine cells [3, 4], it bears only a slight resemblance to its normal cell of origin. Although most small cell cancers exhibit one or several of the APUD (Amino Precursor Uptake and Decarboxylase activity) characteristics [11] typical of normal pulmonary endocrine cells, they have a much poorer cytoplasmic differentiation. Generally, such cancer cells have only a little cytoplasm that is poor in membrane bound organelles (endoplasmic reticulum, mitochondria). In keeping with this morphology, their lack of xenobiotic metabolizing enzymes [2] is logical. In contrast, the two cell lines used in this experiment demonstrated ultrastructural features of well differentiated pulmonary endocrine cells. They represent the only system worldwide that allows one to study functional aspects of pulmonary endocrine cells under controlled *in vitro* conditions.

The data generated with this set of experiments are of paramount importance. They provide fundamental evidence that the cell type of origin of small cell cancer—the pulmonary endocrine cell—is extremely potent in metabolizing nitrosamines. The level of DEN metabolism found in these two cell lines was substantially above the levels reported under similar conditions with well differentiated human

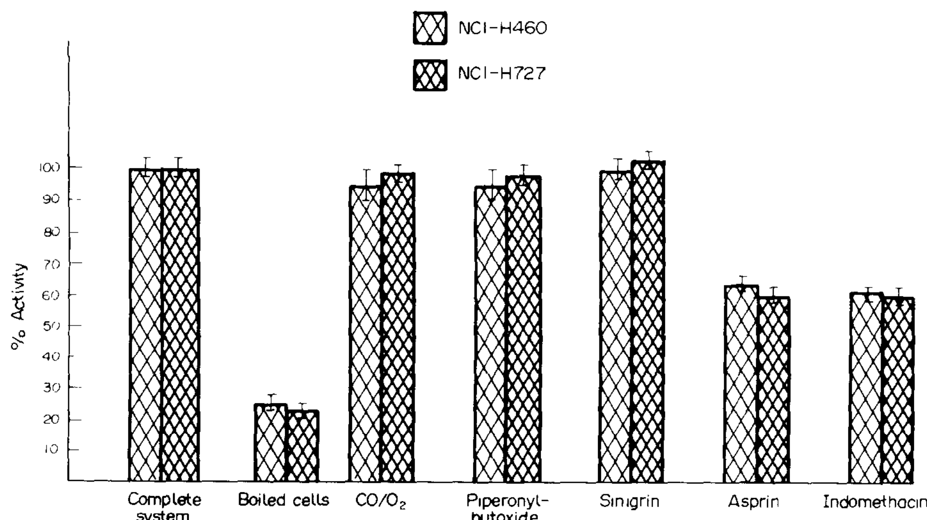

Fig. 4. Effect of enzyme inhibitors on DEN metabolism as assessed by evolution of $^{14}CO_2$. Conditions were as in Fig. 3; preincubation with inhibitor (at 0.1 mM concentration) was for 10 min and incubation with [^{14}C]DEN for 10 min.

lung cancer cell lines of Clara cell and alveolar type II cell morphology [2]. Our findings explain, at least in part, why pulmonary small cell cancer is such a prevalent type of cancer in cigarette smokers. The cell type of origin of this type of cancer (pulmonary endocrine cell) is apparently able to metabolically activate nitrosamines (and possibly other indirect carcinogens, e.g. polycyclic aromatic hydrocarbons) contained in cigarette smoke to a much greater extent than other lung cell types. Of particular interest is our finding that cytochrome P-450 enzymes do not play a major role in the metabolic activation of DEN in pulmonary endocrine cells. As our metabolism–inhibition studies show, inhibitors of the fatty acid cyclooxygenase of prostaglandin endoperoxide synthetase (aspirin, indomethacin) yield a highly significant reduction of DEN metabolism in this cell type. This is in contrast to the nitrosamine metabolism–inhibition data obtained in other systems such as liver *in vivo* [12–14], hamster lung *in vivo* [15], and human lung cancer cell lines of Clara cell and alveolar type II cell morphology [2]. However, our findings are in accord with known physiological aspects of normal pulmonary endocrine cells which are involved in the synthesis, storage, and degradation of 5-hydroxytryptamine (serotonin) [16]. Prostaglandins have been identified as one of the possible mediators of this pathway [17].

Our data give further support to the hypothesis that there are pronounced cell type specific differences not only in the metabolic competence for nitrosamines in general but also for the modality of such metabolic pathways. In light of these findings, generalized concepts on the mechanisms of nitrosamine carcinogenesis derived from studies in a given organ or cell type may not be of significant relevance for another organ or cell type.

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