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Mechanisms of Amiodarone and Desethylamiodarone Cytotoxicity in Nontransformed Human Peripheral Lung Epithelial Cells

Jeanne E. Mulder, James F. Brien, William J. Racz, Takashi Takahashi, and Thomas E. Massey

Department of Pharmacology and Toxicology, Queen's University, Kingston, Ontario, Canada (J.E.M., J.F.B., W.J.R., T.E.M.); and Division of Molecular Carcinogenesis, Center for Neurological Diseases and Cancer, Nagoya University Graduate School of Medicine, Nagoya, Japan (T.T.)

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ABSTRACT

Amiodarone (AM) is a potent antidysrhythmic agent that can cause potentially life-threatening pulmonary fibrosis, and *N*-desethylamiodarone (DEA), an AM metabolite, may contribute to AM toxicity. Apoptotic cell death in nontransformed human peripheral lung epithelial 1A (HPL1A) cells was assessed by annexin V-fluorescein isothiocyanate (ann-V) staining and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL), and necrotic cell death was assessed by propidium iodide (PI) staining. The percentage of cells that were PI-positive increased more than six times with 20 μM AM and approximately doubled with 3.5 μM DEA, relative to control. The percentage of cells that were ann-V-positive decreased by more than 80% after 24-h exposure to 10 μM AM but more than doubled after 24-h incubation with 3.5 μM DEA. Incubation for 24 h with 5.0 μM DEA increased the percentage of cells that were TUNEL-positive more than six times. Incubation with AM (2.5 μM) or DEA (1–2 μM) for 24 h did not significantly alter angiotensinogen mRNA levels. Furthermore, angiotensin II (100 pM–1 μM) alone or in combination with AM or DEA did not alter cytotoxicity, and pretreatment with the angiotensin-converting enzyme inhibitor and antioxidant captopril (3–6 μM) did not protect against AM or DEA cytotoxicity. In conclusion, AM activates primarily necrotic pathways, whereas DEA activates both necrotic and apoptotic pathways, and the renin-angiotensin system does not seem to be involved in AM or DEA cytotoxicity in HPL1A cells.

Introduction

Amiodarone (AM), an iodinated benzofuran, is considered to be the most efficacious antidysrhythmic drug currently available (Lafuente-Lafuente et al., 2009). However, long-term treatment with AM is associated with several adverse effects, the one of greatest concern being AM-induced pulmonary toxicity (AIPT), because it can progress to potentially life-threatening pulmonary fibrosis. Recent studies have reported that the incidence of AIPT occurs in 5 to 13% of patients treated with AM in a dose- and duration-dependent manner (Oyama et al., 2005). The prognosis of a patient with AIPT is poor, with a 10 to 23% mortality rate (Vrobel et al., 1989; Oyama et al., 2005).

AM and its major pharmacological active metabolite, desethylamiodarone (DEA), have large apparent volumes of distribution and slow clearances from adipose tissue, liver, lungs, and lymph nodes and therefore accumulate to high concentrations in these tissues (Freedman and Somberg, 1991). After long-term therapy, AM can accumulate in lung to >1 mmol/kg wet tissue (Brien et al., 1987). In addition, DEA has greater cytotoxic potency than AM and can accumulate in lung to up to four times greater levels than AM (Broekhuysen et al., 1969; Wilson and Lippmann, 1990; Reasor and Kacew, 1996). Hence, there is compelling evidence that DEA plays a role in AIPT.

The underlying etiology of AIPT is unknown; however, both indirect inflammatory processes and direct toxic effects have been proposed previously (Reasor and Kacew, 1996). AM and DEA are directly toxic to bovine arterial endothelial

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ABBREVIATIONS: AM, amiodarone; AIPT, AM-induced pulmonary toxicity; DEA, desethylamiodarone; RAS, renin-angiotensin system; Ang II, angiotensin II; ann-V, annexin-V-fluorescein isothiocyanate; AGTR, angiotensin receptor; PI, propidium iodide; HPL1A, human peripheral lung epithelial cells; PCR, polymerase chain reaction; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography; ANOVA, analysis of variance.

cells, alveolar macrophages, interstitial lung fibroblasts (Martin and Howard, 1985), human pulmonary arterial endothelial cells (Powis et al., 1990), bronchial epithelial cells (Colgan et al., 1984), and hepatocytes (Gross et al., 1989). Many mechanisms have been hypothesized to cause the direct toxicity of AM and DEA, including increased intracellular influx of Ca^{2+} (Powis et al., 1990), mitochondrial disruption (Bolt et al., 2001), free radical and reactive oxygen species formation (Pollak, 1999), and up-regulation of the renin-angiotensin system (RAS) (Uhal et al., 2007).

Studies have indicated that rat lung epithelial cells and human lung adenocarcinoma cells have an intrinsic RAS, with the ability to generate angiotensin II (Ang II) de novo (Li et al., 2003). Ang II signaling, mediated via the angiotensin receptors AGTR1 and AGTR2, plays a role in tissue remodeling in fibrosis (Königshoff et al., 2007). However, the downstream effects of AGTR1 and AGTR2 activation are quite different. The classic physiologic effects of Ang II, including vasoconstriction, aldosterone and vasopressin release, sodium and water retention, and cell proliferation, are mediated by AGTR1, whereas the established role of AGTR2 includes modulation of biological processes involved in development, cell differentiation, tissue repair, and apoptosis (Kaschina and Unger, 2003). Ang II can induce concentration-dependent apoptosis in human lung cancer epithelial cells and in primary type II pneumocytes isolated from adult Wistar rats, an effect that can be abrogated by the nonselective AGTR antagonist saralasin (Wang et al., 1999b). In addition, treatment with the angiotensin-converting enzyme inhibitor captopril can attenuate apoptosis in human lung adenocarcinoma cells and primary rat alveolar epithelial cells treated with AM or DEA (Bargout et al., 2000) and can inhibit alveolar wall collagen formation in lungs of AM-treated rats (Uhal et al., 2003). Furthermore, a retrospective review of patients taking AM suggests that patients who developed AIPT were administered a lower dose of RAS inhibitor than those who did not develop AIPT (Nikaido et al., 2008). Thus, the RAS may play a contributing role in the initiation and/or progression of AIPT.

Surfactant-secreting type II alveolar epithelial cells provide antioxidant defense, local immunomodulation, and a stem cell reserve for alveolar epithelial repair and are critical for normal re-epithelialization and healing without fibrosis of the alveolar surface (Thannickal et al., 2004). Histological analysis of lung tissue from patients treated with AM demonstrates alveolar interstitial damage, including hyperplasia of type II pneumocytes (Brien et al., 1987). In addition, AM is toxic to epithelial cells in vitro (Bargout et al., 2000), thereby implicating epithelial injury in the initiation of AIPT. To better understand the etiology of AIPT, the present study investigated the cytotoxic pathways activated by AM or DEA individually and whether an intrinsic RAS is linked to AM- or DEA-induced cell death. HPL1A cells, which were established by immortalization from a normal adult lung specimen, were employed because they retain morphological and biochemical features characteristic of normal adult human peripheral lung epithelial cells (Masuda et al., 1997).

Materials and Methods

Reagents. Chemicals and reagents were obtained as follows: AM HCl (98% purity), bovine insulin, fetal bovine serum, hydrocortisone, HEPES, Ang II, captopril, propidium iodide (PI), and trypan blue from Sigma-Aldrich (Oakville, ON, Canada); annexin V-fluorescein isothiocyanate (ann-V) from BD Biosciences (Mississauga, ON, Canada); glacial acetic acid, KH_2PO_4, NaCl, $NaHCO_3$, NaOH, and $Na_2HPO_4{\cdot}7H_2O$ from Thermo Fisher Scientific (Nepean, ON, Canada); and antibiotic-antimycotic, L-glutamine, Ham's F-12 nutrient mixture, human transferrin, and trypsin-EDTA from Invitrogen Canada Inc. (Burlington, ON, Canada). DEA HCl (99.9% purity) was synthesized by Dr. Manlio Alessi (Department of Chemistry at Queen's University, Kingston, ON). All other reagents were of analytical grade and were purchased from standard commercial suppliers. Stock solutions of 4.0 mM AM, 1.0 mM DEA, 4.8 mM Ang II, and 2.0 mM captopril were prepared fresh in reverse osmosis purified distilled water at 65°C (AM and DEA) or room temperature (Ang II and captopril).

Cell Culture. HPL1A cells (Masuda et al., 1997) were cultured in Ham's F-12 nutrient mixture medium, pH 7.2, supplemented with 1.18 g/liter sodium bicarbonate, 1% fetal bovine serum, 15 mM HEPES buffer, 1× antibiotic-antimycotic, 100 nM hydrocortisone, 0.13 ng/ml triiodothyronine, 5.0 μg/ml human transferrin, and 5.0 μg/ml bovine insulin (HPL1A medium). Cells were grown in T-75 flasks (Corning Inc., Corning, NY). Culture medium was replaced every 3 to 4 days, and cells were subcultured approximately every 7 days between passages 8 and 12. Cells were incubated at 37°C under 95% air, 5% CO_2. After reaching 80 to 90% confluence, cells were removed from tissue culture flasks by washing twice with 10 ml of phosphate-buffered saline (PBS) (0.2 g/liter KH_2PO_4, 0.8 g/liter NaCl, 2.16 g/liter $Na_2HPO_4{\cdot}7H_2O$, pH 7.3), and then treated with 0.1% trypsin and 1.06 mM EDTA in PBS. The cells were then incubated at 37°C for 5 to 10 min and resuspended in HPL1A medium. Cells were seeded at a density of 2.1×10^5 cells/well in 12-well plates or 2.8×10^6 cells/100-mm dish and allowed to acclimatize for approximately 24 h before drug treatment.

Quantification of AM and DEA in HPL1A Cells. To determine whether HPL1A cells are able to convert AM to DEA, the amount of AM and DEA within HPL1A cells after 24 h of treatment was determined by high-performance liquid chromatography (HPLC). In brief, cells in 100-mm dishes were treated with AM for 24 h. Cells were then harvested and centrifuged at 700*g* for 10 min at 4°C. The supernatant was removed, and the cell pellet was resuspended in 1.0 ml of 4°C PBS and then centrifuged at 700*g* for 5 min at 4°C. The supernatant was removed, and the resulting cell pellet was immediately frozen in liquid nitrogen and stored at −80°C until analysis.

The amount of AM and DEA in cell pellets was measured as described by Bolt et al. (1998). In brief, each cell pellet was thawed, and 100 μl of mobile phase [acetonitrile/5% aqueous acetic acid, 8:2 (v/v) adjusted to pH 5.9 with ammonium hydroxide] was added. Cell pellets were mixed in the mobile phase for 1 min, and the mixture was centrifuged at 16,000*g* for 3 min at room temperature. The supernatants were analyzed quantitatively for AM and DEA by reverse-phase HPLC with UV-visible spectrophotometric detection at 254 nm, with a with-in day precision of 7.00% (Brien et al., 1983, 1987). The percentage conversion of AM to DEA for each treatment condition was calculated. The mean value from three independent experiments was used to calculate the overall percent conversion of AM to DEA for each treatment condition. The lower limit of quantifiable detection for DEA was 0.20 μg/ml (0.31 μM). The concentrations of AM and DEA injected onto the HPLC column from biological samples were within the range of standards employed (11.3–180 μg/ml for AM and 0.25–4.00 μg/ml for DEA).

Annexin-V-Fluorescein Isothiocyanate and Propidium Iodide Dual Staining. HPL1A cells were stained with ann-V to indicate apoptosis and PI to indicate necrosis. For AM and DEA cytotoxicity experiments, HPL1A cells in 12-well plates were treated with AM or DEA for 6, 12, or 24 h. For Ang II cytotoxicity experiments, HPL1A cells in 12-well plates were treated with AM or DEA in combination with Ang II for 24 h. Ang II was administered every 12 h in the 24-h treatment period. For captopril protection experiments,

HPL1A cells in 12-well plates were pretreated for 2 h with captopril to allow captopril to enter the cells and interact with angiotensin-converting enzyme before the addition of AM or DEA. After captopril pretreatment, AM or DEA was added for an additional 24 h. After treatment, culture medium was removed, and cells were washed once with PBS. Cells were detached by incubation with trypsin-EDTA for 5 min at 37°C. The original culture medium, PBS wash, and cell suspension were combined to retain all dead and living cells for analysis. The resulting cell suspension was centrifuged for 4 min at 215*g* at room temperature. The supernatant was removed, and the cell pellet was resuspended in 0.5 ml of a buffer consisting of 10 mM HEPES, 140 mM NaCl, and 5 mM $CaCl_2$. The cell suspension was then treated with 5 μl of stock ann-V solution (BD Biosciences) and 5 μl of 0.5 mg/ml PI and placed on ice in the dark for 20 min. Samples were then centrifuged for 4 min at 215*g*, and the cell pellets were resuspended in 0.5 ml of PBS. Analysis was completed via flow cytometry (EPICS ALTRA; Beckman Coulter, Mississauga, ON, Canada). In addition, total cell death percentages were fitted to a sigmoidal dose-response curve (variable slope), and the concentrations of drug producing 50% cell viability loss (LC_{50}) were interpolated using Prism (ver. 5.0; GraphPad Software, San Diego, CA). Analysis was performed on the mean values of triplicates (values from three individual wells) from three or four independent experiments.

Modified Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling Assay. A modified fluorometric terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) kit was used to detect fragmented DNA, a characteristic of apoptotic cells, according to the manufacturer's protocol (Promega, Madison, WI). Cells in 12-well plates were treated for 24 h with AM or DEA. After treatment, the culture medium was removed, and cells were washed once with PBS. Cells were detached by incubation with trypsin-EDTA for 5 min at 37°C and centrifuged at 215*g* at 4°C for 5 min, and the cell pellet was resuspended in 0.5 ml of 4°C PBS. Cells were fixed using 3.5 ml of 1% paraformaldehyde for 20 min on ice. After fixation, cells were centrifuged and resuspended in 0.5 ml of PBS. Seventy percent of ice-cold ethanol (3.5 ml) was then added to the cell suspensions, and samples were stored at −20°C overnight.

The next day, cells were washed with PBS, resuspended in 80 μl of equilibration buffer (200 mM potassium cacodylate, 25 mM Tris-HCl, 0.2 mM dithiothreitol, 0.25 mg/ml bovine serum albumin, and 2.5 mM cobalt chloride) and left to incubate for 5 min at room temperature. Cells then were resuspended in 50 μl of reverse terminal deoxynucleotidyl transferase incubation buffer and left to incubate in a 37°C water bath for 60 min. The reaction was terminated by adding 1.0 ml of 20 mM EDTA, followed by centrifugation at 175*g* for 6 min. Cells were resuspended in 1.0 ml of 0.1% Triton X-100 solution in PBS containing 5 mg/ml bovine serum albumin, followed by centrifugation at 175*g* for 5 min. Cells were resuspended in 0.5 ml of propidium iodide containing 250 μg of DNase-free RNase A and left to incubate at room temperature in the dark for 30 min. Cell samples were analyzed by flow cytometry, with fluorescein-12-dUTP measured at 520 ± 20 nm and PI measured at >620 nm. Analysis was performed on the mean values of triplicates (values from three individual wells) from three independent experiments.

Trypan Blue Exclusion. HPL1A cells in 12-well plates were treated with Ang II for 24 h. After treatment, cells were detached by incubation with trypsin-EDTA for 5 min at 37°C. Cells were then stained with 0.5% trypan blue dye. Cell viability was assessed by trypan blue exclusion, using a hemocytometer and light microscope (Reichert Scientific Instruments, Buffalo, NY). Analysis was performed on the mean values of triplicates (values from three individual wells) from three independent experiments.

Angiotensinogen mRNA Levels. Total RNA was isolated from HPL1A cells using the RNeasy Mini Kit (QIAGEN, Valencia, CA), with an additional on-column DNase treatment step in accordance with the manufacturer's instructions. The quality of the RNA samples was determined by electrophoretic analysis of 1 μg of RNA on a denaturing gel. Ethidium bromide staining of the gel revealed distinct 28S and 18S rRNA bands, with an intensity ratio of 28S:18S of at least 2. The UV absorbance ratio (260/280 nm) ranged from 1.9 to 2.1 for all RNA samples. cDNA was synthesized from 1 μg of total RNA in a reaction volume of 20 μl using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) in accordance with manufacturer's instructions. For quantitative real-time PCR, 2.0-μl aliquots of cDNA were amplified using an angiotensinogen TaqMan primer and probe set (Applied Biosystem Assay ID Hs00174854_m1) according to the manufacturer's recommendations. Amplification, detection, and analysis were performed using Smart Cycler II instrumentation and software (Cepheid, Sunnyvale, CA). For mRNA quantitation, the standard curve method of relative quantitation was used. PCR product specificity was verified by agarose gel electrophoresis with ethidium bromide staining.

Data Analysis. Data are expressed as the mean ± S.E.M. for each experimental group. Experiments were conducted in the same culture of cells sequentially. To assess viability of treated and untreated cells at multiple concentrations, repeated-measures one-way analysis of variance (ANOVA) was performed, followed by Student Newman-Keuls post hoc test. In all cases, analysis was performed using Prism software, and statistical significance was defined as $p < 0.05$.

Results

Conversion of Amiodarone to Desethylamiodarone in HPL1A Cells. Treatment of HPL1A cells with 5 to 20 μM AM for 24 h resulted in minimal production of DEA; with 5 μM AM, 1.86 ± 0.42% AM was converted to DEA, whereas 0.99 ± 0.36 and 0.92 ± 0.35% AM were converted to DEA after treatment of 10 and 20 μM, respectively. Treatment of HPL1A cells with 20 μM AM for 0 h (treated medium was added to cultures and cultures were immediately harvested for HPLC analysis) resulted in no detectable DEA (i.e., <0.31 μM), confirming that the conversion of AM to DEA occurred during 24-h incubation and was not artifactual. The recovery of AM from the cell pellet after 24-h incubation was ≥85% for the concentrations of AM tested.

AM- and DEA-Induced Necrosis and Apoptosis. Treatment of HPL1A cells with 1 to 20 μM AM for 6 or 12 h increased the percentage of PI-positive cells (necrosis) and decreased the percentage of ann-V-positive cells (apoptosis) (Fig. 1, A and B). Treatment with 10 or 20 μM AM for 24 h increased the percentage of cells that were PI-positive >3- and 6-fold, respectively, relative to control (Fig. 1C). However, at 24 h, a significant decrease in ann-V-positive cells was observed only with 20 μM AM (Fig. 1C).

In contrast, 6- or 12-h exposure to DEA resulted in no change in ann-V staining and an increase in PI-positive cells only with 5 μM DEA (Fig. 2, A and B). At 24 h, the percentage of cells that were PI-positive increased 2- and >6-fold after incubation with 3.5 and 5 μM DEA, respectively, compared with control, whereas a significant increase in ann-V-positive cells occurred with 3.5 μM DEA (Fig. 2C). The concentration of DEA producing 50% cell viability loss (LC_{50}) after 24-h treatment was significantly lower than that of AM (4.67 ± 2.40 and 12.7 ± 1.42 μM, respectively). The concentrations of AM and DEA used in the subsequent captopril and Ang II experiments were 10 and 3.5 μM, respectively, which are the approximate LC_{44} for each drug (AM LC = 44.9 ± 2.92; DEA LC = 42.4 ± 3.78). Representative dot plots obtained from

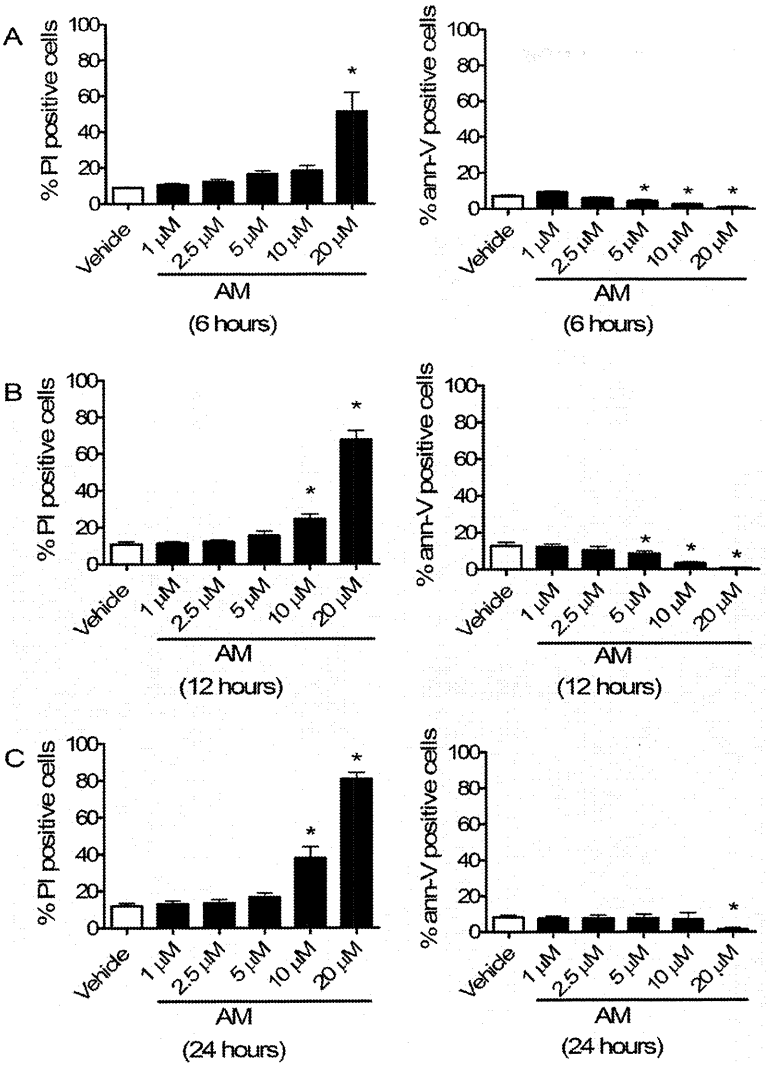

Fig. 1. Percentage of HPL1A cells that were PI-positive (necrotic) and ann-V-positive (apoptotic) after incubation with AM for 6 (A), 12 (B), or 24 h (C). * significantly different from vehicle control, $p < 0.05$ (repeated measures one-way ANOVA with student Newman-Keuls post hoc test; $n = 4$).

flow-cytometric analysis of ann-V- and PI-stained cells illustrate the shift of AM-treated cells predominantly to necrosis, and the shift of some DEA-treated cells to apoptosis and some DEA-treated cells to necrosis (Fig. 3).

Flow-cytometric dot plots from TUNEL staining (Fig. 4, A–D) show a similar trend for vehicle and AM (no shift in the cell population) and a similar trend for the apoptosis-positive control (hydrogen peroxide) and DEA (upward shift in the cell population, indicating apoptosis). After treatment of HPL1A cells with 0.03% hydrogen peroxide for 30 min, the mean percentage of TUNEL-positive cells ranged from 44 to 65% (Fig. 4, E and F). In contrast to quantification of apoptosis by ann-V staining, treatment with 1 to 20 μM AM or 1 to 3.5 μM DEA for 24 h caused no change in apoptosis as determined by the percentage of TUNEL-positive cells (Fig. 4, A, C, E, and F); however, 5 μM DEA increased TUNEL-positive cells from 4.31 ± 2.00 (control) to 26.7 ± 3.72% (Fig. 4 A, D, and F).

Angiotensinogen mRNA Levels. Treatment of HPL1A cells with 2.5 μM AM, 1.0 μM DEA, or 2.0 μM DEA for 24 h caused a trend toward an increase in angiotensinogen levels, which fell just short of statistical significance ($p = 0.0504$, one-way ANOVA; Fig. 5). Higher concentrations of AM or DEA caused substantial cytotoxicity, which precluded recovery of high-quality RNA for analysis.

Assessment of Ang II Cytotoxicity. For initial Ang II cytotoxicity experiments, 0.5% trypan blue exclusion (indicative of plasma membrane integrity) was used to rapidly determine whether Ang II affected cell viability. After 24 h of treatment of HPL1A cells with 100 pM to 1 μM Ang II, no significant cell death was observed relative to vehicle control, with the overall range of cell viability for all concentrations tested being 94.5 to 97.8%. Light microscopic examination also did not reveal any apparent damage to the cells.

To assess the effects of Ang II on apoptosis and necrosis induced by AM and DEA, ann-V and PI dual staining was performed. As expected, the percentage of PI-positive cells increased more than 2-fold after 24-h treatment with 10 μM AM compared with control, whereas no change was seen in ann-V-positive cells (Fig. 6 A). Treatment for 24 h with 3.5 μM DEA increased PI- and ann-V-positive cells (Fig. 6B). However, coincubation of HPL1A cells for 24 h with 10 μM AM or 3.5 μM DEA plus 100 pM to 1 μM Ang

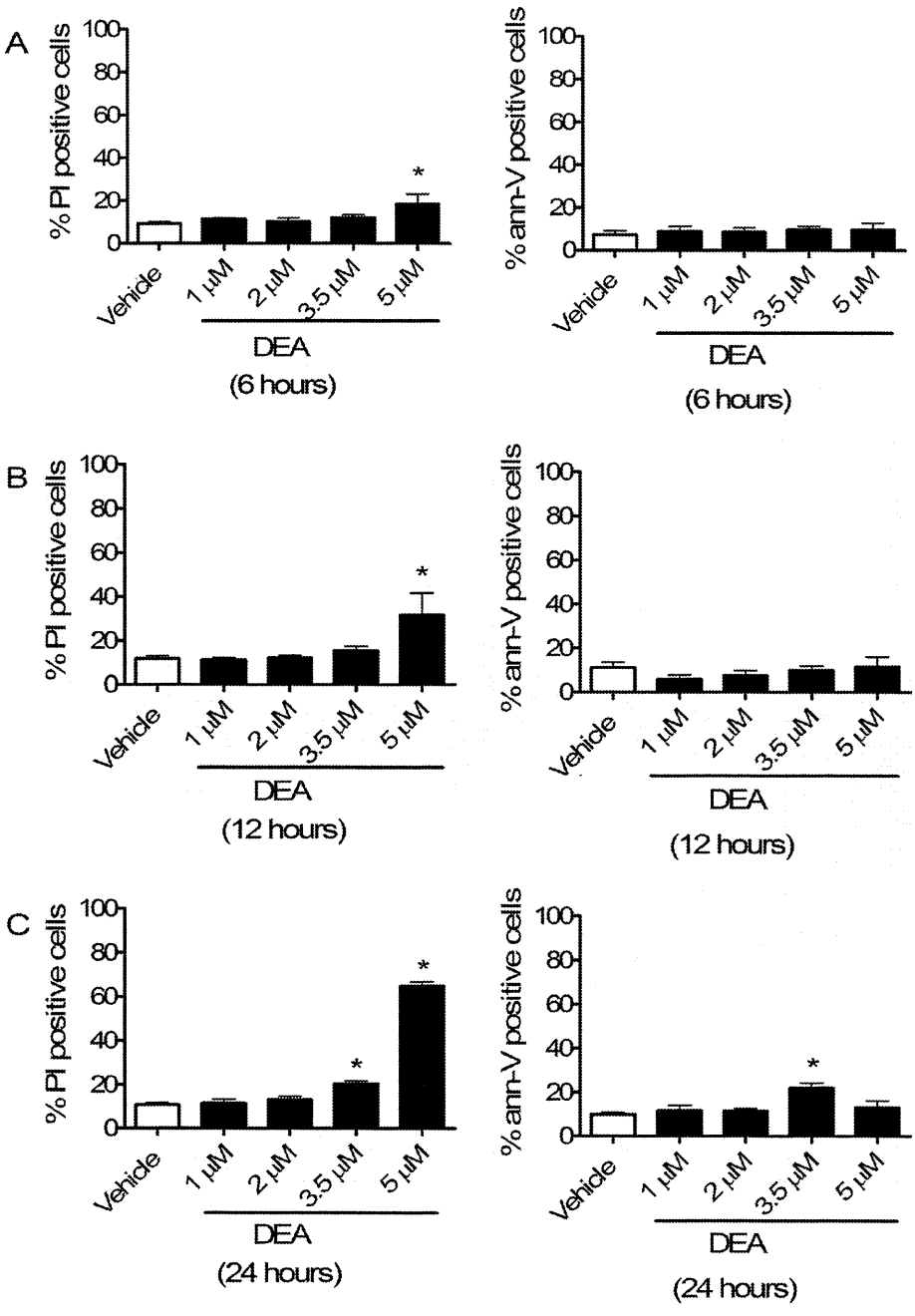

Fig. 2. Percentage of HPL1A cells that were PI-positive (necrotic) and ann-V-positive (apoptotic) after incubation with DEA for 6 (A), 12 (B), or 24 h (C). * significantly different from vehicle control, $p < 0.05$ (repeated measures one-way ANOVA with Student Newman-Keuls post hoc test; $n = 4$).

II caused no significant alterations in PI- or ann-V-positive cells compared with AM or DEA alone (Fig. 6, A and B).

Evaluation of Protection against AM or DEA Cytotoxicity by Captopril. The angiotensin-converting enzyme inhibitor captopril was ineffective at attenuating AM- or DEA-induced cytotoxicity. Consistent with previous experiments (Figs. 2 and 6), PI-positive cells increased more than 3-fold after 24-h treatment with 10 μM AM compared with control, whereas no change occurred in ann-V-positive cells (Fig. 7A). Treatment of cells for 24 h with 3.5 μM DEA increased the percentage of PI- and ann-V-positive cells (Fig. 7B). Pretreatment of cells for 2 h with 3 to 6 μM captopril, before the addition of 10 μM AM or 3.5 μM DEA, caused no significant change in percentages of PI- or ann-V-positive cells compared with AM alone or DEA alone (Fig. 7).

Discussion

DEA has greater cytotoxic potency than AM and accumulates in tissues to a greater extent than AM after long-term treatment of humans with AM (Broekhuysen et al., 1969; Wilson and Lippmann, 1990). Given the high toxicity of DEA and that DEA is a major metabolite of AM, it is possible that many of the initiating processes of AIPT are not solely the result of AM but DEA as well. After long-term therapy, up to

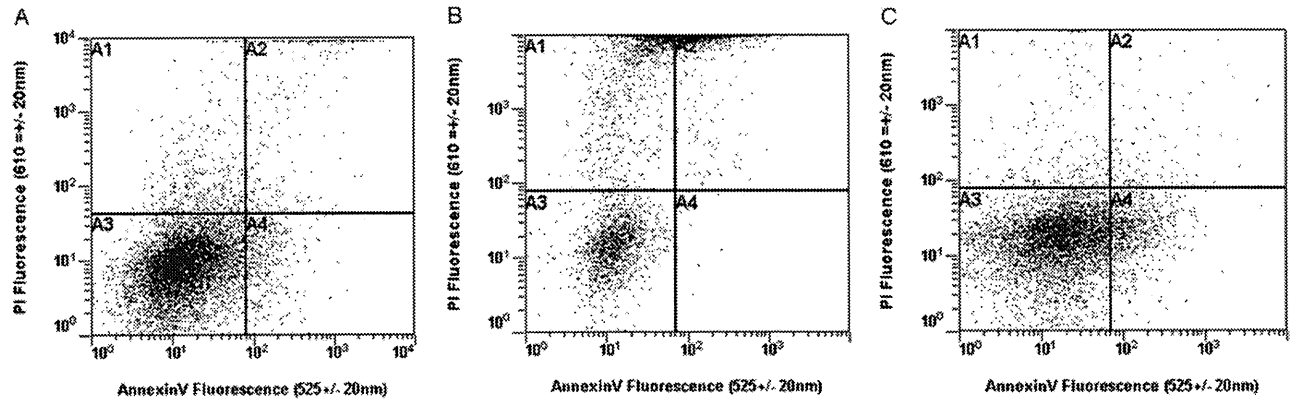

Fig. 3. Representative dot plots depicting the percentage of HPL1A cells that were ann-V-positive (apoptotic) and PI-positive (necrotic) after incubation with vehicle control (24 h) (A), 20 μM AM (24 h) (B), or 3.5 μM DEA (24 h) (C). Quadrant A3 is unstained cells, quadrant A4 is ann-V-only stained cells, quadrant A1 is PI-only stained cells, and quadrant A2 contains cells stained with both PI and ann-V. The percentages of necrotic (quadrant A1 plus quadrant A2) and apoptotic (quadrant A4) cells from triplicates of three independent experiments were transposed to bar graphs (Figs. 1 and 2).

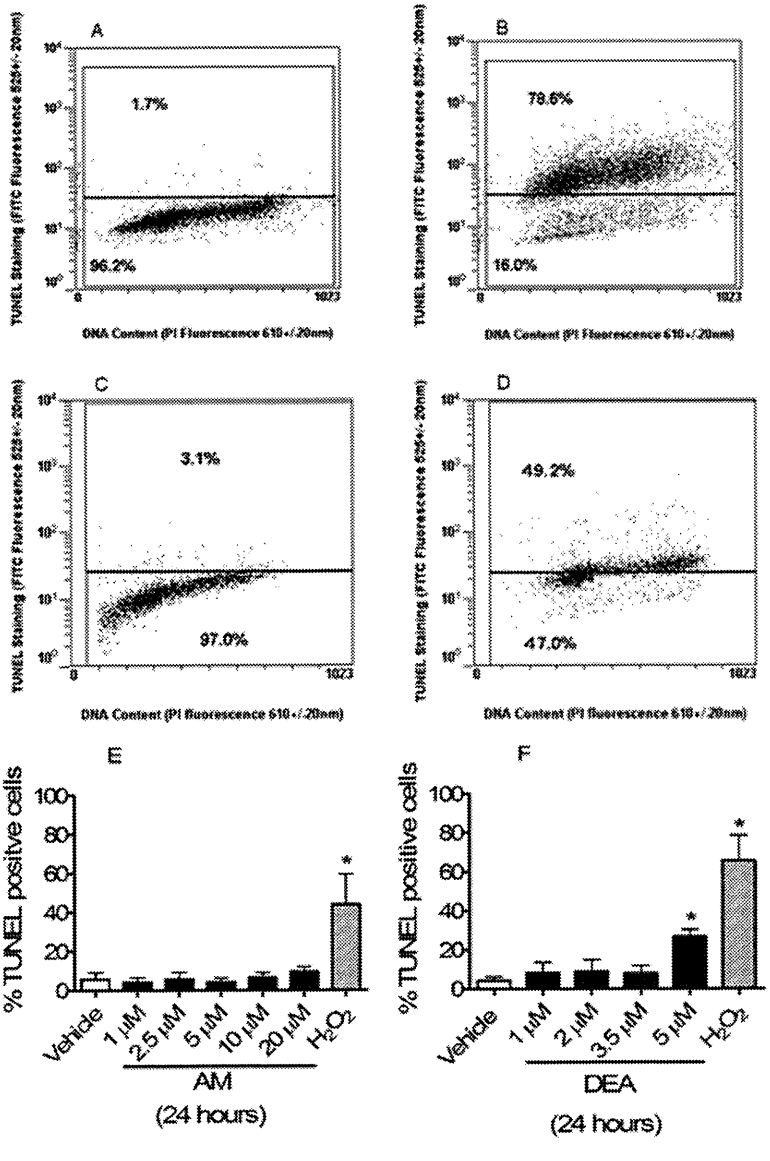

Fig. 4. A to D, representative dot plots depicting the percentage of HPL1A cells that were TUNEL-negative (live) and TUNEL-positive (apoptotic) (below and above the horizontal line, respectively) after incubation with vehicle control (24 h) (A), positive control (0.03% H_2O_2, 30 min) (B), 20 μM AM (24 h) (C), or 5 μM DEA (24 h) (D). E and F, the percentages of apoptotic cells from triplicates of three independent experiments were transposed to bar graphs, shown for AM (E) and DEA (F) treatment. * significantly different from vehicle control, $p < 0.05$ (repeated measures one-way ANOVA with student Newman-Keuls post hoc test; $n = 3$).

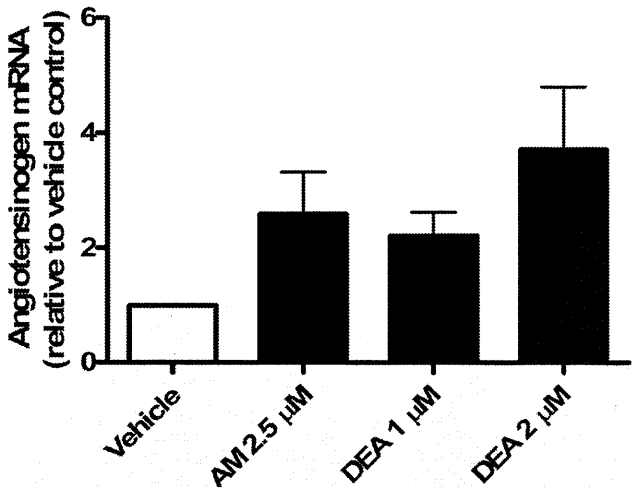

Fig. 5. Angiotensinogen mRNA levels relative to vehicle control after 24-h treatment with AM or DEA, as detected by quantitative real-time PCR. No statistically significant effect was observed between treatments and vehicle control (repeated-measures one-way ANOVA; $n = 4$).

millimolar concentrations of AM and DEA can be found in lung, making the concentrations used in the in vitro studies well within the therapeutically relevant range. In addition, patients who develop amiodarone-induced fibrosis are on the same treatment protocols as those patients who achieve millimolar concentrations of AM and DEA.

In vitro studies investigating cell types in isolation are useful for differentiating the effects of a xenobiotic versus its metabolite(s) if metabolism of the xenobiotic does not occur appreciably in the cells of interest. In humans, AM is metabolized to DEA predominantly by cytochrome P450 enzymes 3A4, 2C8, and 1A1 (Ohyama et al., 2000), which are expressed primarily in the liver, although evidence exists on mRNA and protein of these enzymes in human lung (Macé et al., 1998; Nishimura et al., 2003).

In HPL1A cells, less than 2% AM was converted to DEA over 24 h. The fact that the percentage conversion of AM to DEA decreased with increasing AM concentration was probably attributed to the direct cytotoxicity of AM at the higher concentrations rather than saturation of biotransforming enzymes. The K_M value for cytochrome P450 3A production of DEA in liver microsomes was 0.33 mM (Fabre et al., 1993), a concentration well above those used in this study. Because AM is not converted to DEA to an appreciable extent during the incubation times employed, the HPL1A cell culture model can be used to study the effects of AM and DEA independently on lung epithelial cells.

As observed previously in other systems (Broekhuysen et al., 1969; Wilson and Lippmann, 1990; Bolt et al., 2001; Nicolescu et al., 2008), DEA proved to be a more potent and rapidly acting cytotoxicant than AM in HPL1A cells. Both agents caused HPL1A cell death predominantly by necrosis. Other studies investigating apoptotic and necrotic pathways induced by AM or DEA in other cell types have also found a concentration-dependent increase in necrosis (Bargout et al., 2000; Yano et al., 2008). After AM treatment, we also found a concentration-dependent decrease in apoptosis at 6 and 12 h, but not at 24 h, as reflected by ann-V staining. The percentage of HPL1A cells that underwent apoptosis, however, was small (1.6–12.3%). Therefore, necrosis predominates over apoptosis in AM toxicity. Apoptosis and necrosis can occur in a continuum, whereby cells can undergo processes that have the potential to lead to apoptosis but can ultimately later undergo necrosis, depending on the magnitude of the insult, duration of toxicant exposure, energy

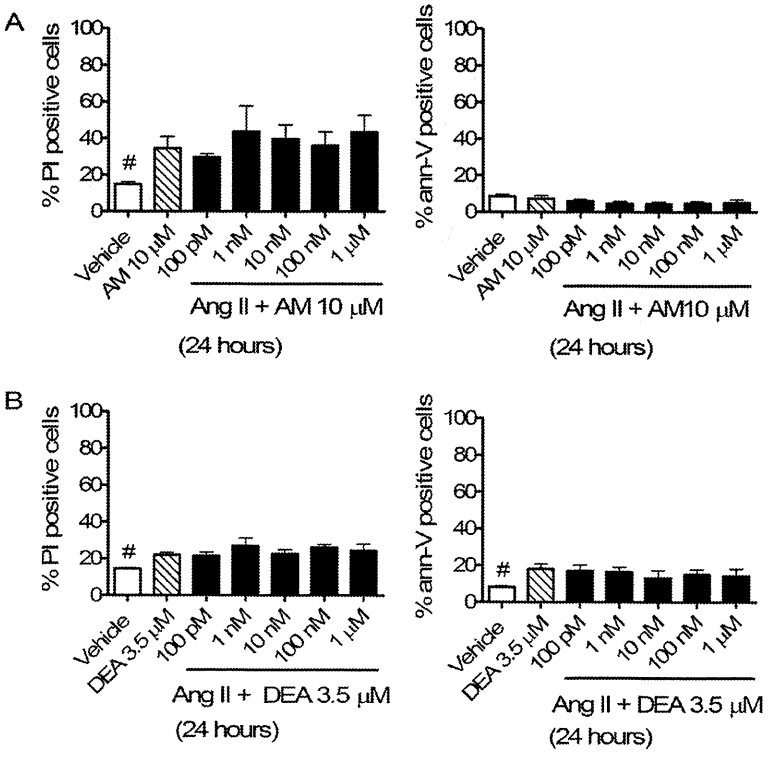

Fig. 6. Percentage of HPL1A cells that were PI-positive (necrotic) and ann-V-positive (apoptotic) after 24-h treatment with Ang II plus 10 μM AM (A), or Ang II plus 3.5 μM DEA (B). #, significantly different from AM or DEA alone (repeated measures one-way ANOVA with student Newman-Keuls post hoc test; $n = 3$).

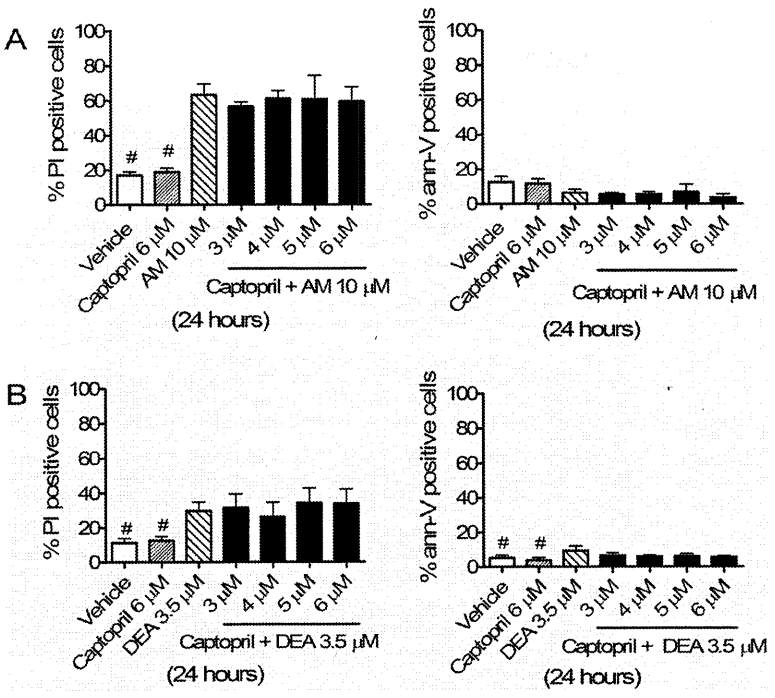

Fig. 7. Percentage of HPL1A cells that were PI-positive (necrotic) and ann-V-positive (apoptotic) after 2-h pretreatment with captopril, followed by the addition of 10 μM AM (A) or 3.5 μM DEA (B) for 24 h. #, significantly different from AM or DEA alone (repeated measures one-way ANOVA with Student Newman-Keuls post hoc test; $n = 3$ for A and $n = 4$ for B).

requirements, and so on. The ann-V-staining results suggest that the pronounced cytotoxicity of the higher concentrations of AM resulted in a shift in the apoptotic-necrotic continuum, resulting in virtually all cells dying via necrosis. This finding in the nontransformed HPL1A human lung epithelium-derived cells contrasts with the results from A549 human lung adenocarcinoma cells and rat alveolar epithelial cells (Bargout et al., 2000; Yano et al., 2008), in which AM caused a concentration-dependent increase in apoptosis. However, in both the previous studies and the present study, both apoptosis and necrosis were observed after exposure to AM or DEA, and necrosis predominated.

Unlike AM, DEA induced apoptosis in HPL1A cells, although the lowest concentration at which apoptosis was detected differed depending on the assay used (Figs. 2C and 4F). This difference can be attributed to the different end points assayed in that ann-V staining indicates phosphatidylserine externalization, an early event in apoptosis relative to DNA fragmentation, which is detected by TUNEL staining (Jetzek-Zader et al., 2007). This is consistent with the fact that the percentage of TUNEL-positive control cells was lower than the percentage of ann-V-positive control cells (Figs. 2C and 4F). If a longer incubation time were to be tested, the percentage of control cells containing fragmented DNA would be expected to increase. The initial induction of ann-V-positive cells was not sustained at higher concentrations of DEA, which is consistent with the marked toxicity associated with 5 μM DEA, a concentration that caused an even greater proportion of the cells to undergo necrosis. These DEA results are also consistent with those of other studies (Bargout et al.,2000; Waldhauser et al., 2006), in which DEA increased both apoptosis and necrosis.

Taken together, the results from the present study suggest that the cytotoxic pathways induced by AM and DEA differ somewhat in HPL1A cells. AM induces necrotic cell death, whereas DEA induces both necrotic and apoptotic cell death despite being the more potent cytotoxicant. DEA caused an increase in PI-positive cells before an increase in ann-V-positive cells. Because PI enters cells when the plasma membrane becomes permeable during necrosis, this suggests that DEA-induced necrosis occurred before apoptosis and that the two cell death processes are occurring simultaneously in different cell populations, which has been proposed previously (Leist et al., 1997; Yano et al., 2008). This concept is best illustrated in Fig. 3, wherein four cell populations can be observed in each dot plot; of significance are the populations of cells that stained only with PI and the distinct populations of cells that stained only with ann-V, indicating that necrosis and apoptosis were occurring simultaneously but in different cells. This phenomenon is not unique to AM and DEA; for example, in ischemia reperfusion and liver damage induced by toxicants, necrotic and apoptotic cell death occur simultaneously (Leist et al., 1997).

A549 cells possess an intrinsic RAS with the ability to generate Ang II de novo (Wang et al., 1999a). Ang II has been reported to induce concentration-dependent apoptosis in A549 cells (0.01–100 μM Ang II) and in rat primary type II pneumocytes (0.005–100 μM Ang II), an effect that is diminished by the angiotensin-converting enzyme inhibitor captopril (Uhal et al., 1998; Wang et al., 1999b). In the present study, treatment of HPL1A cells with AM or DEA caused a nonsignificant trend toward an increase in angiotensinogen mRNA levels. Based on the equivocal effect of AM and DEA on angiotensinogen expression and the evidence supporting RAS involvement in AM toxicity in other systems, the relevance of the RAS was investigated further. The fact that a very broad range of concentrations of Ang II itself was neither cytotoxic nor able to enhance AM or DEA cytotoxicity strongly suggests that a functional intrinsic RAS linked to cell death does not exist in HPL1A cells. Furthermore, the inability of captopril to prevent AM and DEA cytotoxicity at concentrations that occur in plasma during clinical pharma-

cotherapy (Nonoguchi et al., 2008) and that are inhibitory in other systems (Bargout et al., 2000), further precludes the involvement of the RAS in the cell death caused by those two toxicants in HPL1A cells. Hence, a functional RAS is not requisite for cytotoxicity of AM and DEA at concentrations similar to those used in other cell systems, and is well within the range found in lungs of patients treated clinically with AM (Plomp et al., 1984; Brien et al., 1987). Therefore, a RAS-independent mechanism seems to be responsible for AM and DEA cytotoxicity in nontransformed human lung epithelial cells; however, our findings do not preclude the involvement of Ang II and the RAS in the progression of AIPT in vivo.

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Authorship Contributions

Participated in research design: Mulder, Brien, Racz, and Massey.
Conducted experiments: Mulder.
Contributed new reagents or analytic tools: Takahashi.
Performed data analysis: Mulder.
Wrote or contributed to the writing of the manuscript: Mulder, Brien, and Massey.

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Address correspondence to: Dr. Thomas E. Massey, Department of Pharmacology and Toxicology, Rm. 556, Botterell Hall, Queen's University, Kingston, ON, Canada. E-mail: masseyt@queensu.ca

International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society International Multidisciplinary Classification of Lung Adenocarcinoma

William D. Travis, MD, Elisabeth Brambilla, MD, Masayuki Noguchi, MD, Andrew G. Nicholson, MD, Kim R. Geisinger, MD, Yasushi Yatabe, MD, David G. Beer, PhD, Charles A. Powell, MD, Gregory J. Riely, MD, Paul E. Van Schil, MD, Kavita Garg, MD, John H. M. Austin, MD, Hisao Asamura, MD, Valerie W. Rusch, MD, Fred R. Hirsch, MD, Giorgio Scagliotti, MD, Tetsuya Mitsudomi, MD, Rudolf M. Huber, MD, Yuichi Ishikawa, MD, James Jett, MD, Montserrat Sanchez-Cespedes, PhD, Jean-Paul Sculier, MD, Takashi Takahashi, MD, Masahiro Tsuboi, MD, Johan Vansteenkiste, MD, Ignacio Wistuba, MD, Pan-Chyr Yang, MD, Denise Aberle, MD, Christian Brambilla, MD, Douglas Flieder, MD, Wilbur Franklin, MD, Adi Gazdar, MD, Michael Gould, MD, MS, Philip Hasleton, MD, Douglas Henderson, MD, Bruce Johnson, MD, David Johnson, MD, Keith Kerr, MD, Keiko Kuriyama, MD, Jin Soo Lee, MD, Vincent A. Miller, MD, Iver Petersen, MD, PhD, Victor Roggli, MD, Rafael Rosell, MD, Nagahiro Saijo, MD, Erik Thunnissen, MD, Ming Tsao, MD, and David Yankelewitz, MD

Introduction: Adenocarcinoma is the most common histologic type of lung cancer. To address advances in oncology, molecular biology, pathology, radiology, and surgery of lung adenocarcinoma, an international multidisciplinary classification was sponsored by the International Association for the Study of Lung Cancer, American Thoracic Society, and European Respiratory Society. This new adenocarcinoma classification is needed to provide uniform terminology and diagnostic criteria, especially for bronchioloalveolar carcinoma (BAC), the overall approach to small nonresection cancer specimens, and for multidisciplinary strategic management of tissue for molecular and immunohistochemical studies.

Methods: An international core panel of experts representing all three societies was formed with oncologists/pulmonologists, pathologists, radiologists, molecular biologists, and thoracic surgeons. A systematic review was performed under the guidance of the American Thoracic Society Documents Development and Implementation Committee. The search strategy identified 11,368 citations of which 312 articles met specified eligibility criteria and were retrieved for full text review. A series of meetings were held to discuss the development of the new classification, to develop the recommendations, and to write the current document. Recommendations for key questions were graded by strength and quality of the evidence according to the Grades of Recommendation, Assessment, Development, and Evaluation approach.

Results: The classification addresses both resection specimens, and small biopsies and cytology. The terms BAC and mixed subtype adenocarcinoma are no longer used. For resection specimens, new concepts are introduced such as adenocarcinoma in situ (AIS) and minimally invasive adenocarcinoma (MIA) for small solitary adenocarcinomas with either pure lepidic growth (AIS) or predominant lepidic growth with ≤5 mm invasion (MIA) to define patients who, if they undergo complete resection, will have 100% or near 100% disease-specific survival, respectively. AIS and MIA are usually nonmucinous but rarely may be mucinous. Invasive adenocarcinomas are classified by predominant pattern after using comprehensive histologic subtyping with lepidic (formerly most mixed subtype tumors with nonmucinous BAC), acinar, papillary, and solid patterns; micropapillary is added as a new histologic subtype. Variants include invasive mucinous adenocarcinoma (formerly mucinous BAC), colloid, fetal, and enteric adenocarcinoma. This classification provides guidance for small biopsies and cytology specimens, as approximately 70% of lung cancers are diagnosed in such samples. Non-small cell lung carcinomas (NSCLCs), in patients with advanced-stage disease, are to be classified into more specific types such as adenocarcinoma or squamous cell carcinoma,

Affiliations are listed in the appendix.

Disclosure: Valerie W. Rusch, MD, is an active member of the IASLC Staging Committee. Georgio Scagliotti, MD, has received honoraria from Sanofi Aventis, Roche, Eli Lilly, and Astrozeneca. David Yankelevitz, MD, is a named inventor on a number of patents and patent applications relating to the evaluation of diseases of the chest, including measurement of nodules. Some of these, which are owned by Cornell Research Foundation (CRF) are non-exclusively licensed to General Electric. As an inventor of these patents, Dr. Yankelevitz is entitled to a share of any compensation which CRF may receive from its commercialization of these patents. The other authors declare no conflicts of interest.

Address for correspondence: William Travis, MD, Department of Pathology, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, NY 10065. E-mail: travisw@mskcc.org

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whenever possible for several reasons: (1) adenocarcinoma or NSCLC not otherwise specified should be tested for epidermal growth factor receptor (*EGFR*) mutations as the presence of these mutations is predictive of responsiveness to EGFR tyrosine kinase inhibitors, (2) adenocarcinoma histology is a strong predictor for improved outcome with pemetrexed therapy compared with squamous cell carcinoma, and (3) potential life-threatening hemorrhage may occur in patients with squamous cell carcinoma who receive bevacizumab. If the tumor cannot be classified based on light microscopy alone, special studies such as immunohistochemistry and/or mucin stains should be applied to classify the tumor further. Use of the term NSCLC not otherwise specified should be minimized.

Conclusions: This new classification strategy is based on a multidisciplinary approach to diagnosis of lung adenocarcinoma that incorporates clinical, molecular, radiologic, and surgical issues, but it is primarily based on histology. This classification is intended to support clinical practice, and research investigation and clinical trials. As *EGFR* mutation is a validated predictive marker for response and progression-free survival with EGFR tyrosine kinase inhibitors in advanced lung adenocarcinoma, we recommend that patients with advanced adenocarcinomas be tested for *EGFR* mutation. This has implications for strategic management of tissue, particularly for small biopsies and cytology samples, to maximize high-quality tissue available for molecular studies. Potential impact for tumor, node, and metastasis staging include adjustment of the size T factor according to only the invasive component (1) pathologically in invasive tumors with lepidic areas or (2) radiologically by measuring the solid component of part-solid nodules.

Key Words: Lung, Adenocarcinoma, Classification, Histologic, Pathology, Oncology, Pulmonary, Radiology, Computed tomography, Molecular, *EGFR*, *KRAS*, *EML4-ALK*, Gene profiling, Gene amplification, Surgery, Limited resection, Bronchioloalveolar carcinoma, Lepidic, Acinar, Papillary, Micropapillary, Solid, Adenocarcinoma in situ, Minimally invasive adenocarcinoma, Colloid, Mucinous cystadenocarcinoma, Enteric, Fetal, Signet ring, Clear cell, Frozen section, TTF-1, p63.

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RATIONALE FOR A CHANGE IN THE APPROACH TO CLASSIFICATION OF LUNG ADENOCARCINOMA

Lung cancer is the most frequent cause of major cancer incidence and mortality worldwide.[1,2] Adenocarcinoma is the most common histologic subtype of lung cancer in most countries, accounting for almost half of all lung cancers.[3] A widely divergent clinical, radiologic, molecular, and pathologic spectrum exists within lung adenocarcinoma. As a result, confusion exists, and studies are difficult to compare. Despite remarkable advances in understanding of this tumor in the past decade, there remains a need for universally accepted criteria for adenocarcinoma subtypes, in particular tumors formerly classified as bronchioloalveolar carcinoma (BAC).[4,5] As enormous resources are being spent on trials involving molecular and therapeutic aspects of adenocarcinoma of the lung, the development of standardized criteria is of great importance and should help advance the field, increasing the impact of research, and improving patient care. This classification is needed to assist in determining patient therapy and predicting outcome.

NEED FOR A MULTIDISCIPLINARY APPROACH TO DIAGNOSIS OF LUNG ADENOCARCINOMA

One of the major outcomes of this project is the recognition that the diagnosis of lung adenocarcinoma requires a multidisciplinary approach. The classifications of lung cancer published by the World Health Organization (WHO) in 1967, 1981, and 1999 were written primarily by pathologists for pathologists.[5–7] Only in the 2004 revision, relevant genetics and clinical information were introduced.[4] Nevertheless, because of remarkable advances over the last 6 years in our understanding of lung adenocarcinoma, particularly in area of medical oncology, molecular biology, and radiology, there is a pressing need for a revised classification, based not on pathology alone, but rather on an integrated multidisciplinary platform. In particular, there are two major areas of interaction between specialties that are driving the need for our multidisciplinary approach to classification of lung adenocarcinoma: (1) in patients with advanced non-small cell lung cancer, recent progress in molecular biology and oncology has led to (a) discovery of epidermal growth factor receptor (*EGFR*) mutation and its prediction of response to EGFR tyrosine kinase inhibitors (TKIs) in adenocarcinoma patients[8–11] and (b) the requirement to exclude a diagnosis of squamous cell carcinoma to determine eligibility patients for treatment with pemetrexed, (because of improved efficacy)[12–15] or bevacizumab (because of toxicity)[16,17] and (2) the emergence of radiologic-pathologic correlations between ground-glass versus solid or mixed opacities seen by computed tomography (CT) and BAC versus invasive growth by pathology have opened new opportunities for imaging studies to be used by radiologists, pulmonologists, and surgeons for predicting the histologic subtype of adenocarcinomas,[18–21] patient prognosis,[18–23] and improve preoperative assessment for choice of timing and type of surgical intervention.[18–26]

Although histologic criteria remain the foundation of this new classification, this document has been developed by pathologists in collaboration with clinical, radiology, molecular, and surgical colleagues. This effort has led to the development of terminology and criteria that not only define pathologic entities but also communicate critical information that is relevant to patient management (Tables 1 and 2). The classification also provides recommendations on strategic handling of specimens to optimize the amount of information to be gleaned. The goal is not only longer to solely provide the most accurate diagnosis but also to manage the tissue in a way that immunohistochemical and/or molecular studies can be performed to obtain predictive and prognostic data that will lead to improvement in patient outcomes.

For the first time, this classification addresses an approach to small biopsies and cytology in lung cancer diagnosis (Table 2). Recent data regarding *EGFR* mutation predicting responsiveness to EGFR-TKIs,[8–11] toxicities,[16] and therapeutic efficacy[12–15] have established the importance of distinguishing squamous cell carcinoma from adenocarcinoma and non-small cell lung carcinoma (NSCLC) not otherwise specified (NOS) in patients with advanced lung cancer. Approximately 70% of lung cancers are diagnosed and

TABLE 1. IASLC/ATS/ERS Classification of Lung Adenocarcinoma in Resection Specimens

Preinvasive lesions
Atypical adenomatous hyperplasia
Adenocarcinoma in situ (≤3 cm formerly BAC)
Nonmucinous
Mucinous
Mixed mucinous/nonmucinous
Minimally invasive adenocarcinoma (≤3 cm lepidic predominant tumor with ≤5 mm invasion)
Nonmucinous
Mucinous
Mixed mucinous/nonmucinous
Invasive adenocarcinoma
Lepidic predominant (formerly nonmucinous BAC pattern, with >5 mm invasion)
Acinar predominant
Papillary predominant
Micropapillary predominant
Solid predominant with mucin production
Variants of invasive adenocarcinoma
Invasive mucinous adenocarcinoma (formerly mucinous BAC)
Colloid
Fetal (low and high grade)
Enteric

BAC, bronchioloalveolar carcinoma; IASLC, International Association for the Study of Lung Cancer; ATS, American Thoracic Society; ERS, European Respiratory Society.

staged by small biopsies or cytology rather than surgical resection specimens, with increasing use of transbronchial needle aspiration (TBNA), endobronchial ultrasound-guided TBNA and esophageal ultrasound-guided needle aspiration.[27] Within the NSCLC group, most pathologists can identify well- or moderately differentiated squamous cell carcinomas or adenocarcinomas, but specific diagnoses are more difficult with poorly differentiated tumors. Nevertheless, in small biopsies and/or cytology specimens, 10 to 30% of specimens continue to be diagnosed as NSCLC-NOS.[13,28,29]

Proposed terminology to be used in small biopsies is summarized in Table 2. Pathologists need to minimize the use of the term NSCLC or NSCLC-NOS on small samples and aspiration and exfoliative cytology, providing as specific a histologic classification as possible to facilitate the treatment approach of medical oncologists.[30]

Unlike previous WHO classifications where the primary diagnostic criteria for as many tumor types as possible were based on hematoxylin and eosin (H&E) examination, this classification emphasizes the use and integration of immunohistochemical (i.e., thyroid transcription factor [TTF-1]/p63 staining), histochemical (i.e., mucin staining), and molecular studies, as specific therapies are driven histologic subtyping. Although these techniques should be used whenever possible, it is recognized that this may not always be possible, and thus, a simpler approach is also provided when only H&E-stained slides are available, so this classification may be applicable even in a low resource setting.

METHODOLOGY

Objectives

This international multidisciplinary classification has been produced as a collaborative effort by the International Association for the Study of Lung Cancer (IASLC), the American Thoracic Society (ATS), and the European Respiratory Society. The purpose is to provide an integrated clinical, radiologic, molecular, and pathologic approach to classification of the various types of lung adenocarcinoma that will help to define categories that have distinct clinical, radiologic, molecular, and pathologic characteristics. The goal is to identify prognostic and predictive factors and therapeutic targets.

Participants

Panel members included thoracic medical oncologists, pulmonologists, radiologists, molecular biologists, thoracic surgeons, and pathologists. The supporting associations nominated panel members. The cochairs were selected by the IASLC. Panel members were selected because of special interest and expertise in lung adenocarcinoma and to provide an international and multidisciplinary representation. The panel consisted of a core group (author list) and a reviewer group (Appendix 1, see **Supplemental Digital Content 1** available at http://links.lww.com/JTO/A59, affiliations for coauthors are listed in appendix).

Evidence

The panel performed a systematic review with guidance by members of the ATS Documents Development and Implementation Committee. Key questions for this project were generated by each specialty group, and a search strategy was developed (Appendix 2, see **Supplemental Digital Content 2** available at http://links.lww.com/JTO/A60). Searches were performed in June 2008 with an update in June 2009 resulting in 11,368 citations. These were reviewed to exclude articles that did not have any relevance to the topic of lung adenocarcinoma classification. The remaining articles were evaluated by two observers who rated them by a predetermined set of eligibility criteria using an electronic web-based survey program (www.surveymonkey.com) to collect responses.[31] This process narrowed the total number of articles to 312 that were reviewed in detail for a total of 141 specific features, including 17 study characteristics, 35 clinical, 48 pathologic, 16 radiologic, 16 molecular, and nine surgical (Appendix 2). These 141 features were summarized in an electronic database that was distributed to members of the core panel, including the writing committee. Articles chosen for specific data summaries were reviewed, and based on analysis of tables from this systematic review, recommendations were made according to the Grades of Recommendation, Assessment, Development, and Evaluation (GRADE).[32–37] Throughout the rest of the document, the term GRADE (spelled in capital letters) must be distinguished from histologic grade, which is a measure of pathologic tumor differentiation. The GRADE system has two major components: (1) grading the strength of the recommendation and (2) evaluating the quality of the evidence.[32] The strength of recommendations is based on weighing estimates of benefits versus downsides. Evidence was rated as high, moderate, or low or very low.[32] The

TABLE 2. Proposed IASLC/ATS/ERS Classification for Small Biopsies/Cytology

2004 WHO Classification	SMALL BIOPSY/CYTOLOGY: IASLC/ATS/ERS
ADENOCARCINOMA Mixed subtype Acinar Papillary Solid	*Morphologic adenocarcinoma patterns clearly present:* Adenocarcinoma, describe identifiable patterns present (including micropapillary pattern not included in 2004 WHO classification) Comment: If pure lepidic growth – mention an invasive component cannot be excluded in this small specimen
Bronchioloalveolar carcinoma (nonmucinous)	Adenocarcinoma with lepidic pattern (if pure, add note: an invasive component cannot be excluded)
Bronchioloalveolar carcinoma (mucinous)	Mucinous adenocarcinoma (describe patterns present)
Fetal	Adenocarcinoma with fetal pattern
Mucinous (colloid)	Adenocarcinoma with colloid pattern
Signet ring	Adenocarcinoma with (describe patterns present) and signet ring features
Clear cell	Adenocarcinoma with (describe patterns present) and clear cell features
No 2004 WHO counterpart – most will be solid adenocarcinomas	*Morphologic adenocarcinoma patterns not present (supported by special stains):* Non-small cell carcinoma, favor adenocarcinoma
SQUAMOUS CELL CARCINOMA Papillary Clear cell Small cell Basaloid	*Morphologic squamous cell patterns clearly present:* Squamous cell carcinoma
No 2004 WHO counterpart	*Morphologic squamous cell patterns not present (supported by stains):* Non-small cell carcinoma, favor squamous cell carcinoma
SMALL CELL CARCINOMA	Small cell carcinoma
LARGE CELL CARCINOMA	Non-small cell carcinoma, not otherwise specified (NOS)
Large cell neuroendocrine carcinoma (LCNEC)	Non-small cell carcinoma with neuroendocrine (NE) morphology (positive NE markers), possible LCNEC
Large cell carcinoma with NE morphology (LCNEM)	Non-small cell carcinoma with NE morphology (negative NE markers) – see comment Comment: This is a non-small cell carcinoma where LCNEC is suspected, but stains failed to demonstrate NE differentiation.
ADENOSQUAMOUS CARCINOMA	*Morphologic squamous cell and adenocarcinoma patterns present:* Non-small cell carcinoma, with squamous cell and adenocarcinoma patterns Comment: this could represent adenosquamous carcinoma.
No counterpart in 2004 WHO classification	*Morphologic squamous cell or adenocarcinoma patterns not present but immunostains favor separate glandular and adenocarcinoma components* Non-small cell carcinoma, NOS, (specify the results of the immunohistochemical stains and the interpretation) Comment: this could represent adenosquamous carcinoma.
Sarcomatoid carcinoma	Poorly differentiated NSCLC with spindle and/or giant cell carcinoma (mention if adenocarcinoma or squamous carcinoma are present)

IASLC, International Association for the Study of Lung Cancer; ATS, American Thoracic Society; ERS, European Respiratory Society; WHO, World Health Organization; NSCLC, non-small cell lung cancer; IHC, immunohistochemistry; TTF, thyroid transcription factor.

quality of the evidence expresses the confidence in an estimate of effect or an association and whether it is adequate to support a recommendation. After review of all articles, a writing committee met to develop the recommendations with each specialty group proposing the recommendations, votes for or against the recommendation, and modifications were conducted after multidisciplinary discussion. If randomized trials were available, we started by assuming high quality but down-graded the quality when there were serious methodological limitations, indirectness in population, inconsistency in results, imprecision in estimates, or a strong suspicion of publication bias. If well-done observational studies were available, low-quality evidence was assumed, but the quality was upgraded when there was a large treatment effect or a large association, all plausible residual confounders would diminish the effects, or if there was a dose-response gradient.[36] We developed considerations for good practice related to interventions that usually represent necessary and standard procedures of health care system—such as history taking and physical examination helping patients to make informed decisions, obtaining written consent, or the importance of good communication—when we considered them helpful. In that case, we did not perform a grading of the quality of evidence or strength of the recommendations.[38]

Meetings

Between March 2008 and December 2009, a series of meetings were held, mostly at Memorial Sloan Kettering Cancer Center, in New York, NY, to discuss issues related to

lung adenocarcinoma classification and to formulate this document. The core group established a uniform and consistent approach to the proposed types of lung adenocarcinoma.

Validation

Separate projects were initiated by individuals involved with this classification effort in an attempt to develop data to test the proposed system. These included projects on small biopsies,[39,40] histologic grading,[41–43] stage I adenocarcinomas,[44] small adenocarcinomas from Japan, international multiple pathologist project on reproducibility of recognizing major histologic patterns of lung adenocarcinoma,[45] molecular-histologic correlations, and radiologic-pathologic correlation focused on adenocarcinoma in situ (AIS), and minimally invasive adenocarcinoma (MIA).

The new proposals in this classification are based on the best available evidence at the time of writing this document. Nevertheless, because of the lack of universal diagnostic criteria in the literature, there is a need for future validation studies based on these standardized pathologic criteria with clinical, molecular, radiologic, and surgical correlations.

PATHOLOGIC CLASSIFICATION

Histopathology is the backbone of this classification, but lung cancer diagnosis is a multidisciplinary process requiring correlation with clinical, radiologic, molecular, and surgical information. Because of the multidisciplinary approach in developing this classification, we are recommending significant changes that should improve the diagnosis and classification of lung adenocarcinoma, resulting in therapeutic benefits.

Even after publication of the 1999 and 2004 WHO classifications,[4,5] the former term BAC continues to be used for a broad spectrum of tumors including (1) solitary small noninvasive peripheral lung tumors with a 100% 5-year survival,[46] (2) invasive adenocarcinomas with minimal invasion that have approximately 100% 5-year survival,[47,48] (3) mixed subtype invasive adenocarcinomas,[49–53] (4) mucinous and nonmucinous subtypes of tumors formerly known as BAC,[50–52,54,55] and (5) widespread advanced disease with a very low survival rate.[4,5] The consequences of confusion from the multiple uses of the former BAC term in the clinical and research arenas have been the subject of many reviews and editorials and are addressed throughout this document.[55–61]

Pathology Recommendation 1

We recommend discontinuing the use of the term "BAC." Strong recommendation, low-quality evidence.

Throughout this article, the term BAC (applicable to multiple places in the new classification, Table 3), will be referred to as "former BAC." We understand this will be a major adjustment and suggest initially that when the new proposed terms are used, it will be accompanied in parentheses by "(formerly BAC)." This transition will impact not only clinical practice and research but also cancer registries future analyses of registry data.

CLASSIFICATION FOR RESECTION SPECIMENS

Multiple studies have shown that patients with small solitary peripheral adenocarcinomas with pure lepidic growth may have 100% 5-year disease-free survival.[46,62–68] In addition, a growing number of articles suggest that patients with lepidic predominant adenocarcinomas (LPAs) with minimal invasion may also have excellent survival.[47,48] Recent work has demonstrated that more than 90% of lung adenocarcinomas fall into the mixed subtype according to the 2004 WHO classification, so it has been proposed to use comprehensive histologic subtyping to make a semiquantitative assessment of the percentages of the various histologic components: acinar, papillary, micropapillary, lepidic, and solid and to classify tumors according to the predominant histologic subtype.[69] This has demonstrated an improved ability to address the complex histologic heterogeneity of lung adenocarcinomas and to improve molecular and prognostic correlations.[69]

The new proposed lung adenocarcinoma classification for resected tumors is summarized in Table 1.

TABLE 3. Categories of New Adenocarcinoma Classification Where Former BAC Concept was Used

1. Adenocarcinoma in situ (AIS), which can be nonmucinous and rarely mucinous
2. Minimally invasive adenocarcinoma (MIA), which can be nonmucinous and rarely mucinous
3. Lepidic predominant adenocarcinoma (nonmucinous)
4. Adenocarcinoma, predominantly invasive with some nonmucinous lepidic component (includes some resected tumors, formerly classified as mixed subtype, and some clinically advanced adenocarcinomas formerly classified as nonmucinous BAC)
5. Invasive mucinous adenocarcinoma (formerly mucinous BAC)

BAC, bronchioloalveolar carcinoma.

Preinvasive Lesions

In the 1999 and 2004 WHO classifications, atypical adenomatous hyperplasia (AAH) was recognized as a preinvasive lesion for lung adenocarcinoma. This is based on multiple studies documenting these lesions as incidental findings in the adjacent lung parenchyma in 5 to 23% of resected lung adenocarcinomas[70–74] and a variety of molecular findings that demonstrate a relationship to lung adenocarcinoma including clonality,[75,76] *KRAS* mutation,[77,78] *KRAS* polymorphism,[79] *EGFR* mutation,[80] p53 expression,[81] loss of heterozygosity,[82] methylation,[83] telomerase overexpression,[84] eukaryotic initiation factor 4E expression,[85] epigenetic alterations in the *Wnt* pathway,[86] and FHIT expression.[87] Depending on the extensiveness of the search, AAH may be multiple in up to 7% of resected lung adenocarcinomas.[71,88]

A major change in this classification is the official recognition of AIS, as a second preinvasive lesion for lung adenocarcinoma in addition to AAH. In the category of preinvasive lesions, AAH is the counterpart to squamous dysplasia and AIS the counterpart to squamous cell carcinoma in situ.

Atypical Adenomatous Hyperplasia

AAH is a localized, small (usually 0.5 cm or less) proliferation of mildly to moderately atypical type II pneumocytes and/or Clara cells lining alveolar walls and sometimes, respiratory bronchioles (Figures 1*A*, *B*).[4,89,90] Gaps are

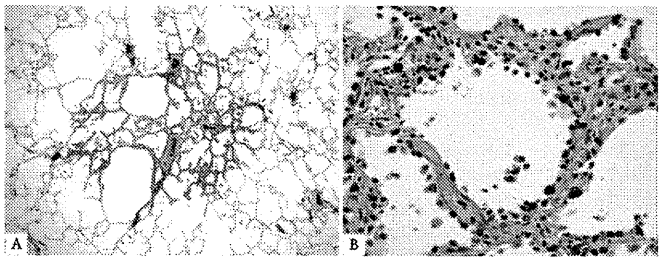

FIGURE 1. Atypical adenomatous hyperplasia. *A*, This 3-mm nodular lesion consists of atypical pneumocytes proliferating along preexisting alveolar walls. There is no invasive component. *B*, The slightly atypical pneumocytes are cuboidal and show gaps between the cells. Nuclei are hyperchromatic, and a few show nuclear enlargement and multinucleation.

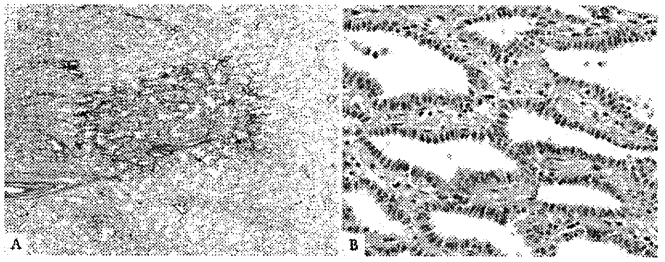

FIGURE 2. Nonmucinous adenocarcinoma in situ. *A*, This circumscribed nonmucinous tumor grows purely with a lepidic pattern. No foci of invasion or scarring are seen. *B*, The tumor shows atypical pneumocytes proliferating along the slightly thickened, but preserved, alveolar walls.

usually seen between the cells, which consist of rounded, cuboidal, low columnar, or "peg" cells with round to oval nuclei (Figure 1*B*). Intranuclear inclusions are frequent. There is a continuum of morphologic changes between AAH and AIS.[4,89,90] A spectrum of cellularity and atypia occurs in AAH. Although some have classified AAH into low- and high-grade types,[84,91] grading is not recommended.[4] Distinction between more cellular and atypical AAH and AIS can be difficult histologically and impossible cytologically.

AIS, Nonmucinous, and/or Mucinous

AIS (one of the lesions formerly known as BAC) is a localized small (≤3 cm) adenocarcinoma with growth restricted to neoplastic cells along preexisting alveolar structures (lepidic growth), lacking stromal, vascular, or pleural invasion. Papillary or micropapillary patterns and intraalveolar tumor cells are absent. AIS is subdivided into nonmucinous and mucinous variants. Virtually, all cases of AIS are nonmucinous, consisting of type II pneumocytes and/or Clara cells (Figures 2*A, B*). There is no recognized clinical significance to the distinction between type II or Clara cells, so this morphologic separation is not recommended. The rare cases of mucinous AIS consist of tall columnar cells with basal nuclei and abundant cytoplasmic mucin; sometimes they resemble goblet cells (Figures 3*A, B*). Nuclear atypia is absent or inconspicuous in both nonmucinous and mucinous

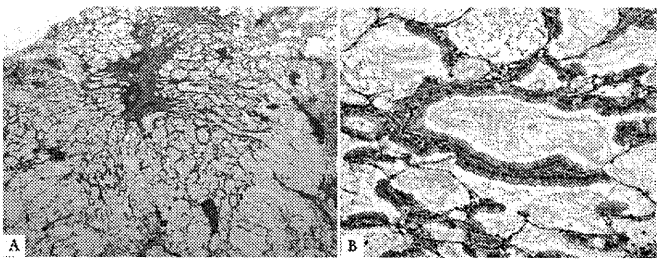

FIGURE 3. Mucinous adenocarcinoma in situ. *A*, This mucinous AIS consists of a nodular proliferation of mucinous columnar cells growing in a purely lepidic pattern. Although there is a small central scar, no stromal or vascular invasion is seen. *B*, The tumor cells consist of cuboidal to columnar cells with abundant apical mucin and small basally oriented nuclei. AIS, adenocarcinoma in situ.

AIS (Figures 2*B* and 3*B*). Septal widening with sclerosis is common in AIS, particularly the nonmucinous variant.

Tumors that meet criteria for AIS have formerly been classified as BAC according to the strict definition of the 1999 and 2004 WHO classifications and type A and type B adenocarcinoma according to the 1995 Noguchi classification.[4,46] Multiple observational studies on solitary lung adenocarcinomas with pure lepidic growth, smaller than either 2 or 3 cm have documented 100% disease-free survival.[46,62–68] Although most of these tumors are nonmucinous, 2 of the 28 tumors reported by Noguchi as types A and B in the 1995 study were mucinous.[46] Small size (≤3 cm) and a discrete circumscribed border are important to exclude cases with miliary spread into adjacent lung parenchyma and/or lobar consolidation, particularly for mucinous AIS.

Pathology Recommendation 2

For small (≤3 cm), solitary adenocarcinomas with pure lepidic growth, we recommend the term "Adenocarcinoma in situ" that defines patients who should have 100% disease-specific survival, if the lesion is completely resected (strong recommendation, moderate quality evidence).

Remark: Most AIS are nonmucinous, rarely are they mucinous.

MIA, Nonmucinous, and/or Mucinous

MIA is a small, solitary adenocarcinoma (≤3 cm), with a predominantly lepidic pattern and ≤5 mm invasion in greatest dimension in any one focus.[47,48,92] MIA is usually nonmucinous (Figures 4*A–C*) but rarely may be mucinous (Figures 5*A, B*).[44] MIA is, by definition, solitary and discrete. The criteria for MIA can be applied in the setting of multiple tumors only if the other tumors are regarded as synchronous primaries rather than intrapulmonary metastases.

The invasive component to be measured in MIA is defined as follows: (1) histological subtypes other than a lepidic pattern (i.e., acinar, papillary, micropapillary, and/or solid) or (2) tumor cells infiltrating myofibroblastic stroma. MIA is excluded if the tumor (1) invades lymphatics, blood vessels, or pleura or (2) contains tumor necrosis. If multiple microinvasive areas are found in one tumor, the size of the largest invasive area should be measured in the largest dimension, and it should be ≤5 mm

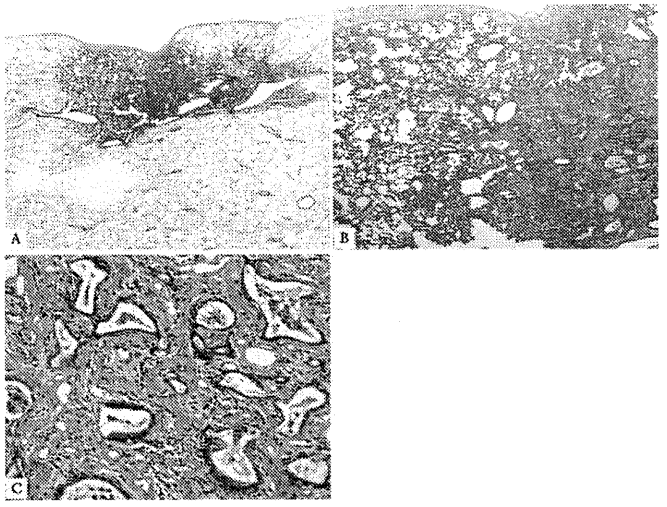

FIGURE 4. Nonmucinous minimally invasive adenocarcinoma. *A*, This subpleural adenocarcinoma tumor consists primarily of lepidic growth with a small (<0.5 cm) central area of invasion. *B*, To the left is the lepidic pattern and on the right is an area of acinar invasion. *C*, These acinar glands are invading in the fibrous stroma.

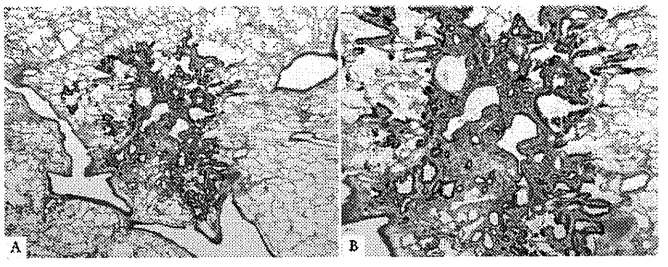

FIGURE 5. Mucinous minimally invasive adenocarcinoma. *A*, This mucinous MIA consists of a tumor showing lepidic growth and a small (<0.5 cm) area of invasion. *B*, The tumor cells consist of mucinous columnar cells growing mostly in a lepidic pattern along the surface of alveolar walls. The tumor invades the areas of stromal fibrosis in an acinar pattern. MIA, minimally invasive adenocarcinoma.

in size. The size of invasion is not the summation of all such foci, if more than one occurs. If the manner of histologic sectioning of the tumor makes it impossible to measure the size of invasion, an estimate of invasive size can be made by multiplying the total percentage of the invasive (nonlepidic) components times the total tumor size.

Evidence for a category of MIA with 100% disease-free survival can be found in the 1995 article by Noguchi et al., where vascular or pleural invasion was found in 10% of the small solitary lung adenocarcinomas that otherwise met the former definition of pure BAC. Even these focally invasive tumors also showed 100% disease-free survival.[46] Subsequent articles by Suzuki et al. and Sakurai et al.[19,21] defined subsets of small lung adenocarcinomas with 100% disease-free survival using scar size less than 5 mm and stromal invasion in the area of bronchioloalveolar growth, respectively. More recently, articles by Yim et al., Borczuk et al., and Maeshima et al.[47,48,92] have described patients with MIA defined similar to the above criteria, and these have demonstrated near 100% disease specific or very favorable overall survival. There is very limited data regarding mucinous MIA; however, this seems to exist. A mucinous MIA with a minor mixture of a nonmucinous component is being reported.[44] The recent report by Sawada et al.[93] of localized mucinous BAC may have included a few cases of mucinous AIS or MIA, but details of the pathology are not specific enough to be certain. A recent series of surgically resected solitary mucinous BAC did not document histologically whether focal invasion was present or not, so AIS versus MIA status cannot be determined, but all eight patients with tumors measuring ≤3 cm had 100% overall 5-year survival rates.[94] Presentation as a solitary mass, small size, and a discrete circumscribed border is important to exclude cases of miliary involvement of adjacent lung parenchyma and/or lobar consolidation, particularly for mucinous AIS.

Pathology Recommendation 3

For small (≤3 cm), solitary, adenocarcinomas with predominant lepidic growth and small foci of invasion measuring ≤0.5 cm, we recommend a new concept of "Minimally invasive adenocarcinoma" to define patients who have near 100%, disease-specific survival, if completely resected (strong recommendation, low-quality evidence).

Remark: Most MIA are nonmucinous, rarely are they mucinous.

Tumor Size and Specimen Processing Issues for AIS and MIA

The diagnosis of AIS or MIA cannot be firmly established without entire histologic sampling of the tumor. If tumor procurement is performed, it should be done strategically as discussed in the molecular section.

Because most of the literature on the topic of AIS and MIA deal with tumors 2.0 or 3.0 cm or less, there is insufficient evidence to support that 100% disease-free survival can occur in completely resected, solitary tumors suspected to be AIS or MIA that are larger than 3.0 cm. Until data validate 100% disease-free survival for completely resected, solitary, adenocarcinomas larger than 3.0 cm suspected to be AIS or MIA after complete sampling, the term "lepidic predominant adenocarcinoma, suspect AIS or MIA" is suggested. In such a tumor larger than 3.0 cm, particularly if it has not been completely sampled, the term "lepidic predominant adenocarcinoma" is best applied with a comment that the clinical behavior is uncertain and/or that an invasive component cannot be excluded.

Invasive Adenocarcinoma

As invasive adenocarcinomas represent more than 70 to 90% of surgically resected lung cases, one of the most important aspects of this classification is to present a practical way to address these tumors that are composed of a complex heterogeneous mixture of histologic subtypes. This complex mixture of histologic subtypes has presented one of the greatest challenges to classification of invasive lung adenocarcinomas. In recent years, multiple independent research groups have begun to classify lung adenocarcinomas according to the most predominant subtype.[43,44,69,95–102] This approach provides better stratifi-

cation of the "mixed subtype" lung adenocarcinomas according to the 1999/2004 WHO Classifications and has allowed for novel correlations between histologic subtypes and both molecular and clinical features.[43,44,69,95–102]

In the revised classification, the term "predominant" is appended to all categories of invasive adenocarcinoma, as most of these tumors consist of mixtures of the histologic subtypes (Figures 6*A–C*). This replaces the use of the term adenocarcinoma, mixed subtype. Semiquantitative recording of the patterns in 5% increments encourages the observer to identify all patterns that may be present, rather than focusing on a single pattern (i.e., lepidic growth). This method provides a basis for choosing the predominant pattern. Although most previous studies on this topic used 10% increments, using 5% allows for greater flexibility in choosing a predominant subtype when tumors have two patterns with relatively similar percentages; it also avoids the need to use 10% for small amounts of components that may be prognostically important such as micropapillary or solid patterns. Recording of these percentages also makes it clear to the reader of a report when a tumor has relatively even mixtures of several patterns versus a single dominant pattern. In addition, it provides a way to compare the histology of multiple adenocarcinomas (see later).[102] This approach may also provide a basis for architectural grading of lung adenocarcinomas.[43] A recent reproducibility study of classical and difficult selected images of the major lung adenocarcinoma subtypes circulated among a panel of 26 expert lung cancer pathologists documented kappa values of 0.77 ± 0.07 and 0.38 ± 0.14, respectively.[45] This study did not test recognition of predominant subtype.

Pathology Recommendation 4

For invasive adenocarcinomas, we suggest comprehensive histologic subtyping be used to assess histologic patterns semiquantitatively in 5% increments, choosing a single predominant pattern. Individual tumors are then classified according to the predominant pattern and the percentages of the subtypes are also reported (weak recommendation, low-quality evidence).

Histologic Comparison of Multiple Adenocarcinomas and Impact on Staging

Comprehensive histologic subtyping can be useful in comparing multiple lung adenocarcinomas to distinguish multiple primary tumors from intrapulmonary metastases. This has a great impact on staging for patients with multiple lung adenocarcinomas. Recording the percentages of the various histologic types in 5% increments, not just the most predominant type, allows these data to be used to compare multiple adenocarcinomas, particularly if the slides of a previous tumor are not available at the time of review of the additional lung tumors.[102] In addition to comprehensive histologic subtyping, other histologic features of the tumors such as cytologic (clear cell or signet ring features) or stromal (desmoplasia or inflammation) characteristics may be helpful to compare multiple tumors.[102]

Pathology Recommendation 5

In patients with multiple lung adenocarcinomas, we suggest comprehensive histologic subtyping may facilitate in the comparison of the complex, heterogeneous mixtures of histologic patterns to determine whether the tumors are metastases or separate synchronous or metachronous primaries (weak recommendation, low-quality evidence).

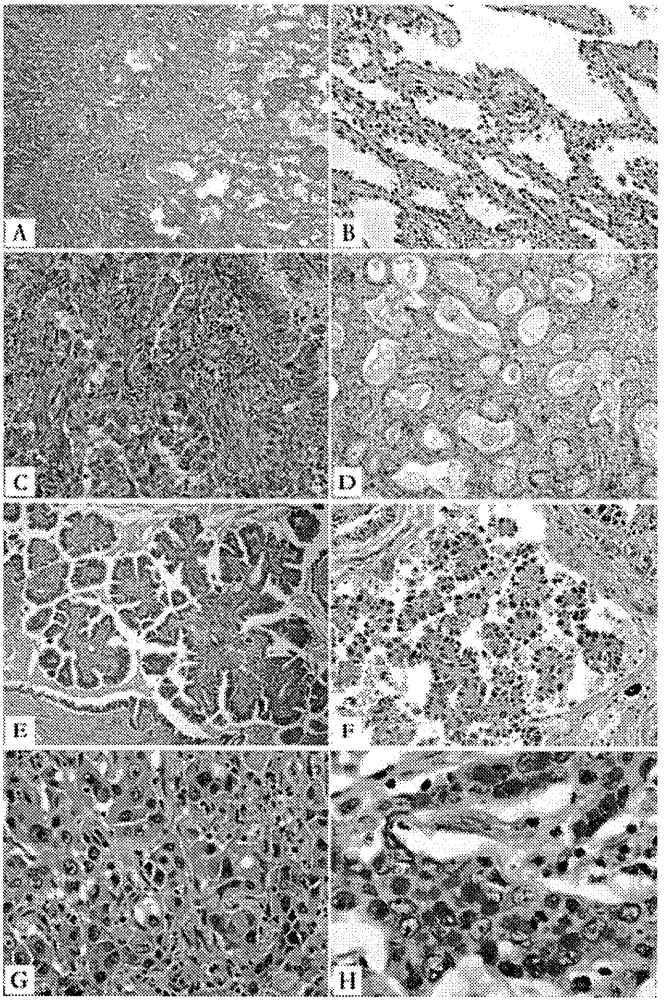

FIGURE 6. Major histologic patterns of invasive adenocarcinoma. *A*, Lepidic predominant pattern with mostly lepidic growth (right) and a smaller area of invasive acinar adenocarcinoma (left). *B*, Lepidic pattern consists of a proliferation type II pneumocytes and Clara cells along the surface alveolar walls. *C*, Area of invasive acinar adenocarcinoma (same tumor as in *A* and *B*). *D*, Acinar adenocarcinoma consists of round to oval-shaped malignant glands invading a fibrous stroma. *E*, Papillary adenocarcinoma consists of malignant cuboidal to columnar tumor cells growing on the surface of fibrovascular cores. *F*, Micropapillary adenocarcinoma consists of small papillary clusters of glandular cells growing within this airspace, most of which do not show fibrovascular cores. *G*, Solid adenocarcinoma with mucin consisting of sheets of tumor cells with abundant cytoplasm and mostly vesicular nuclei with several conspicuous nucleoli. No acinar, papillary, or lepidic patterns are seen, but multiple cells have intracytoplasmic basophilic globules that suggest intracytoplasmic mucin. *H*, Solid adenocarcinoma with mucin. Numerous intracytoplasmic droplets of mucin are highlighted with this DPAS stain. DPAS, diastase-periodic acid Schiff.

LPA typically consists of bland pneumocytic cells (type II pneumocytes or Clara cells) growing along the surface of alveolar walls similar to the morphology defined in the above section on AIS and MIA (Figures 6*A*, *B*). Invasive adenocarcinoma is present in at least one focus measuring more than 5 mm in greatest dimension. Invasion is defined as (1) histological subtypes other than a lepidic pattern (i.e., acinar, papillary, micropapillary, and/or solid) or (2) myofibroblastic stroma associated with invasive tumor cells (Figure 6*C*). The diagnosis of LPA rather than MIA is made if the tumor (1) invades lymphatics, blood vessels, or pleura or (2) contains tumor necrosis. It is understood that lepidic growth can occur in metastatic tumors and invasive mucinous adenocarcinomas. Nevertheless, the specific term "Lepidic predominant adenocarcinoma (LPA)" in this classification defines a nonmucinous adenocarcinoma that has lepidic growth as its predominant component, and these tumors are now separated from invasive mucinous adenocarcinoma. The term LPA should not be used in the context of invasive mucinous adenocarcinoma with predominant lepidic growth.

In the categories of mixed subtype in the 1999/2004 WHO classifications and type C in the Noguchi classification,[4,46] there was no assessment of the percentage of lepidic growth (former BAC pattern), so in series diagnosed according to these classification systems, most of the LPAs are buried among a heterogeneous group of tumors that include predominantly invasive adenocarcinomas. Nevertheless, several studies have shown lepidic growth to be associated with more favorable survival in small solitary resected lung adenocarcinomas with an invasive component.[47,64,103–105] One recent study of stage I adenocarcinomas using this approach demonstrated 90% 5-year recurrence free survival.[44]

Pathology Recommendation 6

For nonmucinous adenocarcinomas previously classified as mixed subtype where the predominant subtype consists of the former nonmucinous BAC, we recommend use of the term LPA and discontinuing the term "mixed subtype" (strong recommendation, low-quality evidence).

Acinar predominant adenocarcinoma shows a majority component of glands, which are round to oval shaped with a central luminal space surrounded by tumor cells (Figure 6*D*).[4] The neoplastic cells and glandular spaces may contain mucin. Acinar structures also may consist of rounded aggregates of tumor cells with peripheral nuclear polarization with central cytoplasm without a clear lumen. AIS with collapse may be difficult to distinguish from the acinar pattern. Nevertheless, when the alveolar architecture is lost and/or myofibroblastic stroma is present, invasive acinar adenocarcinoma is considered present. Cribriform arrangements are regarded as a pattern of acinar adenocarcinoma.[106]

Papillary predominant adenocarcinoma shows a major component of a growth of glandular cells along central fibrovascular cores (Figure 6*E*).[4] This should be distinguished from tangential sectioning of alveolar walls in AIS. If a tumor has lepidic growth, but the alveolar spaces are filled with papillary structures, the tumor is classified as papillary adenocarcinoma. Myofibroblastic stroma is not needed to diagnose this pattern.

Micropapillary predominant adenocarcinoma has tumor cells growing in papillary tufts, which lack fibrovascular cores (Figure 6*F*).[4] These may appear detached and/or connected to alveolar walls. The tumor cells are usually small and cuboidal with minimal nuclear atypia. Ring-like glandular structures may "float" within alveolar spaces. Vascular invasion and stromal invasion are frequent. Psammoma bodies may be seen.

The micropapillary pattern of lung adenocarcinoma was cited in the 2004 WHO classification in the discussion,[4] but there were too few publications on this topic to introduce it as a formal histologic subtype.[107–109] Although most of the studies have used a very low threshold for classification of adenocarcinomas as micropapillary, including as low as 1 to 5%,[108,109] it has recently been demonstrated that tumors classified as micropapillary according to the predominant subtype also have a poor prognosis similar to adenocarcinomas with a predominant solid subtype.[44] All articles on the topic of micropapillary lung adenocarcinoma in early-stage patients have reported data indicating that this is a poor prognostic subtype.[95,108–119] Additional evidence for the aggressive behavior of this histologic pattern is the overrepresentation of the micropapillary pattern in metastases compared with the primary tumors, where it sometimes comprises only a small percentage of the overall tumor.[43]

Pathology Recommendation 7

In patients with early-stage adenocarcinoma, we recommend the addition of "micropapillary predominant adenocarcinoma," when applicable, as a major histologic subtype due to its association with poor prognosis (strong recommendation, low-quality evidence).

Solid predominant adenocarcinoma with mucin production shows a major component of polygonal tumor cells forming sheets, which lack recognizable patterns of adenocarcinoma, i.e., acinar, papillary, micropapillary, or lepidic growth (Figure 6*G*).[4] If the tumor is 100% solid, intracellular mucin should be present in at least five tumor cells in each of two high-power fields, confirmed with histochemical stains for mucin (Figure 6*H*).[4] Solid adenocarcinoma must be distinguished from squamous cell carcinomas and large cell carcinomas both of which may show rare cells with intracellular mucin.

Variants

Rationale for Changes in Adenocarcinoma Histologic Variants

Rationale for separation of invasive mucinous adenocarcinoma (formerly mucinous BAC) from nonmucinous adenocarcinomas. Multiple studies indicate that tumors formerly classified as mucinous BAC have major clinical, radiologic, pathologic, and genetic differences from the tumors formerly classified as nonmucinous BAC (Table 4).[55,77,120,121,125–127,136,145–148] In particular, these tumors show a very strong correlation with *KRAS* mutation, whereas nonmucinous adenocarcinomas are more likely to show *EGFR* mutation and only occasionally *KRAS* mutation (Table 4). Therefore, in

TABLE 4. Difference between Invasive Mucinous Adenocarcinoma and Nonmucinous Adenocarcinoma In Situ/Minimally Invasive Adenocarcinoma/Lepidic Predominant Adenocarcinoma

	Invasive Mucinous Adenocarcinoma (Formerly Mucinous BAC)	Nonmucinous AIS/MIA/LPA (Formerly Nonmucinous BAC)
Female	49/84 (58%)[52,120–123]	101/140 (72%)[52,120–123]
Smoker	39/87 (45%)[52,120–122,124]	75/164 (46%)[52,120–122,124]
Radiographic appearance	Majority consolidation; air bronchogram[125] Frequent multifocal and multilobar presentation[56,125–128]	Majority ground-glass attenuation[23,56,58,103,129–134]
Cell type	Mucin-filled, columnar, and/or goblet[50–52,125,135]	Type II pneumocyte and/or Clara cell[50–52,125,135]
Phenotype		
CK7	Mostly positive (~88%)[a54,55,136–139]	Positive (~98%)[a54,55,136–139]
CK20	Positive (~54%)[a54,55,136–139]	Negative (~5%)[a54,55,136–139]
TTF-1	Mostly negative (~17%)1[a54,55,120,137–139]	Positive (~67%)[a54,55,120,137–139]
Genotype		
KRAS mutation	Frequent (~76%)[a55,94,121,127,140–144]	Some (~13%)[a55,121,127,140–144]
EGFR mutation	Almost none (~3)[a55,121,127,140–142]	Frequent (~45%)[a55,121,127,140–142]

[a] Numbers represent the percentage of cases that are reported to be positive.

BAC, bronchioloalveolar carcinoma; AIS, adenocarcinoma in situ; MIA, minimally invasive adenocarcinoma; LPA, lepidic predominant adenocarcinoma; EGFR, epidermal growth factor receptor; TTF, thyroid transcription factor.

the new classification, these tumors are now separated into different categories (Table 1). The neoplasms formerly termed mucinous BAC, now recognized to have invasive components in the majority of cases, are classified as invasive mucinous adenocarcinoma (formerly mucinous BAC).[149]

Rationale for including mucinous cystadenocarcinoma in colloid adenocarcinoma. Tumors formerly classified as "Mucinous cystadenocarcinoma" are very rare, and they probably represent a spectrum of colloid adenocarcinoma. Therefore, we suggest that these adenocarcinomas that consist of uni- or oligolocular cystic structures by imaging and/or gross examination be included in the category of colloid adenocarcinoma.[150] For such tumors, a comment could be made that the tumor resembles that formerly classified as mucinous cystadenocarcinoma.

Rationale for removing clear cell and signet ring carcinoma as adenocarcinoma subtypes. Clear cell and signet ring cell features are now regarded as cytologic changes that may occur in association with multiple histologic patterns.[151,152] Thus, their presence and extent should be recorded, but data are not available that show a clinical significance beyond a strong association with the solid subtype. They are not considered to be specific histologic subtypes, although associations with molecular features are possible such as the recent observation of a solid pattern with more than 10% signet ring cell features in up to 56% of tumors from patients with echinoderm microtubule-associated protein-like 4 (*EML4*) and anaplastic lymphoma kinase (ALK) gene fusions (*EML4-ALK*).[153]

Rationale for adding enteric adenocarcinoma. Enteric adenocarcinoma is added to the classification to draw attention to this rare histologic type of primary lung adenocarcinoma that can share some morphologic and immunohistochemical features with colorectal adenocarcinoma.[154] Because of these similarities, clinical evaluation is needed to exclude a gastrointestinal primary. It is not known whether there are any distinctive clinical or molecular features.

Histologic Features

Invasive mucinous adenocarcinoma (formerly mucinous BAC) has a distinctive histologic appearance with tumor cells having a goblet or columnar cell morphology with abundant intracytoplasmic mucin (Figures 7*A, B*). Cytologic atypia is usually inconspicuous or absent. Alveolar spaces often contain mucin. These tumors may show the same heterogeneous mixture of lepidic, acinar, papillary, micropapillary, and solid growth as in nonmucinous tumors. The clinical significance of reporting semiquantitative estimates of subtype percentages and the predominant histologic subtype similar to nonmucinous adenocarcinomas is not certain. When stromal invasion is seen, the malignant cells may show less cytoplasmic mucin and more atypia. These tumors differ from mucinous AIS and MIA by one or more of the following criteria: size (>3 cm), amount of invasion (>0.5 cm), mul-

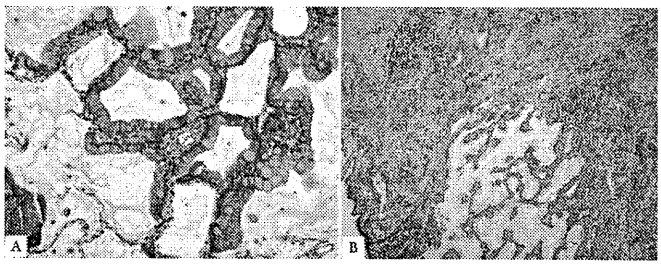

FIGURE 7. Invasive mucinous adenocarcinoma. *A,* This area of invasive mucinous adenocarcinoma demonstrates a pure lepidic growth. The tumor consists of columnar cells filled with abundant mucin in the apical cytoplasm and shows small basal oriented nuclei. *B,* Nevertheless, elsewhere this tumor demonstrated invasion associated with desmoplastic stroma and an acinar pattern.

tiple nodules, or lack of a circumscribed border with miliary spread into adjacent lung parenchyma.

There is a strong tendency for multicentric, multilobar, and bilateral lung involvement, which may reflect aerogenous spread. Mixtures of mucinous and nonmucinous tumors may rarely occur; then the percentage of invasive mucinous adenocarcinoma should be recorded in a comment. If there is at least 10% of each component, it should be classified as "Mixed mucinous and nonmucinous adenocarcinoma." Invasive mucinous adenocarcinomas (formerly mucinous BAC) need to be distinguished from adenocarcinomas that produce mucin but lack the characteristic goblet cell or columnar cell morphology of the tumors that have historically been classified as mucinous BAC. When mucin is identified by light microscopy or mucin stains in adenocarcinomas that do not meet the above criteria, this feature should be reported in a comment after classifying the tumor according to the appropriate terminology and criteria proposed in this classification. This can be done by adding a descriptive phrase such as "with mucin production" or "with mucinous features" rather than the term "invasive mucinous adenocarcinoma."

Pathology Recommendation 8

For adenocarcinomas formerly classified as mucinous BAC, we recommend they be separated from the adenocarcinomas formerly classified as nonmucinous BAC and depending on the extent of lepidic versus invasive growth that they be classified as mucinous AIS, mucinous MIA, or for overtly invasive tumors "invasive mucinous adenocarcinoma" (weak recommendation, low-quality evidence).

Colloid adenocarcinoma shows extracellular mucin in abundant pools, which distend alveolar spaces with destruction of their walls (Figure 8*A*). The mucin pools contain clusters of mucin-secreting tumor cells, which may comprise only a small percentage of the total tumor and, thus, be inconspicuous (Figure 8*A*).[155,156] The tumor cells may consist of goblet cells or other mucin secreting cells. Colloid adenocarcinoma is found more often as a mixture with other adenocarcinoma histologic subtypes rather than as a pure pattern. A tumor is classified as a colloid adenocarcinoma when it is the predominant component; the percentages of other components should be recorded.[150] Cystic gross and histologic features are included in the spectrum of colloid adenocarcinoma, but in most cases, this is a focal feature. Cases previously reported as mucinous cystadenocarcinoma are extremely rare, and now these should be classified as colloid adenocarcinoma with cystic changes. The cysts are filled with mucin and lined by goblet or other mucin secreting cells (Figure 8*B*). The lining epithelium may be discontinuous and replaced with inflammation including a granulomatous reaction or granulation tissue. Cytologic atypia of the neoplastic epithelium is usually minimal.[157]

Fetal adenocarcinoma consists of glandular elements with tubules composed of glycogen-rich, nonciliated cells that resemble fetal lung tubules (Figure 8*C*).[4] Subnuclear vacuoles are common and characteristic. Squamoid morules may be seen within lumens. Most are low grade with a favorable outcome. High-grade tumors occur. When mixtures

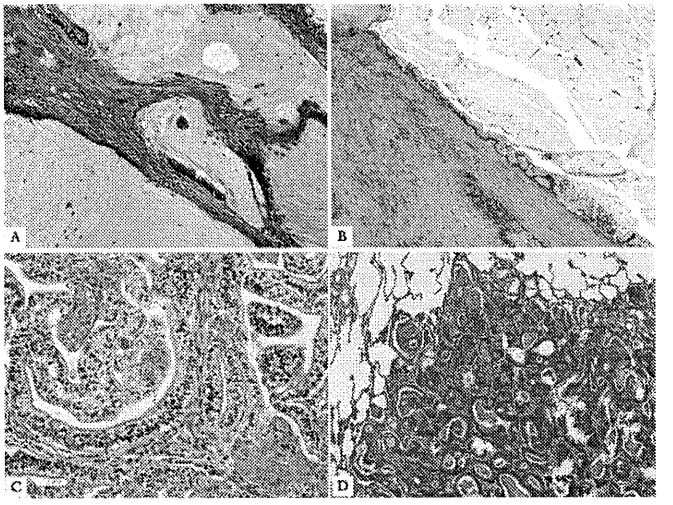

FIGURE 8. Adenocarcinoma, variants. *A*, Colloid adenocarcinoma consists of abundant pools of mucin growing within and distending airspaces. Focally well-differentiated mucinous glandular epithelium grows along the surface of fibrous septa and within the pools of mucin. Tumor cells may be very inconspicuous. *B*, This colloid adenocarcinoma contains a cystic component surrounded by a fibrous wall that is filled with pools of mucin; such a pattern was previously called mucinous cystadenocarcinoma. The surface of the fibrous wall is lined by well-differentiated cuboidal or columnar mucinous epithelium. *C*, Fetal adenocarcinoma consists of malignant glandular cells growing in tubules and papillary structures. These tumor cells have prominent clear cytoplasm, and squamoid morules are present. *D*, Enteric adenocarcinoma consists of an adenocarcinoma that morphologically resembles colonic adenocarcinoma with back-to-back angulated acinar structures. The tumor cells are cuboidal to columnar with nuclear pseudostratification.

occur with other histologic subtypes, the tumor should be classified according to the predominant component.[158] This tumor typically occurs in younger patients than other adenocarcinomas. Uniquely, these tumors appear driven by mutations in the beta-catenin gene, and the epithelial cells express aberrant nuclear and cytoplasmic staining with this antibody by immunohistochemistry.[159,160] Nakatani et al. and Sekine et al.[159,160] have suggested that up-regulation of components in the Wnt signaling pathway such as β-catenin is important in low-grade fetal adenocarcinomas and in biphasic pulmonary blastomas in contrast to high-grade fetal adenocarcinomas.

Enteric differentiation can occur in lung adenocarcinoma, and when this component exceeds 50%, the tumor is classified as pulmonary adenocarcinoma with enteric differentiation. The enteric pattern shares morphologic and immunohistochemical features with colorectal adenocarcinoma.[154] In contrast to metastatic colorectal adenocarcinoma, these tumors are histologically heterogeneous with some component that resembles primary lung adenocarcinoma such as lepidic growth. Recording of the percentages of these other components may be useful. The enteric pattern consists of glandular and/or papillary structures sometimes with a cribriform pattern, lined by tumor cells that are mostly tall-