Human Lung Tumor–associated Antigen Identified as an Extracellular Matrix Adhesion Molecule

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Summary

A single chain glycoprotein with an estimated molecular mass of 160 kD (gp160) was previously identified as a human lung tumor–associated antigen. This tumor marker is shown here to be associated noncovalently with a second 130-kD protein. Sequential immunoprecipitation studies of surface iodinated lung tumor cell lysates reveal that this heterodimeric complex is indistinguishable serologically and structurally from the integrin VLA-2, found originally on activated T lymphocytes and platelets. The VLA-2–like complex expressed on the lung tumors possesses similar characteristic Mg^{2+} dependent binding of collagen and laminin as observed with VLA-2 on normal cells. RNA analysis indicates that human lung tumors express at least 20 times more VLA-2 α chain message than normal adult human lung tissue. The results presented here raise the possibility that the overproduction of VLA-2 may be involved in the pathogenesis of human lung tumors by modulating the invasive and metastatic potential of the tumor.

In view of the fact that primary cancers of the lung are the leading cause of cancer related deaths in men and women in the United States, it is surprising that there are relatively few reports on the molecular and functional characterization of human lung tumor–associated antigens (TAA)[1] (1–5). These reports have provided limited characterizations of the molecules with regard to structure, and none of these studies have been able to postulate a possible functional role of the TAA with respect to tumor pathogenesis. Previous studies using a mAb, 5E8, derived from the fusion of spleen cells from mice repeatedly immunized with fresh human lung tumor biopsy tissue, led to the identification of a cell surface glycoprotein (gp160) that was associated with a high proportion of various histological types of human lung cancers (6, 7). This TAA has been detected on 75–80% of the non–small cell human lung cancer biopsies and lung tumor cell lines, and was originally characterized as a single polypeptide chain with an estimated molecular mass of 160 kD (7), with characteristics and distribution patterns similar to the epidermal growth factor receptor (8–10). Our objective here was to characterize this molecule more completely in order to gain some insight regarding its molecular identity and possible function. The results presented here establish that gp160 is a subunit of a heterodimeric complex that is indistinguishable from a collagen binding integrin, with regard to subunit structure and ligand specificity, and that the expression of this molecule is upregulated in non–small cell lung tumors. Adhesion

molecules such as VLA-2 determine the specificity of cell-to-cell and cell-to-extracellular matrix associations. They have provided insights into embryonic development (11, 12) in normal tissue and cellular invasion and metastasis (13, 14) of neoplastic tissue. The process of tumor invasion and metastasis, like that of normal embryonic development or the inflammation and subsequent repair of damaged tissue, requires a complex coordinated set of changes in cell-to-cell and cell-to-substratum interactions (15, 16). We, therefore, consider the possibility that the over expression of VLA-2 reported here may be directly involved in the pathogenesis of non–small cell primary lung tumors either through effects on the tumor's ability to invade normal tissue or to metastasize.

Materials and Methods

Cell Lines and Tissues. Human lung cancer cell lines, expressing or lacking the lung tumor–associated antigen (gp160) used in this study include A549, a gp160-positive human alveolar carcinoma cell line (7, 17), and QU-DB, a gp160-negative large cell carcinoma cell line (18), obtained from Dr. Susan P. C. Cole (Dept. of Microbiology and Immunology, Queen's University, Kingston, Ontario, Canada). Cells were cultured in RPMI 1640 containing 10% FCS and harvested with 0.05% trypsin and 0.04% EDTA in PBS.

Human primary lung tumor biopsies including adjacent normal lung tissues, were obtained by surgical biopsy of patients from the Roswell Park Cancer Institute (RPCI) Departments of Thoracic Surgery and Surgical Oncology.

Monoclonal Antibodies. mAbs used in this study include 5E8, a mouse $\gamma_1\kappa$ mAb, specific for gp160 (7, 8), 5C7, a mouse $\gamma_1\kappa$ mAb that binds to a cell surface molecule found on human adenocar-

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[1] *Abbreviations used in this paper:* CEA, carcinoembryonic antigen; gp, glycoprotein; RαMγG, rabbit anti–mouse gammaglobulins; TAA, tumor associated antigen.

cinomas of the lung (6); and 12F1, a mouse IgG2a mAb, specific for the α chain of the VLA-2 heterodimer (19), a gift from Dr. M. E. Hemler (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA).

Immunoprecipitation. A549 cells were surface iodinated as described previously (20, 21). The labeled cells or membrane fractions were washed in PBS and then lysed in PBS containing 1% NP-40 and 1 mM PMSF. After centrifugation (30,000 g for 15 min), the soluble material from the cell lysate was immunoprecipitated according to the following protocol. 20 μl of either normal mouse gammaglobulin (NMγG) or mAb (at a concentration of 0.8–1.0 mg/ml) was added to 300–400 μl of the radiolabeled lysates. After a 12–16-h incubation at 4°C, 10 μl of rabbit anti–mouse gammaglobulin (RαMγG) antisera (2 mg/ml) was added and incubated at 4°C for 15 min. Precipitation of the immune complex was accomplished by the addition of 150 μl of a 10% suspension of *Staphylococcus aureus* microorganisms.

In some cases the supernatants of radiolabeled cell lysates were subjected to a second immunoprecipitation. In these sequential immunoprecipitation protocols, the labeled cell lysate was first immunoprecipitated by one mAb followed by RαMγG and *S. aureus* and after centrifugation the supernatant of the labeled lysate was reprecipitated with a second mAb followed by RαMγG and *S. aureus*. Some labeled cell lysates were subjected to a pulse of either high or low pH and then adjusted to pH 7.0 (with NaOH or HCl) before immunoprecipitation.

Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) Analysis. One-dimensional, discontinuous, SDS-PAGE analysis was performed according to Laemmli (22) with a 10% acrylamide concentration for the separating gel. Each immunoprecipitate was extracted by boiling for 2 min in the sample buffer (0.0625 M Tris-HCl, pH 6.8, containing 1% SDS, 10% glycerol, and 0.001% bromophenol blue) with or without 1% β-ME.

Magnesium Dependent Collagen or Laminin Binding Assays. 96-well microtiter plates (3915; Falcon Plastics, Cockeysville, MD) were coated with either collagen type IV (from human placenta, C-7521; Sigma Chemical Co., St. Louis, MO) or laminin (from mouse EHS sarcoma, L-2020; Sigma Chemical Co.) by incubating the plates overnight at 4°C with 100 μl of a collagen or laminin solution (5 μg of protein per ml of PBS). The plates were then rinsed to remove the unbound protein and incubated with a 1% solution of BSA in PBS for 2 h at room temperature to saturate the surface area of the plates with protein. A549 cells (4 × 10⁶ cells/ml) were added to the plate (200 μl of cell suspension per well in PBS with 2 mM MgCl₂ for collagen binding and 8 mM MgCl₂ for laminin binding). After 1 h at 37°C, nonadherent cells were removed by washing the plates with PBS containing MgCl₂. The bound cells were fixed by the addition of paraformaldehyde (3% in PBS) at room temperature for 10 min and stained with a 1:1 (vol/vol) mixture of crystal violet (1% in methanol) and paraformaldehyde (3% in PBS) for 3 min at room temperature. After washing in PBS the plates were air dried and the binding of cells to the immobilized ligand was monitored photometrically at 540 nm using an ELISA plate reader as previously described (23), except with using crystal violet (24) to stain cells instead of toluidine blue. Antibodies (5E8, 5C7, and 12F1) and soluble ligands (collagen and laminin) were tested for their ability to inhibit the binding to either immobilized collagen or laminin by adding the inhibitor (at the concentrations indicated) to the cell suspension before and during the incubation of the cells on the protein-coated microtiter plates. The percentage of attachment was determined as follows: percent of attachment = (OD₅₄₀ of binding with inhibitor − OD₅₄₀ of nonspecific binding*)/(OD₄₅₀ of maximum binding‡ − OD₅₄₀ of nonspecific

binding*); where (*) A549 cells bound to immobilized ligand in absence of MgCl₂; and (‡) A549 cells bound to immobilized ligand in optimal concentration of MgCl₂ with no inhibitor.

VLA-2 α Chain Specific mRNA Analysis. A cDNA probe specific for the VLA-2 α chain message was prepared from a plasmid (2.72L) obtained from Dr. M. Hemler. The 2.72L plasmid contains cDNA from base 1,792 to 5,373 (COOH half of the coding area and the 3′-untranslated region) of VLA-2 α chain gene (25). *Escherichia coli* DH5 bacteria transformed with 2.72 L were grown under selection conditions, i.e., in Luria-Bertani medium (26) plus ampicillin, and plasmid DNA extraction was done according to a modified alkaline lysis procedure on a miniprep scale (27). The plasmid DNA was then precipitated by adding 0.6 volumes of isopropanol and washed once with 70% (vol/vol) ethanol. The plasmid DNA preparation was digested with two restriction enzymes EcoR1 and Bgl1 (Bethesda Research Laboratories), and the DNA fragments were separated on a 1% agarose gel. DNA in the gel was visualized by ethidium bromide staining (26). The VLA-α₂ cDNA fragment was excised and the DNA was electroeluted from the gel slice and ethanol precipitated (26). The specificity of this probe for VLA-α₂ mRNA was established by a PCR and by Northern blot analysis of mRNA derived from cells expressing VLA-2 (A549) and cells on which VLA-2 is not detectable (QU-DB).

For the PCR, purified cDNA from 2.72L was mixed with two oligo-nucleotide primers that were designed based upon the published sequence of the VLA-2 gene (25). The 18mer and 20mer primers were prepared by the DNA Synthesis Facility at RPCI and were labeled at the 5′-end with T4 polynucleotide kinase in the presence of [γ-³²P]ATP (7,000 Ci/mM). Double-stranded cDNA was synthesized and amplified 21 cycles in the presence of DNA polymerase. The last five cycles were run with the labeled primers and the labeled DNA was extracted, ethanol precipitated, subjected to electrophoresis, and then exposed for autoradiography. The resulting autoradiograph revealed a single oligonucleotide band with the predicted size, i.e., 465 bases.

For the Northern blot, total cellular RNA was isolated by modification of the guanidinium monothiocyanate method of Cathala et al. (28). Briefly, A549 or QU-DB cells (3 × 10⁸ cells) or snap frozen lung tumor specimens or nontumor lung tissue (2–6 g each) were lysed in 20–40 ml of lysis buffer containing 5 M guanidine monothiocyanate, 10 mM EDTA, 50 mM Tris, pH 7.5, and 8% (vol/vol) β-ME. The lysis mixture was vigorously shaken to obtain a clear solution. Total RNA was precipitated with 140–280 ml of 4 M lithium chloride followed by incubation at 4°C, overnight. The sample was transferred to 30-ml Corex tubes and RNA was sedimented by centrifugation at 11,000 g for 90 min. The RNA pellet in each tube was resuspended in 10 ml of 3 M lithium chloride to dissolve residual DNA and RNA and repelleted by centrifugation at 11,000 g for 60 min. The RNA pellet in each tube was then dissolved in 10 ml of RNA solubilization buffer (0.1% SDS, 1 mM EDTA, and 10 mM Tris, pH 7.5). The resulting solution was treated with 100 μg/ml proteinase K at 37°C for 2 h followed by three extractions with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1). The aqueous layer was adjusted to 100 mM NaCl and the RNA precipitated with 2.5 volumes of ethanol. Poly(A)⁺ RNA was purified by affinity chromotography on an oligo (dT) cellulose column (29).

RNA samples were dot blotted onto nitrocellulose paper directly for hybridization or were separated in a 1% agarose gel containing formaldehyde (20 mM boric acid, pH 8.3, 1 mM EDTA, 3% formaldehyde) which was then dried for in situ agarose gel hybridization as described (30) or stained with ethidium bromide.

For ethidium bromide staining, the agarose gel containing the RNA sample was sequentially soaked in 50 mM NaOH, 0.5 M Tris (pH 7.0), and 0.5 µg/ml ethidium bromide for 30 min each, then exposed to an UV light for fluorescence photography. The RNA was probed using the 2.72L cDNA (described above) labeled with ^{32}P (31). The dot blot hybridization technique was based upon a previously reported protocol (32) and the in situ agarose gel hybridization technique was a modification of a protocol reported by Ahmad et al. (30) in which we used SSC (0.15 M NaCl, 0.015 M sodium citrate at pH 7.0) in lieu of SSPE (0.18 M NaCl, 10 mM NaPO$_4$, 1 mM EDTA).

Results

Lung Tumor–associated Antigen Identified As A Noncovalently Linked Heterodimeric Molecule. Previous reports identified a cell surface molecule present on ~75% of all human non-small cell lung cancers that was not readily detectable on adult normal human lung tissue. A previous report indicated that this molecule was recognized by a mAb, 5E8, that immunoprecipitates a single glycoprotein chain with an estimated molecular mass of 160 kD (7). Fig. 1 illustrates that at pH 7.0, the 5E8 antibody consistently immunoprecipitates, from the detergent lysate of surface iodinated lung tumor cell line A549, a heterodimeric protein complex in nonreducing and reducing gels (Fig. 1, *A* and *C*), and that incubation of the cell lysates at either high pH (pH 11.5; see Fig. 1 *E*) or low pH (pH 4.5; data not shown) before immunoprecipitation results in the precipitation of only one of the two proteins of this complex, i.e., the heavier 160-kD molecule. The original conclusion that gp160 consisted of a single polypeptide chain was based upon the presence of only one major radiolabeled band in the SDS gels of lung tumor cell lysates immunoprecipitated with the mAb 5E8 (7). However, in this original report and in a subsequent report on gp160 (8), a second faster migrating band was observed. One possibility was that this second minor band with an estimated molecular mass of 130 kD was a proteolytic fragment of the larger protein. An alternative explanation for the faster migrating protein observed with the lung TAA was that gp160 was noncovalently as-sociated with a second protein. Pursuant to this possibility a careful review of the original labeling and immunoprecipitation protocols revealed that the cell lysates were inadvertently exposed to low pH (a condition that could readily dissociate noncovalently associated molecules) before immunoprecipitation. This low pH (~4.5) resulted from the vendor of radioactive iodine (ICN) switching from using 0.1 N to 0.01 N NaOH in the stock iodine preparation. Since investigators were not aware of this change, they continued to add equal volumes of 0.1 N HCl to neutralize the stock iodine preparation before iodination of cells, which resulted in a 10-fold excess of acid required to neutralize the new stock preparation of iodine thereby inadvertently dissociating the 160-kD and 130-kD polypeptide chains. It was concluded from the results presented in Fig. 1 that the 5E8 antibody recognizes a determinant on the 160-kD subunit, and that this larger protein is noncovalently associated with a protein that migrates faster in the gel. It was also noted that both bands that are immunoprecipitated at a neutral pH migrate slightly slower in reducing compared to nonreducing gels, an observation that is consistent with molecules that have multiple intrachain disulfide bonded cysteines.

Lung Tumor–associated Molecule Comigrates with VLA-2 in SDS-PAGE. The estimated molecular masses of the lung tumor–associated heterodimer, i.e., 160 and 130 kD in reducing gels, and the faster migration patterns of both protein chains in nonreducing gels was strikingly similar to that which had been observed and reported for the heterodimeric collagen-binding integrin VLA-2 which was originally detected on a portion of normal human T lymphocytes following activation (33, 34). Based upon these similarities, radioiodinated cell lysates of A549 lung tumor cells were immunoprecipitated with a mAb, 12F1, which is specific for the α chain of VLA-2 (19). Analysis of the labeled immunoprecipitates in SDS-PAGE revealed two bands that were indistinguishable from the two bands immunoprecipitated by 5E8 both in nonreducing and reducing gels (Fig. 1, compare lanes *A* with *B*, and *C* with *D*). As was observed with 5E8, the anti–VLA-2 α$_2$ antibody (12F1) immunoprecipitated only
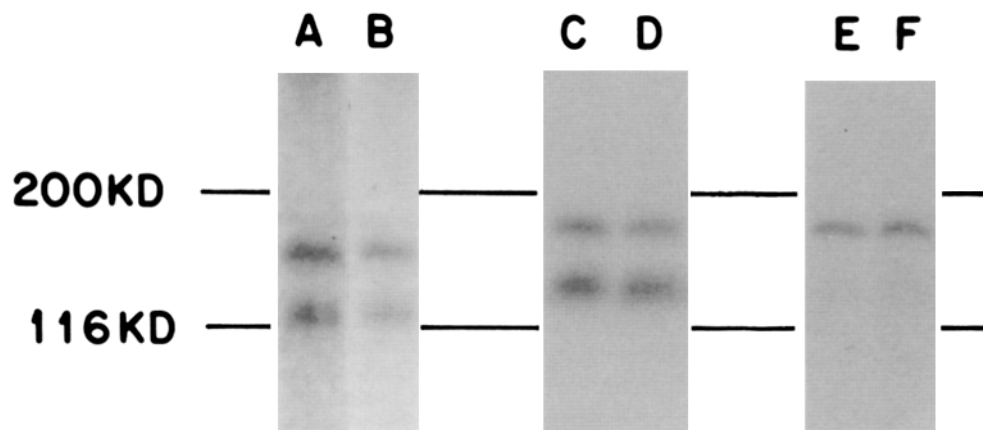

Figure 1. SDS-PAGE analysis of surface iodinated A549 cell lysates immunoprecipitated with mAbs specific for gp160 (5E8) or VLA-2 α chain (12F1). Following immunoprecipitation with mAb 5E8 (lanes *A*, *C*, and *E*) and 12F1 (*B*, *D*, and *F*) the immunoprecipitates were analysed: *A* and *B*, on a non-reducing gel; *C*, *D*, *E*, and *F*, on a reducing gel. All A549 cell lysates were precleared by NMγG before immunoprecipitation with mAb. Cell lysates analyzed in *E* and *F* were exposed to pH 11.5 for 30 min and readjusted to pH 7.0 before immunoprecipitation. All other cell lysates were maintained at pH 7. Under reducing conditions (lanes *C* and *D*) two bands at ~160 and ~130 kD are revealed, which run slightly faster under nonreducing conditions (lanes *A* and *B*). After exposure to high pH before immunoprecipitation (lanes *E* and *F*) only the higher molecular mass band is observed.

the 160-kD α chain when the cell lysates were first exposed to high pH (Fig. 1 F) or low pH (data not shown), indicating that the association of the α chain of VLA-2 (and the lung TAA) with the β chain is noncovalent and pH labile.

Sequential and Reciprocal Immunoprecipitations Support the Notion that TAA Heterodimer and VLA-2 Are the Same Molecule Complex. The results presented in Fig. 1 were consistent with the notion that VLA-2 and the lung tumor–associated molecular complex recognized by 5E8 were either very similar coexpressed molecules or that they were the same molecular complexes. The latter possibility was previously suggested by others (Hemler, M. E., and C. Crouse, unpublished results, see reference 25). An attempt was made to resolve this question by a sequential immunoprecipitation of surface iodinated A549 cell lysates first with the TAA-specific antibody (5E8) and subsequently with the VLA-2 α chain–specific antibody (12F1). After immunoprecipitation of A549 cell lysates with 5E8, we found that nothing further could be immunoprecipitated by 12F1 (see Fig. 2, lane 8). The reciprocal sequential immunoprecipitation revealed the same result (Fig. 2, lane 11). Densitometric scanning of the gel revealed that 5E8 eliminated 97% and 12F1 eliminated 93% of the VLA-2–like molecule. The elimination of the target molecule with either 5E8 or 12F1 suggested that these two antibodies were recognizing the same molecular complex. The failure of a control mAb (5C7) to eliminate the target molecule (Fig. 2, lanes 9 and

10) confirmed the specificity of the sequential immunoprecipitation experiments.

VLA-2–like Complex on Lung Tumors Binds Collagen and Laminin. Since the sequential immunoprecipitations established that the tumor-associated molecular complex and VLA-2 were serologically indistinguishable and their patterns of migration in SDS gels identical, it was of interest to determine whether the VLA-2–like molecule on human lung tumors was also functionally similar to VLA-2 expressed on normal cells in terms of ligand binding. Previous studies showed that VLA-2 binds to collagen (36, 37) or collagen and laminin (37, 38). We first established that the human lung tumor cell line A549 binds to human collagen type IV and laminin and that this binding is magnesium dependent (data not shown) as has been reported for VLA-2 on normal cells (39). The data presented in Fig. 3 illustrate that 80% of the binding of the tumor cells to collagen was inhibited and ~30% of the laminin binding was inhibited by the 5E8 mAb. This suggests that the majority of the collagen binding and a smaller, but significant proportion of the laminin binding by the lung tumor cells is mediated by the VLA-2–like molecule. It is of interest that the 12F1 antibody which immunoprecipitates VLA-2 does not inhibit collagen or laminin binding (Fig. 3 A) suggesting that while these two antibodies both recognize determinants on the α chain (Fig. 1, E and F) only the 5E8 antibody recognizes a determinant within
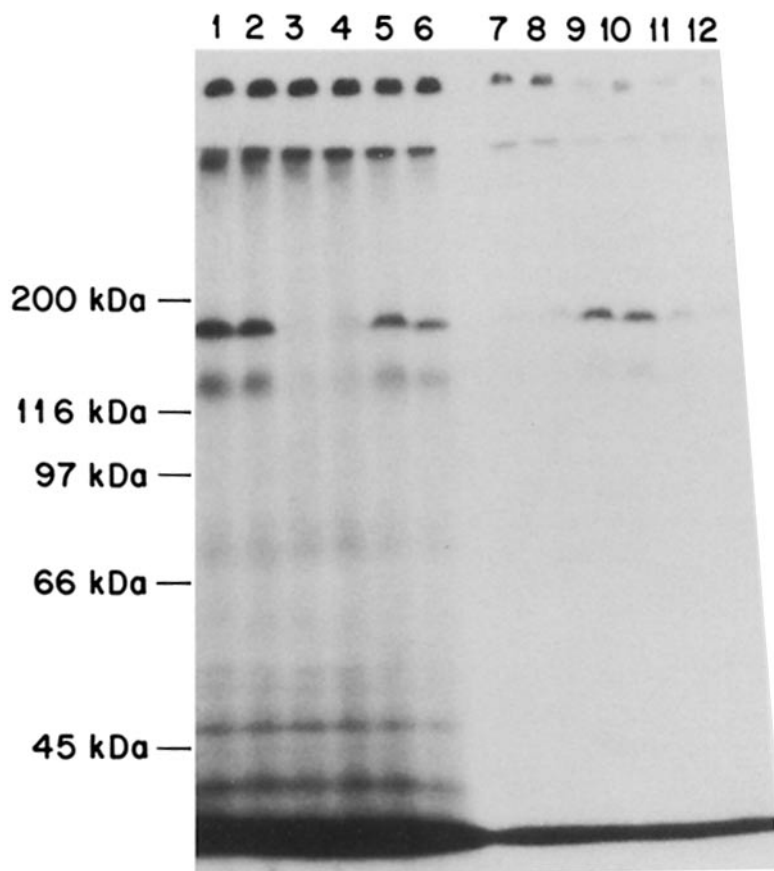

Figure 2. SDS-PAGE analysis of surface iodinated A549 cell lysates sequentially and reciprocally immunoprecipitated with mAbs specific for gp160 (5E8) or VLA-2 α chain (12F1). In lanes *1–6*, surface iodinated cell lysates were first immunoprecipitated with one of three mAbs: lanes *1* and *2*, 5E8; lanes *3* and *4*, 5C7 (a control mAb); lanes *5* and *6*, 12F1. Two bands at ~160 and ~130 kD are present in lanes *1*, *2*, *5*, and *6*. These bands are not present in lanes *3* and *4*. In lanes *7–12*, the supernatants from the first immunoprecipitations (lanes *1–6*, respectively) were subjected to a second immunoprecipitation with either 5E8 (lanes 7, *9*, and *11*) or 12F1 (lanes 8, *10*, and *12*). With the exception of lanes *9* and *10*, no specific bands are observed in the reprecipitation protocol.

A. Collagen coated plate   B. Laminin coated plate
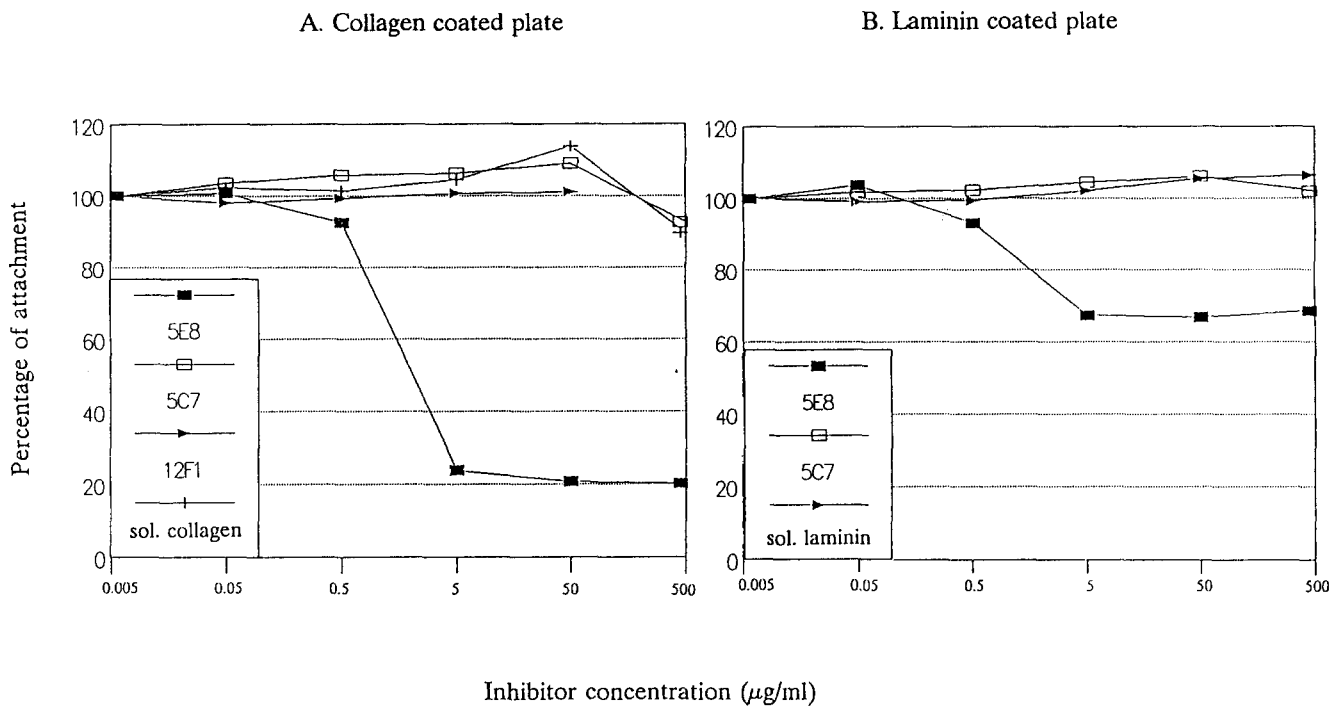

Figure 3. Binding of A549 to immobilized collagen (*A*) or laminin (*B*) in the presence of various mAbs or soluble collagen or laminin. The optimal magnesium concentration for A549 cell attachment to each ligand, 2 mM $MgCl_2$ for collagen binding and 8 mM for laminin binding, was first established by a direct cell attachment assay.

or close to the ligand binding site of this extracellular matrix receptor. The failure of collagen or laminin in solution to block the binding of these cells to the ligand immobilized on plastic surfaces (Fig. 3, *A* and *B*) suggests that VLA-2 has a much higher affinity for the immobilized ligand than for the same molecule in solution.

VLA-2–like Molecule Over Expressed on Primary Lung Tumors. Having established the presence of a molecular complex on the surface of human lung tumors that was indistinguishable from VLA-2 (found on activated T cells and platelets) with regard to subunit structure, antigenicity and ligand specificity, it was important to determine whether or not this molecular complex was expressed by normal human lung tissue and either over expressed or aberrantly expressed by neoplastic lung tissue. Previous immunofluorescent and immunocytochemical studies utilizing the 5E8 antibody revealed minimal staining of normal adult human lung tissue (6), although restricted areas of normal lung (primarily the terminal bronchial epithelium) did stain above background with the 5E8 antibody (7). The data presented in Fig. 3, which indicate that the 5E8 antibody binds to a determinant within or close to the ligand binding site, cast some doubt on the conclusions drawn from these previous studies since it is conceivable that some or all of the VLA-2 combining sites may have been occupied by the extracellular matrix ligand thereby blocking the binding of the 5E8 antibody. Moreover, immunohistochemical studies of VLA-2 expression using an antibody that recognizes a determinant on the α chain of VLA-2 outside of the collagen-binding site suggested that in addi-

tion to bronchial epithelium, the alveolar septa, a portion of the type II pneumocytes and the mesothelial lining of the pleura expressed staining levels for VLA-2 above background (40). Therefore, the question of VLA-2 expression in normal and neoplastic lung tissues was now focused upon the levels of α_2-specific mRNA in order to circumvent potential pitfalls associated with immunofluorescence and immunocytochemistry. A Northern hybridization analysis of poly(A)$^+$ RNA extracted from two different gp160-positive human tumor cell lines, A431 and A549 (using a ^{32}P-labeled, VLA$_2$ α-chain–specific cDNA probe 2.72L, an α_2 cDNA subclone from EcoR1 site near base 1800 through end of 3′-untranslated region of α_2 cDNA clone 2.72 [reference 25]) identified a single 7.3-kb band (Fig. 4 *A*, lanes *1* and *3*). This same probe did not hybridize with poly(A)$^+$ RNA extracted from a gp160-negative human lung tumor cell line, QU-DB (Fig. 4 *A*, lane *2*). These results further support the presence of VLA-2 α chain on the gp160-positive human tumor cell lines, and confirm the specificity of the cDNA probe 2.72L. Total RNA extracted from (*a*) matched sets of patients' lung tumor biopsies and normal lung tissue, and (*b*) gp160-positive and -negative lung tumor cell lines, was dot blotted onto nitrocellulose membranes and probed with the ^{32}P-labeled cDNA clone 2.72L. The probe hybridized to RNA from lung tumor biopsy tissue and a gp160-positive lung tumor cell line, but evidence of hybridization was not detectable with the RNA from normal lung tissue or a gp160 negative lung tumor cell line. Representative results are shown in Fig. 4 *B*. A positive signal was observed with as little as 2 μg of lung tumor RNA
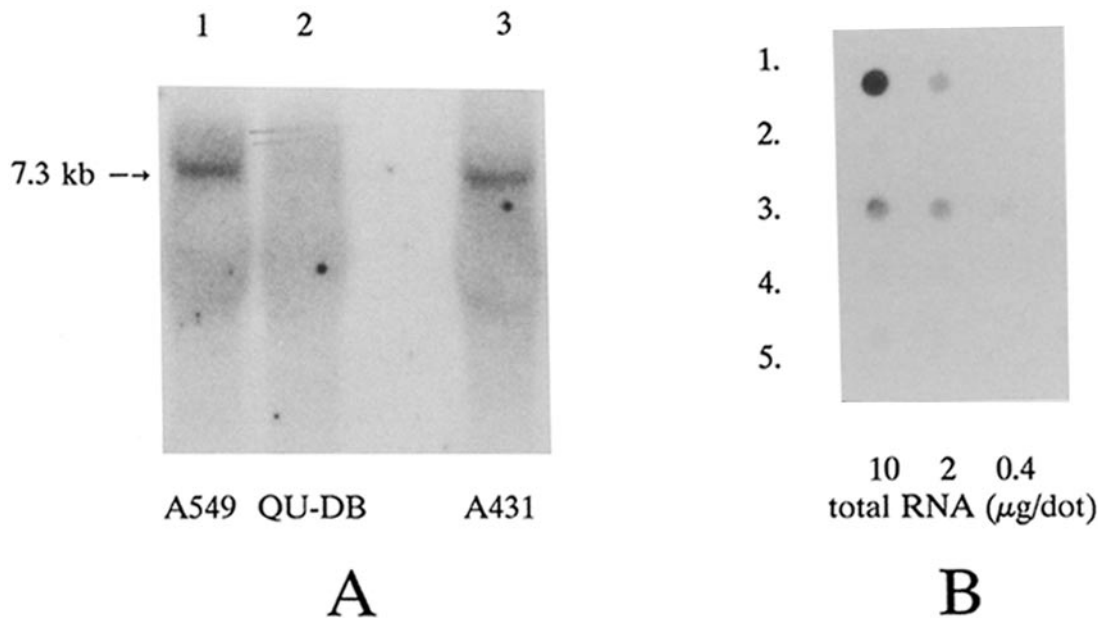
Figure 4. VLA-2 α chain–specific mRNA analysis. (A) Northern hybridization (in situ). Poly(A)⁺ RNA was separated in a 1% agarose gel and hybridized in situ with the ³²P-labeled VLA-2 α chain cDNA clone 2.72L. The poly(A)⁺ RNA samples were extracted from: lane *1*, A549 cells (VLA-2⁺); *2*, QU-DB cells (VLA-2⁻); *3*, A431 cells (VLA-2⁺). One specific band at 7.3 kb is present in lanes *1* and *3*, while absent in lane *2*. (B) Dot blot analysis of total cellular RNA probed with ³²P-labeled VLA-2 α chain cDNA clone 2.72L. The RNA was isolated from: (*1*) A549 cells; (*2*) normal lung tissue, (*3*) lung tumor tissue from the same patient as *2*; (*4*) QU-DB cells; (*5*) normal lung tissue from another patient. VLA-2 α chain–specific mRNA is detected only in *1* and *3*.

and no positive signal was observed with as much as 10 μg of RNA from normal lung. These same results have been observed four times using matched sets of normal and neoplastic lung tissue. Based upon this data and upon the fact that about four times more RNA was extracted from a gram of lung tumor than from a gram of normal adult lung tissue, we estimate that lung tumors express at least twenty times more VLA-2 α chain message than normal adult human lung tissue.

Discussion

The results presented here establish that the overexpression of VLA-2 by human lung tumors represents a new lung tumor–associated molecular complex with a well-defined subunit structure and possible function. Previous studies have illustrated that the overproduction of a molecule by human tumors may represent a common cellular phenotypic change that occurs as a function of cellular transformation. Carcinoembryonic antigen (CEA) is a member of a family of cell surface glycoproteins that are produced in excess in essentially all human colon carcinomas (41) and in a high proportion of carcinomas of many other sites, including lung (42, 43). These observations have led to the development of extremely useful diagnostic and prognostic clinical assays that are now used extensively in oncology. However, the functions and possible significance of these tumor-associated molecules with regard to malignant cell behavior have remained largely unknown for many years. Recently, CEA was identified as an intercellular adhesion molecule and the overproduction of this molecule was postulated to be directly involved in colon car-

cinogenesis either through effects on metastasis or on tissue architecture (44). Since evidence supports the notion that integrin-matrix interactions facilitate the invasion of fibroblasts, lymphoid cells, and epithelial cells during wound healing (45), it is tempting to speculate that the overexpression of an integrin such as VLA-2 by human lung tumors contributes to the tumor's ability to invade normal tissue and to metastasize to other tissues. The association of increases in certain β1 integrins with increases in tumorigenicity has been reported for human osteosarcomas (46) and the β3 integrin subunit was found to be strongly expressed by human malignant melanomas, but not in benign melanocytes (47, 48). The potential importance of integrin-mediated adherence in the metastasis of tumors is also illustrated by the ability of RGD-containing peptides, which inhibit the binding of many integrins to their extracellular matrix ligands (49) to reduce the number of metastatic nodules found within the lungs of mice injected with B16F10 melanoma cells (50) and the inhibition of the movement of human melanoma cells through an amnionic basement membrane (51). These findings and the information presented here provide an opportunity and a rationale to explore the role of VLA-2 in the pathogenesis of human lung cancer.

The data presented here are the first to demonstrate the overproduction of an integrin by primary human lung tumors. While these data have not causally linked the overexpression of VLA-2 to tumor invasion or metastasis in view of the experimental data linking integrins to tumor growth, it is reasonable to postulate that their overexpression in lung tumors is associated with tumor progression. One of the initial and

requisite steps in tumor invasion involves the tumor cell's penetration through a basement membrane. This step requires the attachment of the neoplastic cell to components of the basement membrane, primarily laminin and type IV collagen. These two molecules are recognized as ligands for VLA-2 on normal cells (39) and are demonstrated here to be ligands that are bound by the VLA-2–like molecule on human lung tumors. The role of VLA-2 (as well as other integrins) in the pathogenesis of tumor must now be addressed directly by the comparison of VLA-2-positive and VLA-2-negative variants of human lung tumors for their tumorigenicity in immunodeficient mice. The role of these integrins may also

be addressed by gene transfer, followed by analysis of invasiveness, metastasis, and homing of cells after the cells' engraftment into immunodeficient mice. The finding that human lung tumor cell lines (52) as well as human lung tumor biopsies (53) can be engrafted into the CB.17-SCID mouse make such experiments entirely feasible. The observation that after implantations of SCID mice with tumor biopsy pieces, mice are coengrafted with the tumor and the infiltrating leukocytes (54) opens the possibility of investigating the complex and interactive role of integrins on the tumor and host immunocompetent cells in the progression of a tumor in vivo.

References

1. Brown, D.T., and M. Moore. 1982. Monoclonal antibodies against two human carcinoma cell lines. *Br. J. Cancer.* 46:794.
2. Varki, N.M., R.A. Reisfield, and L.E. Walker. 1984. Antigens associated with human lung adenocarcinoma defined by monoclonal antibodies. *Cancer Res.* 44:681.
3. Mazauric, T., K.F. Mitchell, G.J. Letchworth III, and H. Koprowski. 1982. Monoclonal antibody-defined human lung cell surface protein antigens. *Cancer Res.* 42:150.
4. Mulshine, J.L., G. Cuttitta, M. Bibro, J. Fedorko, S. Fargion, C. Little, D.N. Carney, A.F. Gazdar, and J.D. Minna. 1983. Monoclonal antibodies that distinguish non-small cell lung cancer from small cell lung cancer. *J. Immunol.* 131:497.
5. Radosevich, J.A., Y. Ma, I. Lee, H.R. Salwen, V.E. Gould, and S.T. Rosen. 1985. Monoclonal antibody 44-3A6 as a probe for a novel antigen found on human lung carcinomas with glandular differentiation. *Cancer Res.* 45:5808.
6. Saji, S., S. Zylstra, B.S. Schepart, S.K. Ghosh, Y.H. Jou, H. Takita, and R.B. Bankert. 1984. Monoclonal antibodies specific for two different histological types of human lung carcinoma. *Hybridoma.* 3(2):119.
7. Zylstra, S., F.A. Chen, S.K. Ghosh, E.A. Repasky, U. Rao, H. Takita, and R.B. Bankert. 1986. Membrane-associated glycoprotein (gp160) identified on human lung tumors by a monoclonal antibody. *Cancer Res.* 46:6446.
8. Chen, F.A., E.A. Repasky, H. Takita, B.S. Schepart, and R.B. Bankert. 1989. Cell surface glycoprotein associated with human lung tumors that is similar to but distinct from the epidermal growth factor receptor. *Cancer Res.* 49:3642.

9. Veale, D., N. Kerr, G.J. Gibson, and A.L. Harris. 1989. Characterization of epidermal growth factor receptor in primary human non-small cell lung cancer. *Cancer Res.* 49:1313.
10. Reubi, J.C., B. Waser, M. Sheppard, and V. Macaulay. 1990. Somatostatin receptors are present in small-cell but not in non-small-cell primary lung carcinomas: relationship to EGF-receptors. *Int. J. Cancer.* 45:269.
11. Edelman, G.M. 1988. Morphoregulatory molecules. *Biochemistry.* 27:3533.
12. Takeichi, M. 1988. The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. *Development (Camb).* 102:639.
13. Nicolson, G.L. 1988. Organ specificity of tumor metastasis: role of preferential adhesion, invasion and growth of malignant cells at specific secondary sites. *Cancer Metastasis Rev.* 7:143.
14. Albelda, S.M., and C.A. Buck. 1990. Integrins and other cell adhesion molecules. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 4:2868.
15. Juliano, R. 1987. Membrane receptors for extracellular matrix macromolecules: relationship to cell adhesion and tumor metastasis. *Biochim. Biophys. Acta.* 907:261.
16. Ruoslahti, E., and F.G. Giancotti. 1989. Integrins and tumor cell dissemination. *Cancer Cells (Cold Spring Harbor).* 1:119.
17. Lieber, M., B. Smith, A. Szakal, W. Nelson-Rees, and G. Todaro. 1976. A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. *Int. J. Cancer.* 17:62.
18. Cole, S.P.C., B.G. Campling, D.F Dexter, J.J.A. Holden, and J.C. Roder. 1986. Establishment of a human large cell lung

tumor line (QU-DB) with metastatic properties in athymic mice. *Cancer (Phila).* 58:917.

19. Pischel, K.D., M.E. Hemler, C. Huang, H.G. Sluestein, and V.L. Woods, Jr. 1987. Use of the monoclonal antibody 12F1 to characterize the differentiation antigen VLA-2. *J. Immunol.* 138(1):226.

20. Kubo, R.T., and M.L. Pelanne. 1983. Tunicamycin inhibits the expression of membrane IgM in the human lymphoblastoid cell line Daudi. *Mol. Immunol.* 20:67.

21. Kloppel, T.M., R.T. Kubo, P.S. Cain, C.P. Brown, S.M. Colon, J.F. Kearney, and H.M. Grey. 1981. Structural analysis of the μ-chain synthesized by fetal liver hybridomas. *J. Immunol.* 126:1346.

22. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680.

23. Ruoslahti, E., E.G. Hayman, M. Pierschbacher, and E. Engvall. 1982. Fibronectin: Purification, Immunochemical Properties, and Biological Activities. *Methods Enzymol.* 82:803.

24. Takada, Y., E.A. Wayner, W.G. Carter, and M.E. Hemler. 1988. Extracellular matrix receptors, EMCRII and ECMRI, for collagen and fibronectin correspond to VLA-2 and VLA-3 in the VLA family of heterodimers. *J. Cell. Biochem.* 37:385.

25. Takada, Y., and M.E. Hemler. 1989. The primary structure of the VLA-2/collagen receptor α2 subunit (platelet GPIa): homology to other integrins and the presence of a possible collagen-binding domain. *J. Cell Biol.* 109:397.

26. Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 545 pp.

27. Morelle, G. 1989. A plasmid extraction procedure on a miniprep scale. *Focus (Idaho).* 11(1):7.

28. Cathala, G., S. Jean-Francois, M. Bernadita, B.L. West, M. Karin, J.A. Martial, and J.D. Baxter. 1983. A method for isolation of intact translationally active ribonucleic acid. *DNA (NY).* 2:329.

29. Preparation of poly(A⁺) RNA. 1987. In Current Protocol in Molecular Biology. F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, editors. John Wiley and Sons, Inc., New York. pp. 4.5.1–4.5.2.

30. Ahmad, I., J.A. Finkelstein, and A.W. Steggles. 1990. The analysis of RNA by in situ agarose gel hybridization is more sensitive than the equivalent northern blot analysis. *BioTechniques.* 8(2):162.

31. Feinberg, A.P., and B. Vogelstein. 1984. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 137:266.

32. Zeff, R.A., and J. Geliebter. 1987. Oligonucleotide probes for genomic DNA blots. *Focus (Idaho).* 9(2):1.

33. Hemler, M.E., C.F. Ware, and J.L. Strominger. 1983. Characterization of a novel differentiation antigen complex recognized by a monoclonal antibody (A-1A5): unique activation-specific molecular forms on stimulated T cells. *J. Immunol.* 131(1):334.

34. Hemler, M.E., J.G. Jacobson, M.B. Brenner, D. Mann, and J.L. Strominger. 1985. VLA-1: a T cell surface antigen which defines a novel late stage of human T cell activation. *Eur. J. Immunol.* 15:502.

35. Reference deleted in proof.

36. Santoro, S.A., S.M. Rajpara, W.D. Staatz, and V.L. Woods, Jr. 1988. Isolation and characterization of a platelet surface collagen binding complex related to VLA-2. *Biochem. Biophys. Res. Commun.* 153(1):217.

37. Elices, M.J., and M.E. Hemler. 1989. The human integrin VLA-2 is a collagen receptor on some cells and a collagen/laminin receptor on others. *Proc. Natl. Acad. Sci. USA.* 86:9906.

38. Languino, L.R., K.R. Gehlsen, E. Wayner, W.G Carter, E. Engvall, and E. Ruoslahti. 1989. Endothelial cells use α2β1 integrin as a laminin receptor. *J. Cell Biol.* 109:2455.

39. Staatz, W.D., S.M. Rajpara, E.A. Wayner, W.G. Carter, and S.A. Santoro. 1989. The membrane glycoprotein Ia-IIa (VLA-2) complex mediates the Mg⁺⁺-dependent adhesion of platelets to collagen. *J. Cell Biol.* 108:1917.

40. Zutter, M.M., and S.A. Santoro. 1990. Widespread histologic distribution of the α2β1 integrin cell-surface collagen receptor. *Am. J. Pathol.* 137(1):113.

41. Shuster, J., D.M.P. Thomsom, A. Fuks, and P. Gold. 1980. Immunologic approaches to the diagnosis of malignancy. *Prog. Exp. Tumor Res.* 25:89.

42. Zimmerman, M., B. Ortlieb, R. Friedrich, and S. von Kleist. 1987. Isolation and characterization of cDNA clones encoding the human carcinoembryonic antigen reveal a highly conserved repeating structure. *Proc. Natl. Acad. Sci. USA.* 84:2960.

43. Cournoyer, D., N. Beauchemin, D. Boucher, S. Benchimol, A. Fuks, and C.P. Stanners. 1988. Transcription of genes of the carcinoembryonic antigen family in malignant and nonmalignant human tissues. *Cancer Res.* 48:3153.

44. Behcnimol, S., A. Fuks, S. Jothy, N. Beuchemin, K. Shirota, and C.P. Stanners. 1989. Carcinoembryonic antigen, a human tumor marker, functions as an intercellular adhesion molecule. *Cell.* 57:327.

45. Cassiman, J.J. 1989. The involvement of the cell matrix receptors, or VLA integrins, in the morphogenetic behavior of normal and malignant cells is gradually being uncovered. *Cancer Genet. Cytogenet.* 41:19.

46. Dedhar, S., and R. Saulnier. 1990. Alterations in integrin receptor expression on chemically transformed human cells: specific enhancement of laminin and collagen receptor complexes. *J. Cell Biol.* 110:481.

47. McGregor, B.C., J.L. McGregor, L.M. Weiss, G.S. Wood, C.H. Hu, H. Boukerche, and R.A. Warnke. 1989. Presence of cytoadhesins (IIb-IIa–like glycoproteins) on human metastatic melanomas but not on benign melanocytes. *Am. J. Clin. Pathol.* 92:495.

48. Albelda, S.M., S.A. Mette, D.E. Elder, R.M. Stewart, L. Damjanovich, M. Herlyn, and C.A. Buck. 1990. Integrin distribution in malignant melanoma: association of the β2 subunit with tumor progression. *Cancer Res.* 50:6757.

49. Ruoslahti, E., and M.D. Pierschbacher. 1987. New perspectives in cell adhesion: RGD and integrins. *Science (Wash. DC).* 238:491.

50. Humphries, M.J., K.M. Yamada, and K. Olden. 1988. Investigation of the biological effects of anti-cell adhesive synthetic peptides that inhibit experimental metastasis of B16-F10 murine melanoma cells. *J. Clin. Invest.* 81:782.

51. Gehlsen, K.R., W.S. Argraves, M.D. Pierschbacher, and E. Ruoslahti. 1988. Inhibition of in vitro tumor cell invasion by Arg-Gly-Asp–containing synthetic peptides. *J. Cell Biol.* 106:925.

52. Reddy, S., D. Piccione, H. Takita, and R.B. Bankert. 1987. Human lung tumor growth established in the lung and subcutaneous tissue of mice with severe combined immunodeficiency. *Cancer Res.* 47:2456.

53. Phillips, R.A., and G.M. Fulop. 1989. Pleiotropic effects of the scid mutation: effects on lymphoid differentiation and on

repair of radiation damage. *In* Current Topics in Microbiology and Immunology (The Scid Mouse). M.J. Bosma, R.A. Phillips, and W. Schuler, editors. Springer-Verlag, Inc., New York. pp. 11–17.

54. Bankert, R.B., T. Umemoto, Y. Sugiyama, F.A. Chen, E. Repasky, and S. Yokota. 1989. Human lung tumors, patients'

peripheral blood lymphocytes and tumor infiltrating lymphocytes propagated in scid mice. *In* Current Topics in Microbiology and Immunology (The Scid Mouse). M.J. Bosma, R.A. Phillips, and W. Schuler, editors. Springer-Verlag, Inc., New York. pp. 201–210.