A fluorescence-based method using the cell sorter has been devised to separate rat lung fibroblasts into subpopulations. Type I or type III collagen antiserum was used as the primary antibody to react with parent rat lung fibroblasts. This was followed by a fluorescein-conjugated secondary antibody. Specificity of the primary collagen antibody was determined using a monoclonal $\beta$-actin antibody and purified IgG as the primary antibodies. The fluorescent shift of parent rat lung fibroblasts was optimized for the amount of primary collagen antibody and secondary fluorescein-conjugated antibody. An increase in slot blot intensity was observed for pro-$\alpha1$(I), pro-$\alpha2$(I), and pro-$\alpha1$(III) mRNAs with increasing amounts of cellular RNA. When precipitating with type I collagen antibodies, the total cellular steady-state levels of type I procollagen mRNAs were increased in the high intensity cells as compared with the low intensity cells. Alternatively, when the type III collagen antibodies were used to precipitate the rat lung fibroblasts, the low intensity cells had increased type I procollagen mRNAs while the high intensity cells had increased type III procollagen mRNA. The subpopulations of rat lung fibroblasts after isolation using the fluorescent cell sorter were readily propagated for at least four passages.

The fact that specific cells synthesize specific proteins is the major criterion for development and differentiation. Collagen is the major connective tissue protein synthesized by fibroblasts which are responsible for the production and maintenance of an extracellular matrix component and for repair following tissue injury. Fibroblasts synthesize approximately 80% type I procollagen, 15-20% type III procollagen, and, to a much lesser extent, type V procollagen (1-4). Several studies involving normal and disease state fibroblasts indicate these cells are composed of functionally heterogeneous subpopulations. These subpopulations have been characterized on the basis of growth (5-8), morphology (6, 8-11), and metabolic differences including the relative synthesis levels of collagens, fibronectin, collagenolytic enzymes, prostaglandin E$_2$, and tissue factor (6, 9, 10, 12, 13). Increased evidence of this heterogeneity has implicated the interstitial lung fibroblast subpopulations in normal growth and development. Furthermore, these subpopulations display differential sensitivity to cytokines, eicosanoids, growth factors, and other substances. A select subpopulation of fibroblasts may show increased proliferative capacity and collagen production during fibrosis and wound healing (5-7, 14, 15). Fibroblast subsets could also influence the outcome of tissue injury if one fibroblast subset was more affected than another.

The identification of heterogeneous fibroblast subpopulations has previously been approached by culturing explants. Different cell types within a population have been visualized by immunofluorescent staining and electron microscopy. The in vitro isolation of pure subpopulations has previously been achieved by a limited dilution technique. However, the fluorescence-activated cell sorter allows a more defined procedure for isolating subpopulations based on antigenic determinants expressed on the cell surface. This approach has been used by Bordin et al. (7, 16) to separate 10% of a gingival explant fibroblast population with a high density of specific membrane receptors which bind the collagenous portion of C1q complement factor. This interesting finding demonstrated that high receptor density cells when bound to C1q have increased cell growth and elevated levels of collagen types III and V. This offers a unique role for one fibroblast subpopulation in fibroblast growth and collagen synthesis following tissue injury. However, the separation of these cell subpopulations in the present study has based on collagen type which reflected aggregate gene expression. The present study is more mechanistic in nature.

The present study employed a fluorescence activated cell sorter to separate rat lung fibroblasts into subpopulations based on cell surface-associated type I collagen or type III collagen. Since other studies demonstrated cell surface collagen receptors, we envisioned that collagen would coat the cell membrane and would be good primary antigen to separate cell subpopulations. Three integral membrane proteins on MG-63 osteosarcoma cells recognize and bind specifically native type I collagen through the Arg-Gly-Asp-containing sequence within the triple helical region (17). Collagen receptor proteins have been isolated from various cells (17-20). These putative cell surface receptors for collagen have been thought to be necessary for the binding of soluble procollagen secreted by fibroblasts and subsequent enzymatic processing to native collagen for fibril assembly (18). The subpopulations in our study were labeled with rabbit anti-rat type I or type III collagen serum. A fluorescein isothiocyanate (FITC)$^1$-conjugated goat anti-rabbit IgG antibody was then used as a secondary antibody. Unlike the C1q separation of fibroblasts in which the cells were differentiated based on collagen types (7), the high intensity subpopulation in our study demonstrates increased steady-state levels of pro-$\alpha1$(I) and pro-$\alpha2$(I) messenger RNAs, and the low intensity subpopulation has an increased steady-state level of pro-$\alpha1$(III) messenger RNAs.

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Subpopulations of Rat Lung Fibroblasts with Different Amounts of Type I and Type III Collagen mRNAs*

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RNA. The novelty of the present study is that rat lung fibroblasts may be separated into subpopulations based on cell surface type I and type III collagen which can then be correlated with differences in the cellular ratio of type I and type III procollagen mRNAs. The hypothesis which forms the basis of this study is that differences of collagen types synthesized by the fibroblast subpopulations result from the unique mRNA content of these cell subsets.

**EXPERIMENTAL PROCEDURES**

**Fibroblast Cell Cultures**—Fisher 344 male rats weighting 200–250 g were anesthetized with an intraperitoneal injection of pentobarbital (0.2 ml/100 g of body weight). After opening the thoracic cavity, the animals were bled by cardiac puncture, and the lungs were perfused with sterile phosphate-buffered saline (PBS). The lungs were removed, rinsed in sterile PBS, and then placed in cold Hank's balanced salt solution (HBSS) containing 500 units/ml penicillin, 300 μg/ml streptomycin, and 7.5 μg/ml Fungizone (3 X PSF) for 15 min. Lung tissue was placed in HBSS containing one-third the above concentration of antibiotics and minced to approximately 1 mm³. The minced tissue was rinsed twice in calcium- and magnesium-free HBSS (CMF-HBSS) plus 1 X PSF. Enzyme solution (0.1% collagenase and 0.2% trypsin in CMF-HBSS) and DNase (4 mg/ml in CMF HBSS) was added to the minces, and the tissue was digested for 20 min at 37 °C in a shaking water bath. The supernatant was strained through 100-μm nylon mesh into cold HBSE and 1 X PSF plus 10% fetal bovine serum. The enzyme digestion steps were repeated twice. The supernatants were centrifuged for 10 min at 500 × g, pooled, and resuspended in minimal essential medium containing 10% fetal bovine serum and antibiotics. Cell numbers were determined, and viability was assessed by trypan blue dye exclusion. The cells (1 × 10⁶) were seeded into 75-cm² flasks in 25 ml of complete minimal essential medium and were incubated at 37 °C in a shaking water bath. The supernatant was harvested during the late log phase of growth when essentially all of the cells were in the G1 resting phase. A typical positive fluorescent shift obtained when either type I or type III antisera was used as primary antibody was distributed over a wide range of fluorescent intensities (Fig. 1). To ensure the specificity of the collagen

**Flow Cytometry and Cell Sorting**—Rat lung fibroblasts exist as a heterogeneous population of cells. Subpopulations of these cells were separated through the use of the fluorescence-activated cell sorter which allows one to sort and analyze individual cells based on a specific antigenic determinant expressed on the surface of their cell membrane. In the following experiments, cultures of rat lung fibroblasts were reacted with rabbit antiserum against collagen type I or collagen type III. Second, fluorescein-labeled antibodies were used to tag the surface-associated collagens. Each cell culture analyzed by flow cytometry was harvested during the late log phase of growth when essentially all of the cells were in the G1 resting phase. A typical positive fluorescent shift obtained when either type I or type III antiserum was used as primary antibody was distributed over a wide range of fluorescent intensities. To ensure the specificity of the collagen

**RESULTS**

**Flow Cytometry Analysis**—Single cell analysis and sorting were performed on an Ortho Cytofluorograf 50-H (Ortho Diagnostics, Westwood, MA). The excitation source was an argon laser operating at 488-nm wavelength and 250-milliwatt power output. The three fluorescence emission measured through a 530 bandpass filter. Correlated forward angle scatter, 90° scatter, and the fluorescence emission measured through a 530 bandpass filter. Correlated forward angle scatter, 90° scatter, and the fluorescence emission measured through a 530 bandpass filter. Correlated forward angle scatter, 90° scatter, and the fluorescence emission measured through a 530 bandpass filter. Correlated forward angle scatter, 90° scatter, and the fluorescence emission measured through a 530 bandpass filter. Correlated forward angle scatter, 90° scatter, and the fluorescence emission measured through a 530 bandpass filter.

**Antibody Reactions with Fibroblasts and Immunofluorescence**—Late log phase rat lung fibroblasts of passages 3–5 were harvested with 0.25% (w/v) trypsin/EDTA solution at 37 °C. The cells were suspended in a solution of 1% (w/v) bovine serum albumin, 10% (v/v) fetal calf serum, and PBS (RFP). The cells (1 X 10⁶) were then incubated with primary antibody on ice for 60 min. The cells were washed three times with PBS and then incubated with FITC-conjugated goat anti-rabbit IgG antibody for 30 min on ice. The cells were washed again three times to remove excess unbound antibodies and then suspended in RFP solution. The cells were directly analyzed by flow cytometry and sorted. The primary antibodies used in these experiments were rabbit anti-rat type I collagen antiserum (DMI, Westbrook, ME), rabbit anti-rat type III collagen antiserum (Chen,ilon, El Segundo, CA), purified rabbit IgG antibody (R & D Systems, Minneapolis, MN), and a mouse monoclonal antibody against chicken gizzard actin (Mab C4) which was a gift from Dr. J. L. Lessard, University of Cincinnati (21).

**Isolation of Total Cellular RNA**—A single-step method was employed to isolate total cellular RNA from rat lung fibroblasts (22).

**Recombinant DNA**—The pro-a1(1) and pro-a2(1) cDNA recombinant plasmids, pc1R1 and pc2R2, respectively, were generous gifts of Drs. D. Rowe, University of Connecticut (23). The genomic pro-a1(III) plasmid, pMSC-1, was a gift from Dr. D. de Crombrugghe, M. D. Anderson Hospital and Tumor Institute, University of Texas System Cancer Center, Houston, TX (24). The recombinant plasmids were characterized by DNA sequencing and restriction mapping (23, 24). The plasmid amplification and plasmid CsCl gradient isolation were carried out essentially as described by Maniatis et al. (25). For hybridization the plasmids were digested with the appropriate restriction enzyme and electrophoresed in 0.8% low melting point agarose using 0.5 ng/ml ethidium bromide. The insert bands were excised from the gel, boiled, and stored at −20 °C. Plasmid DNAs were subsequently oligolabeled by a modification of the method of Feinberg and Vogelstein (26). Recombinant DNA techniques were done under P1 containment.

**Slot Blot Hybridizations**—One μg of total cellular RNA was spotted onto nitrocellulose using a Minifold II slot blot system (Schleicher & Schuell). The nitrocellulose was baked for 2 h at 80 °C and hybridized as previously described. The specific activities of the probes were 1.6-3.0 X 10⁶ cpm/μg DNA. Band intensities were determined by densitometric scanning using a dual wavelength TLC scanner, model C5-930 (Shimadzu Corp., Kyoto, Japan).

**FIG. 1.** Specificity of the fluorescent shift of rat lung fibroblasts using flow cytometry and different primary antibodies. Late log cultures of rat lung fibroblasts were reacted (or not reacted) with the following primary antibodies: rabbit antiserum against collagen type I or collagen type III. Second, fluorescein-labeled antibodies were used to tag the surface-associated collagens. Each cell culture analyzed by flow cytometry was harvested during the late log phase of growth when essentially all of the cells were in the G1 resting phase. A typical positive fluorescent shift obtained when either type I or type III antiserum was used as primary antibody was distributed over a wide range of fluorescent intensities (Fig. 1). To ensure the specificity of the collagen
antibodies, rat lung fibroblasts were stained with the FITC-conjugated IgG antibody alone as a negative control (Fig. 1, *top panel*). The positive fluorescent shift was then gated above this background of autofluorescence and nonspecific staining. As other controls, antibodies directed against normal IgG and a monoclonal anti-β-actin antibody were used as primary antibodies. No positive fluorescent shift was noted when these antibodies were used.

The optimal amounts of the anti-rat type I collagen serum (Fig. 2) and the FITC-conjugated anti-IgG antibody (Fig. 3) were determined for flow cytometry and later sorting. Various amounts of anti-rat type I collagen serum ranging from 0.7 to 10.0 mg per labeling reaction were analyzed by flow cytometry (Fig. 2). The data demonstrate a proportional shift of fluorescent intensity with increasing amounts of primary antibody. Saturation of the cell membrane receptors with type I collagen antibody occurred at 5.0 mg of antiserum/1 × 10^6 cells.

Accordingly, 5.0 mg was used as the amount of primary antibody. The optimal amount of FITC-conjugated anti IgG was also determined by analyzing the maximum fluorescent intensity shift (Fig. 3). The maximum shift was observed at 60 μg of antibody/1 × 10^6 cells. In all subsequent sorting experiments the high and low intensity cells were separated, and the upper and lower 25% of the cells were collected.

**Total Cellular RNA Versus Autoradiographed Slot Blots**—Analysis of the steady-state levels of type I and type III procollagen mRNAs in either the high or low intensity fibroblasts requires that each mRNA be assayed in a range where total cellular RNA is related to slot blot intensity. As can be seen in Fig. 4 there is a broad range of slot blot intensities for the various cDNA and genomic probes hybridized. One μg of total cellular RNA was used. Each mRNA was assayed for low and high intensity cells separately using different probes and different conditions of hybridization.

**The Steady-state Cellular Levels of Procollagen Type I and Type III mRNAs**—In order to assess the mechanism of aggregate gene expression, the steady-state levels of type I and type III procollagen mRNAs were determined in the high and low intensity rat lung fibroblast subpopulations. Slot blot hybridization of the total cellular RNA from the subpopulations revealed that the high intensity cells have elevated steady-
state levels of pro-α1(I) and pro-α2(I) mRNA as compared with the low intensity cells (Fig. 5). Alternately, the low intensity cells demonstrated an increase in the steady-state level of pro-α1(III) mRNA as compared with the high intensity cells. In Fig. 6 in which rat lung fibroblasts were sorted with type III antiserum as the primary antibody, an expected reversal of the data in Fig. 5 was observed. The low intensity cells demonstrated increased pro-α1(I) and pro-α2(I) mRNA while the high intensity cells were enriched with pro-α1(III) mRNA. Rat lung fibroblasts, which were sorted using type I collagen antiserum, were passed four times in culture (Fig. 7). During propagation of these rat lung fibroblasts, the high intensity cells maintained the highest levels of type I collagen mRNAs while the low intensity cells maintained the highest level of type III collagen mRNA. In these last three figures comparisons can only be made within each frame since the different recombinant probes had different lengths and different specific activities. In addition, different autoradiographic times were used for the three different probes. Comparison may be made only between low and high intensity rat lung fibroblasts for pro-α1(I), pro-α2(I), and pro-α1(III) mRNAs.
Lung Fibroblasts with Increased Type I or III Collagen mRNAs

Collagens exist as a heterogeneous group of molecules of the extracellular matrix (27). Certain collagens interact with cells and also with other components of the extracellular matrix including elastin, glycoproteins, and protein polysaccharides. As a result of these interactions, cells exhibit certain functions. Changes in the composition of the extracellular matrix result in the alterations of cell-matrix interactions, cell proliferation, cell function, and cell-cell interactions. Recently, solubilized membrane proteins with an affinity for collagens have been isolated from various cells (17, 19, 20). These proteins are considered to be collagen receptors. Collagen receptors exist on the cell membrane of hepatocytes as well as fibroblasts (28). Although binding of these diverse cell types to collagen occurs, there may be differences in collagen-cell interactions. This results in receptor-immobilized collagen molecules on the outer surface of the cell membrane. Since collagen is the most abundant connective tissue protein of the extracellular matrix and probably directs the future of cells, the extracellular matrix may be viewed as a network of collagen-cell interactions.

The present study uses the cell sorter to separate rat lung fibroblasts into subpopulations based on the defined adhesion of collagen to specific cellular receptors. Lung fibroblasts exist as a heterogeneous population of cells. For example, cultures of WI-38 human lung fibroblasts exist as a heterogeneous group of cells with defined subpopulations (29–32). Cells of intermediate passages were found to differ in both their morphological and biochemical characteristics (33). Two distinct cell sizes and shapes, a typical fibroblast type and a much larger non-fusiform type, were identified (34). This heterogeneity has also been observed in IMR-90 human embryonic lung diploid fibroblasts which have been separated into subsets by flow sorting (11). This resulted in two distinct subpopulations of cells with different proliferative potentials. Absher and Absher (35, 36) reported wide variations in intermediate time and proliferative capacity of individual IMR-90 human lung fibroblasts taken from young or old cultures. In addition human lung fibroblasts are heterogeneous in cell volume, cell density, a nuclear size (37, 38).

In the present study a heterogeneous population of rat lung fibroblasts was separated into two distinct subpopulations with different ratios of type I to type III collagens associated with their outer cell membrane surfaces. Soluble type I collagen binds specifically to a receptor in the plasma membrane of fibroblasts (18, 20). Three integral membrane proteins have been identified which specifically interact with a sequence in the triple helical region of the native type I collagen molecule (17). One subpopulation of rat lung fibroblasts has a high density of type I collagen surface receptors and a relatively low density of type III collagen surface receptors. The low intensity cells have a high density of type III collagen surface receptors and a relatively low density of type I collagen surface receptors. The high intensity cells have elevated cellular steady-state levels of pro-α1(I) and pro-α2(I) mRNA while the low intensity cells have an increased cellular steady-state level of pro-α1(III) mRNA.

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