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LR8 expression in fibroblasts of healthy and fibrotic human tissues

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ABSTRACT

LR8 gene was first reported in a subpopulation of cultured human lung fibroblasts expressing the receptor for C1q-globular domain, and it was not detectable in cultured endothelial cells and smooth muscle cells. LR8 mRNA levels were higher in fibrotic lungs. In this study we assessed LR8 production in human tissues and determined if the distribution of fibroblasts producing LR8 is affected in fibrosis. Normal and fibrotic tissue sections from human liver, lung and kidneys were immunostained with antibodies to LR8 and examined for the presence of fibroblasts staining positively and negatively. The cells were also examined for co-expression of α-smooth muscle actin (SMA), a marker for myofibroblasts. The results showed that LR8 was expressed by fibroblasts, smooth muscle cells, endothelial cells, bile duct cells, pulmonary alveolar cells and distal and proximal kidney tubule cells. Connective tissues of normal and fibrotic tissues contained fibroblasts staining positively and negatively with anti- LR8 antibody. The number of LR8-positive cells was higher in fibrotic tissues, but differences were not statistically significant. Fibroblasts producing both LR8 and SMA were present in higher numbers in fibrotic tissues as compared to normal tissues and the differences were statistically significant ($p < 0.05$). Our results show that fibroblast subtypes differing in LR8 expression are present in human tissues, and that in fibrotic tissues cells co-expressing LR8 and SMA are present. Our results indicate that LR8 expressing cells may participate in the early stages of fibrotic diseases and that fibroblasts expressing LR8, not LR8 negative cells, have potential to become myofibroblasts in fibrotic tissues.

1. Introduction

Fibrosis is a pathological phenomenon in which excessive deposition of collagen and other extra cellular matrix (ECM) components leads to loss of normal tissue architecture and function. Fibrosis is believed to be due to dysregulated wound healing response to chronic and progressive tissue injury, and inflammation is believed to play a significant role in many types of fibrosis. The degree of inflammation and repair varies depending on the etiology, and host and tissue responses. Injury activates inflammation and in most cases ongoing chronic inflammation is the major cause for the progression of fibrosis. Patients respond poorly to anti-inflammatory therapies because there is little or no inflammation in advanced stages of fibrosis. In certain types of fibrosis, intrinsic defects in the wound healing can also lead to chronic fibrosis. Fibroblasts are the major cell type responsible for the synthesis of ECM components in normal and fibrotic connective tissues. Fibroblasts from normal and diseased tissues and cells from different anatomic locations have been shown to manifest phenotypic differences, and fibroblast subpopulations have been separated based on differences in the expression of thymocyte 1 antigen (Thy 1) and receptors for the collagen- and globular-domains of C1q [1–8]. The fibroblasts expressing receptors for C1q-globular domain have the phenotype expected of cells participating in inflammation and wound healing [7].

A gene product, LR8 (accession no. AF115384), has been identified in human lung fibroblast subpopulation with the receptor for C1q-globular domain [9]. This product is either not detectable or only minimally expressed in other fibroblasts, and it was not detected in cultured endothelial cells, epithelial cells or alveolar macrophage. The LR8/TMEM176B gene is mapped to chromosome 7q32 in humans and it is located on chromosome 6 in the mouse genome [10,11]. Human LR8 gene is about 12 kb long and contains a 772 base pair long open reading frame. The LR8 protein belongs to the CD-20 superfamily (NCBI Conserved Domain Database), and it appears to be involved in the control of dendritic cell maturation, differentiation of myoblasts into an osteoblast lineage and regulation of immune cells [10,12,13].

LR8 expression is upregulated in human lungs with idiopathic pulmonary fibrosis and bleomycin-induced fibrotic mouse lungs [9].

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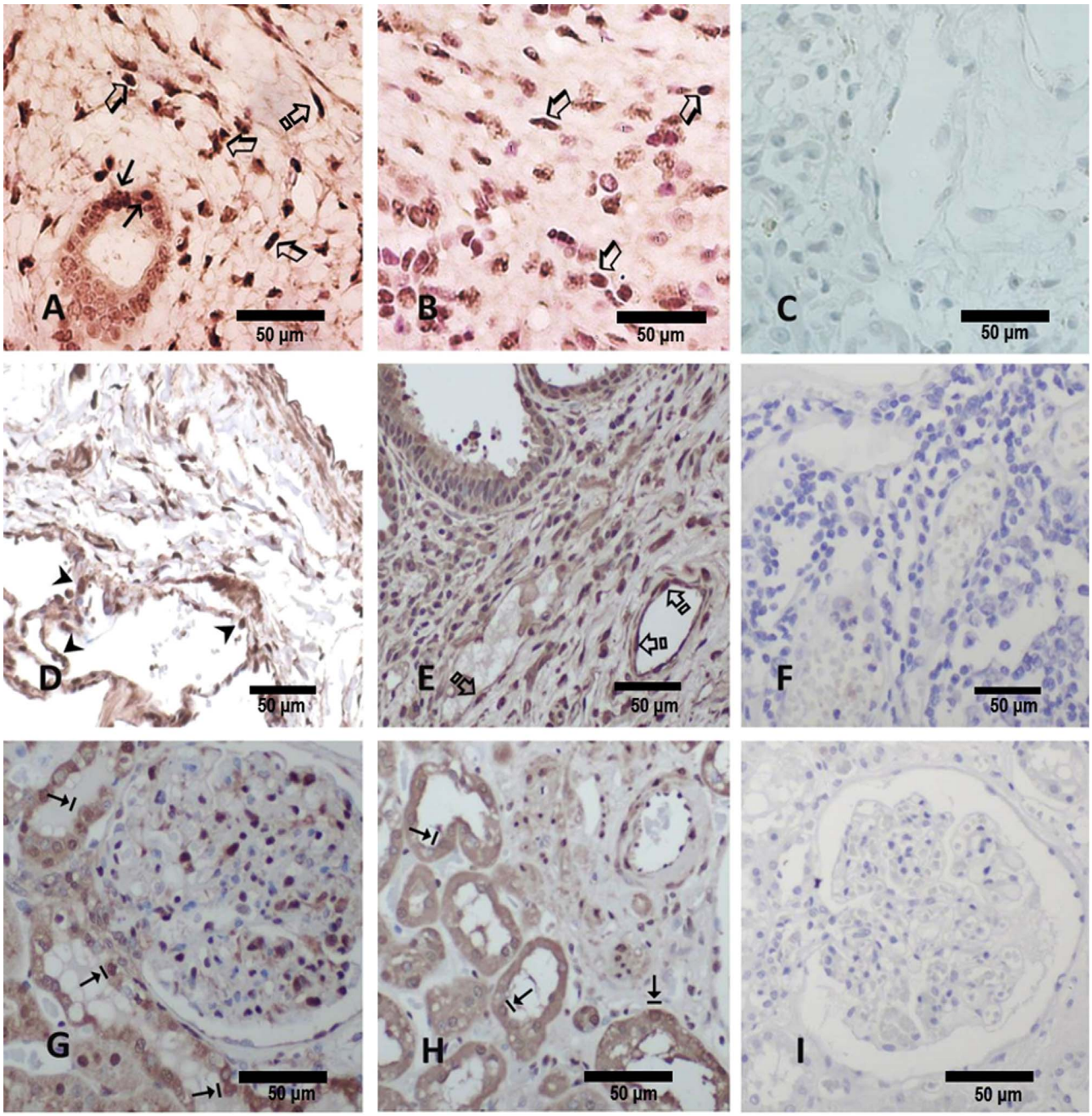

Fig. 1. Photomicrographs of human tissue sections immunostained with anti-LR8 antibody. A. Normal liver B. Fibrotic liver C. Control, preimmune serum D. Normal lung E. Fibrotic lung F. Control, preimmune serum G. Normal kidney F. Fibrotic kidney I. Control, preimmune serum. Cells staining positively are shown. ⇦- Hepatocytes; ←- Bile duct cells; ◀-Alveolar cells; ⇪- Endothelial cells; ⭳ - Nephrons.

LR8 expression is not detectable in gingival fibroblasts cultured from some human patients [14], whereas cells from all patients with phenytoin induced gingival overgrowth express LR8 [data not shown]. These observations indicate that fibroblasts are heterogeneous with respect to LR8 expression and that LR8-expressing cells may participate in the evolution of fibrosis. In order to examine these possibilities, we determined LR8 expression in normal and fibrotic human tissues. Our objectives were to determine if fibroblasts in tissues are heterogeneous in LR8 expression, and if the distribution of LR8 expressing cells is affected in fibrosis. LR8 expressing gingival fibroblasts also express α-smooth muscle actin (SMA) and there is a positive correlation between the expression of LR8 and SMA [14]. The SMA is a component of microfilaments of myofibroblasts, which are believed to be activated fibroblasts and associated with excessive connective tissue synthesis in fibrosis and inflammation [15]; therefore we also determined if LR8 expressing fibroblasts in tissues express SMA.

2. Materials and methods

2.1. Materials

Rabbit polyclonal antibody produced against carboxyl terminus of LR8 protein was obtained as a generous gift from Dr. Math Cuajungco, California State University, Fullerton [16]. Paraffin embedded normal and fibrotic human lung, liver and kidney tissue sections were obtained from the Department of Pathology, University of Washington Medical Center, after approval by University of Washington Human Subjects Committee. Fibrotic liver tissues were obtained from patients with HCV

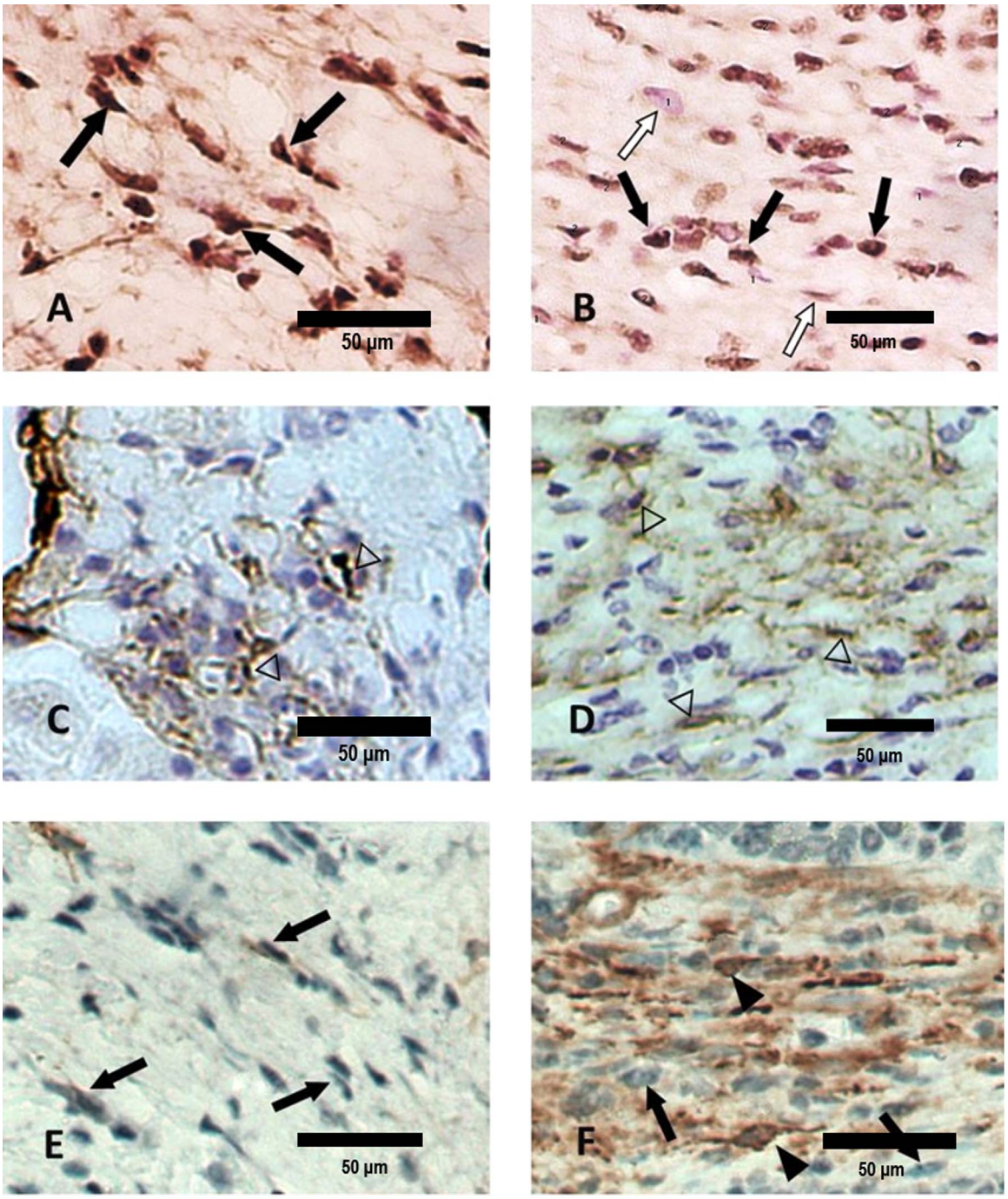

Fig. 2. Photomicrographs of human liver tissue sections immunostained with anti-LR8 (visualized in blue/gray) and anti-SMA (visualized in red) antibodies. Controls were similar to Fig. 1C, F and I, respectively (not shown). A. Normal liver, anti-LR8 antibody. B. Fibrotic liver, anti-LR8 antibody C. Normal liver, anti-SMA antibody D. Fibrotic liver, anti-SMA antibody E. Normal liver, anti-LR8 and anti-SMA antibodies F. Fibrotic liver, anti-LR8 and anti-SMA antibodies. ⇦- Cells staining negatively for anti-LR8 antibody; ⬅ - Cells staining positively with anti-LR8 antibody; Δ - Cells staining positively with anti-SMA antibody; ▲- Cells staining positively with anti-LR8- and anti-SMA antibodies.

cirrhosis and NASH cirrhosis, and normal liver tissue was taken for unrelated causes. The fibrotic kidneys were transplant rejection nephrectomies and the normal kidneys were tissues from a site remote from tumor after nephrectomy. The sections of the fibrotic human lungs were from patients who met the 2011 diagnostic criteria for Idiopathic pulmonary fibrosis [22]; these patients were referred to University of Washington Medical center for diagnosis and management of pulmonary fibrosis of unknown causes, and the surgical lung biopsies were obtained for clinically relevant reasons for ascertaining the diagnosis before treatment. At least 3 specimens each of normal and fibrotic tissues were examined.

2.2. Immunohistochemistry

The tissue sections were deparaffinized, rehydrated in ethanol, and blocked using 3% hydrogen peroxide followed by 2.5% horse serum by conventional procedures. They were then incubated with primary antibodies (rabbit polyclonal anti-LR8 antibody [16] or mouse monoclonal antibody to SMA, Clone 1A4, Dako (Dako, Carpenteria, CA)) in 1% bovine serum albumin for overnight at 4 °C. The sections were washed with phosphate-buffered saline (PBS) and then incubated with secondary antibodies: HRP-conjugated anti-rabbit polymer (ImmPress, Vector Laboratories, Burlingame CA) for LR8 and HRP conjugated anti-

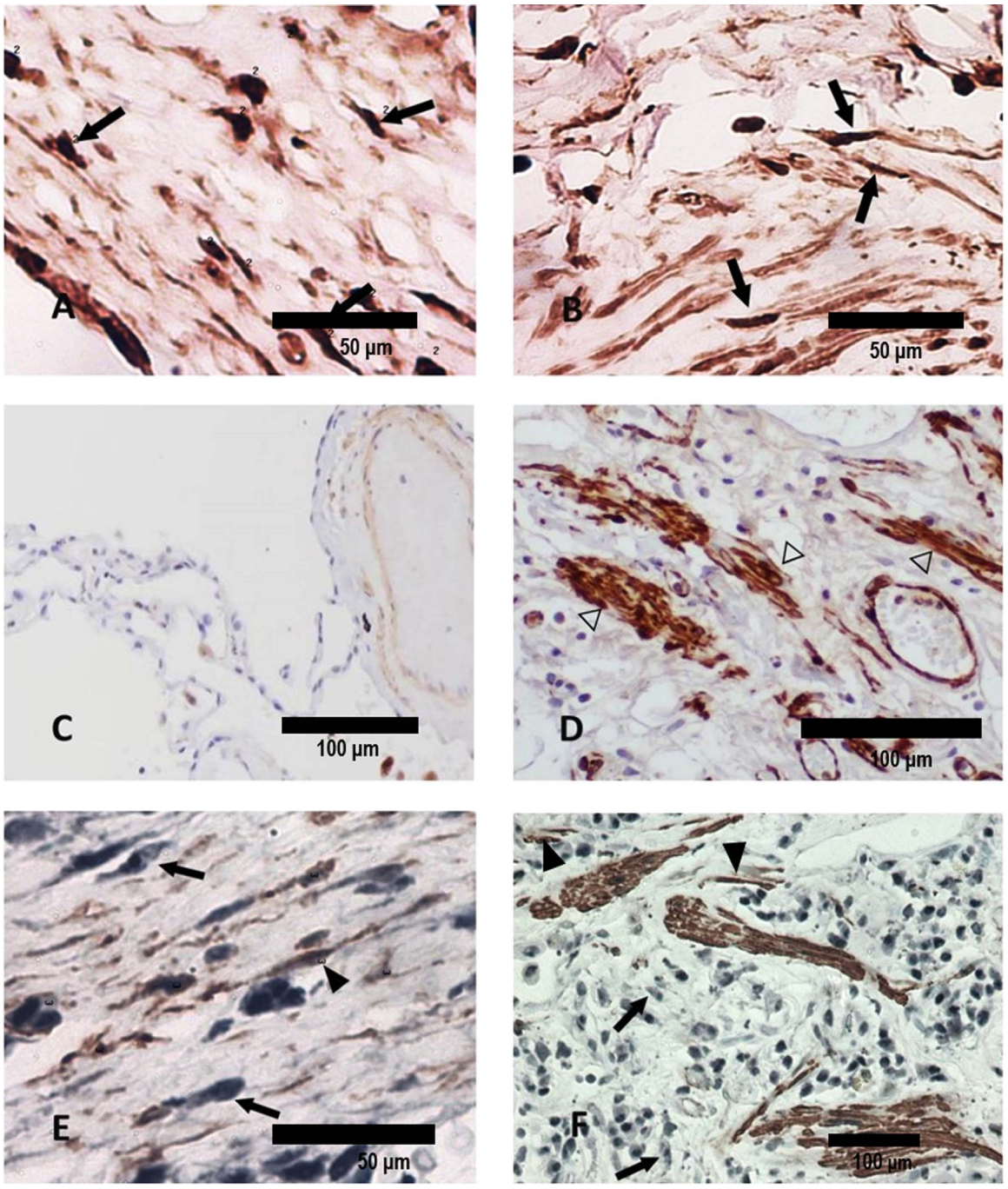

Fig. 3. Photomicrographs of human lung tissue sections immunostained with anti-LR8 (visualized in blue/gray) and anti–SMA (visualized in red) antibodies. Controls were similar to Fig. 1C, F and I, respectively (not shown). A. Normal lung, anti-LR8 antibody B. Fibrotic lung, anti-LR8 antibody C. Normal lung, anti-SMA antibody D. Fibrotic lung, anti-SMA antibody E. Normal lung, anti-LR8 and anti-SMA antibodies F. Fibrotic lung, anti-LR8 and anti-SMA antibodies. ⇦- Cells staining negatively for anti-LR8 antibody; ⬅ - Cells staining positively with anti-LR8 antibody; Δ - Cells staining positively with anti-SMA antibody; ▲- Cells staining positively with anti-LR8- and anti-SMA antibodies.

IgG2a antibody (Pharmingen, San Diego, CA, USA) for SMA for 30 min. Staining was visualized in diaminobenzidine reagent for 15 s to 1 min. The sections were then counterstained in hematoxylin. Double staining for LR8 and SMA antibodies was performed using horseradish peroxidase (HRP) activity in red and blue/gray using, respectively, Vector® NovaRed™ stain and Vector® SG substrate kit (Vector Laboratories, Burlingame, CA) for the peroxidase stains. The tissue sections were first stained for SMA in red followed by staining for LR8 in blue/gray. Dako® N- Universal Negative control for rabbit/mouse primary antibodies was used as negative control. The histology slides were analyzed using Nikon Eclipse E400 microscope and the digital images were taken using a SPOT camera mounted on the microscope. MetaVue™ Imaging System software was used to count the stained cells manually. The number of these cells was quantified in three fields each of normal and fibrotic specimens.

We used preimmune serum (for LR8) and normal, non immune-mouse serum (for SMA) and Dako® N- Universal Negative control as controls. The results for controls were similar and representative data are shown in the Fig. 1.

2.2.1. *Statistical analysis*

The values obtained from quantifying the number of cells staining positively with anti-LR8 antibody in single staining of normal and fibrotic tissue sections, and for both anti-LR8 and anti-SMA antibodies

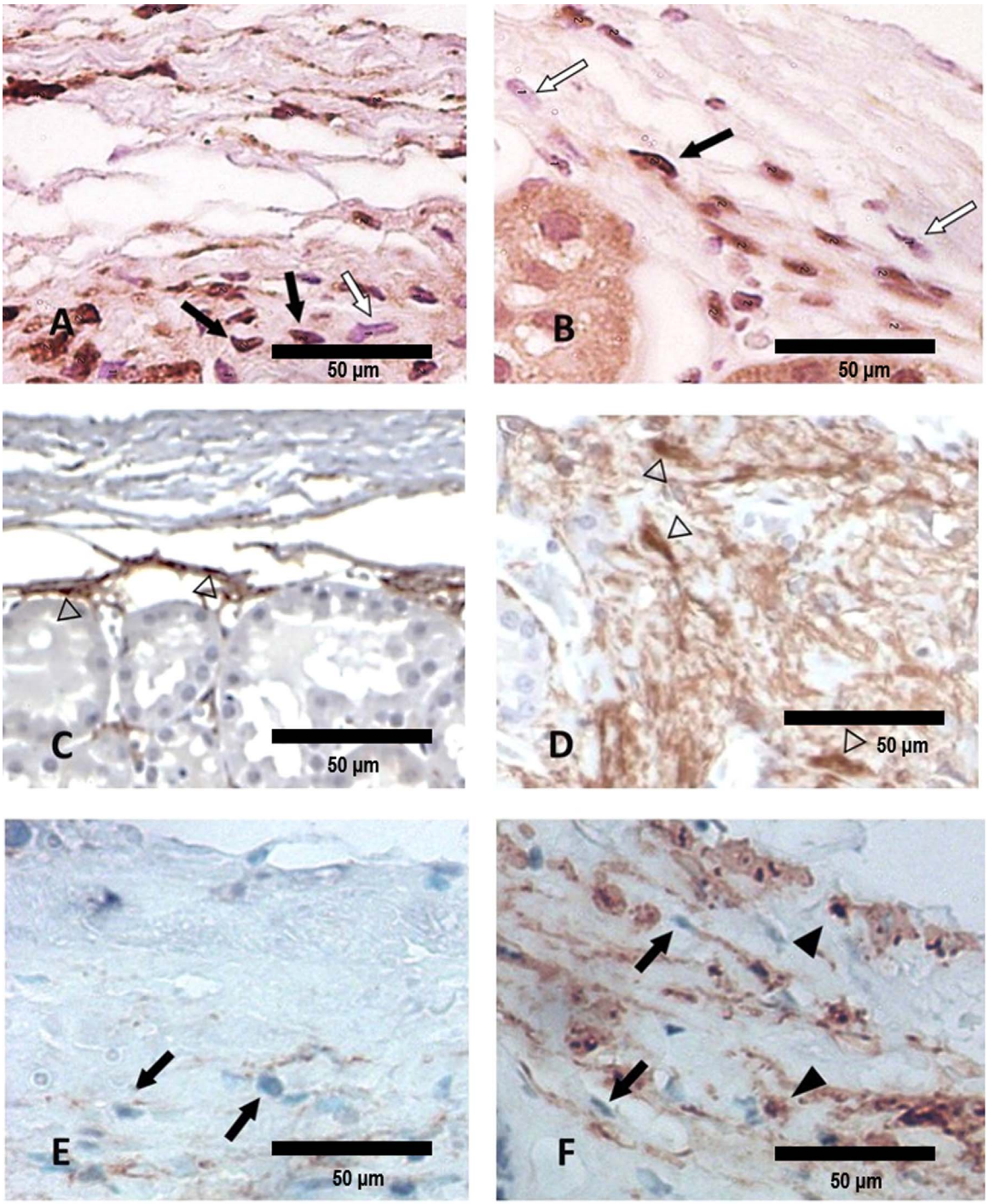

Fig. 4. Photomicrographs of human kidney tissue sections immunostained with anti-LR8 (visualized in blue/gray) and anti–SMA (visualized in red) antibodies. Controls were similar to Fig. 1 C, F and I, respectively (not shown). A. Normal kidney, anti-LR8 antibody B. Fibrotic kidney, anti-LR8 antibody C. Normal kidney, anti-SMA antibody D. Fibrotic kidney, anti-SMA antibody E. Normal kidney, anti-LR8 and anti-SMA antibodies F. Fibrotic kidney, anti-LR8 and anti-SMA antibodies. ⇦- Cells staining negatively for anti-LR8 antibody; ⬅ - Cells staining positively with anti-LR8 antibody; Δ - Cells staining positively with anti-SMA antibody; ▲- Cells staining positively with anti-LR8- and anti-SMA antibodies.

in double staining, were subjected to Students T-test, and p values ≤ 0.05 were considered statistically significant.

3. Results

In previous studies we observed that LR8 mRNA levels were higher in fibrotic human and mouse lungs [9]. To substantiate these results we performed real time-PCR; however, the results were inconclusive with considerable variability in LR8 expression, and differences were not statistically significant. One reason for this could be expression of LR8 in tissues by cells other than fibroblasts which are not affected by disease; therefore, we performed immunostaining to determine the distribution of fibroblasts producing LR8. The results showed that normal human liver, lung and kidneys contain fibroblasts staining positively as well as negatively for LR8 (Fig. 1, also see later). Endothelial cells lining blood vessels were strongly positive, especially in the liver and lungs (Fig. 1). Smooth muscle cells, bile duct cells, hepatocytes in the liver and alveolar cells in the lungs were also positive (Fig. 1A, B, D, and E). In the kidneys, proximal and distal tubule cells within nephrons stained positively (Fig. 1G, H).

Table 1
Comparison of number of fibroblasts immunostained for antibodies to LR8 and SMA in normal and fibrotic human tissue sections*.

Tissue	LR8^{negative}		LR8$^{positive@}$		LR8 ^{positive} and SMA^{positive}	
	Normal	fibrotic	Normal	fibrotic	Normal	fibrotic
Liver	2 ± 1	20 ± 2	43 ± 10$^{\#}$	60 ± 25$^{\#}$	5 ± 2**	36 ± 9**
Lung	0	0	30 ± 3$^{\#}$	38 ± 2$^{\#}$	8 ± 4**	23 ± 9**
Kidney	15 ± 5	11 ± 1	19 ± 7$^{\#}$	17 ± 6$^{\#}$	1 ± 1**	9 ± 2**

* Cells were quantified in three fields each of normal and fibrotic tissue sections. Data are presented as mean ± SD.

@ Percentage of LR8 positive cells in normal liver, lung and kidney were 96 ± 2, 100 ± 0 and, 55 ± 12, respectively. Corresponding values for fibrotic tissues were 74 ± 11, 100 ± 0 and 61 ± 7, respectively.

Number of LR8 positive cells in normal tissues vs. fibrotic tissues: t = 1.4826, p = 0.2348. (Number of LR8 positive cells in normal liver vs. fibrotic liver: t = 1.0936, p = 0.3356. Number of LR8 positive cells in normal lung vs. fibrotic lung: t = 3.8431, p = 0.0184. Number of LR8 positive cells in normal kidney vs. fibrotic kidney: t = 0.3757, p = 0.7262).

** Number of LR8 and SMA positive cells in normal tissues vs. fibrotic tissues: t = 4.478, p = 0.0208. (Number of LR8 and SMA positive cells in normal liver vs. fibrotic liver: t = 5.8239, p = 0.0043. Number of LR8 and SMA positive cells in normal lung vs. fibrotic lung: t = 2.6379, p = 0.0577. Number of LR8 and SMA positive cells in normal kidney vs. fibrotic kidney: t = 6.1968, p = 0.0034).

Because fibroblasts are the major cell type which synthesizes ECM in normal and fibrotic connective tissues, and fibroblast cultures contain populations which differ in LR8 expression [9,14], we examined LR8 production by fibroblasts in tissues. We did not use markers to identify fibroblasts, and based on their morphology and location, we refer to these cells as fibroblast-like cells. The results showed that in healthy and fibrotic liver fibroblast-like cells staining positively and negatively for LR8 were present (Fig. 2A, B). These cells were mostly negative for SMA in healthy liver, while fibrotic liver contained SMA-positive cells (Fig. 2C, D).

Normal and fibrotic lungs also contained fibroblasts staining positively and negatively for LR8 (Fig. 3A, B). Only endothelial cells stained positively for SMA in normal lungs, while some fibroblast-like cells were positive for SMA in fibrotic lungs (Fig. 3C, D). The SMA positive cells also stained positively for LR8, and these cells were larger and contained microfilament-structures (Fig. 3F). These cells were not detectable in normal lungs (Fig. 3E). Cells which were positive only for LR8 did not contain microfilaments (Fig. 3).

In normal and fibrotic kidneys, cells in the capsule displayed heterogeneity in staining for LR8; some were positive with variable staining intensities while others were negative (Fig. 4A, B). Fibroblast-like cells in normal kidney cortex were positive for staining with anti-LR8 antibody (Fig. 4A), but negative for anti-SMA antibody (Fig. 4C), while SMA positive cells were present only in fibrotic kidney (Fig. 4D). Some of these cells staining positively for LR8 were also positive for SMA in fibrotic kidneys (Fig. 4F), while in normal kidney fibroblast-like cells stained positively only for LR8 (Fig. 4A, C and E). Cells negative for LR8 as well as SMA were also present in normal and fibrotic kidneys.

The number of fibroblast-like cells staining for LR8 and SMA is compared in Table 1. LR8 positive and negative cells were present in normal tissues. The number of LR8 positive cells was greater in fibrotic liver and kidney, but differences were not statistically significant (Table 1). However, cells staining positively for both LR8 and SMA were present in significant number in fibrotic tissue sections ($p < 0.02$, Table 1). The number of fibroblast-like cells expressing only SMA was also higher in fibrosis (data not shown).

4. Discussion

Although the heterogeneous nature of fibroblasts has been known for more than 40 years, distinction of subpopulations based on expression of specific protein markers have not been achieved for fibroblasts at one location in normal tissues [4]. Recently, diversity has been demonstrated based on complex pattern of protein markers in cancer associated fibroblasts [17], and in fibroblasts at different anatomic locations [18]. To our knowledge the LR8 gene product was the first marker that distinguished human fibroblast subpopulations from one anatomical location.

Immunostaining of human liver, lung and kidney with anti-LR8 antibody revealed the presence of fibroblasts staining positively and negatively, indicating heterogeneity in LR8 expression by these cells. This is consistent with previous results obtained for cultured cells [9,14], and with the fibroblast heterogeneity hypothesis [1–7]. These cells appear to be present in higher number in fibrotic lungs; this is consistent with the fact that LR8 was first identified in and isolated from a subpopulation of fibroblasts cultured from human lungs [9]. Previously we observed that LR8 expression was not detectable in cultured human smooth muscle cells, epithelial cells and macrophages [9]. However, results presented here show that LR8 is detectable in smooth muscle cells, endothelial cells, bile duct cells, hepatocytes, alveolar cells and proximal and distal tubule cells within nephrons (Fig. 1). This difference may be due to differences in regulation mechanisms between tissues and cultured cells. The methodology used for detecting LR8, Northern blots vs. immunostaining, may be another reason. Existence of more than one splice variant of LR8 mRNA, which are differentially expressed and affected in fibrotic tissues, may be another reason; indeed, RT-PCR using three sets of primers from different LR8 domains showed tissue specific differences in LR8 expression between human liver and gingiva (data not shown).

The number of cells expressing LR8 appeared higher in fibrotic liver and lungs, but the differences were not statistically significant. These cells were largely negative for SMA in healthy tissues; in contrast, LR8 expressing cultured gingival fibroblasts also expressed SMA [14]. The difference between these two observations may be due to the methodology (RT-PCR vs. immunostaining), differences in regulatory mechanisms, or tissue location of these cells.

In kidney there was strong positive staining for LR8 in the proximal tubule cells. The proximal tubule cells play important role in renal damage in proteinuria. In proteinuria, interstitial inflammation activates the fibroblasts to synthesize ECM proteins. In a study using gene expression profiling of renal proximal tubules in proteinuria, GS188, a gene closely related to LR8, was found to be upregulated [11]. Since these genes are located very close to one another on human chromosome 7, it is likely that these are regulated coordinately and the LR8 may possibly be involved in the regulation of proteinuria and renal fibrosis [11]. This is consistent with the increase in mRNA levels observed in fibrotic lungs [9].

Fibroblasts are involved in normal homeostasis and ECM remodeling, and they are believed to undergo activation and transformation to SMA-positive myofibroblasts during wound healing and fibrosis [15]. Results presented here demonstrate the presence of fibroblasts co-expressing LR8 and SMA in fibrotic tissues, especially in lungs and kidneys, and their number increases in fibrosis. Fibroblasts have been shown to express SMA during the initial wave of dermal wound healing [19]; based on the presence of these cells in fibrotic tissues, and their relative absence (or presence in relatively lower numbers) in healthy tissues, it is tempting to speculate that these cells represent putative, not yet differentiated, myofibroblasts. It is likely that such cells are selected by ligands present in the environmental *milieu* [2,3,7]. The LR8 protein, which has receptor domains [9], may play a role in this process. Such a mechanism can account for the presence of fibroblasts with characteristic phenotypes in fibrotic tissues [20,21].

5. Conclusion

We believe that fibroblast subtypes differing in LR8 expression are present in human tissues and that in fibrotic tissues fibroblasts co-expressing LR8 and SMA appear and increase in number. Our results

indicate that fibroblasts expressing LR8, not LR8 negative cells, have potential to become myofibroblasts in fibrotic tissues.

Acknowledgement

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Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2017.03.012.

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