Lipoxin A₄ Modifies Platelet-Derived Growth Factor-Induced Profibrotic Gene Expression in Human Renal Mesangial Cells

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Lipoxins (LXs), endogenously produced eicosanoids, possess potent anti-inflammatory, proresolution bioactivities. We investigated the potential of LXA₄ (1 to 10 nmol/L) to modify the effects of platelet-derived growth factor (PDGF)-induced gene expression in human renal mesangial cells (hMCs). Using oligonucleotide microarray analysis we profiled profibrotic cytokines and matrix-associated genes induced in response to PDGF. LXA₄ modulated the expression of many PDGF-induced genes, including transforming growth factor- β 1, fibronectin, thrombospondin, matrix metalloproteinase 1, and several collagens. Analysis of both transcript and protein levels confirmed these findings. Because the activated glomerulus is frequently a source of injurious mediators that contribute to tubulointerstitial damage, we investigated the effect of hMC-secreted products on the integrity of renal proximal tubular epithelial cells using an *in vitro* model of progressive renal disease. Cell supernatant from PDGF-stimulated hMCs caused morphological and genetic changes in proximal tubular epithelial cells, consistent with a profibrotic phenotype. Interestingly, supernatant from cells pre-exposed to LXA₄ and PDGF did not induce these effects. These results suggest a novel role for LXA₄ as a potent modulator of matrix accumulation and profibrotic change and suggest a potential protective role in progressive renal disease. (*Am J Pathol* 2005, 167:683–694)

Cellular proliferation, migration, and extracellular matrix expansion are fundamental responses of mesangial cells to glomerular injury. The affected glomerulus initiates a

host of reactions involving the release of proinflammatory mediators including proteases, extracellular matrix products, eicosanoids, and growth factors. Resolution of such appropriate host defense is a dynamically regulated process that may be subverted in chronic inflammation.¹ Sustained inflammatory and growth factor responses result in the development of progressive glomerular diseases characterized by tubulointerstitial fibrosis. Although the mechanisms underlying these changes are incompletely understood, many studies have implicated a major role for cytokines and growth factors including platelet-derived growth factor (PDGF), basic fibroblast growth factor, and transforming growth factor (TGF)- β ₁.^{2,3}

PDGF is implicated in the progression of renal disease, being synthesized by infiltrating macrophages and platelets, as well as resident mesangial cells in response to multiple stimuli including endothelin, thrombin, and angiotensin II.³ PDGF bioactivity encompasses increased cellular proliferation,⁴ mesangial matrix expansion,⁵ and increased expression of the profibrotic cytokine TGF- β ₁.^{6,7} The roles of both PDGF and TGF- β ₁ in renal disease have been well characterized. However, current therapeutic interventions to regulate chronic renal inflammation are limited and PDGF has been proposed as a potential therapeutic target.^{8,9}

There is growing evidence that lipoxins (LXs), endogenously produced eicosanoids, may have significant anti-inflammatory and proresolution bioactions. Biphasic lipid mediator production has been demonstrated in the context of an effective host defense. Initial proinflamma-

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tory mediator production is superseded by the production of anti-inflammatory, proresolution mediators including LXs, resolvins, and docosatrienes.^{10,11} LXs are well documented to inhibit neutrophil chemotaxis, adhesion, and transmigration¹² and to stimulate monocyte chemotaxis.¹³ More recent evidence of their anti-inflammatory roles includes modulation of eosinophil activation.¹⁴ A role for LX promoting the resolution of inflammation has been demonstrated by stimulating phagocytic clearance of apoptotic neutrophils *in vivo*¹⁵ and *in vitro*.¹² In the context of renal inflammation we have previously reported that human mesangial cells (hMC) express a G-protein-coupled receptor that binds LXA₄, known as the ALXR.¹⁶ We have reported LXA₄ inhibition of PDGF and epidermal growth factor (EGF)-induced hMC mitogenesis.^{4,6} These effects are mediated by modulation of receptor activation and inhibition of specific downstream signaling pathways, including the Akt/PKB pathway.⁶ Given the ability of LXA₄ to inhibit the effects of PDGF receptor activation in hMCs, it was of further interest to elucidate the effects of LXA₄ on the induction of PDGF-mediated gene expression changes. Here we report that PDGF stimulates the expression of multiple genes associated with matrix expansion and fibrosis, and that LXA₄ modulates PDGF-induced gene expression. We report that soluble factors released by PDGF-stimulated hMCs can induce a profibrotic response in renal epithelia characterized by epithelial-to-mesenchymal transformation (EMT). The equivalent profibrotic response was prevented in supernatants from hMCs treated with LXA₄ and PDGF. These data suggest that LXA₄ may have distinct anti-fibrotic actions in human renal disease.

Materials and Methods

Materials

LXA₄ was obtained from Biomol (Plymouth Meeting, PA). Human recombinant PDGF-BB was acquired from Upstate Biotechnology, Milton Keynes, UK. Anti-thrombospondin and anti-fibronectin monoclonal antibodies were from Calbiochem (Nottingham, UK). Enzyme-linked immunosorbent assay (ELISA) kits for TGF- β_1 and matrix metalloproteinase (MMP)-1 were from R&D (Abingdon, Oxon, UK) and Amersham (Buckinghamshire, UK) respectively. Anti- α -smooth muscle actin monoclonal antibody was from Sigma-Aldrich (Tallaght, Dublin, Ireland) and anti-E-cadherin monoclonal antibody was obtained from BD Biosciences (Oxford, UK), alternatively, for immunoblotting anti-E-cadherin (U3254) from Sigma-Aldrich was used. Fluorescein isothiocyanate-conjugated phalloidin was obtained from Molecular Probes (Eugene, OR).

Mesangial Cell Culture

hMCs were isolated from a nephrectomy sample obtained from the Mater Misericordiae University Hospital in accordance with institutional ethical guidelines. As previously described by Mitchell and colleagues,⁶ a sample

of cortex was isolated and differentially sieved to extract the glomeruli, which were subsequently grown on collagen-coated plates. Cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μ g/ml), which was selective for mesangial cell growth. These cells retained the phenotypic characteristics of hMCs, including stellate morphology, positive staining for vimentin and α -smooth muscle actin, and negative staining for ZO-1 and occludin.¹⁷

In Vitro Model of EMT

Murine cortical tubular (MCT) cells were grown in Dulbecco's modified Eagle's medium-F12 Hams supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μ g/ml) and were stimulated in Dulbecco's modified Eagle's medium-F12 Hams supplemented with insulin-transferrin-selenium supplement (Sigma), L-glutamine, penicillin (100 U/ml), streptomycin (100 μ g/ml), and hydrocortisone (K1 media). Supernatant from hMCs treated with vehicle, LXA₄ (1 nmol/L), PDGF (10 ng/ml), or LXA₄ (15 minutes) pretreatment followed by PDGF were removed after 24 hours. MCT cells were stimulated with supernatants from pre-exposed hMCs, diluted in K1 media (Figure 1). To control for variability in mesangial cell number in PDGF versus vehicle-treated cells, supernatants were diluted accordingly. PDGF treatment was associated with a twofold increase in cell number as compared to vehicle, therefore conditioned media was diluted 1:3 with K1 media. Vehicle-treated supernatant was alternatively diluted 1:1 with K1 media.

Oligonucleotide Microarray Analysis

hMCs were serum restricted (RPMI 1640 supplemented with 0.2% fetal calf serum) for 48 hours before serum starving (RPMI 1640 supplemented with 0% fetal calf serum) for a further 1 hour before stimulating. Cells were subsequently treated with PDGF (10 ng/ml) $\pm$ LXA₄ (1 nmol/L) in 0% fetal calf serum-RPMI 1640 for 24 hours. RNA from three independent experiments was pooled after isolation using lysis buffer, in accordance with the Qiagen minicolumn preparation (Qiagen, Valencia, CA). Complementary DNA (cDNA) synthesis, *in vitro* transcription, and microarray analysis were performed as we have previously reported.¹⁸ Briefly, cDNA was synthesized from total RNA using the Superscript Choice kit (Invitrogen, Carlsbad, CA). Biotin-labeled cRNA prepared from template cDNA was fragmented and hybridized to Affymetrix HGU133A arrays according to the Affymetrix protocol (Affymetrix, Santa Clara, CA). Arrays were then fluorescently labeled before scanning with a confocal scanner (Affymetrix). This process was repeated to obtain a duplicate chip from which data were compiled and analyzed.

Image files were obtained through Affymetrix GeneChip software (MAS5). Robust multichip analysis (RMA) was subsequently performed. RMA is a technique that analyzes directly from the Affymetrix microarray and is

Figure 1. Experimental model of hMC-induced changes in MCT cells.

comprised of three steps; background adjustment, quantile normalization, and summarization. RMAexpress was used to make the data accessible to a Microsoft Windows operating system for further analysis, as per Sadlier and colleagues.¹⁹

The data from each microarray were collected and expression data for each condition were compared to control. Genes altered by PDGF, causing a 0.5 signal log ratio (SLR) or greater change, with respect to control on both microarrays (equivalent to a fold change in expression of 1.4 or greater) were termed significant and further analyzed for differential expression. Using unsupervised hierarchical cluster analysis as described in Eisen and colleagues²⁰ a visual representation of genomic differential expression was attained. Furthermore, genes could be categorized using a web-based ontology program (Onto-Express).²¹ This program assigns genes a category based on current known biological function, however redundancy of genes between several categories may exist.

Promoter and Transcription Factor Analysis

Genes significantly altered by PDGF were analyzed using the web-based software Genomatix (Genomatix Software GmbH, Munich, Germany). Genes mapped to loci with experimentally verified promoter regions were further analyzed for common transcription factor binding sites.²² A random expectation value (re-value) was assigned to each transcription factor (the program assigns an expectation value for the number of transcription factor binding site matches per 1000 bp of random DNA sequence). The actual occurrence and random expectation of a given transcription factor were compared to confirm the presence of a binding site. Furthermore, we examined the binding of stimulating protein 1 (SP-1) to consensus

SP-1 binding sites using the TransAM SP-1 kit (Active Motif, Rixensart, Belgium). hMCs were treated with PDGF (10 ng/ml) $\pm$ LXA₄ (1 nmol/L) for 24 hours before extraction of nuclear lysate. Ten μ g of nuclear lysate protein was added to each well and the assay was conducted according to the manufacturer's protocol.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Quantitative real-time PCR was performed using TaqMan universal PCR master mix (P/N 4304437; Applied Biosystems, Weiterstadt, Germany) as per the manufacturer's protocol. Samples were run in duplicate and were analyzed using the ABI Prism 7700 sequence detection system (Applied Biosystems). TaqMan PCR reactions were multiplexed for the gene of interest and VIC-labeled 18S rRNA, as an endogenous control (P/N 4310893E, Applied Biosystems). FAM-labeled forward primers, reverse primers, and TaqMan probes are listed below (Table 1).

Western Blot Analysis

hMCs were treated as follows; cells were serum-starved for 24 hours before the addition of stimuli, they were then pretreated with LXA₄ (1 nmol/L) or vehicle for 15 minutes before addition of PDGF (10 ng/ml). The treatment was for the indicated time periods (24 to 72 hours). Supernatants were retained and lysates were harvested in RIPA buffer (150 mmol/L NaCl, 50 mmol/L Tris, 5 mmol/L ethylenediamine tetraacetic acid, 10 mmol/L NaF, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate). The lysates were clarified by centrifugation at $10,621 \times g$ for 10 minutes, the supernatant fraction was retained, and the protein concentration quantified using a

Table 1. Primer Sequences for Quantitative RT-PCR

Primer	Forward	Reverse	Probe
Human fibronectin	CCGCCGAATGTAGGACAAGA	TGCCAACAGGATGACATGAAA	CAACCATCTCATGGGCCCCATTCC
Human collagen I α 1	TGTTGGCCCAAGAGGTCCT	CACCGGGCTCTCCCTTATC	TGGCCCAACAAGGCATTTCGTGG
Rat fibronectin	AAACAGGTCTGGACTCCCCA	CAGAATGCTCGGCGTGATG	TCTTCTGATGTCACCGCCAACCTATTCA
Mouse VEGF	AACGATGAAGCCCTGGAGTG	TTGATCCGCATGATCTGCAT	TGCCACGTCAGAGAGCAACATCAC
Mouse CTGF	CCCACACAAGGGCCTCTTC	CCATCTTTGGCAGTGACAC	CCCCCGCCAACCGCAAGATT

Bradford protein assay. MCT cells were conditioned in K1 media, TGF- β_1 (10 ng/ml) and EGF (10 ng/ml) were added as a positive control for EMT. MCT cells were treated with pretreated hMC supernatant, the volume of supernatant from each condition was determined by hMC number. Cells were subsequently harvested as above and protein levels were determined by the Bradford assay. Specific protein levels were detected by immunoblotting lysates from hMC or MCT cell culture.

For Western blot analysis, 20 to 40 μ g of hMC protein or 30 μ g of MCT protein were loaded into each lane under reducing conditions on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and subsequently transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) by electroblotting. Nonspecific antibody binding was reduced by blocking the membrane in 3% bovine serum albumin (Sigma, Dublin, Ireland) for 1 hour at room temperature. The antibody was then added at a concentration according to the manufacturer's instructions and incubated on a rocking platform at 4°C, overnight. Membranes were subsequently incubated with a horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature and visualized using enhanced chemiluminescence (Santa Cruz, Heidelberg, Germany) and X-ray film. To check for equal loading the membranes were stripped using sodium dodecyl sulfate, blocked and reprobed with anti- β -actin monoclonal antibody (Sigma).

Quantitation of TGF- β_1 Production

Quiescent hMCs were treated as indicated and at 72 hours the supernatant was retained and assayed for TGF- β_1 release by ELISA (R&D Systems) as per manufacturer's protocol.

Quantitation of MMP-1 Production

Quiescent hMCs were treated as above, at 6, 18, 24, 48 and 72 hours time-points the supernatant was retained and assayed for MMP-1 release by ELISA (Amersham Biosciences, Bucks, UK) as per the manufacturer's protocol.

Immunofluorescent Microscopy

MCT cells were cultured on chamber slides (Nalge Nunc, Naperville, IL), and subsequently stimulated with pretreated hMC supernatant, as detailed above. Cells were then washed and fixed with 4% paraformaldehyde added

directly to the wells. After washing in phosphate-buffered saline (PBS), cells were permeabilized in 0.5% Triton-X for 10 minutes, blocking agent (PBS containing 1% goat serum and 3% bovine serum albumin) was then added. Cells were kept on a rocking platform for 1 hour at room temperature. The primary antibody was subsequently added at concentrations according to the manufacturer's instructions and incubated overnight on a rocking platform at 4°C. After washing, a fluorescein isothiocyanate-conjugated secondary antibody, Goat anti-mouse (Molecular Probes) was added at room temperature for 1 hour, after this, cells were washed in PBS containing 4,6-diamidino-2-phenylindole (DAPI) stain for nuclei visualization. Slides were then mounted with coverslips and analyzed using phase contrast ($\times 40$ magnification) and also using Axiovert 200 fluorescent microscopy DAPI and fluorescein isothiocyanate filters (Carl Zeiss, Jena, Germany).

Results

PDGF Induces Expression of a Distinct Cohort of Genes in hMCs

Differential gene expression was examined using oligonucleotide microarrays (HG133A), cDNA was extracted from hMCs exposed to 10 ng/ml PDGF for 24 hours with or without LXA₄ pretreatment (15 minutes, 1 nmol/L). This procedure allowed whole genome analysis of hMCs in normal and growth factor-treated cells, identifying signatures of the conditions.²⁰ Data obtained from each condition were normalized with respect to control (ie, vehicle-stimulated cells). An overview of the information acquired is represented in Figure 2A, PDGF significantly altered (0.5 SLRs or greater) 4.16% of genes represented on the array (926 of 22,283 genes). Consistent with the modulation of PDGF signaling, pretreatment with LXA₄ diminished the effects of PDGF. LXA₄ pretreatment of the PDGF-stimulated response prevented significant PDGF responses in almost 40% of the 926 genes. Of the 582 genes increased significantly by PDGF, pretreatment of hMCs with LXA₄ diminished this response to 353 genes stimulated by PDGF. Genes decreased by PDGF consisted of 344 transcripts, again LXA₄ pretreatment diminished this to 205 genes. To gain more insight into the significance of these changes, genes are given functionality based on current known biological process using Onto-Express,²¹ a web-based ontological program (Figure 2B). Categories include

embryogenesis, cell proliferation, fibrosis-related, immune response, and lipid metabolism. LXA₄ decreased the number of PDGF-driven genes, with particular attention to cell proliferation (42% reduction) and fibrosis-related (49% reduction).

Data obtained from RMA analysis of the oligonucleotide microarray were organized using pairwise average linkage clustering that arranges data into unsupervised hierarchical clusters, whereby each gene represented is equally weighted. This was achieved using Eisen-Lab²⁰ web-based cluster software (Figure 2C). Genes significantly altered by PDGF (less than or equal to 0.5 SLR and greater than and equal to 0.5 SLR) were represented. Genome-wide data were clustered using statistical algorithms to arrange genes according to similarity in patterns of expression with co-expressed genes believed to have functional similarity.²⁰ The primary cluster represented all genes significantly altered by PDGF, normalized with respect to control. Additional to this, each ontological category was clustered. The cell proliferation cluster displayed 65 transcripts, of these, 36 were significantly altered in the presence of LXA₄, including various cyclins and p53-induced proteins. Of the 35 fibrosis transcripts, 18 were significantly altered by LXA₄ pretreatment. Fibronectin, thrombospondin, and TGF- β_1 were among these. Immune response, embryogenesis, and lipid metabolism clusters also displayed altered transcript expression with LXA₄ pretreatment.

Of particular interest to us was whether we could define specific transcription factors (TFs) activated in hMCs when exposed to PDGF and whether these might further be subdivided into LXA₄-regulated and LXA₄-independent genes. Analysis of gene promoter regions was conducted using Genomatix²² software. Genes significantly affected by PDGF were mapped to loci containing experimentally verified promoter regions. Subsequently, common TF binding regions were extracted using MatInspector software. Several ubiquitously expressed transcription factor-binding sites were found including cAMP response element binding site (CREB), early growth response factor (EGRF), and stimulating protein 1 (SP-1) (Figure 2D). SP-1 was found to be present on almost half of all PDGF-stimulated genes. Each TF is assigned a random-expectation (re) value based on the estimated number of binding sites for that TF per 1000 bp of sequence. For the SP-1 TF, the re-value was predicted at 1.7, however actual occurrence was averaged at 2.8. Subsequently, we analyzed SP-1 TF binding using a TransAM assay that measures SP-1 and posttranslationally modified SP-1. We found that PDGF significantly elevated levels of SP-1 (1.6-fold) and that this response was attenuated with LXA₄ pretreatment (1.18-fold) (Figure 2E).

Validation of Oligonucleotide Microarray Findings

A cohort of genes was selected for further analysis to validate gene expression changes identified by oligonucleotide microarray. In Figure 3a quantitative RT-PCR data displayed PDGF-induced (10 ng/ml) increases in the expression of fibronectin and collagen

type I $\alpha 1$ transcripts at 24 hours normalized with respect to 18S-ribosomal RNA (1.2- and 1.7-fold induction, respectively). Pretreatment with LXA₄ (1 nmol/L) prevented PDGF-induced gene expression increases. PDGF also induced alterations in the protein levels of fibronectin, decorin, and thrombospondin (Figure 3b). Stimulation of mesangial cells with PDGF for 24 hours resulted in elevated levels of fibronectin and thrombospondin, a TGF- β_1 activator.²³ Corresponding decreases were observed of the small proteoglycan decorin, a known inhibitor of the profibrotic cytokine TGF- β_1 .²⁴ These changes in protein expression were consistent with gene expression array findings and were altered with the pretreatment of hMCs with LXA₄ (1 nmol/L).

Transforming growth factor (TGF)- β_1 and MMP-1 secretion from hMCs was also measured in response to PDGF (10 ng/ml) with or without LXA₄ (1 nmol/L) (Figure 3c). Supernatant from stimulated cells was collected at the indicated time points and assayed using ELISA. TGF- β_1 levels were increased significantly by PDGF at 72 hours (2.1-fold change) with LXA₄ pretreatment ablating this effect (1.1-fold change). Consistent with this, secreted MMP-1 levels were significantly enhanced in response to PDGF at 6, 18, and 24 hours, (1.2-, 1.5-, and 1.6-fold change, respectively) whereas LXA₄ achieved significant diminution of the PDGF effect at 6 and 18 hours only (0.9- and 0.7-fold change).

Epithelial-to-Mesenchymal Transformation of Proximal Tubular Epithelial Cells

It has been proposed that pleiotropic mediators released from the inflamed glomerulus play an important role in progressive renal disease by tubulointerstitial fibrosis.^{25,26} Tubulointerstitial fibrosis is frequently characterized by EMT. Consistent with this we observed that supernatants from hMCs treated with PDGF (10 ng/ml, 24 hours) induced a loss of E-cadherin expression concomitant with increased levels of α -smooth muscle actin in MCT cells (Figure 4b). These phenotypic changes mirrored those seen with the prototypic inducers of EMT, TGF- β_1 , and EGF (Figure 4a).²⁷ MCT cells treated with cell supernatant from vehicle or LXA₄-treated hMCs retained much of their epithelial phenotype. Remarkably, this observed change in MCT phenotype was less substantial after treatment with supernatant from PDGF-stimulated and LXA₄-pretreated hMCs. Pretreatment with LXA₄ diminished the effects of PDGF thereby impeding the indicated transformation. These changes were verified using Western blot analysis of MCT lysate (Figure 4c). PDGF-stimulated hMC supernatant caused decreases in E-cadherin and corresponding increases in α -SMA levels. Again, supernatant from hMCs pretreated with LXA₄ diminished this effect.

Changes in MCT gene expression of fibronectin, vascular endothelial growth factor (VEGF), and connective tissue growth factor (CTGF) were measured using quantitative real-time RT-PCR. The positive control (TGF- β_1 and EGF) displayed significant increased expression of

Figure 2. Global changes in gene expression in hMCs, representative ontological data examining biological functionality, and transcription factor analysis of genes. Gene expression changes in hMCs treated with PDGF (10 ng/ml) alone or PDGF with LXA₄ (1 nmol/L) pretreatment were analyzed. Three independent experiments were pooled for hybridization to each microarray. Duplicate experiments using HGU133A Affymetrix microarrays are illustrated, data are representative of genes differentially expressed by 0.5 SLRs or greater (≥ 0.5 SLR and ≤ 0.5 SLR) from the average of duplicate arrays. **A:** An overview of the numbers of differentially expressed genes. **B:** Changes in gene expression within major functional families using OntoExpress.

each gene, as compared to K1 media alone (Figure 5A). Consistent with this, supernatant from PDGF-stimulated (10 ng/ml, 24 hours) hMCs caused an increase in the expression of VEGF, CTGF, and fibronectin in MCT cells. Supernatant from hMCs pretreated with LXA₄ (1 nmol/L, 15 minutes) and subsequently PDGF-stimulated, diminished these observed changes in gene expression (Figure 5; B, C, and D), consistent with LXA₄ modulation of PDGFR signals. Expression of all three genes was continuously decreased with LXA₄-pretreated supernatant. Changes in epithelial cell gene expression induced by hMC supernatants were not due to residual PDGF in the media. This was verified by the addition of the tyrophostin PDGFR kinase inhibitor AG1296 (10 nmol/L) to hMC su-

pernatant. Addition of the inhibitor did not significantly effect observed gene expression changes (data not shown). Furthermore, direct stimulation of the MCT cells with PDGF-BB did not induce significant expression of fibronectin, VEGF, or CTGF (Figure 5A).

Discussion

The pathology of progressive renal disease in glomerulonephritis (GN) involves mesangial cell proliferation and migration, matrix expansion, and cytokine production, which left unchecked may result in end-stage renal disease characterized by tubulointerstitial fibrosis. Initial in-

Figure 2. *Continued.* **C:** Unsupervised hierarchical cluster analysis of the average of duplicate arrays and ontological clustering. **D:** Transcription factor consensus binding sequences on PDGF-altered genes, random-expectation values, and actual binding site occurrence are shown. **E:** SP-1 expression in hMC nuclear lysate after treatment with PDGF (10 ng/ml), LXA₄ (1 nmol/L), vehicle, or LXA₄ (15 minutes) followed by PDGF. Data are mean ± SEM of duplicate measurements from three independent experiments (**P* < 0.05 compared to vehicle, **P* < 0.05 compared to PDGF).

Figure 3. Altered expression levels of hMC DNA, protein, and secreted protein in response to PDGF $\pm$ LXA₄, validating changes demonstrated by Affymetrix microarray. hMCs were treated with PDGF (10 ng/ml), LXA₄ (1 nmol/L), vehicle, or LXA₄ (15 minutes) followed by PDGF. **a:** Changes in the expression of collagen I α I and fibronectin cDNA after 24 hours, as measured by real-time PCR. The average of two independent experiments performed in duplicate is shown. **b:** Altered levels of fibronectin, thrombospondin 1, and decorin protein measured by immunoblotting after 24 hours, corresponding β -actin blots demonstrate equal loading. Data are representative of three independent experiments. **c:** TGF- β ₁ and MMP-1 secretion from hMCs as measured by ELISA at the indicated time points. Data shown are mean $\pm$ SEM of duplicate measurements from three independent experiments (* P < 0.05, ** P < 0.005).

sult to the glomerulus causes a host of inflammatory reactions resulting in the progressive accumulation of extracellular matrix components, thus decreasing filtration surface area.²⁸ The release of inflammatory mediators and cytokines/growth factors into the surrounding interstitium accompanies the development of fibrosis and tubular atrophy. Previous reports have suggested the involvement of PDGF in mediating these effects.^{7,29}

LXs have been shown to exert potent anti-inflammatory actions in the early stages of GN mediated through inhibition of leukocyte infiltration and or adhesion³⁰ and are proposed to act as endogenously produced proresolution agents in host defense and inflammation.³¹ As observed in intestinal enterocytes, expression of the LXA₄ receptor was up-regulated in response to cytokines, fur-

thermore addition of LXA₄ or its stable, synthetic analogues inhibited interleukin-8 chemokine release, thereby modulating the initiation of inflammation.³² Previous data suggest that the anti-proliferative effects of LXA₄ are mediated via intracellular mechanisms associated with modulation of growth factor receptor (PDGF and EGF) activation,^{4,6} and recruitment of specific SH-2 containing signaling molecules (Mitchell D, Gaffney A, Crean JK, Kinsella BT, Godson C, submitted). Here we demonstrate that LXA₄ can counteract PDGF-induced gene expression. In agreement with other models investigating LXA₄-induced differential gene expression,^{18,33,34} we report that LXA₄ modulated the expression of the NAB1 co-repressor, up-regulating expression by 0.3 SLRs. The stable synthetic analogue 15-epi-16-(para-fluorophe-

(a) Immunocytochemical Expression of Cell Markers α -SMA and E-cadherin in MCT Cells in response to EGF + TGF β 1

(b) Immunocytochemical Expression of Cell Markers α -SMA and E-cadherin in MCT Cells in response to pre-treated hMC supernatant

(c)

Expression of Cell Markers α -SMA and E-cadherin in MCT Cells in Response to MC Supernatant

Figure 4. Altered morphology and expression in MCT cells in response to pretreated hMC supernatant. **a:** Immunocytochemistry of MCT cells was used to illustrate altered expression in E-cadherin and α -SMA after the induction of EMT by TGF- β 1 + EGF treatment throughout 72 hours. **b:** Changes in MCT cells after 72 hours of incubation with pretreated hMC supernatant, measured by phalloidin, E-cadherin, and α -SMA. hMCs were stimulated throughout a 24-hour period with PDGF (10 ng/ml), LXA₄ (1 nmol/L), vehicle or LXA₄ (15 minutes) followed by PDGF. Cell nuclei are DAPI stained (blue) and antibodies are fluorescein isothiocyanate conjugated (green). **c:** MCT cells were treated as indicated above, whole cell lysates were extracted from three independent experiments. Immunoblots for E-cadherin, α -SMA, and β -actin are displayed.

noxy)-lipoxin A (4)-methyl ester has been shown to exert remarkable renoprotection in a murine model of ischemia-reperfusion injury. Several of the genes differentially expressed in this model were also observed here, including interleukin-6, thrombospondin-1, metallothionein 1, transgelin, phosphoserine aminotransferase, and Enigma. LXA₄ diminished the effects of PDGF-stimulated genes responsible for biological functions such as fibrosis, cell proliferation, immune response, lipid metabolism, and embryogenesis. Among the numerous genes identified as PDGF-responsive, those contributing to fibrosis-related changes in hMCs were clustered and examined further. We report that LXA₄ modulates the PDGF-induced expression of several matrix-associated genes, including collagens, transforming growth factors, and fibronectin. Moreover, PDGF-increased expressions of matrix-associated proteins, thrombospondin, and fibronectin, were reduced by LXA₄ to levels that were not significantly different to those of baseline. Such proteins contain multiple domains that bind proteoglycans, collagens, integrins, and cytokines, including TGF- β 1 and are therefore important in the control of cell viability, mitogenesis, and motility. Fibronectin is present basally within the mesangial matrix functioning in supporting mesangial cell viability,³⁵ increases in fibronectin have been demonstrated in fibrotic disease processes.³⁶ TSP is transiently expressed in matrix during development and repair^{23,37} and has previously been shown to respond to PDGF, TGF- β 1, and basic fibroblast growth factor,³⁷ it is a known regulator of TGF- β 1 activity.^{23,38} We further verify that levels of decorin, a small leucine-rich proteoglycan that acts as an endogenous inhibitor of TGF- β 1,³⁹ are decreased in response to PDGF and are recovered by LXA₄ pretreatment. Overall, the up-regulation of both RNA and protein levels of the various fibrosis-associated genes, suggests an expansion of glomerular matrix and associated cytokines. The decreased expression of a negative regulator (decorin) and a corresponding increase in a positive regulator (thrombospondin) of TGF- β 1 demonstrates that PDGF-induced profibrotic activity in hMCs may be associated with increases in TGF- β 1. In contrast, LXA₄ significantly reduced this PDGF-induced profibrotic activity observed in hMCs. These data further support the observed reduction in PDGF-induced secretion of TGF- β 1 by LXA₄ at 24, 48,⁶ and 72 hours. The diverse changes in matrix-associated proteins occurring throughout time, relate to initial injury and long-term damage sustained and the ultimate healing process thereafter. Secreted MMP-1 (collagenase 1) levels were significantly enhanced in response to PDGF at 6, 18, and 24 hours, whereas LXA₄ achieved diminution of the PDGF effect only at 6 and 18 hours. However, consistent with this MMP-1 RNA levels in response to PDGF were not significantly altered by LXA₄ at 24 hours.

The observed dynamics of extracellular matrix synthesis and degradation are consistent with various models of glomerular and tubulointerstitial renal disease. Adhikary and colleagues⁴⁰ examined various extracellular matrix components in anti-glomerular basement membrane nephritis, mRNA for fibronectin and collagens I and IV were elevated in glomeruli from day 15, throughout the disease

Figure 5. Altered fibronectin, VEGF, and CTGF gene expression levels in MCT cells, as measured by quantitative real-time PCR. **A:** Differences in fibronectin, VEGF, and CTGF mRNA in MCT cells treated with a combination of TGF- β_1 and EGF or PDGF-BB for 72 hours. **B–D:** Altered mRNA levels of fibronectin, VEGF, and CTGF, respectively. RNA was extracted from MCT cells treated with pretreated hMC supernatant for 72 hours as previously indicated. Results are displayed as normalized relative quantity, data are mean $\pm$ SEM of duplicate measurements from three independent experiments (* P < 0.05 compared to vehicle, # P < 0.05 compared to PDGF). All values have been normalized to respective 18s rRNA.

course. TIMP-1 and TGF- β_1 mRNA were also enhanced. A similar profile of extracellular matrix components and TGF- β_1 was observed in the cortex, increasing more gradually from day 15 to day 29 as tubular damage progressed.⁴⁰

The reduction in secreted levels of both MMP-1 and TGF- β_1 in LX₄ pretreated cells may reflect reduced autokinase activity of the receptor tyrosine kinases (RTKs) and/or enhanced tyrosine phosphatase activity (Mitchell D, Gaffney A, Crean JK, Kinsella BT, Godson C, submitted). Significant evidence for the cross-talk between the LX G-protein-coupled receptor activated by LX₄ (ALXR) and the RTKs for both PDGF and EGF has been shown.^{4,6} In the context of LX₄ modulation of profibrotic changes, it is noteworthy that a role for the ALXR has recently been proposed in the anti-fibrotic treatment of a lung fibrosis model in mice.⁴¹ In these experiments LX₄ also significantly prevented enhanced proliferation of NIH3T3 fibroblasts and collagen expression by TGF- β_1 . Furthermore Sodin-Semrl and colleagues⁴² described a role for LX₄ in regulating human synovial fibroblast activation, levels of MMP-1 and MMP-3 were diminished in response to LX₄ treatment of stimulated fibroblasts.

The effects of LX₄ on PDGFR activation are relatively specific, being restricted to recruitment to precise phosphotyrosine residues (Mitchell D, Gaffney A, Crean JK, Kinsella BT, Godson C, submitted). In this regard it was of interest to investigate whether LX₄ might modulate transcription factors downstream of specific signal transduction processes. It has been reported that LX₄ can mediate NF- κ B-induced gene expression in intestinal epithelial cells.⁴³ Using MatInspector, many ubiquitous TF binding sequences were observed in the promoters of PDGF-induced genes implicated in both matrix-associated and proliferative gene regulation. Verrecchia and colleagues⁴⁴ showed that inhibition of the SP-1 TF prevented the expression of extracellular matrix genes in dermal fibroblasts. Although SP-1 is a ubiquitous TF, it has also been shown to respond to several growth factors, MAPK and glucose.⁴⁵ Posttranslational modification of SP-1 may have a significant role in TF activation. In our study we measured the binding of nuclear SP-1 from hMCs treated with PDGF and PDGF pretreated with LX₄, we found that SP-1 was significantly increased in response to PDGF. LX₄ consistently diminished this effect.

The translation of a relatively modest glomerular injury to devastating tubulointerstitial fibrosis is increasingly appreciated. Such fibrosis can reflect on a combination of EMT of resident epithelia or progenitor cells, infiltration of circulating or proliferation of resident fibroblasts.^{46,47} Alterations in the mediators present in the interstitium and in the glomerular filtrate contribute to the development of tubulointerstitial fibrosis.²⁶ Tubulointerstitial fibrosis encompasses loss of epithelium polarity, adherens junctions, tight junctions, desmosomes, and cytokeratin intermediate filaments to rearrange their F-actin stress fibers and express filopodia and lamellipodia.⁴⁸ Epithelial cells gain plasticity during the remodeling process, promoting healing or scarring as a response to injury.⁴⁹ Induction of EMT is associated with the expression of cytokines and the proteolytic degradation of epithelial basement membrane.⁵⁰ Matrix metalloproteinases or membrane assembly inhibitors dismantle the membrane, whereas local expression of TGF- β , EGF, IGF-II, or FGF-2 facilitates EMT.^{48,51,52} These cytokines contribute to elevated levels of matrix metalloproteinases and alter the cell proteome. Higgins and colleagues⁵³ observed increases in the expression of collagen I and PAI-1, among others, in EMT using the *in vivo* model of unilateral ureteral obstruction and the *in vitro* model of stimulated murine proximal tubular (MCT) cultured cells. Signature markers for the disease were identified using oligonucleotide microarray.

We report here that PDGF-treated hMC supernatant caused a morphological change in renal tubular MCT cells, similar to that of TGF- β ₁ and EGF. Loss of epithelial tight junction marker E-cadherin and gain of mesenchymal actin cytoskeleton marker α -SMA in MCT cells treated with PDGF-stimulated hMC supernatant was observed. Interestingly, these effects were diminished by LXA₄ pretreatment. LXA₄ pretreatment could alter these effects perhaps by altering PDGF-induced receptor activation in the hMCs, changing the spectrum of soluble factors present in the ultrafiltrate. Furthermore, immunoblotting for E-cadherin and α -SMA under the same conditions confirmed these findings. Previously, we have reported the inhibition of PDGF and EGF mediated PKB/Akt phosphorylation by LXA₄.⁶ This pathway may be involved in the release of cytokines or mediators from hMCs, which we observe to cause morphological changes in MCT cells, a role for this pathway in fibrosis is well established. Stimulation of fibroblasts with endothelin-1 promotes enhanced contractile phenotype or activation, this is prevented by blockade of the PI3K/Akt pathway.⁵⁴ In agreement with this Vittal and colleagues⁵⁵ observed that inhibition of PKB/Akt in bleomycin-induced lung fibrosis markedly reduced accumulation of α -smooth muscle actin-expressing myofibroblasts.

Supernatant from PDGF-stimulated hMCs stimulated an increase in RNA expression of VEGF, CTGF, and fibronectin. This effect was modulated by LXA₄ pretreatment. To control for the possibility that residual PDGF in the media from pretreated hMCs might have induced profibrotic changes in the epithelial cells, these cells were treated with the PDGF-specific phosphotyrosine inhibitor, AG1296. However, in these cells changes in profibrotic gene expression persisted. Similarly, treatment of

MCT cells with PDGF ligand did not induce profibrotic gene expression. Soluble factors released from hMCs in response to PDGF in our *in vitro* model may indicate the progression of renal disease seen *in vivo*.

Collectively these data demonstrate LXA₄ as a potential anti-fibrotic agent, preventing growth factor-induced mesangial matrix production and the progression of renal disease, by alleviating the effect of hMC products on tubular cells. Further investigation into these bioactivities of LXA₄ and its stable synthetic analogues will include examining effects on hMCs and tubular cells in an *in vivo* model of progressive renal disease.

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