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Thrombin induces collagen gel contraction partially through PAR1 activation and PKC-ε

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ABSTRACT: The ability of fibroblasts to contract three-dimensional collagen gels has been used as an *in vitro* model of the tissue contraction which characterises both normal repair and fibrosis. Among its actions, thrombin can activate the protease-activated receptor (PAR)1 and, thereby, stimulate inflammation and repair. The current study evaluated whether thrombin could stimulate fibroblast-mediated collagen gel contraction by activating PAR1 and whether its downstream signalling depends on protein kinase C (PKC)-ε.

Human foetal lung fibroblasts (HFL-1) were cultured in three-dimensional collagen gels and the area of the gels was measured by image analyser.

Both thrombin and TFLLR, a selective PAR1 agonist, stimulated collagen gel contraction mediated by HFL-1. After RNA interference-mediated PAR1 knockdown in HFL-1, both thrombin and the PAR1 agonist-induced gel contraction were partially inhibited (by 22.4±2.2% and 17.6±5.6%, respectively). The gel contraction stimulated by thrombin was also reduced by a nonspecific PKC inhibitor and a calcium-independent PKC-ε inhibitor. Both thrombin and TFLLR significantly increased PKC-ε activity, and this effect was blocked by PAR1 knockdown.

Thrombin stimulates collagen gel contraction at least partially through activation of protease-activated receptor 1 and protein kinase C-ε, and may contribute to tissue remodelling in inflammatory airway and lung diseases.

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Alterations in tissue structure are characteristic features of many chronic lung diseases. In interstitial lung diseases, fibroblasts accumulate together with the collagenous extracellular matrix they produce. Importantly, this fibrotic alteration is associated with disruption of normal tissue architecture. Characteristically, fibrotic tissues contract, distorting normal anatomy and altering function [1]. Similar tissue remodelling is now recognised to occur in airways disease as well, where it may be a major contributor to airway narrowing and fixed airflow limitation [2, 3]. Identification of the mechanisms which lead to these tissue alterations is a major goal in developing strategies to alter the natural history of many chronic lung disorders.

While the mechanisms that lead to tissue remodelling are incompletely understood, fibroblasts are believed to play a pivotal role. In this regard, the ability of fibroblasts to contract three-dimensional collagen gels has been used as an *in vitro* model of the tissue contraction which characterises fibrosis [4]. Many mediators, including platelet-derived growth factor, transforming growth factor (TGF), prostaglandin D_2, lysophosphatidic acid and bradykinin, can stimulate fibroblast-mediated collagen gel contraction, and have been suggested to have a pro-fibrotic role [5–9]. While the signal transduction pathways directly responsible for stimulating collagen gel contraction remain to be defined completely, several stimulators of gel contraction appear to act through protein kinase C (PKC)-ε [7]. This novel, calcium-independent member of the PKC family is known to regulate cytoskeletal reorganisation and to modulate cell spreading and adhesion [10]. Conversely, many mediators, including interleukin-1, tumour necrosis factor-α, prostaglandin E_2 and nitric oxide, can inhibit fibroblast-mediated collagen gel contraction and may have an anti-fibrotic function [11, 12]. The balance among these various mediators may be crucial in determining whether tissue injury is followed by inadequate, excessive or effective tissue repair.

Thrombin is one of the mediators probably present in the inflammatory milieu in the airway. Thrombin is a serine protease activated from pro-thrombin as part of the clotting cascade. In addition to cleaving fibrinogen and serving as the final effector for blood clotting, thrombin has other actions on many cell types. In particular, it can stimulate a number of functions in fibroblasts and other cells consistent with a pro-repair function [13]. Thrombin, moreover, has been reported to be elevated in several chronic inflammatory diseases, including lung diseases characterised by fibrotic tissue remodelling, such as scleroderma and asthma [14, 15].

Thrombin is capable of activating cells by a number of mechanisms. Through its proteolytic action, thrombin can generate secondary mediators. Thrombin can also cleave protease-activated receptors (PARs) on the cell surface. The PARs are G-protein-coupled receptors that are also

substrates for proteolytic cleavage. Upon cleavage, a novel site on the receptor is exposed that can result in auto-activation of the receptor. To date, four types of PARs have been identified and thrombin activates at least three members of the PAR family, including PAR1, PAR3 and PAR4. Much evidence accumulated to date has highlighted the importance of PAR1 as an activator of a number of cell types [16, 17].

The current study was, therefore, designed to determine whether thrombin could stimulate fibroblast-mediated collagen gel contraction by activating the PAR1 receptor. Furthermore, the role of PKC-ε as a downstream mediator of thrombin acting through PAR1 to direct contraction was assessed.

Materials and methods

Materials

Type I collagen was extracted from rat tail tendons (RTTC) as previously described [18, 19]. Briefly, tendons were excised from rat tails, and the tendon sheath and other connective tissues were removed carefully. Repeated washing with Tris-buffered saline (0.9% NaCl, 10 mM Tris, pH 7.5) was followed by dehydration and sterilisation with 50, 75, 95 and 100% ethanol. Type I collagen was extracted in 6 mM hydrochloric acid at 4°C for 24 h. The supernatant was harvested by centrifugation at 2,000×g for 2 h at 4°C. Protein concentration was determined by weighing a lyophilised aliquot from each lot of collagen solution. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) routinely demonstrated no detectable proteins other than type I collagen. The RTTC was stored at 4°C until use.

Thrombin from human plasma and EGTA were purchased from Sigma (St. Louis, MO, USA). The human PAR1 agonists, TFLLR and TFLLR-NH_2, PAR1 agonist negative control, RLLFT-NH_2, and the human PAR4 agonist, GYPGQV, were synthesised by Genemed Synthesis Inc. (South San Francisco, CA, USA). Calphostin C, BAPTA/AM, Ro-31-8220, Gö6976 and TMB-8 were obtained from Calbiochem (San Diego, CA, USA). TGF-β1 was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Tissue culture supplements, FBS and media were purchased from GIBCO (Life Technologies, Grand Island, NY, USA).

Cell culture

Human foetal lung fibroblasts (HFL-1) were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in 100-mm^2 tissue culture dishes (FALCON; Becton-Dickinson Labware, Franklin Lakes, NJ, USA) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 50 $U \cdot mL^{-1}$ penicillin, 50 $\mu g \cdot mL^{-1}$ streptomycin and 1 $\mu g \cdot mL^{-1}$ fungizone. The fibroblasts were passaged every 3–5 days. Subconfluent fibroblasts were trypsinised (trypsin-EDTA; 0.05% trypsin, 0.53 mM EDTA-4Na) and used for collagen gel culture. Fibroblasts used in these experiments were between cell passage 14 and 19.

Collagen gel preparation and contraction assay

Collagen gels were prepared as described previously [19]. Briefly, the appropriate amount of RTTC was mixed with distilled water, 4× concentrated DMEM and the cell suspension, so that the final mixture resulted in 0.75 $mg \cdot mL^{-1}$ of collagen, 3.0×10^5 $cells \cdot mL^{-1}$ and a physiological ionic strength. Fibroblasts were routinely added last to minimise damage during the preparation of collagen gels. One half millilitre of the mixture was cast into each well of 24-well tissue culture plates (FALCON; Becton Dickinson Labware). Gelation occurred in ~20 min at room temperature, after which the gels were released and transferred to 60-mm^2 tissue culture dishes, containing 5 mL of serum-free DMEM with or without thrombin or the PAR agonists in the presence or absence of inhibitors. The floating gels were then incubated at 37°C in a 5% CO_2 atmosphere for various periods of time. The areas of floating gels were measured using an image analyser (Optomax, Burlington, MA, USA). Gel contraction was measured in triplicate gels within each experiment and no less than three separate experiments were performed for each unique parameter.

Preparation and transfection of siRNA targeting PAR1

Short interfering RNA (siRNA) targeting human PAR1 was designed according to the methods as described by ELBASHIR *et al.* [20]. The siRNA sequence targeting PAR1 was from position 288–308, relative to the first nucleotide of the start codon (GeneBank accession number BC002464). Twenty-one-nt RNAs were chemically synthesised by Dharmacon (Lafayette, CO, USA). The sequences of each siRNA pair were as follows: 5′-CAA AUG CCA CCU UAG AUC CdTdT-3′ and 5′-GGA UCU AAG GUG GCA UUU GdTdT-3′. Annealing of siRNAs was performed as described by the manufacturer. Briefly, equal content of each siRNA oligo was incubated in annealing buffer (100 mM KOAc, 30 mM HEPES-KOH, pH 7.4, 2 mM MgOAc) for 1 min at 90°C, followed by 1 h at 37°C. The final concentration of the siRNA duplex was 20 μM in 1× annealing buffer. In addition, commercially available PAR1 siRNA SMARTpool and siRNA SMARTpool negative control were purchased from Dharmacon. Transfection with *Trans*IT-TKO (Mirus Corporation, Madison, WI, USA) was performed as described previously [20]. Twenty-four hours after transfection, the efficacy of knockdown was assessed by Western blot. Cell viability was evaluated by calcein AM and ethidium homodimev-1, a two-colour fluorescence-based method, using the LIVE/DEAD Kit, following the manufacturer's instructions (Molecular Probe, Eugene, OR, USA).

Western blotting

Cells were washed with PBS twice and lysed with lysing buffer containing 35 mM Tris-HCl, pH 7.4, 0.4 mM EGTA, 10 mM $MgCl_2$, 0.1% Triton X-100 and protease inhibitors (3 $\mu g \cdot mL^{-1}$ aprotinin, 1 μM phenylmethanesulphonylfluoride and 10 $\mu g \cdot mL^{-1}$ leupeptin). The cells were then gently scraped and the lysate was transferred to a microcentrifuge tube. After sonication, the samples were centrifuged at 10,000×g for 10 min at 4°C. The protein content of the supernatants was measured and 10 μg of each sample was analysed by SDS-PAGE in 10% polyacrylamide gels. After separation by electrophoresis, the proteins were transferred to polyvinyl difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked in 5% nonfat milk in PBS-Tween at room temperature for 1 h and then probed with anti-PAR1, as well as anti-PAR2, anti-PAR3 and anti-PAR4 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Target proteins were subsequently detected using horseradish peroxidase-conjugated second antibody in conjunction with enhanced chemiluminescence detection system

(ECL; Amersham Biosciences UK Ltd, Little Chalfont, UK). The anti-PAR1 and its second antibody were then stripped from the membranes using stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-Cl, pH 6.7) at 50°C for 30 min. Vimentin expression was detected using anti-vimentin mAb (DAKO Corporation, Carpinteria, CA, USA).

PKC-ε activity assay

PKC-ε activity was determined in crude whole-cell fractions. The assay employed was a modification of procedures previously described [21], using 900 μM PKC-ε-specific substrate peptide (Calbiochem), 8 μM phosphatidyl-L-serine, 24 $\mu g \cdot mL^{-1}$ PMA, 30 mM dithiothreitol, 150 μM ATP, 45 mM $Mg(C_2H_3O_2)_2$ and 10 $\mu Ci \cdot mL^{-1}$ [γ-^{32}P] ATP in a Tris-HCl buffer (pH 7.5). Samples (20 μL) were added to 40 μL of the above reaction mixture and incubated for 15 min at 30°C. Incubations were halted by spotting 50 μL of each sample onto P-81 phosphocellulose papers (Whatman). Papers were then washed five times for 5 min each in phosphoric acid (75 mM), washed once in ethanol, dried and counted in non-aqueous scintillant, as previously described [22]. Kinase activity was expressed in relation to total cellular protein assayed and calculated in $pmol \cdot min^{-1} \cdot mg^{-1}$. All samples were assayed in triplicate and each experiment was repeated on no less than three separate occasions.

Statistical analysis

Individual experiments included triplicate gels within an experiment or for all experimental conditions. Results were always confirmed by repeating each experiment on separate occasions at least three times. Statistical comparisons were made from all experiments, including both the within- and between-group variance. PKC-ε was expressed as fold change in activation compared to control, untreated cells for all experiments. Group data were analysed by one-way ANOVA. Differences between series of data that appeared statistically different were corrected by Tukey's test. A $p<0.05$ was considered significant.

Results

Effect of thrombin on collagen gel contraction

Under control conditions, HFL-1 contracted the collagen gels progressively over the 5-day culture period. Collagen gel contraction was augmented by thrombin over the entire 5-days incubation period (fig. 1). At day 1, the size of gels treated with thrombin (2, 10 and 50 nM) was 42.9±2.1, 35.1±1.6 and 32.3±1.5% of initial gel size, respectively ($p<0.01$, compared with control gels 78.0±2.8%). After 5 days of culture, the size of gels treated with thrombin (2, 10 and 50 nM) was 29.3±3.9, 8.6±0.4, 5.9±0.4% of original size, respectively ($p<0.01$, compared with control gels 46.4±2.8%).

Effect of the proteolytic activity on thrombin-induced collagen gel contraction

To examine whether proteolytic activity of thrombin was required for thrombin-induced collagen gel contraction, hirudin, a thrombin inhibitor, was used. Hirudin itself had no effect on collagen gel contraction. In contrast, the stimulatory effect of thrombin on collagen gel contraction was completely blocked by hirudin (fig. 2).

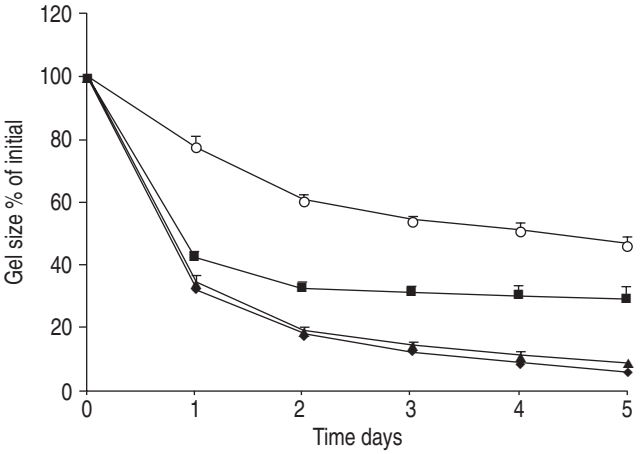

Fig. 1. – Effect of thrombin (2 nM: ■; 10 nM: ▲; 50 nM: ◆) on collagen gel contraction mediated by human foetal lung fibroblasts compared to control (○). Data are presented as mean±SEM for three separate experiments, each of which included triplicate gels for each condition.

Role of PAR1 in thrombin-induced collagen gel contraction

Two separate approaches were used to evaluate the role of PAR1 in mediating thrombin-induced collagen gel contraction. First, a PAR1-selective agonist was utilised to mimic the effects of thrombin. Secondly, the technique of RNA interference (RNAi) was used to decrease PAR1 expression on cultured fibroblasts.

To determine whether PAR1 activation was involved in thrombin-induced collagen gel contraction, the effect of the selective PAR1 agonist, TFLLR, was measured. TFLLR, at a concentration of 600 μM, significantly augmented collagen gel contraction over the 5-day culture period (fig. 3a, $p<0.01$). TFLLR augmented fibroblast-mediated collagen gel contraction at all time points observed. At day 1, gel size was 58.5±1.7% of initial gel size in the presence of TFLLR *versus* 81.6±1.2% for control gels ($p<0.01$). After 5 days, this

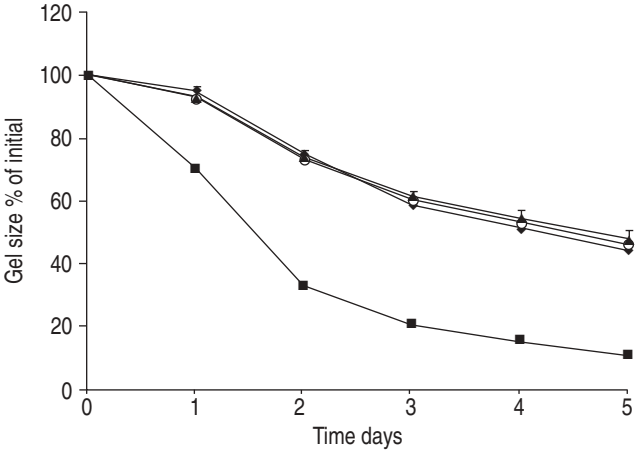

Fig. 2. – Effect of hirudin (◆) on thrombin-induced collagen gel contraction (■) mediated by human foetal lung fibroblasts compared to control (○). The effect of hirudin alone (▲) is also shown. Data are presented as mean±SEM for three separate experiments, each of which included triplicate gels for each condition.

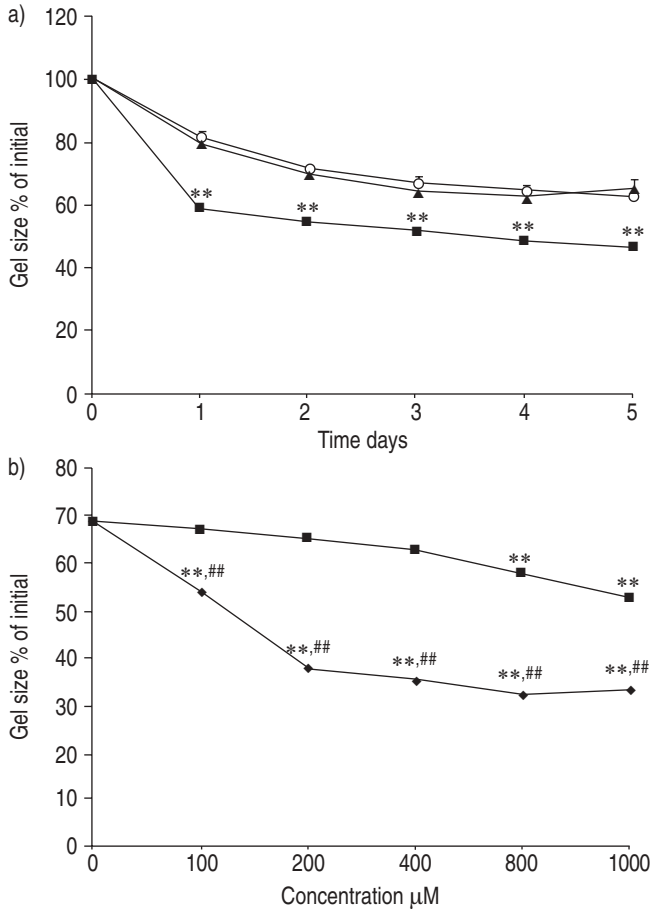

Fig. 3. – Effect of protease-activated receptor (PAR)1 agonist on collagen gel contraction mediated by human foetal lung fibroblasts. a) Effect of PAR1 agonist (TFLLR; ■) and PAR4 agonist (GYPGQV; ▲) compared with control (○). **: $p<0.01$. b) Effect of PAR1 agonist (TFLLR-NH_2; ◆) and its negative control peptide (RLLFT-NH_2; ■). **: $p<0.01$ as compared with control; ##: $p<0.01$ as compared with RLLFT-NH_2. Data are presented as mean±SEM for three separate experiments, each of which included triplicate gels for each condition.

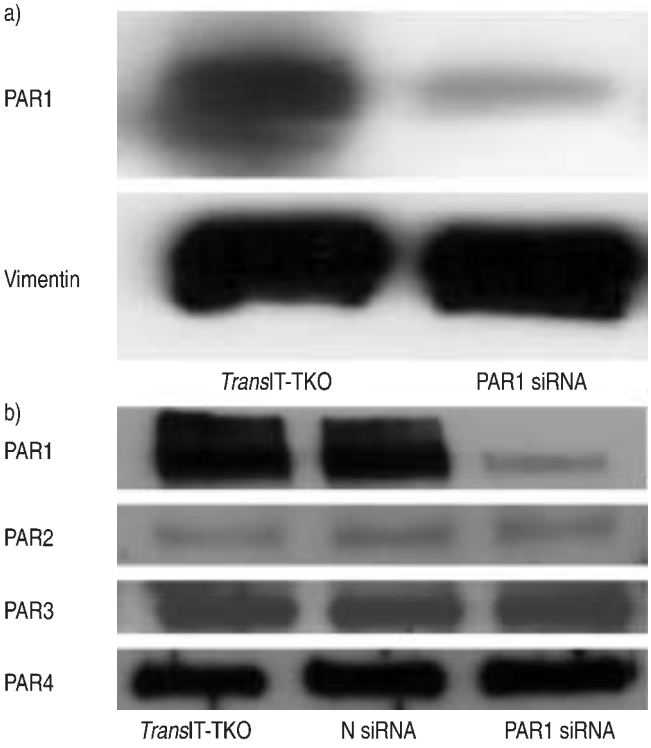

Fig. 4. – Effect of protease-activated receptor (PAR) short interfering RNA (siRNA) on PAR expression in human foetal lung fibroblasts. a) Effect of single PAR1 siRNA transfection on PAR1 and vimentin expression. b) Effect of PAR1 siRNA SMARTpool (PAR siRNA) and siRNA SMARTpool negative control (N siRNA) transfection on PAR1, PAR2, PAR3 and PAR4 expression compared to control (*Trans*IT-TKO).

difference was maintained (46.5±2.0 *versus* 62.3±2.7%, $p<0.01$). No effect on gel contraction was observed with the PAR4 agonist GYPGQV at 600 μM. Amide modification of the PAR1 agonist (TFLLR-NH_2), which is known to increase potency, and its scrambled negative control (RLLFT-NH_2) were also tested (fig. 3b). TFLLR-NH_2 (100–1,000 μM) significantly stimulated gel contraction ($p<0.01$). No effect on gel contraction was observed with RLLFT-NH_2 at lower concentrations. RLLFT-NH_2 at 800 and 1,000 μM also stimulated gel contraction, although the potency was far less than TFLLR-NH_2 ($p<0.01$).

The second approach to confirm the role of PAR1 in mediating thrombin-stimulated fibroblast contraction of three-dimensional collagen gels was to use RNAi to specifically suppress PAR1 expression in cultured HFL-1. Following transfection of PAR1-specific single siRNA, Western blotting confirmed suppression of PAR1 expression without alteration in vimentin expression (fig. 4a). PAR1 expression was also reduced after cells were transfected with a mixture of PAR1 siRNAs (SMARTpool). No inhibitory effect on PAR2, PAR3 or PAR4 expression was observed. The siRNA SMARTpool negative control had no effect on PARs expression (fig. 4b). Transfection did not affect cell viability (data not shown). Cells with or without PAR1 siRNA transfection were then cast into three-dimensional collagen gels and the ability of thrombin to augment collagen gel contraction was assessed. Transfection reagent alone had no effect on fibroblast-mediated collagen gel contraction. In contrast, transfection with either PAR1 single siRNA (fig. 5a) or PAR1 siRNA SMARTpool (fig. 5b) significantly inhibited the augmented contraction induced by both thrombin and by the PAR1 agonist. In contrast, siRNA to PAR1 had no effect on TGF-β1-induced contraction.

Effects of PKC and calcium inhibitors on thrombin-induced collagen gel contraction

To examine the potential role of the PKC pathway on thrombin-induced collagen gel contraction, collagen gels were stimulated with thrombin in the presence or absence of PKC inhibitors. A nonspecific PKC inhibitor, calphostin C, partially blocked the stimulatory effect of thrombin on collagen gel contraction. No inhibitory effect was observed with the conventional PKC isozyme inhibitor Gö6976 (fig. 6). In contrast, the PKC-ε inhibitor Ro-31-8220 partially but significantly decreased collagen gel contraction augmented by thrombin. As novel PKC isozymes are independent of calcium, the effects of three kinds of calcium inhibitors on thrombin-induced collagen gel contraction were also measured. An intracellular calcium chelator (BAPTA/AM), EGTA and an inhibitor of intracellular calcium mobilisation (TMB8) had no effect on collagen gel contraction augmented by thrombin (data not shown).

The role of PKC-ε was further evaluated by determining both the ability of thrombin and TFLLR to stimulate PKC-ε

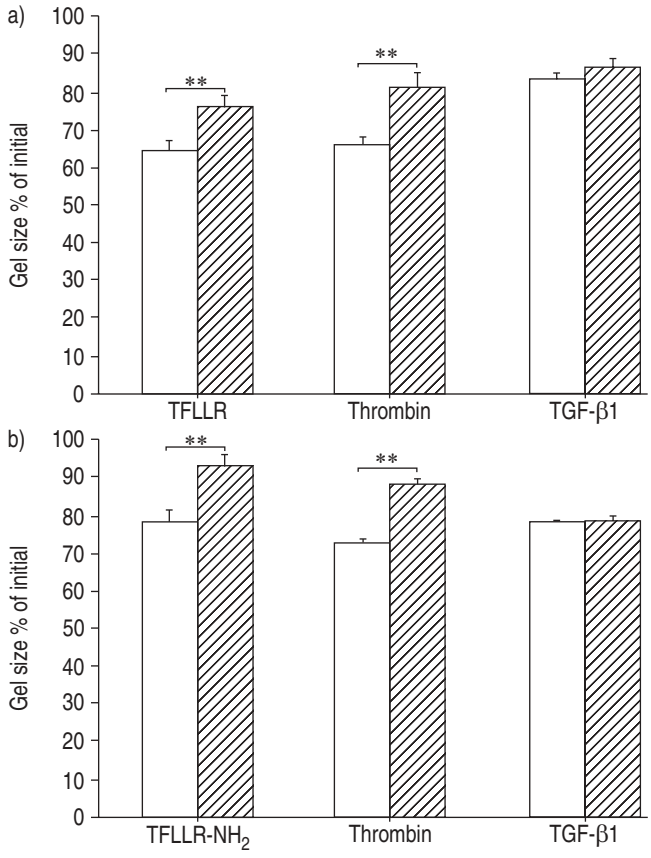

Fig. 5. – a) Effect of protease-activated receptor (PAR)1 knockdown mediated by a single PAR1 short interfering RNA (siRNA; ▨) on PAR1 agonist (TFLLR)-, thrombin- and transforming growth factor (TGF)-β1-induced collagen gel contraction (□) in human foetal lung fibroblasts. b) Effect of PAR1 knockdown mediated by PAR1 siRNA SMARTpool (▨) on TFLLR-NH_2-, thrombin- and TGF-β1-induced collagen gel contraction (□) in human foetal lung fibroblasts. **: $p<0.01$. Data are presented as mean±SEM for three separate experiments, each of which included triplicate gels for each condition.

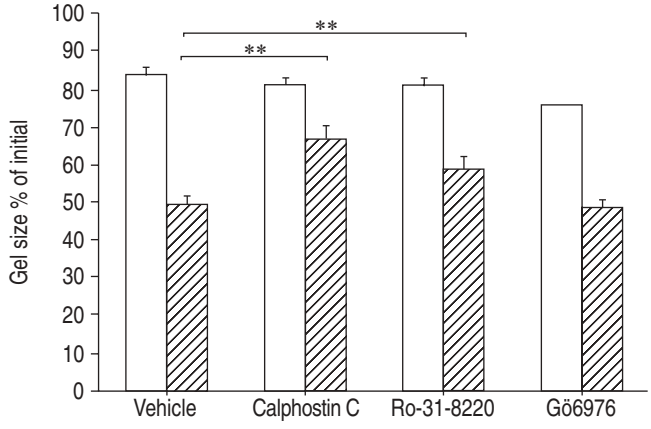

Fig. 6. – Effect of protein kinase C inhibitors (□) on thrombin-induced collagen gel contraction (▨). Data are presented as mean± SEM for three separate experiments, each of which included triplicate gels for each condition. **: $p<0.01$.

activity and to determine whether it could be blocked by siRNA targeting PAR1 (fig. 7). Both thrombin and TFLLR significantly stimulated PKC-ε activity measured directly in

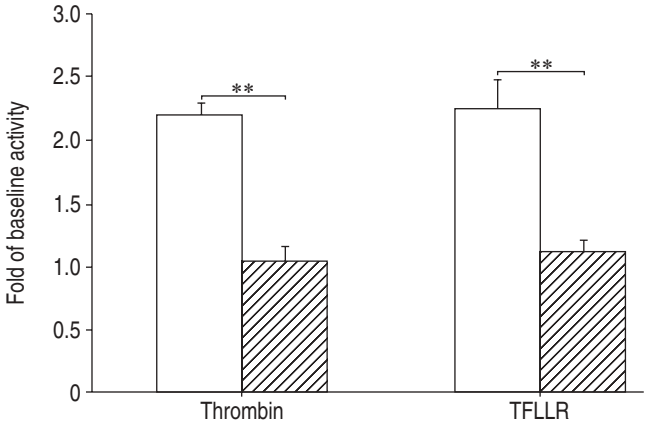

Fig. 7. – Effect of protease-activated receptor (PAR)1 knockdown (▨) on protein kinase C-ε activity stimulated with thrombin and PAR1 agonist (TFLLR) (□) in human foetal lung fibroblasts. Data are presented as mean±SEM for three separate experiments, each of which included triplicate gels for each condition. **: $p<0.01$.

HFL-1. In the presence of siRNA targeting PAR1, however, neither thrombin nor the PAR1 agonist stimulated PKC-ε activity.

Discussion

The current study demonstrates that PAR1 partially mediates thrombin-augmented contraction of three-dimensional collagen gels. Both thrombin and the PAR1 agonist augmented contraction, and this was partially inhibited when PAR1 expression was suppressed by RNAi. Augmented gel contraction appears to depend on PKC-ε activity, as both thrombin and TFLLR led to augmented PKC-ε activation, and this was also blocked by RNAi. Inhibition of PKC-ε, moreover, was able to partially inhibit thrombin-augmented contraction, indicating that PKC-ε is a downstream mediator in the pathway. Finally, the ability of thrombin to augment collagen gel contraction requires the proteolytic activity of the thrombin.

Activation of the blood coagulation system and generation of thrombin is common in the processes of tissue injury, inflammation and repair. In addition to its important role in haemostasis and thrombosis, thrombin mediates multiple other cellular functions. These include stimulating proliferation of various cell types [23–25]. In addition, thrombin can affect extracellular matrix deposition and degradation by promoting pro-collagen production from smooth muscle cells and fibroblasts, and activation of matrix metalloproteinase-2 produced by vascular smooth muscle cells [26, 27]. Thrombin may participate in tissue remodelling by several additional methods. Thrombin has been reported to stimulate fibroblast-mediated contraction of three-dimensional matrices [28], an *in vitro* model of the tissue contraction which characterises fibrosis [4]. The current study supports a role for thrombin as a mediator of tissue remodelling through stimulating collagen gel contraction.

The PARs belong to the family of G-protein-coupled receptors. They are activated by a unique process: serine proteases cleave the extracellular NH_2-terminal extension. This generates a new NH_2 terminus, which serves as a tethered ligand that can interact intramolecularly and bind to the cleaved receptor inducing activation. Four members of the PAR family (PAR1, PAR2, PAR3 and PAR4) are known to date. PAR1, PAR3 and PAR4 are activated by thrombin.

However, while the full spectrum of PAR-induced cellular activities remains to be defined, studies in a number of systems have suggested that PAR1 plays a crucial role in mediating many effects induced by thrombin [16, 17, 29].

Several methods have been used to explore the role of specific PAR receptors. Peptides corresponding to the tethered ligands have been developed. In this context, TFLLR is considered to be the most selective and essential peptide to activate PAR1 [30, 31] and has been used in many studies to help define the role of PAR1 [31–33]. This peptide, however, interacts with relatively low-binding affinity and, therefore, requires relatively high concentrations. Presumably the *in vivo* tethered ligand, by virtue of being retained near the receptor, can be effective even with the relatively low binding affinity. In the current study, TFLLR and TFLLR-NH_2 were used. Both had a stimulatory effect on collagen gel contraction and more potency was observed with TFLLR-NH_2.

An alternate method to evaluate the receptor is the use of PAR1-gene-deficient (knockout) mice [25, 34]. The current study, which used human cells, used a similar method of suppressing PAR1 by exploiting the technique of RNAi [35, 36]. With this method, PAR1 expression could be significantly inhibited and this was associated with inhibition of the effect of both thrombin and the selective PAR1 agonist TFLLR/TFLLR-NH_2. However, neither single PAR1 siRNA nor PAR1 siRNA SMARTpool were completely effective in blocking the effects of either thrombin or PAR1 agonist. This could be due to incomplete suppression of PAR1 expression, which is usually observed with the method of RNAi. Alternatively, an effect of either thrombin or PAR1 agonist on receptors other than PAR1, including other PARs, cannot be excluded. The current study, however, clearly demonstrates that PAR1, at least in part, is responsible for the effect of thrombin.

Like most mediators, thrombin can act through a number of signal transduction pathways. Included among these are the PKCs [13, 16, 37]. PKC-ε, in particular, a member of the novel PKC sub-family, is a calcium-independent serine/threonine kinase, expressed in lung fibroblasts, human airway smooth muscle cells, and many other cell types and tissues [10, 38, 39]. PKC-ε has been shown to participate in the regulation of cytoskeletal reorganisation relating to cell adhesion and motility. PKC-ε is required for cell spreading mediated by integrin β1 and appears to be responsible for the regulation of HeLa cell adhesion to extracellular matrix [40, 41]. Prostaglandin D_2-induced collagen gel contraction has been shown to be mediated through PKC-ε activation [7]. The current study supports this role for PKC-ε and demonstrates that activation of PKC-ε by thrombin occurs through the PAR1 receptor. It also suggests that thrombin acts through what may be a common pathway to promote collagen gel contraction, *i.e.* PKC-ε. Data in the present study, moreover, indicates that thrombin-induced gel contraction is independent of calcium mobilisation, consistent with its action through a novel PKC isozyme.

The present study demonstrates that thrombin-augmented contraction of collagen gels is mediated, at least in part, through PAR1. However, it is possible that other signalling pathways could also contribute. In this regard, the siRNA experiments only partially blocked the thrombin effect. It is not possible to determine if this is due to incomplete suppression of PAR1 expression or to alternate pathways contributing to the thrombin effect. Moreover the current study was performed with the HFL-1 cell strain [42]. This strain of normal human lung fibroblasts has been used extensively to assess fibroblast biology. Fibroblasts, however, are heterogeneous, and to what extent various signalling pathways contribute in other types of fibroblasts remains to be determined. The possibility that other pathways also contribute raises the interesting possibility that inhibition of additional mechanisms could synergise with inhibitors of the PAR1 pathway.

Remodelling of structural tissues with the accumulation of fibrosis is a characteristic feature of many interstitial lung diseases. Similar processes also occur in airways diseases such as asthma and COPD [1–3]. It has been suggested that this airway remodelling is a major cause of airway narrowing and fixed airflow limitation. In this context, increased levels of thrombin have been observed in lung diseases, including asthma and scleroderma [14, 15]. The current study supports the concept that thrombin could contribute to the pathogenesis of fibrotic alterations in a variety of lung diseases. However, in addition to thrombin, other proteases can also cleave PARs [43, 44]. The role these proteases may play in PAR-modulated tissue remodelling remains to be defined. However, the fact that various proteases and protease inhibitors could interact through such signalling mechanisms raises interesting possibilities for coordinated control of remodelling processes.

In conclusion, thrombin is capable of stimulating collagen gel contraction mediated by fibroblasts. This action may contribute to the regulation of tissue repair and remodelling responses consequent to the thrombin activation that occurs following inflammation and injury. This effect of thrombin is mediated by the protease-activated receptor 1 and acts through protein kinase C-ε. Defining this action of thrombin and its signalling pathways may help define the mechanisms that underlie tissue remodelling in diseases such as asthma and chronic obstructive pulmonary disease, and may help define new therapies for these disorders.

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