

Novel Functions of Intracellular IL-1ra in Human Dermal Fibroblasts: Implications in the Pathogenesis of Fibrosis

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Intracellular IL-1 receptor antagonist (icIL-1ra) is reportedly involved in functions independent of blocking IL-1 receptor signaling. Fibroblasts derived from the involved skin of patients with systemic sclerosis (SSc) are predominantly of the myofibroblast phenotype, with higher levels of icIL-1ra compared to normal skin fibroblasts. We examined the effect of overexpression of icIL-1ra on the phenotype and function of normal fibroblasts with respect to the expression of alpha smooth muscle actin (α -SMA), a specific marker for myofibroblasts, and plasminogen activator inhibitor (PAI), a protein involved in fibrogenesis and expressed at higher levels in myofibroblasts, and the production of collagenase (matrix metalloproteinase-1 (MMP-1)), the major enzyme involved in the degradation of native collagen in the skin. Normal human foreskin fibroblasts overexpressing icIL-1ra showed higher levels of α -SMA and PAI and had lower levels of collagenase and MMP-1 mRNA induced by inflammatory cytokines. By contrast, levels of mRNA for tissue inhibitor of metalloproteinase-1 in the transfected cells were not different from the control cells. Pretreatment of the ic-IL-1ra-transfected cells with antisense oligonucleotide directed against the mRNA of icIL-1ra restored MMP-1 expression induced by stimulation with IL-1 β . Our data indicate novel functions for icIL-1ra, which might be relevant to the genesis of fibrotic diseases such as SSc.

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INTRODUCTION

Members of the IL-1 family of cytokines are involved in both acute and chronic inflammatory responses (Kolb *et al.*, 2001) with a growing body of evidence indicating a pivotal role of IL-1 in profibrotic responses. Many human and animal studies have shown the presence of IL-1 in chronic inflamed tissues and in tissues undergoing fibrotic responses (Phan and Kunkel, 1992; Thrall and Scalise, 1995; Johnston *et al.*, 1996).

The three well-known constituents of the IL-1 family are IL-1 α , IL-1 β , and IL-1 receptor antagonist (IL-1ra). Cultured dermal fibroblasts from systemic sclerosis (SSc) patients express higher levels of intracellular IL-1 α than fibroblasts from healthy controls (Kawaguchi, 1994; Kawaguchi *et al.*, 1995). SSc dermal fibroblasts also express higher levels of an isoform of intracellular IL-1ra (icIL-1ra) than normal fibroblasts after stimulation with IL-1 β or tumor necrosis factor (TNF)- α (Higgins *et al.*, 1999a). We have previously shown a linkage between the expressions of these two proteins (Higgins *et al.*, 1999a). Normal dermal fibroblasts transfected and overexpressing pre-IL-1 α exhibited four-fold higher levels of icIL-1ra than control fibroblasts. IL-1 α -mediated transcriptional regulation of icIL-1ra has been reported in keratinocytes (La and Fischer, 2001). Studies by Dewberry *et al.* (2000) demonstrated enhanced expression of icIL-1ra type 1 in human coronary artery and umbilical vein endothelial cells in response to transforming growth factor (TGF)- β , lipopolysaccharide, and phorbol myristate acetate (PMA) and postulated a role of icIL-1ra in the development of atherosclerosis. Together, these findings suggest involvement of an intracellular form of IL-1ra in fibroblasts associated with fibrotic lesions.

IL-1ra exists as four isoforms capable of binding to IL-1 receptors (IL-1R) without transducing any signals. One isoform is secreted (sIL-1ra), whereas the other three remain intracellular (Arend and Guthridge, 2000). They are expressed in a variety of cells such as epithelial cells (especially those that line the gastrointestinal tract), keratinocytes,

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Abbreviations: AP-1, activator protein-1; DMEM, Dulbecco's modified minimal essential medium; icIL-1ra, intracellular IL-1 receptor antagonist; IL-1R, IL-1 receptor; MMP-1, matrix metalloproteinase-1; PAI, plasminogen activator inhibitor; PBS phosphate-buffered saline; α -SMA, alpha smooth muscle actin; SSc, systemic sclerosis; TIMP-1, tissue inhibitor of metalloproteinase-1; TNF, tumor necrosis factor; TGF, transforming growth factor

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some lines of human foreskin fibroblasts (our unpublished observation), vascular smooth muscles, vascular endothelial cells, monocytes, and polymorphonuclear leukocytes (Haskill *et al.*, 1991; Muzio *et al.*, 1995; Bocker *et al.*, 1998; Malyak *et al.*, 1998a, b). It is evident that the secreted form functions extracellularly as a competitor of IL-1 for IL-1R binding and hence acts as an anti-inflammatory agent by preventing the IL-1-IL-1R interaction. However, functions of the intracellular isoforms are only beginning to be delineated. Although the report by Irikura *et al.* (2002) on the epistatic inter-relationship between IL-1, IL-1ra, and IL-1R1 concluded that the only essential function of IL-1ra in both health and disease is competitive inhibition of IL-1R1, some reports have shown results independent of IL-1-IL-1R interactions. Watson *et al.* (1995) reported intrinsic biologic activity for icIL-1ra. By overexpressing icIL-1ra in an icIL-1ra-negative cell line, these authors demonstrated significant reduction of steady-state levels of IL-1-inducible growth-related oncogene (GRO) mRNA owing to effects on mRNA stability. Similarly, it has been reported that expression of icIL-1ra type 1 in human intestinal epithelial cell line Caco-2 inhibited IL-1-induced IL-8 expression (Bocker *et al.*, 1998). Another report demonstrated the decrease of NF- κ B activity in icIL-1ra-expressing cells (Wolf *et al.*, 2001; Garat and Arend, 2003). Most recently, Banda *et al.* (2005) reported that icIL-1ra type 1 binds to the third component of the COP9 signalosome and thereby inhibits IL-1-induced cytokine production in keratinocytes, again supporting icIL-1ra action independent of IL-1-IL-1R interaction.

In this study, to further understand the role of icIL-1ra in the genesis of fibrotic lesions, we overexpressed icIL-1ra type 1 in normal foreskin fibroblasts. Infant fibroblasts were used to avoid the problem of senescence resulting from repeated passages during stable transfection. We examined the phenotype of fibroblasts overexpressing icIL-1ra with respect to the expression of alpha-smooth muscle actin (α -SMA), a specific marker of myofibroblasts prevalent in fibrotic lesions (Tomasek *et al.*, 2002), as well as the expression of plasminogen activator inhibitor (PAI), reported to be elevated in myofibroblasts (Higgins *et al.*, 1999b; Offersen *et al.*, 2003). We also examined the expression levels of matrix metalloproteinase-1 (MMP-1), tissue inhibitor of metalloproteinase-1 (TIMP-1), and collagen type I in infant foreskin fibroblasts overexpressing icIL-1ra. Our current data support the idea that icIL-1ra type 1 contributes to the development of the myofibroblast-like phenotype with enhanced expression of α -SMA and PAI and also downregulates the expression of collagenase in response to inflammatory cytokines. The fact that icIL-1ra is able to inhibit TNF-induced MMP-1 expression shows that the actions of icIL-1ra are independent of the IL-1-IL-1R interactions. Collectively, our data indicate possible important novel roles for icIL-1ra.

RESULTS

Expression of icIL-1ra in transfected fibroblasts

Levels of icIL-1ra type 1 mRNA and icIL-1ra protein were assessed in human fibroblasts transfected with PLXSN-icIL-1ra (HF-icIL-1ra) and in human fibroblasts transfected with

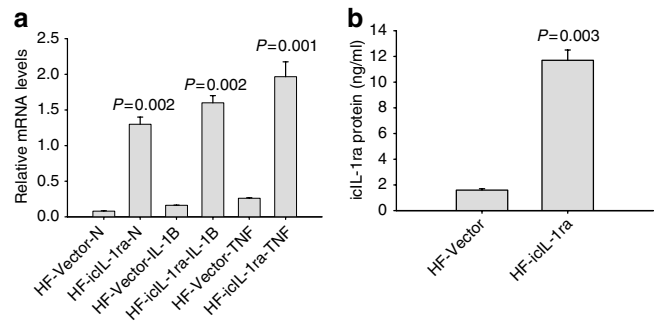


Figure 1. Overexpression of icIL-1ra in HF-icIL-1ra. (a) Equal number of cells (HF-icIL-1ra and HF-Vector) were stimulated with 0 and 1.0 ng of hrIL-1 β or 10 ng/ml of hrTNF- α . After 24 hours, total RNA was extracted and mRNA levels of icIL-1ra and GAPDH were estimated by real-time RT-PCR. Ratios of icIL-1ra type 1 to GAPDH messages are plotted in the graph. (b) Equal numbers of HF-icIL-1ra and HF-Vector maintained in complete DMEM for 48 hours were harvested and lysed. The clarified cell lysates were tested for icIL-1ra type 1 by ELISA. The error bars indicate mean \pm SD of three separate experiments on the same batch of stably transfected fibroblasts.

PLXSN plasmid alone (HF-Vector). Abundant levels of icIL-1ra type 1 mRNA levels were constitutively expressed in HF-icIL-1ra, whereas HF-Vector did not constitutively express detectable levels of icIL-1ra type 1 mRNA (Figure 1a). However, upon stimulation with human recombinant (hr)IL-1 β or hrTNF- α , the HF-Vector did express low levels of icIL-1ra type 1 mRNA (Figure 1a). HF-icIL-1ra expressed high levels of icIL-1ra protein compared to HF-Vector (Figure 1b). The culture medium of HF-icIL-1ra was tested for the presence of secreted IL-1ra and our results presented in Table 1 show that the culture supernatant had very low levels (<30 pg/ml) of IL-1ra compared to the ng/ml levels present in the cell lysates of icIL-1ra-transfected fibroblasts.

Phenotypic features of dermal fibroblasts overexpressing icIL-1ra

Myofibroblasts represent a major cell population in various fibrotic lesions including the involved skin of patients with scleroderma. We have previously observed enhanced expression of icIL-1ra in fibroblasts obtained from the involved skin of patients with scleroderma after IL-1 β or TNF- α stimulation. It was of interest to determine whether normal fibroblasts overexpressing icIL-1ra would have features of myofibroblasts. Therefore, we examined the morphology and α -SMA (a specific marker of myofibroblasts) in HF-Vector and HF-icIL-1ra. For these studies, HF-Vector and HF-icIL-1ra were maintained in culture with complete Dulbecco's modified minimal essential medium (DMEM) for 6 weeks with medium changes every 5th day. HF-Vector retained a spindle-shaped morphology (Figure 2a) with little staining for α -SMA (Figure 2c). In contrast, HF-icIL-1ra assumed a myofibroblast-like appearance (Figure 2b) and stained positive for α -SMA (Figure 2d). Human fibroblasts passed in TGF- β showed myofibroblast morphology (Figure 2e) and more intense staining for α -SMA (Figure 2f).

Table 1. Levels of IL-1ra protein in cell lysates and culture supernatants of human foreskin fibroblasts transfected with icIL-1ra-encoding plasmids and in control fibroblasts

Samples	IL-1ra protein			
	In culture supernatants (pg/ml)		In cell lysates (ng/ml)	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
HFF-Vector	ND	ND	2.2	3.2
HFF-icIL-1ra	12	16	11.5	12.6
HFF-Vector-TNF	8	10	4.7	6.8
HFF-icIL-1ra-TNF	25	28	19.3	20.4
HFF-Vector-IL-1	6	7	3.9	3.5
HFF-icIL-1ra-IL-1	20	26	15.5	16.9

Human fibroblasts transfected with plasmid encoding icIL-1ra (HF-icIL-1ra) and empty vector (HF-Vector) were maintained in DMEM containing 5% FBS. Seventy-two hours after transfection and geneticin selection, culture supernatants were collected and cell lysates were prepared as described in Materials and methods. The levels of IL-1ra were estimated by ELISA using R&D Systems reagents.

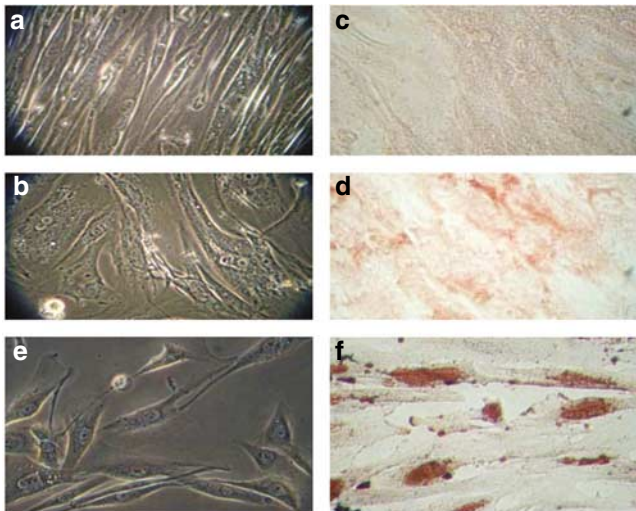


Figure 2. Myofibroblast morphology of HF-icIL-1ra. (a) HF-Vector with normal spindle-shaped fibroblast morphology after culturing in complete DMEM for 6 weeks with medium changes every 5 days. (b) HF-icIL-1ra with myofibroblast-like morphology. (c, d) Cells were fixed and permeabilized (Cytofix/Cytoperm) and were subjected to immunoperoxidase staining with mouse anti-human α -SMA (Sigma) clone 1A 4 monoclonal antibody followed by color reaction developed by a streptavidin-horseradish peroxidase system as described. (c) HF-Vector with little α -SMA staining. (d) HF-icIL-1ra with α -SMA staining. (e) HFF-TGF-passed cells with myofibroblast-like morphology. (f) HFF-TGF with intense staining for α -SMA.

Real-time RT-PCR analysis of constitutive mRNA expression of α -SMA showed significantly enhanced expression in HF-icIL-1ra compared to HF-Vector ($P=0.002$) (Figure 3).

Higgins *et al.* (1999b) have reported that differential regulation of PAI-1 gene expression in human fibroblasts

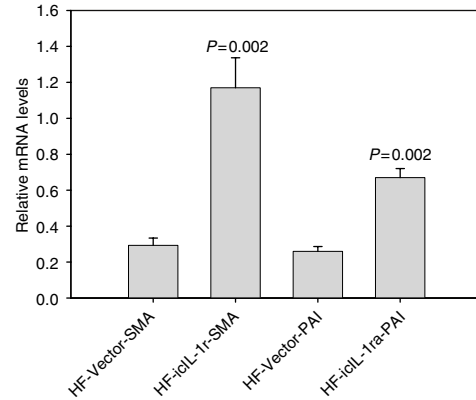


Figure 3. Enhanced levels of α -SMA and PAI mRNA in HF- icIL-1ra.

HF-icIL-1ra and HF-Vector were maintained in complete DMEM for 6 weeks with medium changes every 5 days. Cells were harvested and total cellular RNA was extracted and reverse transcribed. The cDNA thus obtained was amplified by real-time PCR and quantified by SYBR Green using α -SMA primers and PAI primers (Table 4). The values are expressed as ratios of C_t values of α -SMA to those of the housekeeping gene GAPDH. The values indicate mean \pm SD of three independent experiments performed on the same batch of stably transfected fibroblasts.

predisposed to a fibrotic phenotype. To determine the possible association between icIL-1ra and expression of PAI mRNA, HF- icIL-1ra and HF-Vector were examined by real-time RT-PCR. HF-icIL-1ra had increased constitutive levels ($P=0.002$) of PAI mRNA compared to HF-Vector (Figure 3).

Basal expression of MMP-1 mRNA in icIL-1ra type 1 in HF-Vector and HF-icIL-1ra

We examined the basal and inducible levels of MMP-1 in HF-icIL-1ra and HF-Vector. The basal levels of MMP-1 mRNA were remarkably low in HF-icIL-1ra cells compared to HF-Vector (Figure 4).

Expression of MMP-1 in HF-icIL-1ra type 1-transfected fibroblasts stimulated with IL-1 β , TNF- α , or PMA

To determine the impact of overexpression of icIL-1ra type 1 on induced MMP-1 expression, HF-icIL-1ra or HF-Vector fibroblasts were stimulated with hrIL-1 β and hrTNF- α . The MMP-1 mRNA levels were analyzed by real-time RT-PCR. HF-icIL-1ra exhibited a significant decrease in MMP-1 mRNA levels for both basal and stimulated conditions compared to HF-Vector (Figure 5). The effect of another potent stimulant for MMP-1 expression, namely PMA (10 ng/ml), was also examined in cultures of HF-icIL-1ra and HF-Vector. The results from two separate experiments showed up to 50% reduction in MMP-1 mRNA levels in HF-icIL-1ra after PMA stimulation compared to HF-Vector (data not shown).

In addition to measuring MMP-1 mRNA levels, MMP-1 protein was also measured. Cultures of HF-Vector and HF-icIL-1ra fibroblasts were collected after 48 hours of stimulation with 1 ng/ml of hrIL-1 β or 5 ng/ml hrTNF- α . MMP-1 protein levels in culture supernatants were determined by ELISA. Results from two separate experiments presented in Figure 6 show significantly reduced levels of collagenase in HF-icIL-1ra compared to HF-Vector. The

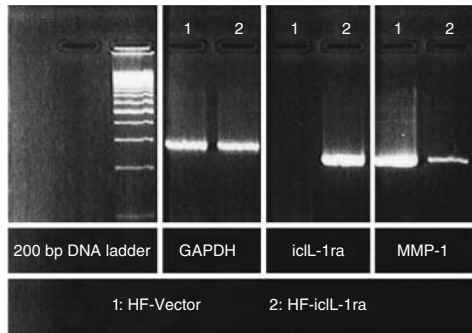


Figure 4. Reduced mRNA in HF-icIL-1ra. HF-icIL-1ra and HF-Vector were maintained in complete DMEM for 6 weeks with medium changes every 5 days. Cells were harvested in Tri-Reagent and total cellular RNA was extracted. One microgram of total RNA was reverse transcribed in a 20 μ l reverse transcription reaction mixture and 5.0 μ l of the cDNA was amplified by 28 cycles of PCR using primers specific for GAPDH and MMP-1. The PCR products were analyzed on a 2% agarose gel, stained with ethidium bromide, and photographed.

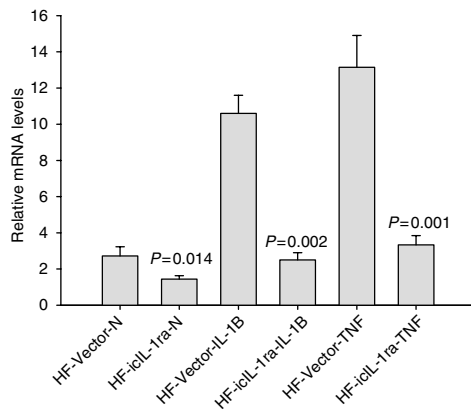


Figure 5. Reduced expression of IL-1- and TNF-induced MMP-1 mRNA in HF-icIL-1ra. HF-icIL-1ra and HF-Vector were stimulated with 1.0 ng/ml IL-1 β or 10 ng/ml TNF- α for 12–16 hours. MMP-1 and GAPDH message levels were estimated using real-time RT-PCR. Three separate experiments were performed on the same batch of stably transfected fibroblasts. The results are represented as the reciprocal of the ratios of the C_t values of MMP-1 to those of GAPDH (the housekeeping gene).

results show that human fibroblasts overexpressing icIL-1ra type 1 are refractory to MMP-1 upregulation when exposed to potent stimulators such as IL-1 β , TNF- α , or PMA.

Two other human fibroblast cell lines transfected with plasmid encoding icIL-1ra also had similar reduced response to collagenase expression upon treatment with TNF- α . The data are depicted in Table 2.

Expression of MMP-1 in fibroblasts treated with rhIL-1ra stimulated prior to stimulation with TNF- α

As we detected low levels of secreted form of IL-1ra in icIL-1ra-transfected fibroblasts, we decided to determine if extracellular presence of IL-1ra could mimic the effects of icIL-1ra on collagenase expression. Fibroblasts were treated with 0, 25 pg/ml, 1 ng/ml, 10 ng/ml, 15 ng/ml, and 50 ng/ml rhIL-1ra prior to TNF- α exposure. The results shown in

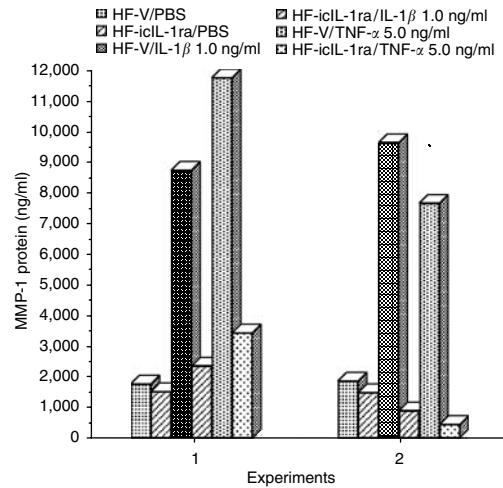


Figure 6. Reduced levels of MMP-1 protein in HF-icIL-1ra type 1. HF-icIL-1ra and HF-Vector were cultured for 48 hours with hrIL-1 β (1.0 ng/ml) or hrTNF- α (5 ng/ml) and MMP-1 protein secreted into the culture medium was measured by ELISA.

Table 2. Reduced collagenase (MMP-1) production in human foreskin fibroblasts derived from two different individuals and expressing icIL-1ra

Samples	Collagenase (ng/ml)			
	HFF-414		HFF-419	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
HFF-Vector	134	129	152	160
HFF-Vector-TNF	1,625	1,605	1,768	2,363
HFF-icIL-1ra	88	60	78.2	—
HFF-icIL-1ra-TNF	233	213	173	—

Two different lines of human foreskin fibroblasts (HFF-414 and HFF-419) were transfected with plasmids encoding icIL-1ra (HFF-icIL-1ra) or empty vector (HFF-Vector). Seventy-two hours after selection with geneticin, the fibroblasts were exposed to TNF or PBS. Forty-eight hours after TNF- α treatment, the supernatants were collected and the levels of collagenase were estimated by ELISA as described in Materials and methods.

Table 3 indicate that presence of rhIL-1ra even up to a concentration of 1.0 ng/ml did not have any significant effect on collagenase expression as determined by ELISA.

Expression of TIMP-1 mRNAs in HF- icIL-1ra or HF-Vector after stimulation with TNF- α

As we observed a substantial reduction in basal and IL-1/TNF/PMA-inducible MMP-1 expression in icIL-1ra-transfected fibroblasts, we wanted to see if the TIMP-1 expression was also affected by icIL-1ra. Interestingly, the mRNA levels of TIMP-1 were not different between TNF- α -stimulated HF-icIL-1ra and HF-Vector (Figure 7).

Specificity of icIL-1ra type 1 action on MMP-1 expression

To further study the direct relationship between icIL-1ra type 1 and MMP-1 expression, an antisense oligonucleotide for

Table 3. TNF-induced collagenase (MMP-1) production in human foreskin fibroblasts exposed to varying concentrations of human recombinant IL-1ra

Samples	MMP-1/collagenase	
	MMP-1 mRNA (relative units)	Collagenase (ng/ml)
HFF+TNF	2.52 ± 0.3	1,202
HFF+25 pg/ml rhIL-1ra+TNF	2.59 ± 0.3	1,069
HFF+1 ng/ml rhIL-1ra+TNF	2.73 ± 0.5	1,574
HFF+10 ng/ml rhIL-1ra+TNF	2.63 ± 0.4	880
HFF+15 ng/ml rhIL-1ra+TNF	2.77 ± 0.6	823
HFF+50 ng/ml rhIL-1ra+TNF	2.42 ± 0.5	901

Human foreskin fibroblasts maintained in DMEM containing 5% FBS were exposed to 0, 25 pg/ml, 1 ng/ml, 10 ng/ml, and 50 ng/ml of rhIL-1ra (R&D Systems). Two hours later, TNF-α was added to a concentration of 5 ng/ml. The fibroblasts from one set of cultures were harvested after 24 h of TNF-α treatment and total RNA was isolated. The mRNA levels of MMP-1 were determined by real-time RT-PCR (Materials and methods). One set of the culture supernatants was collected 48 h later and the collagenase levels were detected by ELISA as described in Materials and methods.

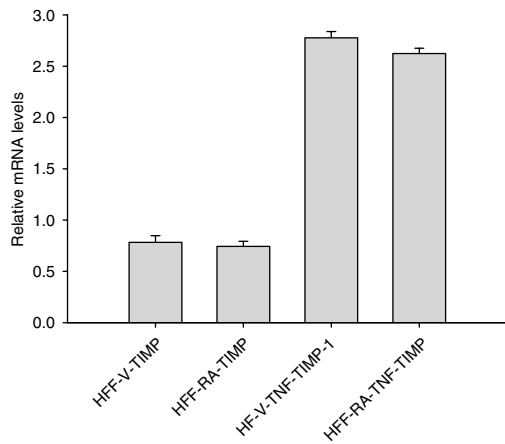


Figure 7. Normal expression of TIMP-1 mRNA in fibroblasts overexpressing icIL-1ra. HF-icIL-1ra type 1 HF-icIL-1ra and HF-Vector were stimulated with 0 or 10 ng/ml TNF-α for 12–16 hours. Total cellular RNA was extracted and TIMP-1 and GAPDH message levels were estimated using SYBR Green real-time RT-PCR. The values are expressed as the reciprocal ratios of Ct values of TIMP-1 mRNA and collagen type I mRNA to those of the housekeeping gene GAPDH. The values indicate mean ± SD of three independent experiments performed on the same batch of stably transfected fibroblasts.

icIL-1ra type 1 mRNA was designed to specifically inhibit the translation of icIL-1ra type 1 into the corresponding protein. HF-icIL-1ra fibroblasts stimulated with 1.0 ng/ml IL-1β were treated with varying concentrations of antisense oligonucleotide directed against icIL-1ra type 1 mRNA. An oligonucleotide with a scrambled sequence was used for the control. Oligonucleotides were mixed with LipofectAMINE reagent to enhance cellular uptake. Results presented in Figure 8a (gel) and Figure 8b (bar graph) show that HF-icIL-1ra treated with 200 μM antisense icIL-1ra type 1 oligonucleotide expressed significantly higher levels of MMP-1 mRNA compared to the

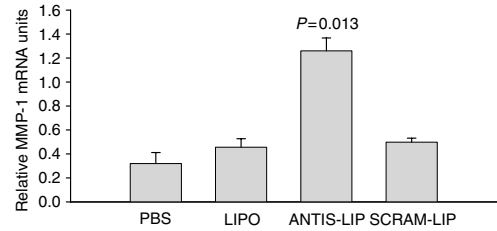


Figure 8. Transfection of HF-icIL-1ra with antisense oligonucleotide directed toward icIL-1ra restores IL-1-induced MMP-1 mRNA expression. Phosphorothioate-derivatized antisense oligodeoxynucleotide complimentary to -6 to +12 of the natural icIL-1ra was synthesized and oligonucleotide with a scrambled sequence was prepared by a similar method as a control. HF- icIL-1ra stimulated with 1.0 ng/ml IL-1β was transfected with 300 μM of antisense icIL-1ra type 1 oligonucleotide (24 hours prior to stimulation with 1.0 ng/ml of IL-1β) using LipofectAMINE. PBS and LipofectAMINE alone served as additional controls. The cells were harvested 12–18 hours after stimulation for RNA extraction. Total RNA was reverse transcribed and MMP-1 mRNA was estimated by real-time RT-PCR. The values indicate mean ± SD of three independent experiments performed on the same batch of transfected geneticin-selected fibroblasts (PBS: PBS alone; LIPO: LipofectAMINE alone; ANTIS-LIP: antisense icIL-1ra oligonucleotide + LipofectAMINE; SCRAM-LIP: scrambled oligonucleotide + LipofectAMINE).

controls (ie HF-icIL-1ra treated with phosphate-buffered saline (PBS), with empty liposome, and the oligonucleotide with the scrambled sequence). The results indicate a direct association between increased icIL-1ra type 1 and decreased MMP-1 expression in fibroblasts stimulated by agents, such as IL-1β, TNF-α, and PMA, that normally upregulate MMP-1.

DISCUSSION

In this study, we present data that attribute to the involvement of icIL-1ra in fibroblasts in some novel functions relating to enhanced α-SMA expression and PAI expression, and its ability to impart resistance to TNF-α-, IL-1-, and PMA-induced MMP-1 expression. Together, these data indicate the role of icIL-1ra in the genesis of fibrosis following an inflammatory response. Our current data are in line with the previous reports on the cardinal involvement of members of the IL-1 family in inflammation-associated fibrotic lesions (Kotecha et al., 1996; Sime and Gaudie, 1996; Mikuniya et al., 1997, 2000; Marshall et al., 1998; Ziegenhagen et al., 1998; Whyte et al., 2000; Kolb et al., 2001).

The observed ability of icIL-1ra to inhibit the TNF-α-induced expression of MMP-1 is evidence toward ascribing an IL-1R-independent icIL-1ra action. Similar IL-1R-independent functions of icIL-1ra have been reported earlier (Watson et al., 1995; Wolf et al., 2001; Banda et al., 2005). icIL-1ra-transfected fibroblasts secrete picogram quantities of IL-1ra into the medium. However, adding recombinant IL-1ra up to a concentration of 10.0 ng/ml did not have any significant effect on TNF-α-induced collagenase expression. We therefore believe that the effects observed in our present study are unique to the intracellular form of IL-1ra. Understanding of the mechanisms by which icIL-1ra type 1 affects MMP-1 expression is only rudimentary and speculative at this juncture. Regulation of MMP-1 expression is complex and may involve interventions at transcription, post-transcriptional

processing of mRNA, and cellular translocation (Vincenti *et al.*, 1996; Cook *et al.*, 2003). Inflammatory cytokines such as IL-1 β and TNF- α used in the current study trigger the ceramide signaling pathways (Spiegel *et al.*, 1996). Three distinctive mitogen-activated protein kinase pathways, that is, extracellular signal-regulated kinase 1/2, stress-activated protein kinase/c-Jun N-terminal kinase, and p38, are involved in the ceramide-dependent expression of MMP-1 (Reunanen *et al.*, 1998). Also, the activator protein-1 (AP-1) pathway is involved in MMP-1 expression. The effect of PMA on MMP-1 stimulation is mediated via the formation of AP-1 complexes and their interaction with two AP-1 sites (referred to as phorbol ester responsive element (TRE)) in the MMP-1 promoter (Angel *et al.*, 1987; Schonthal *et al.*, 1988). Involvement of AP-1 components c-fos and c-jun in IL-1-mediated induction of MMP-1 in gingival fibroblasts has been reported by Hamid *et al.* (2000). It is possible that icLL-1ra has the capability to decrease the expression of AP-1 components or their activity. It is pertinent to note here that icLL-1ra has been shown to decrease the activity of NF- κ B in intestinal epithelial cells (Garat and Arend, 2003). Also, icLL-1ra-mediated reduction in stability and/or degradation of MMP-1 mRNA cannot be ruled out as we measured only steady-state levels of MMP-1 mRNA. A recent study by Reunanen *et al.* (2002) has shown enhancement of MMP-1 and MMP-3 mRNA stabilization by activated p38 α -mitogen-activated protein kinase. Our preliminary microarray data on icLL-1ra-overexpressing fibroblasts have shown reduced levels of p38 mitogen-activated protein kinase mRNA expression (data not shown). The ability of icLL-1ra to reduce mRNA stability of induced message has also been reported by Watson *et al.* (1995). These authors expressed icLL-1ra in an icLL-1ra-negative cell line using a retroviral expression system and measured the half-life of GRO mRNA upon exposure to IL-1 β . The half-life of GRO mRNA was reduced by more than half in the icLL-1ra-expressing cell line compared to the control cell line.

Although there are several potent inducers of MMP-1, there are only a few effective downregulators of MMP-1 expression, and the regulation appears to be cell dependent. IFN- γ downregulates MMP-1 in fibroblasts and macrophages (Shapiro *et al.*, 1990; Varga *et al.*, 1995) but upregulates MMP-1 in keratinocytes (Tamai *et al.*, 1990). The cytokine TGF- β has long been regarded as a key player in fibrotic processes including renal fibrosis, pulmonary fibrosis, and SSc (Leask and Abraham, 2004). It is a potent inhibitor of MMP-1 expression in fibroblasts (White *et al.*, 2000); however, it enhances the expression of MMP-1 in epithelial cells (Strissel *et al.*, 1995). The inhibitory effect of TGF- β has been reported to be mediated through a TGF- β inhibitory element present at -249 in the rabbit MMP-1 promoter, and is conserved at -246 in the human gene (Strissel *et al.*, 1995). The TGF- β inhibitory element functions as a constitutive repressor as well as an antagonist of transcriptional induction by phorbol esters. PMA circumvents specific receptor activation by binding and activating protein kinase C and increasing AP-1 complexes and their interaction with two AP-1 sites (referred to as phorbol ester response element) in

the MMP-1 promoter (Brose and Rosenmund, 2002). As TIMP-1 is a specific inhibitor of MMP-1, we also examined the mRNA levels of TIMP-1 in HF-icLL-1ra and HF-Vector. We did not observe any notable differences in TIMP-1 mRNA expression between HF-icLL-1ra and HF-Vector, thereby ruling out the possibilities of TIMP-mediated inhibition of MMP-1 in HF-icLL-1ra.

The observation that overexpression in normal fibroblasts of icLL-1ra type 1 induces a myofibroblast phenotype is novel and is of potential importance to tissue repair and fibrosis. Myofibroblasts are abundant in granulation tissue of healing wounds where they are responsible for wound contraction (Schmitt-Graff *et al.*, 1994). Myofibroblasts are particularly increased in organs undergoing fibrosis such as lung, kidney, liver, bone marrow, and eye (Schmitt-Graff *et al.*, 1994). They comprise a major portion of fibroblasts in dermal lesions of patients with SSc (>90% patients in 50% of the patients studied; Kirk *et al.*, 1995). In culture, α -SMA-positive fibroblasts can be grown from cloned α -SMA-negative fibroblasts (Schmitt-Graff *et al.*, 1994). Several cytokines and growth factors have been shown to modulate α -SMA expression in fibroblast cultures. TGF- β increases α -SMA expression, whereas platelet-derived growth factor, basic fibroblast growth factor, and IFN- γ decrease α -SMA expression (Schmitt-Graff *et al.*, 1994). Myofibroblasts are also known to express high levels of PAI, a key player in the genesis of fibrosis. Reduction in fibrotic tissue formation in mice genetically deficient in PAI-1 has been reported by Chuang-Tsai *et al.* (2003). Ames *et al.* (1997), in a report on coagulation/fibrinolysis balance in SSc, showed significantly elevated levels of PAI in the plasma of patients with the disease. The overexpression of icLL-1ra type 1 induced a myofibroblast phenotype with characteristic morphology and enhanced expression of α -SMA and PAI. The mechanisms of icLL-1ra-mediated enhancement of α -SMA and PAI expression and myofibroblast morphology in normal fibroblasts are not clear at this time. It is possible that icLL-1ra may have altered actions owing to its retention of the seven additional N-terminal amino acids, which might confer an intracellular agonist's role as speculated by Haskill *et al.* (1991). Further studies at the cellular and molecular levels are necessary to understand the mechanisms involved in icLL-1ra-mediated upregulation of α -SMA and myofibroblast differentiation.

In conclusion, we have presented evidence for novel functions of IL-1ra type 1 that could have a potential role in delineating the molecular events associated with regulation of collagenase expression and fibroblast-myofibroblast differentiation, both of which are believed to be key events in normal tissue repair and in the genesis of fibrosis. Our findings that relate elevated icLL-1ra in fibroblasts to a reduced responsiveness of the MMP-1 pathway seem to be appropriate for the myofibroblast phenotype that is normally committed to matrix deposition.

MATERIALS AND METHODS

All the experiments described in this report were conducted upon approval of the institutional review board of the University of Tennessee Health Science Center Memphis, TN, based on the formal

guarantee (Federal Wide Assurance) provided to the Department of Health and Human Services and in compliance with the institutional biosafety committee.

Cloning of icIL-1ra

The icIL-1ra type 1 cDNA insert was prepared as follows: poly(A)⁺ RNA obtained from THP-1 monocytic cells (ATCC, Manassas, VA) stimulated with 1 µg/ml lipopolysaccharide and 100 ng/ml PMA was reverse transcribed using oligo(dT)₁₈ primers and random hexamers. The cDNA thus obtained was subjected to PCR using 5' and 3' primers corresponding to the coding sequence of icIL-1ra type 1 sequence as reported by Haskill *et al.* (1991). Desired restriction enzyme sites were incorporated into the terminals of each primer for cloning. Correct sequence of the cloned icIL-1ra type 1 was verified by automated dye terminator cycle sequencing (ABI Prism Kit, Perkin Elmer, Foster City, CA) at the University of Tennessee Molecular Resources Center. The sequence of the cloned icIL-1ra corresponds to the isoform designated as icIL-1ra type 1. The cDNA for icIL-1ra type 1 was then cloned into an expression plasmid (pLXSN) carrying a neomycin resistance gene. The icIL-1ra type 1 cDNA was placed under the control of a simian virus 40 promoter (pLXSN icIL-1ra). Unmodified plasmid carrying the neomycin resistance gene served as control (HF-Vector). Plasmids (pLXSN icIL-1ra type 1 and pLXSN-Vector) were amplified in *E. coli* HB101/JM109 and purified using a commercially available plasmid purification kit (Promega, Madison, WI).

Human dermal fibroblast cultures

Dermal fibroblasts were obtained from infant foreskins by conventional explant culture techniques and grown in DMEM containing HEPES buffer, non-essential amino acids, sodium pyruvate, 100 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 9% fetal bovine serum (FBS) (hereafter referred to as "complete DMEM"). Low-passage (5–10th) fibroblasts were used for transfection.

Transfection and expression of icIL-1ra type 1 in human dermal fibroblasts

Fibroblasts were transfected using LipofectAMINE 2000 reagent obtained from Invitrogen Life Technologies Inc. (Gaithersburg, MD) following the manufacturer's protocol. Briefly, 1 day prior to transfection, 2 × 10⁵ fibroblasts were seeded per well (in 500 µl) in a 24-well plate. The cells were maintained in complete DMEM without antibiotics. For transfection of each well, ~1.0 µg of plasmid DNA was taken up in 50 µl of OPTI-MEM with reduced serum (Life Technologies Inc.). For each well of cells to be transfected, 2 µl of LipofectAMINE reagent was added to 50 µl of OPTI-MEM and incubated at ambient temperature for 5 minutes. Diluted plasmid DNA was then combined with the diluted LipofectAMINE reagent and incubated at ambient temperature for 20 minutes to allow DNA-LipofectAMINE complexes to form. The plasmid DNA-LipofectAMINE complex was then added to each well and mixed gently by rocking the plates back and forth. The cells were incubated at 37°C for 4–6 hours. To each well, an additional 500 µl of OPTI-MEM with reduced serum was added and the incubation continued for another 48–72 hours. The medium was then replaced with complete DMEM containing 600 µg/ml of geneticin (Life Technologies Inc.). Cells were incubated for 5–7 days with removal of dead cells and replenishment with complete

medium containing 600 µg/ml geneticin. Several days later and after 3–4 subcultures, only cells resistant to geneticin (600 µg/ml) were remaining. The stably transfected cells were tested for the expression of icIL-1ra type 1 mRNA by real-time RT-PCR and for the expression of icIL-1ra protein by ELISA. Aliquots of cells stably transfected with icIL-1ra type 1 (HF-icIL-1ra) and of control cells (HF-Vector) were preserved for future use by freezing in complete DMEM containing 10% DMSO.

Estimation of total icIL-1ra type 1 protein in transfected fibroblasts

Total icIL-1ra type 1 was measured by ELISA (R&D Systems, Minneapolis, MN). The transfected cells were harvested, washed once in serum-free DMEM, counted manually in a hemocytometer, and lysed by incubation for 30 minutes at 4°C with 50 mM Tris, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (Sigma Aldrich Chemicals, St Louis, MO) and 0.1% Nonidet P-40 pH 7.5, containing protease inhibitors (25 mM benzamide, 1 mM phenylmethanesulfonyl fluoride, 10 mM N-ethylmaleimide, 1 mM EDTA, 1.0 µg/ml leupeptin, 1.0 µg/ml aprotinin, and 1.0 µg/ml pepstatin). The cell lysates were cleared by centrifugation at 18,000 × g for 30 minutes at 4°C. Cleared lysates were stored at –80°C until tested after adding 0.1 volumes of NaCl (to a final concentration of 0.9%) to each sample. The concentration of total icIL-1ra is expressed as ng/1 × 10⁶ vector-transfected (HF-Vector) or icIL-1ra-transfected (HF-icIL-1ra) cells.

Estimation of IL-1ra protein in culture supernatants of transfected fibroblasts

Culture supernatants were collected from the transfected fibroblasts to determine the levels of IL-1ra that was released into the culture medium. IL-1ra protein was estimated by ELISA method as described above using reagents obtained from R&D Systems.

Stimulation of cultured fibroblasts with IL-1β, TNF-α, and PMA

Transfected fibroblasts were continuously maintained in complete DMEM containing 600 µg/ml geneticin. To study the effect of hrIL-1β, hrTNF-α (R&D Systems), or PMA (Promega), fibroblasts were harvested from confluent monolayers by trypsin treatment. Equal numbers of fibroblasts/well were seeded in 12- or 24-well plates and grown to confluence for 72 hours. One day prior to treatment with hrIL-1β, hrTNF-α, or PMA, the initial growth medium was replaced with complete DMEM containing 5% FBS. Separate plates were set up for mRNA and protein analysis. Duplicate wells were set up for each assay condition. Fibroblasts were then exposed to 1.0 ng/ml hrIL-1β, 5 or 10 ng/ml hrTNF, or 10 ng/ml of PMA and were harvested 12–16 hours later for mRNA analysis. One milliliter of Tri-Reagent was added to each well to lyse the fibroblasts. The cell lysates thus obtained were stored at –80°C until analyzed. For MMP-1 protein estimation, the fibroblasts set up as above were incubated for 48 hours with hrIL-1β (1.0 ng/ml) and hrTNF-α (5 ng/ml) after which the cell culture supernatants were collected and stored at –80°C until tested.

Stimulation of normal human foreskin fibroblasts with TNF-α in the presence of rhIL-1ra

Normal human fibroblasts (70–80%) maintained in DMEM containing 5% FBS in 24-well plates were exposed to varying concentrations

(0, 25 pg/ml, 1.0 ng/ml, 10 ng/ml, 15 ng/ml, 50 ng/ml) of rhIL-1ra (R&D Systems) 2 hours prior to the addition of 5 ng/ml TNF- α . This was performed to determine whether extracellular IL-1ra could simulate the effect of intracellular form of IL-1ra on TNF-induced MMP expression. The supernatants from these cultures were collected after 48 hours and were stored at -80°C until tested for collagenase.

Estimation of MMP-1 protein

MMP-1 protein secreted into the culture medium was measured by ELISA, performed as previously described using the protein analysis program of Stoscheck (1987). Rabbit anti-human MMP-1 antibody was kindly provided by Dr George Stricklin, VAMC and Vanderbilt University Medical College, Nashville, TN. Culture supernatants were collected 48 hours after treatment with hrIL-1 β and hrTNF- α and cleared by centrifugation at $18,000 \times g$ for 30 minutes at 4°C . Cleared supernatants were treated with protease inhibitors (25 mM benzamide, 1 mM phenylmethanesulfonyl fluoride, 10 mM N-ethylmaleimide, 1 mM EDTA, 1.0 $\mu\text{g/ml}$ leupeptin, 1.0 $\mu\text{g/ml}$ aprotinin, 1.0 $\mu\text{g/ml}$ pepstatin) and stored at -80°C until tested.

Estimation of mRNA by real-time RT-PCR

Total cellular RNA was isolated from the cells using Tri-Reagent (Sigma-Aldrich, St Louis, MO) followed by chloroform (Sigma-Aldrich) extraction and isopropanol (Sigma-Aldrich) precipitation. The total RNA was used for oligo(dT)-mediated reverse transcription of mRNA species in each sample. Specific messages were amplified and detected by real-time PCR performed using the SYBR Green method (Applied Biosystems, Foster City, CA) using specific sets of forward and reverse primers (Table 4) synthesized at Integrated DNA Technologies Inc. (Coralville, IA). The reactions were performed according to the manufacturer's protocol. Each sample was assayed in duplicate. The PCR product was detected by measuring the increase in fluorescence caused by the binding of the SYBR Green dye to double-stranded DNA. The specificity of the product was confirmed by a specific melting/dissociation curve for each product. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control to normalize for the amount of RNA present in various test samples. The relative value of specific mRNAs in each sample was expressed as a reciprocal ratio of the Ct values of each message to those of the corresponding GAPDH.

Assessment of myofibroblast phenotype

Myofibroblast phenotype was assessed by light microscopy, immunoperoxidase staining for α -SMA, estimation of mRNA levels of α -SMA, and expression of PAI by SYBR Green staining of real-time RT-PCR using specific primers listed in Table 4. For immunoperoxidase staining, HF-Vector and HF-icIL-1ra fibroblasts were grown to subconfluency in 48-well flat-bottom tissue culture plates.

As a control, human foreskin fibroblasts maintained in the presence of rhTGF- β (10 ng/ml) for 2 weeks and plated similarly in 48-well plates were used. The medium was removed and cell layers were incubated with Cytofix/Cytoperm for 20 minutes and then with 0.3% hydrogen peroxidase (Sigma-Aldrich) for 5 minutes. Thereafter, cell layers were washed three times with PermWash (BD Pharmingen, San Diego, CA) and cell monolayers were incubated with PBS containing 2% BSA for 20 minutes and washed three times with PermWash. A 1:25 dilution of Sigma's mouse anti-human α -SMA clone 1A 4 monoclonal antibody was added to the cell monolayers for 30 minutes and washed three times with PermWash. Cell monolayers were then incubated with biotinylated rat anti-mouse IgG (Fab-specific) (Sigma-Aldrich) for 30 minutes and washed three times with PermWash. Streptavidin-horseradish peroxidase was then added to the cell layers for 10 minutes and the cell layers were washed three times with PermWash. Substrate AEC (BD Pharmingen) was added to the cell monolayers for 10 minutes and cell monolayers were washed with distilled water and counterstained with 0.02% Coomassie blue (Sigma-Aldrich) for 20 minutes and finally washed three times with distilled water.

Treatment of transfected human dermal fibroblasts with icIL-1ra type 1 antisense oligonucleotide

A phosphorothioate-derivatized antisense oligodeoxynucleotide complimentary to -6 to $+12$ (5'-CGTCTGTAAGGCATGGG-3') of the natural icIL-1ra type 1 was synthesized and reverse phase high performance liquid chromatography purified by Integrated DNA Technologies Inc. An antisense oligonucleotide with a scrambled sequence was prepared by a similar method and used as a control. Fibroblasts overexpressing icIL-1ra type 1 were transfected with 50, 100, and 300 μM of icIL-1ra type 1 antisense oligonucleotide (24 hours prior to stimulation with 1.0 ng/ml of hrIL-1 β) using the LipofectAMINE protocol as described above. Fibroblasts cultured with PBS or the transfecting agent alone served as additional

Table 4. Primer sequences (5'-3') used in real-time RT-PCR

Target	Sense (5'-3')	Antisense (5'-3')
GAPDH	GCA GGG GGG AGC CAA AAG GG	TGC CAG CCC CAG CGT CAA AG
icIL-1ra type 1	CCA CCA TGG CTT TAG AGA CCA TC	CTA CTC GTC CTC CTG GAA GTA
sIL-1ra	GAA TGG AAA TCT GCA GAG GCC TCC GC	GTA CTA CTC GTC CTC CTG G
MMP-1	ACC TGA AGA ATG ATG GGA GGC AAG T	CAT CAA AAT GAG CAT CTC CTC CAA TAC CT
TIMP-1	AAC CCA CCA TGG CCC CCT TTG AG	GTT CCA CTC CGG GCA GGA TTC AGG
α -SMA	GTC CCC ATC TAT GAG GGC TAT	GCA TTT GCG GTG GAC AAT GGA
PAI	AAG GAC CGC AAC GTG GTT TTC TCA	TGA AGA AGT GGG GCA TGA AGC C
Collagen I ($\alpha 2$)	CAG ATA CTT GAA TGT TGA TGG	CTG CTT GCC CAA GAA ACA AAG C

The oligonucleotides were synthesized and desalted at Integrated DNA Technology Inc., IA.

controls. The fibroblasts were harvested for RNA extraction 12–18 hours after stimulation.

Statistical analysis

Student's *t*-test was performed using Sigmasat 3.0 program (SPSS Inc., Chicago, IL) to compare the two groups (fibroblasts transfected with vector alone and fibroblasts transfected with vector encoding icIL-1ra), both groups having subjected to the same treatment.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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