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OSTEOARTHRITIS and CARTILAGE

Effect of IL-13 on cytokines, cytokine receptors and inhibitors on human osteoarthritis synovium and synovial fibroblasts

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Summary

Objective: In this study we investigated the effect of interleukin-13 (IL-13), an anti-inflammatory cytokine, for potential therapeutic use in osteoarthritis (OA).

Design: We examined the effect of IL-13 on the synthesis and expression of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), IL-1 receptor antagonist (IL-1Ra) and stromelysin-1 on human OA synovial membrane in *ex vivo* cultures. In addition, we explored the effect of IL-13 on both the IL-1 receptor (IL-1R) and TNF-receptor (TNF-R) systems on OA synovial fibroblasts. This included determination of the levels of IL-1β and TNF-α receptor binding, IL-1Ra and TNF-soluble receptors 55 and 75 (TNF-sR55 and TNF-sR75).

Results: In OA synovial membrane treated with LPS, IL-13 inhibited the synthesis of IL-1β, TNF-α and stromelysin-1, but increased IL-1Ra production. In addition, IL-13 reduced the level of IL-1β mRNA and stimulated the level of IL-1Ra mRNA. In synovial fibroblasts, IL-13 decreased the level of IL-1 binding, an effect related to the increased production of IL-1Ra. Although IL-13 had no effect on the TNF-R level, this cytokine markedly decreased the shedding of TNF-R75.

Conclusion: These experiments suggest that IL-13 is potentially useful in the therapeutic treatment of OA, as it could regulate the major pathological process of this disease by reducing the production of proinflammatory cytokines and metalloproteases, and favoring the production of IL-1Ra.

Keywords: IL-13, Osteoarthritis, Synovial membrane, Proinflammatory cytokines.

Introduction

Osteoarthritis (OA) is a disease characterized by progressive cartilage destruction and secondary inflammation of the synovial membrane [1]. Both catabolic and antianabolic mechanisms are believed to play a role in the pathophysiology of this disease. Proinflammatory cytokines produced in OA tissues, such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), may be instrumental in the disease process, since they both induce synthesis of degradative enzymes such as metalloproteases (MMP) [1]. These cytokines have been found in increased amounts in the joint tissues of patients suffering from OA [1–6]. The predominant role of IL-1β in the pathogenesis of this disease has been suggested, both in human, by *ex vivo* experiments [2], and *in vivo,* in an OA animal model [7–9].

During the disease process, conditions may favor proinflammatory cytokine inducibility, the increased level of their receptors and/or the inadequacy of inhibitory factors to block their activity. Variations in the levels of cytokine receptors and/or their soluble forms represent a mechanism by which cellular responses to these cytokines could be controlled. Another mechanism is the production of a specific receptor antagonist. Such a molecule has been identified only for the IL-1 receptor (IL-1R), and named IL-1R antagonist (IL-1Ra) [10]. In arthritic disorders, and more particularly in OA, a relative deficit of IL-1Ra has been suggested as a possible cause for an increased level of IL-1 activity [2, 8, 9].

Another natural avenue to reducing proinflammatory cytokine production and/or activity is through certain cytokines having anti-inflammatory properties. Four such cytokines—namely, transforming growth factor-β (TGF-β), IL-4, IL-10 and IL-13—have been identified as able

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to modulate various inflammatory processes. Their anti-inflammatory potential, however, appears to depend greatly on the target cell. For example, IL-4 reduced IL-6 and TNF-α synthesis in blood monocytes/macrophages, but not in the rheumatoid synovial fibroblasts [11, 12]. Similarly, IL-13 significantly inhibited lipopolysaccharide (LPS)-induced TNF-α production by mononuclear cells from peripheral blood, but not in cells from inflamed synovial fluid [13].

In search of therapeutic agents capable of regulating the inflammatory process, we investigated the effect of an anti-inflammatory cytokine, IL-13, on the human OA synovial membrane and synovial fibroblasts. To date, transcripts for human IL-13 have only been found in T-cells [14]. This human cytokine is an unglycosylated protein of 132 amino acids, with a molecular size of 10 kDa [15]. Although the discovery of IL-13 is recent, many biological activities have already been attributed to this cytokine. Of particular importance, IL-13 inhibited the production of a wide range of proinflammatory cytokines in monocytes/macrophages, B cells, natural killer cells and endothelial cells, while increasing IL-1Ra production [15–18]; yet its effect on OA tissues and cells has not been examined. A study of the effect of this cytokine on OA tissue seems even more relevant, as IL-13 has been identified in the mononuclear cell infiltrate of the sublining layer of OA synovium [19]. We therefore analyzed, using OA synovial membrane explants and synovial fibroblasts, the regulatory effect of IL-13 on two major proinflammatory cytokine systems. More specifically, we studied the effect of IL-13 on the synthesis and expression of IL-1β, TNF-α, IL-1Ra, and stromelysin-1, on human OA synovial membrane. Furthermore, we looked at the effect of IL-13 on both the IL-1R and TNF-R levels, as well as on the production of IL-1Ra and TNF-soluble receptors (TNF-sR) on OA synovial fibrblasts.

Materials and Methods

SPECIMEN SELECTION

Synovial membranes were obtained from 16 OA patients (mean age 70 ± 8 years, 10F/6M) undergoing total knee joint replacement. From the X-rays, they were shown as being at stages III and IV. All OA patients were evaluated by a certified rheumatologist and diagnosed based on the criteria developed by the ACR Diagnostic Subcommittee for OA [20]. At the time of surgery, they were treated with analgesics and/or nonsteroidal anti-inflammatory drugs (NSAIDS). None had intra-articular steroid injections within 3 months prior to surgery.

SYNOVIAL MEMBRANE EXPLANT CULTURES

Each synovial membrane was aseptically dissected from underlying fibrous and fatty tissues and rinsed in cold saline solution as described [2]. The synovial explants of approximately 200 mg each were randomly divided into groups. Tissues were incubated in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL Canadian Life Technologies, Burlington, ON, Canada) containing 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco-BRL), in the presence or absence of 1 μg/ml LPS (*Escherichia coli* serotype 055:B5; Sigma, St Louis, MO, U.S.A.), and with increasing concentrations of human recombinant IL-13 (rhIL-13) (R&D Systems, Minneapolis, MN, U.S.A.). Two sets of experiments were carried out. In the first set, synovial explants were incubated at 37°C for 72 h in a humidified 5% CO_2/95% air mixture, and determinations done on the culture medium as described below. After each experiment, the culture medium were frozen at –80°C before biochemical measurements. In the second set of experiments, synovial membrane explants were incubated for 24 h in a humidified atmosphere, and processed for total RNA extraction.

SYNOVIAL FIBROBLAST CULTURES

Synovial membranes were rinsed and dissected, and cells released as previously described [21]. Sequential enzymatic digestion was performed with 1 mg/ml pronase (Boehringer Mannheim, Indianapolis, IN, U.S.A.) for 1 h, followed by 6 h with 2 mg/ml collagenase (type IA, Sigma) at 37°C in DMEM containing antibiotics and supplemented with 10% heat inactivated fetal calf serum (FCS, Hyclone, Logan, OH, U.S.A.). Before seeding, the cells were incubated for 1 h at 37°C in Primaria tissue culture flasks (Falcon #3824, Lincoln Park, NJ, U.S.A.), allowing the adherence of nonfibroblast cells possibly present in the synovial preparation. In addition, flow cytometric analysis was conducted to confirm that no monocytes/macrophages were present in the fibroblast preparations [21]. Synovial fibroblasts were then seeded in high density in tissue culture flasks, and cultured until confluence in DMEM, supplemented with 10% FCS and antibiotics at 37°C in a humidified atmosphere of 5% CO_2/95% air. At the end of the incubation period, the cells were again counted in representative wells and data normalized to 5×10^5 cells/well. The cells were

incubated in a fresh serum-free medium for 24 h before the experiment, with the experiment conducted under serum-free medium. Only primary culture or first passaged cells were used.

BINDING ASSAY

Receptor binding assays were performed for IL-1β and TNF-α as previously described [21, 22]. Monolayer synovial fibroblast cultures were washed in phosphate buffered saline (PBS) and incubated with the binding buffer (PBS, 10 mM HEPES, 0.5% gelatin, pH 7.4) for 4 h at 4°C with [^{125}I]-rhTNF-α (40 pM) (specific activity, 800–900 Ci/mmole; Dupont Canada Ltd., Mississauga, ON, Canada), or 3 h at 22°C with [^{125}I]-rhIL-1β (40 pM) (specific activity, 1450–3200 Ci/mmole; Dupont Canada Ltd.), with or without a 500-fold relative molar excess of unlabeled human recombinant cytokine. After the incubation period, the cells were washed by immersion in ice-cold PBS, solubilized in 0.5% sodium dodecyl sulfate and radiolabel counted. The number of cells was calculated using parallel cultures in which synovial fibroblasts were detached with trypsin-EDTA and counted using the trypan blue method. Specific binding was calculated as previously described [21, 22].

NORTHERN BLOTTING

Total RNA from synovial membrane explants was extracted using the Trizol® reagent (Gibco-BRL) and processed according to the manufacturer's specifications. The extracted RNA was quantitated spectrophotometrically. Five micrograms (15 μg for TNF-α) of total RNA was resolved on 1.2% agarose formaldehyde gels, and transferred to nylon membranes (Hybond N, Amersham Canada Ltd., Oakville, ON, Canada) in 10 mM sodium acetate buffer, pH 7.8, containing 20 mM Tris and 0.5 mM EDTA, overnight at 4°C. The human probes were as previously described for IL-1β [23], TNF-α [23], IL-1Ra [24], stromelysin-1 [25] and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [23].

Prehybridization was carried out as described [23], and detection performed with a luminescent method using digoxigenin-11-uridyl triphosphate (DIG-11-UTP) (Boehringer Mannheim Biochemica, Mannheim, Germany) with Lumigen PPD [4-methoxy-4-(3-phosphatephenyl) spiro-(1,2-dioxetane-3,2'-adamantane)disodium salt] as substrate for alkaline phosphatase conjugated to anti-DIG antibody (Ab) Fab fragments, as described in the Boehringer Mannheim user's guide. The membranes were then subjected to autoradiography using Kodak XAR5 film (Eastman Kodak Ltd., Rochester, NY, U.S.A.) at room temperature. Autoradiography was subjected to laser scanning densitometry (GS-300 Hoefer Scientific Instruments, San Francisco, CA, U.S.A.) for mRNA measurement, and the results calculated as the relative expression normalized to the level of GAPDH mRNA.

OTHER ANALYSES

IL-1β, TNF-α, IL-1Ra, stromelysin-1, type II IL-1 soluble receptor (IL-1sR), TNF-sR55 and TNF-sR75 proteins were all determined in the culture medium using ELISA assay. All were purchased from R&D Systems, except the stromelysin-1 ELISA assay, which was purchased from Amersham Canada Ltd. The sensitivity for cell culture medium was 0.3 pg/ml for IL-1β, 4.4 pg/ml for TNF-α, 6.5 pg/ml for IL-1Ra, 3.75 ng/ml for stromelysin-1, 10 pg/ml for type II IL-1sR, 1 pg/ml for TNF-sR55 and 0.5 pg/ml for TNF-sR75. The reaction was measured on a micro-ELISA Vmax photometer (Molecular Devices Corp., Menlo Park, CA, U.S.A.). Data were expressed as picograms or nanograms per milligram tissue weight wet for synovial membrane explant experiments, or per 5×10^5 cells when synovial fibroblast cells were used.

Stromelysin activity was measured by the method of Chavira *et al* [26] using azocoll as a substrate, as previously described [27]. Total enzyme activity was measured by the addition of APMA (1 mM) to activate the latent form of the enzyme. The chelator *o*-phenanthroline (5 mM) was used as a blank. Enzyme activity was calculated in units, in which one unit corresponds to hydrolysis of 1 μg of substrate/hour at 37°C. The total enzymatic activity was expressed in units per milligram wet weight.

Results

EFFECTS OF IL-13 ON CYTOKINE AND STROMELYSIN IN OA SYNOVIAL MEMBRANES

Synthesis

OA synovial membranes ($N = 3$ patients) spontaneously produce IL-1β, IL-1Ra, and stromelysin-1, but not TNF-α (Fig. 1). For the latter enzyme and cytokines, LPS treatment increased

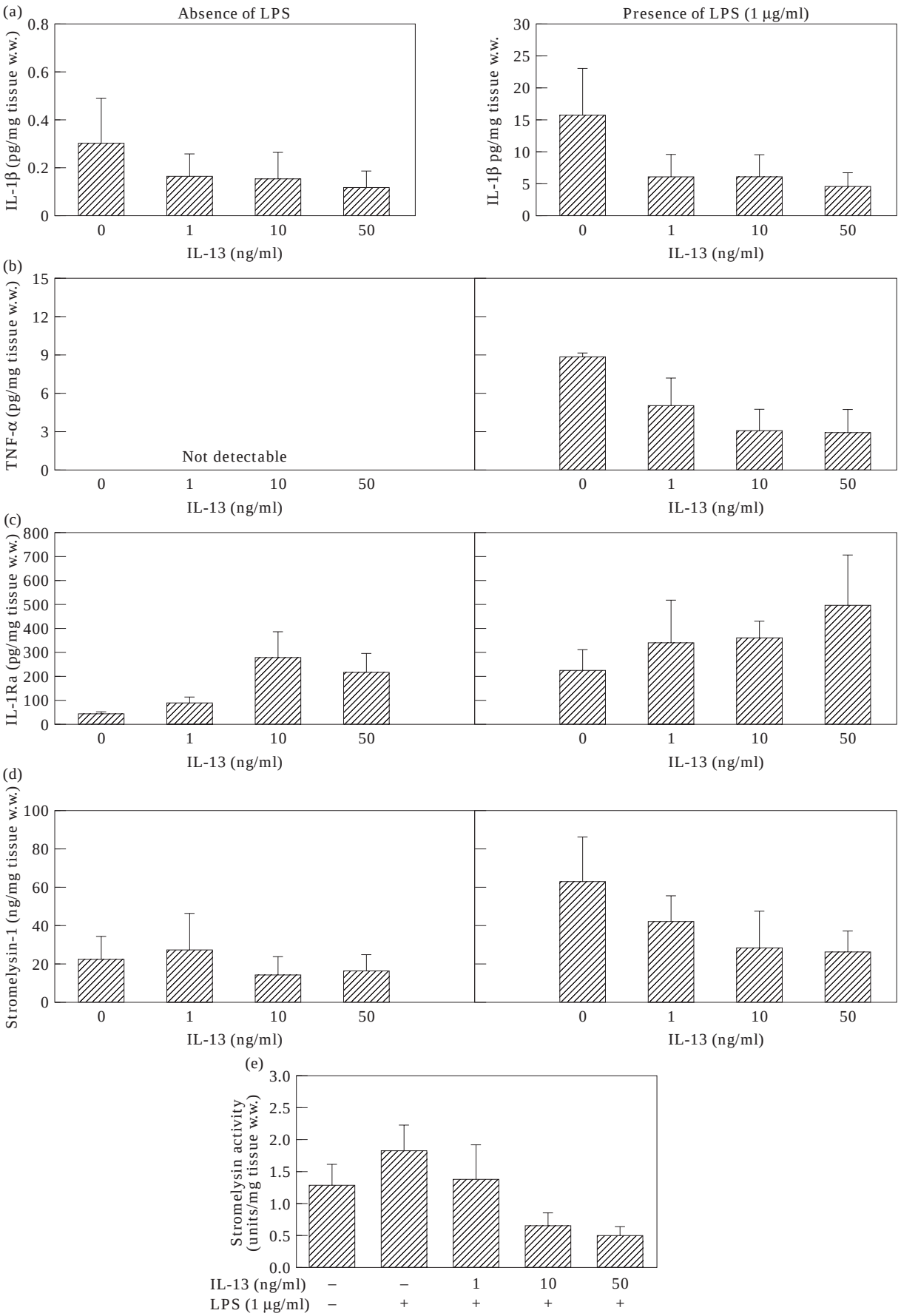

FIG. 1. Human OA synovial membranes. Effect of IL-13 on the synthesis of (a) IL-1β, (b) TNF-α, (c) IL-1Ra, (d) stromelysin-1, and (e) on the activity of stromelysin, in the absence or presence of 1 μg/ml LPS. Values are the mean ± S.E.M. of three independent patients.

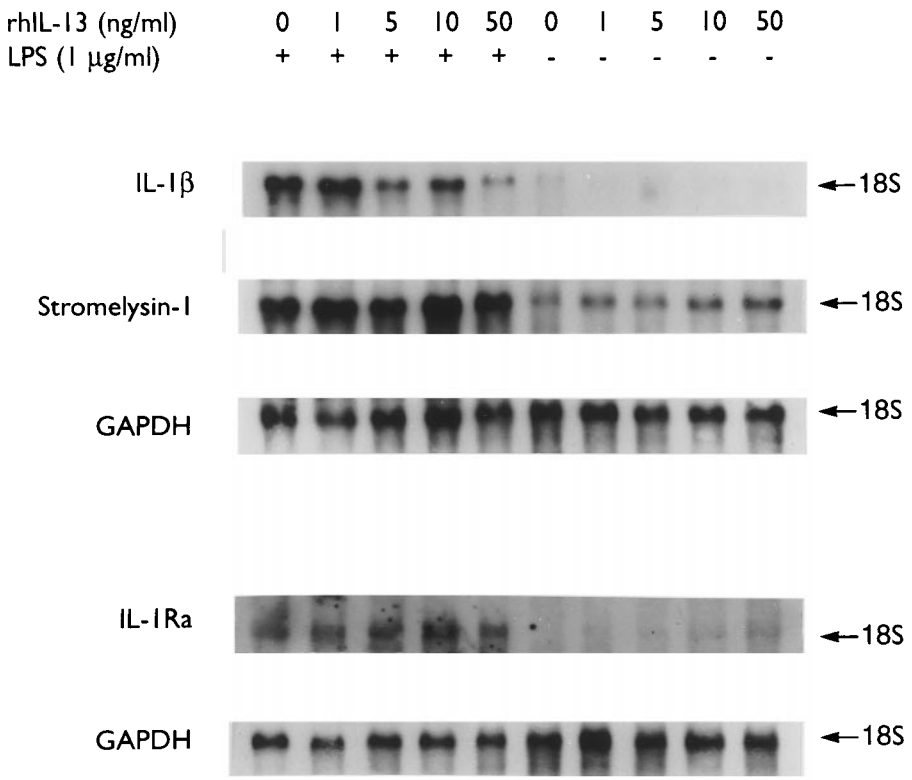

FIG. 2. Representative Northern blotting of IL-1β, stromelysin-1, IL-1Ra and GAPDH mRNA in OA synovial membranes treated with IL-13 in the presence or absence of 1 μg/ml LPS.

their levels; about 51-fold for IL-1β [Fig. 1(a)], to 8 pg/mg tissue for TNF-α [Fig. 1(b)], 4.5-fold for IL-1Ra [Fig. 1(c)] and 2.9-fold for stromelysin-1 [Fig. 1(d)]. As for the synthesis, stromelysin activity also showed an increased level under LPS [Fig. 1(e)].

For unstimulated and LPS-treated OA synovial membrane, IL-13 suppressed the synthesis of IL-1β, TNF-α and stromelysin-1 as well as the stromelysin activity. Conversely, IL-13 induced a dose-dependent increase of the IL-1Ra [Fig. 1(c)].

Gene Expression

Northern blot analysis ($N = 2$ patients) (Fig. 2, Table I) revealed that, under basal conditions, IL-1β expression was weak, while the level increased with LPS treatment. The accumulation of IL-1β mRNA induced by LPS was markedly inhibited in a dose-dependent fashion by IL-13. The level of expression of TNF-α under both basal conditions and LPS treatment was below the level of our detection methods despite the fact that we loaded 15 μg/lane of total RNA and

Table I
Human osteoarthritic synovial membrane. Relative expression of mRNA normalized to GAPDH

rhIL-13 (ng/ml)	Presence of LPS (1 μg/ml)					Absence of LPS				
	0	1	5	10	50	0	1	5	10	50
IL-1β	1.31*	1.11	0.40	0.48	0.19	0.06	0.03	ND†	ND	ND
Stromelysin-1	1.12	1.43	1.23	1.26	0.98	0.68	0.55	0.54	0.41	0.40
IL-1Ra	1.36	1.89	1.69	2.19	1.27	0.15	0.12	0.14	0.21	0.47

*Values are the mean of two independent patients.
†ND = not detectable.

maximized the duration of autoradiography (data not shown).

The level of IL-1Ra mRNA was also low under basal conditions, with a noticeable elevation occurring upon treatment with LPS (Fig. 2, Table I). In explants, and more particularly with those treated with LPS, IL-13 induced an increase in IL-1Ra mRNA.

In contrast to the protein activity level, no effect by IL-13 was noted on the level of stromelysin-1 expression (Fig. 2, Table I) under both untreated and LPS-treated conditions.

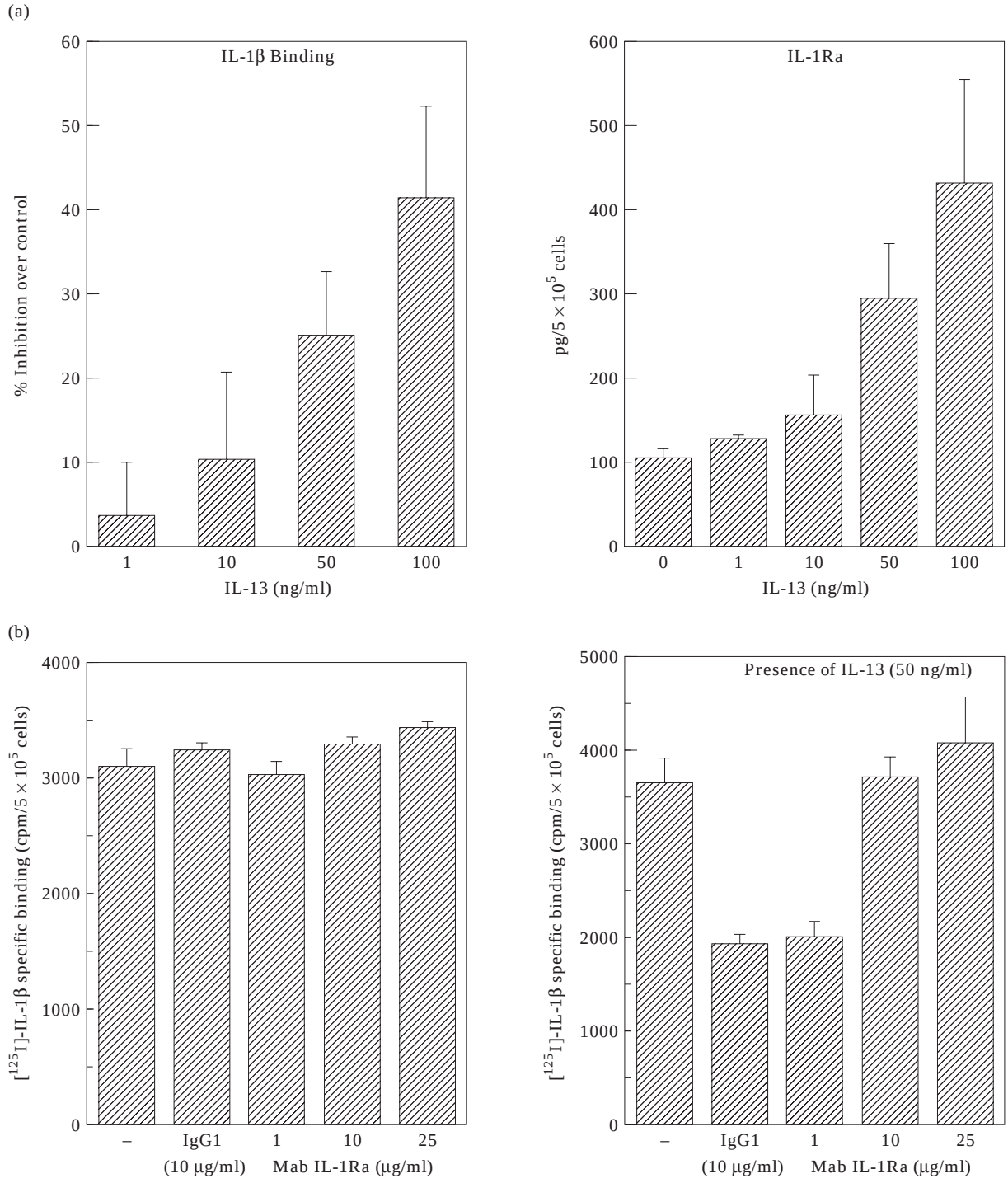

FIG. 3. Human OA synovial fibroblasts. Effect of IL-13 on (a) the inhibition of IL-1 binding and IL-1Ra level ($N = 3$ independent patients), (b) IL-1 binding level in the presence of a monoclonal antibody (mAb) against human IL-1Ra or the IgG_1 as control in the absence ($N = 2$ independent patients) or presence ($N = 2$ independent patients) of rhIL-13. Values are the mean ± S.E.M.

EFFECTS OF IL-13 ON IL-1 AND TNF-α RECEPTOR SYSTEMS IN OA SYNOVIAL FIROBLASTS

To further investigate the role of IL-13 on IL-1 and TNF-α, we looked at its effects on the IL-1 and TNF-α specific receptors. This study was performed on OA synovial fibroblasts, as these cells appear central to the destruction processes in OA [1, 6].

Effects of IL-13 on IL-1R

As illustrated in Fig. 3(a), the level of IL-1 binding ($N = 3$ patients) was substantially reduced with IL-13 and occurred in a dose-dependent manner. At 100 ng/ml, IL-13 induced a 40% inhibition in the level of binding. The decrease in [^{125}I]-IL-1 binding may be the result of factors capable of blocking IL-1 binding to its receptor site (i.e., IL-1Ra) or forming an inactive complex with IL-1 (i.e., release of the type II IL-1R). We therefore examined the effect of IL-13 on the level of these factors.

Results showed that, as for the OA synovial membrane, IL-13 increased the production of IL-1Ra by synovial fibroblasts ($N = 3$ patients) which showed a dose-response, with an approximate four-fold enhancement at the highest IL-13 concentration (100 ng/ml) tested [Fig. 3(a)]. The level of type II IL-1sR was also determined. Results showed that its level in the culture medium with or without IL-13 was below the limit of detection (data not illustrated).

In order to confirm that the increased production of IL-1Ra was responsible for the effect of IL-13 on IL-1β binding, further experiments ($N = 3$ patients) were performed in which a neutralizing IL-1Ra antibody (monoclonal Ab # 10307.1, generously provided by R&D Systems) was added at increasing concentrations (0, 1, 10, 25 μg/ml) [Fig. 3(b)]. IL-1 binding assay was performed, as described in the Materials and Methods section, on OA synovial fibroblasts. As shown in Fig. 3(b), the addition of the IL-1Ra Ab to the culture medium under basal conditions did not affect the [^{125}I]-IL-1 binding. When IL-13 was added in conjunction with the IL-1Ra Ab ($N = 3$ patients), a decrease of the IL-1 binding was noted with complete inhibition reached at a concentration of 10 μg/ml IL-1Ra Ab. Experiments performed with the isotype-matched nonimmune IgG showed no effect on the binding; this was found both in the presence and in the absence of IL-13 [Fig. 3(b)].

Effects of IL-13 on TNF-α receptors

Further experiments were conducted in which the TNF-α binding level was evaluated on OA synovial fibroblasts ($N = 3$ patients) treated with IL-13. Results, as represented in Fig. 4, revealed that IL-13 did not affect the level of binding of TNF-α to OA cells.

As the extracellular portion of each of the two types of TNF-R could be released from the

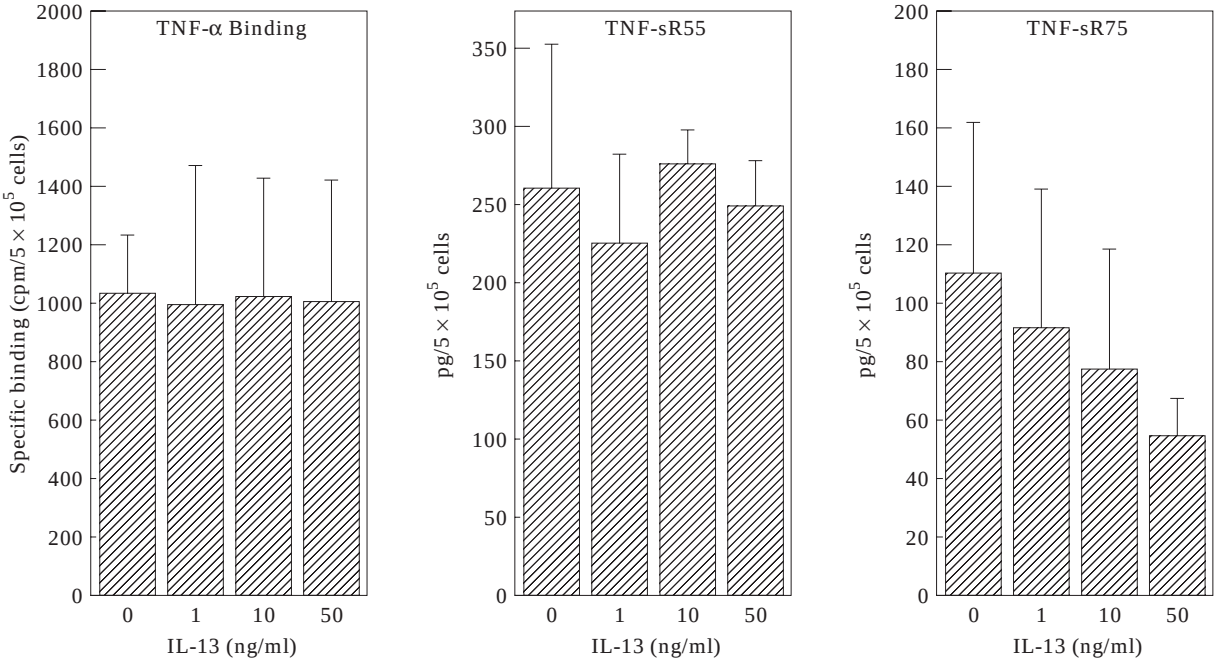

FIG. 4. Human OA synovial fibroblasts. Effect of IL-13 on TNF-α binding and TNF-sR55 and TNF-sR75 levels. Values are the mean ± S.E.M. of three independent patients.

membrane to form soluble receptors, their levels were also determined. Data (Fig. 4) showed, as previously described [28], that both TNF-R55 and TNF-R75 are shed from OA synovial fibroblasts with a greater amount of release for the TNF-R55. IL-13 did not affect the level of TNF-sR55, but induced a dose-dependent decrease for the TNF-sR75 release (Fig. 4).

Discussion

Structural changes and cartilage destruction are key events in the OA process. An imbalance between the synthesis level of inflammatory and anti-inflammatory factors in articular joint tissue may contribute to the degradation of articular cartilage. Therefore, controlling production of some of these factors would be a major achievement in the treatment of OA. In this context, studying the effect of IL-13, a potent anti-inflammatory cytokine was thought to be most relevant. Our results highlight a further potential usefulness for IL-13 in the treatment of OA, as it is capable of inhibiting IL-1β, TNF-α and stromelysin-1 synthesis and stromelysin activity on OA synovial membrane, while simultaneously increasing production of IL-1Ra. Moreover, IL-13 downregulates IL-1β while upregulating IL-1Ra gene expression. Our study also showed that the IL-13-stimulated-IL-1Ra level may decrease the IL-1 binding to its cognate receptor, thereby limiting its biological activity.

In OA, proinflammatory cytokines, as well as MMP, play a pivotal role in mediating pathophysiological mechanisms, and contribute to the evolution of the disease process [1]. IL-1β demonstrated a predominant role in the etiopathology of OA [2, 7–9], in contrast to rheumatoid arthritis where TNF-α appears to play a major role. In OA, the level of TNF-α in the articular joint tissues was modest and variable, and was found in a higher level in severely inflamed synovial membrane [2, 4, 5, 29]. The results of these previous studies are in accordance with the findings of the present study. As these proinflammatory cytokines function as networks, the regulating effect of IL-13 at controlling the expression and synthesis of these cytokines is therefore critical to the outcome of this disease process.

Results of our study showed that IL-13 exerts a suppressive effect on the synthesis of IL-1β, TNF-α and stromelysin-1, agreeing with previous reports on mononuclear cells from peripheral blood [13, 14]. Our data, however, differed from those reported on macrophages that were isolated from synovial fluid of arthritic patients, which did not seem to respond to IL-13, at least in regard to TNF-α [13]. One should note that, in synovial fluid, the macrophages are already activated. However, our results likely reflect that, in OA, we tested a cell population different from the one found in the synovial fluid. Indeed, in contrast to more inflammatory arthritic diseases, in OA, the synovial fibroblasts from the lining cell appear the main cell type responsible for producing proinflammatory cytokines and MMP, rather than the infiltrate monocytes/macrophages.

As the action of one particular factor might occur at various levels of cell metabolism, it seemed important that we investigate the effect of IL-13 on cytokine and MMP gene expression. Results showed that the effect of IL-13 at inhibiting IL-1β production was related to a decrease in gene expression. No effect, however, was noted on the stromelysin-1 mRNA level, indicating that in this case, the IL-13 action may occur at translational or post- translational levels or be indirect and related to the inhibition of pro-inflammatory cytokines. The latter is a likely possibility as additional experi- ments revealed that the inhibition of stromelysin-1 synthesis by IL-13 on synovial membrane could be reversed (∼70%) by the addition of either IL-1β or TNF-α (personal observation).

As a cytokine can regulate the expression of homologous or heterologous receptors, it appeared most appropriate to explore the effect of IL-13 on the IL-1β and TNF-α receptors of OA synovial fibroblasts which participate actively in the development of synovitis in this disease. In OA synovial fibroblasts, IL-13 induced a decreased level of IL-1 binding, but not of TNF-α binding. Additional experiments showed that the effect of IL-13 on reducing IL-1 binding level could be related to the enhancement of the level of IL-1Ra by these cells, but not as reported with polymorphonuclear cells, to an increased release of the type II IL-1R [30]. Although one cannot completely exclude the latter possibility in the synovial fibroblasts, if this process occurs, it probably plays a minor role, as the level of the soluble form of type II IL-R was under the assay limit of detection, both in the presence and absence of IL-13. The actual findings showing the effect of IL-13 at reducing the IL-1 binding level by increasing the IL-IRa level may or may not have relevancy *in situ* in OA tissues. However, the multiple effects of IL-13 on the IL-1 system, namely increasing IL-1Ra at the same time that it reduces the synthesis of IL-1, are certainly creating conditions which are favorable for decreasing the stimulation of OA cells by IL-1.

While IL-13 had no effect on the number of TNF-α binding sites, it was capable of inducing a marked decrease in the shedding of the TNF-R75, a process which might contribute to reducing the response of these cells to stimulation by TNF-α. Although the exact role of TNF-sR in the control of TNF-α action remains controversial, some studies indicate the possibility that TNF-sR75 is involved in facilitating the binding of TNF-α to its receptor and/or stabilizing its ligand [31, 32]. Therefore, the TNF-sR would function as a carrier rather than an inhibitor of TNF-α. On the other hand, the reduced shedding of TNF-sR75 could have a detrimental effect if it was to act as a scavenger to TNF-α, thus allowing for a greater stimulation of cells by the cytokine. The reduction in TNF-sR75 may have somewhat increased the level of TNF-R75 at the cell surface. However, since they do not mediate cell signaling [22], it is therefore unlikely that IL-13 could have increased the cell response to stimulation by TNF-α throughout that mechanism.

In summary, this in vitro study provides useful information on the effect of IL-13 on cellular metabolism and on the potential use in the therapeutic treatment of OA, as it could regulate some of its major pathological processes by reducing the production of proinflammatory cytokines and MMP, and favoring the production of IL-1Ra. Further studies will be needed to explore the usefulness of this anti-inflammatory cytokine in the context of the clinical treatment of this disease.

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