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Truncated thioredoxin in RA synoviocytes

EXPRESSION AND EXTRACELLULAR RELEASE OF TRX80, THE TRUNCATED FORM OF THIOREDOXIN, BY TUMOR NECROSIS FACTOR-ALPHA AND INTERLEUKIN-1 BETA-STIMULATED HUMAN RHEUMATOID SYNOVIOCYTES.

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Running title: Truncated thioredoxin in RA synoviocytes

Abstract

Thioredoxin (Trx) plays several important roles through changes to sulfhydryl reactions and protein interactions in controlling the cellular signaling process in rheumatoid arthritis (RA). The 10-kDa C-terminal truncated form of thioredoxin (Trx80) is a potent mitogenic cytokine and is involved in the Th1 response. We have investigated the ability of synoviocytes from five RA patients to induce the Trx80 form, after *ex vivo* stimulation by the pro-inflammatory cytokines IL-1 β and TNF- α or by hydrogen peroxide (H₂O₂). Synovial cells from five osteoarthritis (OA) patients were used as controls. Immunoprecipitation assays using two different antibodies showed that RA but not OA cells expressed intact Trx80 protein even when not stimulated in culture. Treatments with pro-inflammatory cytokines alone or in combination enhanced this basal production and induced extracellular release of the truncated Trx80 form by all RA cells tested. Under our experimental conditions, the rate of Trx80 release from RA cells was approximately 30% of total Trx production. By contrast, Trx80 was not detected in RA and OA cell lysates and their respective culture supernatants in response to H₂O₂, indicating that the oxidative process induced by hydrogen peroxide in synoviocytes was unable to modify Trx80 release. Moreover, Trx80 induced synovial cell proliferation evaluated by tritiated thymidine incorporation. These results highlight the effect of the inflammatory process on the release of both Trx and Trx80 from synoviocytes in RA, and suggest that the cytokine-induced elevation of Trx80 cell release may constitute a link between inflammation and the immune system in RA disease.

Keywords: Truncated thioredoxin; pro-inflammatory cytokines; oxidative stress; rheumatoid arthritis; synoviocyte.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by progressive and irreversible joint damage, which results in both synovial proliferation and the massive infiltration of immune cells [1]. Macrophages and synoviocytes produce large amounts of tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and reactive oxidative species, that contribute to the development and the maintenance of the disease [1,2]. Several studies have reported that levels of Thioredoxin (Trx), a stress-inducible 12-kDa protein containing a conserved redox-active moiety, are increased in the synovial fluid (SF) and sera of rheumatoid arthritis (RA) patients. Trx induction is associated with both the disease activity score and oxidative stress marker levels [3-5], and previous reports have indicated that high levels of TNF- α and hydrogen peroxide (H₂O₂) enhance the local production of Trx by RA synoviocytes [3]. Trx is now known to be endowed with broad-ranging biological functions, which may be antioxidant, anti-apoptotic or involved in the inflammation process [6,7]. In the event of cellular activation, e.g. inflammation or oxidative stress, Trx expression increases and the protein is rapidly secreted into the extracellular environment via an unknown pathway [8, 9]. Posttranslational modifications to Trx have been reported, especially its truncation into a 10-kDa C-terminal form (called Trx80) containing the first 80 or 84 N-terminal amino acids of the initial protein [9, 10]. Loss of the C-terminal part of Trx confers wide properties to Trx80 which differ in many ways from those of the native form of Trx [11]. Extracellular Trx80, mainly arising from the immune cell system, has been shown to exert striking effects on cell activation and also to exhibit cytokine-like effects such as the induction of proliferation, differentiation and cytokine expression [11-12]. Trx80 also generates phenotypic changes to monocytes, and activates T cells to produce IFN- γ , inducing a Th1-type T-

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cell response [11-12]. Such a response may be beneficial in many respects, such as defending the body against microorganisms, but may also promote the development of autoimmune diseases [11].

The aim of the present study was to determine whether isolated synoviocytes from RA patients were able to produce the truncated Trx80 form of Trx. We also investigated whether stimulating agents such as TNF- α or IL-1 β (as pivotal pro-inflammatory cytokines in the pathogenesis of RA), and hydrogen peroxide (H₂O₂) (as a pro-oxidant molecule) were able to regulate Trx80 expression and release. The results of this study would also highlight the functions of the inflammatory process linked to the production of Trx and Trx80 in a setting of rheumatoid arthritis.

Methods

Patients

Human synovial cells are obtained from five patients with RA (four women and one man; mean age: 63.4 ± 9.8 years - mean duration of the disease: 10.3 ± 4.3 years). All patients had been diagnosed with RA in accordance with the 1987 definition of the American College of Rheumatology [14]. These patients were treated with low-dose glucocorticoids (<5 mg/day) (60%), non-steroidal anti-inflammatory drugs (NSAIDs) (20%), methotrexate (60%) and hydroxychloroquine (20%). Five patients (three women and two men; mean age: 72.3 ± 7.9 years - mean duration of the disease: 8.2 ± 4.6 years) with osteoarthritis (OA) diagnosed in the light of clinical, biological and radiological examinations, were also included and considered as controls.

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Most of these patients were received NSAID (80%). All patients gave informed consent for this study.

Cell isolation and culture

Human synovial cells were isolated from fresh synovium collected during synovectomy or joint replacement surgery, and were cultured as previously described [15]. Briefly, the superficial layer of the synovium is dissected out and is treated with collagenase and trypsin for 2 hours at 37°C. The cells obtained are suspended in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% fetal calf serum (FCS), amphotericin B, netromycin, ceftazidime and vancomycin, and are cultured at a density of 3×10^6 cells per flask in 75 cm² culture flasks at 37°C, in a humidified incubator under 5% CO₂ in air. The culture medium is changed and non-adherent cells are removed every 3 days until subconfluency. Adherent cells are harvested by trypsinization and subcultures are maintained in the same medium. All experiments were performed using synoviocytes between the 2nd and 4th passages.

Immunoprecipitation analysis to detect the truncated form of thioredoxin in synoviocytes

Synoviocytes in Dulbecco's Minimal Essential Medium (DMEM) were treated for 24 hours with human recombinant IL-1 β (1 ng ml⁻¹) or/and TNF- α (0.5 ng ml⁻¹) (R&D Systems, Lille, France), or H₂O₂ (15 or 30 μ M - Sigma Aldrich, L'Isle d'Abeau-Chesnes, France). Cells (2×10^6) were lysed in 0.5 ml ice-cold CelLytic™-M cell lysis (Sigma Aldrich, Saint-Louis, USA) containing a cocktail of protease inhibitors at 4°C for 30 minutes, and then centrifuged at 16,000g for 15 minutes. For immunoprecipitation, 2 μ g of goat polyclonal antibodies raised against epitopes mapping at the N-terminus of Trx (Trx_(N14), sequence 1-14 VKQIESKTAFQEAL, Tebu, Le

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Perray, France), were added per 0.5 ml of cell lysate. The samples were incubated for 2 hours at 4°C before the addition of 20 µl of a suspension of protein A/G-PLUS-Agarose beads (Tebu), and then incubated at 4°C under agitation overnight. Pellets were collected by rapid centrifugation at 4°C, then washed four times with stringent RIPA buffer and resuspended in 40 µl of 2X electrophoresis-reducing Tris-Tricine buffer. Samples (25 µg protein) were boiled for 7 minutes and loaded onto 16.5% Tris-Tricine protein separation gels (Bio-Rad, Marne la Coquette, France). At the completion of electrophoresis, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham, Buckinghamshire, UK). Membranes were blocked with 5% non-fat dried milk in PBS containing 0.1% Tween 20 (TPBS), and then incubated with either mouse antibody against human full-length Trx (clone mAb 2G11) (BD Biosciences, Lincoln Park, N.J., USA) which does not cross-reacted with Trx80, or with Trx80 (clone 4H9) mAbs, as previously described [16] (BD Biosciences), or with goat IgG Trx Ab (R&D Systems) for which these two antibodies recognize human full length Trx and Trx80 (according to the manufacturer's instructions). The membranes were incubated for 1 hour with peroxidase-labeled anti-mouse (Amersham Biosciences) or peroxidase-labeled anti-goat antibodies (Dako, Trappes, France), respectively. Bound antibodies were visualized with an enhanced chemiluminescence ECL kit using Hyperfilm MP (Amersham). Immunoprecipitation procedures were also performed on cell culture supernatants. Band intensities were determined for their band surface areas by the densitometric analysis of exposed film with ImageJ software, using the same sized section of the blot for each scan. Recombinant human Trx and Trx80 (R&D Systems) were used as controls. The limit of detection for Trx was approximately 1 ng under reducing conditions.

Quantitative determination of LDH

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Assays for LDH on cell lysates and supernatants were carried out using an LDH optimized assay kit (Roche Diagnostics, Meylan, France) according to the manufacturer's instructions, on a Roche automated clinical chemistry analyzer MODULAR P[®]. Cell supernatant LDH activities were less than 5% of total LDH cell activity in both non-stimulated and stimulated cells, thus indicating that the treatment of synoviocytes with either proinflammatory cytokines or hydrogen peroxide do not lead to cell lysis under our experimental conditions.

Measurement of synoviocyte proliferation

To determine the effect of human recombinant Trx and human recombinant Trx80 on synoviocyte proliferation, DNA synthesis was measured by [³H]-thymidine incorporation. Synoviocytes were seeded overnight at 5×10^3 cells per well in 96-well tissue culture plates in DMEM/10% FCS. Cell growth was synchronized by culturing synoviocytes in DMEM/0.1% bovine serum albumin (Sigma Aldrich) for 24 hours. Cells were treated with human recombinant Trx (0.01-1 μ g/ml) and human recombinant Trx80 (0.01-1 μ g/ml) for 54 hours prior to cells being pulsed for 18 hours with 0.5 μ Ci/well [³H]-thymidine. Triplicate cultures were used for each determination. Synoviocytes were detached by three successive cycles of congelation/thawing, and harvested using a cell harvester. The radioactivity incorporated into DNA was determined using liquid scintillation counting. The proliferative responses were expressed as cpm \pm SD in stimulated synoviocytes versus cpm \pm SD in unstimulated cells.

Statistical analysis

Data were compared using the nonparametric Mann-Whitney (unpaired data) and Wilcoxon (paired data) tests; p values less than 0.05 were considered significant. All quantitative data are expressed as mean \pm SD.

Results

Immunodetection analysis of Trx80 expression in non-treated synoviocytes

Immunoprecipitation analyses with Trx80 (clone 4H9) mAb and Trx polyclonal goat IgG Ab were performed to determine the intracellular expression and release of the truncated form of Trx in synoviocytes at baseline. As confirmed by purified human recombinants for Trx or Trx80, these two antibodies recognized both full-length Trx (12-kDa) and Trx80 (10-kDa) proteins, whereas the specific full-length Trx (clone 2G11) mAb used as a control did not identify Trx80 protein (Figure 1A). The results in cell lysates of RA synoviocytes revealed a strong immunoreactive band at 12 kDa identified as thioredoxin and an additional band of weaker intensity representing 10 kDa and corresponding to Trx80. The presence of the two specific bands was determined in 4/5 tested RA cells. By contrast, immunoprecipitations in OA cell lysates revealed the presence of the full-length Trx form but we failed to observe the Trx80-specific 10 kDa band, whatever the antibody used. Examination of the culture supernatants showed that the cell release of full-length Trx was clearly higher in RA than in OA synoviocytes. On the other hand, neither RA nor OA cell culture supernatants displayed detectable Trx80 release by non-stimulated cells.

Production and release of a truncated form of Trx by pro-inflammatory cytokine-stimulated synoviocytes

Quantitative densitometric analysis of 12-kDa bands showed that pro-inflammatory cytokines induced a significant Trx expression in RA and OA cells lysates and supernatants (Table 1). The incubation of RA synoviocytes with IL-1 β or/and TNF- α for a period of 24h resulted also in an

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increase in Trx80 expression in the five RA cell lysates studied, whereas such an increase was not detected in OA cell lysates (Figure 1B). In stimulated RA cells, Trx over-expression was associated with the extracellular release of Trx80, but was not observed in stimulated OA synoviocytes (Figure 1C). As shown in Table 2, densitometry analysis indicated significant increases in Trx80 expression in RA cell lysates after IL-1 β or TNF- α treatment, the effects of the two cytokines were quite similar, and we did not observe any additive effects when cells were incubated in the presence of both IL-1 β and TNF- α . Under our experimental conditions, the rate of Trx80 release by stimulated RA synoviocytes could be estimated at approximately 30% of total Trx80 expression (Table 2).

In vitro H₂O₂ treatment does not induce the production and release of truncated Trx in synoviocytes

Pro-inflammatory cytokines trigger a range of cell responses, including induction of the oxidative process. In order to explore the implication of oxidative stress in the production and release of Trx80, we stimulated RA and OA synoviocytes for 24h with H₂O₂ at two different concentrations (15 and 30 μ M), and then performed immunoprecipitation assays using Trx80 (clone 4H9) mAb and Trx polyclonal goat IgG Ab. As indicated in Figure 2A, cell lysates exhibited a strong immunoreactive band at 12-kDa, confirming the effect of reactive oxygen species on Trx regulation in both cell types. Densitometric analysis revealed that H₂O₂ at high dose (30 μ M) induced significant Trx expression in RA and OA cell lysates when compared with respective controls (0.94 ± 0.04 vs. 0.88 ± 0.05 arbitrary densitometric unit $p < 0.05$ for RA cell lysates, and 0.83 ± 0.05 vs. 0.77 ± 0.03 arbitrary densitometric unit, $p < 0.05$ for OA cell lysates). In the culture supernatants from RA and OA synoviocytes, only, H₂O₂ at 30 μ M, induced significant

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release of Trx: 0.42 ± 0.05 vs. 0.32 ± 0.09 arbitrary densitometric unit, $p < 0.05$ for RA, and 0.24 ± 0.03 vs. 0.19 ± 0.04 arbitrary densitometric unit for OA. Any Abs revealed the expression of Trx80 in RA and OA cell extracts and supernatants treated with H_2O_2 , thus clearly demonstrating that the truncated form of Trx was not induced by H_2O_2 in synoviocytes (Figure 2B).

Effects of Trx and Trx80 on synoviocyte proliferation

One of the prominent characteristics of RA synoviocytes is their proliferative activity, leading to pannus formation as well as swelling of the joints. Because Trx80 was known to be a mitogenic cytokine, we assessed the involvement of Trx and Trx80 on synoviocyte proliferation, by the measurement of [3H]-thymidine incorporation after stimulation with various concentrations of human recombinants Trx and Trx80. Data shown in figure 3 indicated at baseline that RA cells exhibited a significant proliferative activity as compared with OA cells (4011 ± 859 cpm vs. 2678 ± 240 cpm, $p < 0.01$). There were no effect on cell proliferation with human recombinant Trx. In contrast, the effect of Trx80 on proliferation increased dose-dependently in RA and OA cells, showing that Trx80 is a potent mitogenic cytokine for synoviocytes.

Discussion

During the present study, we investigated whether pro-inflammatory cytokines (IL- 1β and TNF- α) and hydrogen peroxide were able to induce both the expression and cell release of Trx80 in RA synoviocytes; the experimental controls were synoviocytes from patients with OA, a disease associated with a weak inflammatory process and low levels of cell proliferation. Two different antibodies, Trx80 (clone 4H9) mAb and goat IgG Trx Ab, both recognizing human full-length

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Trx and Trx80 proteins, were initially tested using immunoprecipitation analysis. A full-length Trx (clone 2G11) mAb that did not cross-react with Trx80 was also used to verify the specificity of our determinations. Firstly, our investigations indicate that non-stimulated synoviocytes from RA patients expressed intracellular Trx at higher levels when compared with OA cells. Secondly, in line with the findings of a previous study [3], we confirmed that the expression of Trx was strongly induced by both pro-inflammatory cytokines and H₂O₂. Furthermore, we report for the first time that stimulated RA synoviocytes released Trx at the extracellular level to a greater extent than OA cells. These data are consistent with the significantly elevated levels of Trx found in synovial fluid from the inflamed joints of RA patients when compared with other joint diseases such as OA, gout and reactive arthritis [3, 4, 17] and suggest that synovial cells may constitute a major source of Trx.

It has been suggested that elevated extracellular Trx levels may represent a potential reservoir for Trx80 [11]. With a few exceptions, the cells which produce Trx80 are limited to the immune system, and monocytes appear to constitute the principal source for this protein [12, 18]. Our results demonstrate that a 10-kDa band corresponding to the Trx80 protein was expressed at baseline in RA cells but not in OA cells. We also demonstrate that IL-1 β or/and TNF- α -stimulation of RA synoviocytes for 24h resulted in an increase in Trx80 cell expression. By contrast, H₂O₂ treatment at two different concentrations did not induce Trx80 expression, suggesting that the increase in Trx expression was insufficient to favor Trx80 protein formation. We conclude that Trx80 formation in synoviocytes probably does not result from a redox-dependent pathway. According to data in the literature, the most likely mechanism leading to the generation of a truncated Trx form is the cleavage of full-length Trx by an inducible but as yet unknown protease [19]. Clearly, one model accounting for the generation of Trx80 in RA

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synoviocytes is the involvement of pro-inflammatory cytokines such as IL-1 β and TNF- α in inducing proteolytic cleavage of the 12-kDa precursor Trx.

In addition, our results confirm that RA (but not OA) synoviocytes, stimulated with IL-1 β or/and TNF- α , were able to secrete Trx80 into the extracellular compartment, thus constituting a source of truncated Trx in synovial fluid. Under our experimental conditions, the rate of released Trx80 was estimated to account for approximately 30% of total Trx80 production, as these cells are stimulated by IL-1 β , TNF- α or both. This suggests the existence of a feedback loop sensing the level of Trx80 in or out cells, a mechanism that has also been described for full-length Trx [20]. We can thus hypothesize that, at the site of joint inflammation, synoviocytes stimulated by pro-inflammatory cytokines may produce both full-length Trx and Trx80, the chemoattractant properties of which may result in the recruitment of a massive infiltration of immune cells [21].

The mechanism of action of Trx80 appears to differ from that of full-length Trx insofar as it is independent of thiol-disulfide reactions, and it is more probably mediated *via* a receptor-mediated pathway [11,12]. One of the principal functions of Trx80 is to increase the monocyte expression of the various antigens (including CD14, CD40 and ICAM-1) that play an essential role in inflammatory diseases by controlling the immune response and disrupting the Th1/Th2 balance. Trx80 thus induces phenotypic changes to monocytes that influence the activation of T-cells, macrophages and Th1 T cells [13]. Trx80 can also potentiate release of the Th1 inducer cytokines IL-12 and IFN- γ from PBMC, which may contribute to the development of autoimmune diseases [22]. All these properties confer on extracellular synoviocyte-secreted Trx80 a major role in the establishment and/or progression of autoimmune RA disease. Trx80 has also been reported to be a mitogenic cytokine for peripheral mononuclear blood cells (PBMC) inducing cell differentiation and proliferation and cytokine expression. The present study demonstrates the

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ability of human recombinant Trx80 to stimulate the proliferation of cultured RA and OA synoviocytes in dose-dependent manner, establishing that the synoviocyte is a potent responder to mitogenic effect of Trx80.

In accordance with previous study, our results show that Trx alone is not capable to induce the proliferation of normal cells [11].

In conclusion, the present data show that both the expression and release of Trx by RA synoviocytes are significantly increased in responses to an inflammatory process (as studied through IL-1 β or/and TNF- α stimulation). This increase involves the release of Trx80, the truncated form of Trx, thus possibly contributing to the positive feedback regulation of a self-perpetuating inflammatory response. The novel association that we suggest between Trx and inflammation in synoviocytes, may constitute a link between inflammation and the immune system in RA disease. Moreover, we demonstrate that Trx80 has significant effects on RA synoviocyte proliferation, suggesting that the truncated form of native Trx may be an important contributor to synovial hyperplasia in RA.

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Table 1. Trx protein expression appreciated by quantitative densitometric analysis after 24h of pro-inflammatory treatment in RA and OA synoviocytes.

	Cell lysates		RA vs. OA p values	Culture supernatants		RA vs. OA p values
	RA	OA		RA	OA	
Control	0.88 ± 0.05	0.77 ± 0.03	0.01	0.32 ± 0.09	0.19 ± 0.04	0.03
IL-1 β (1 ng/ml)	0.91 ± 0.05 *	0.81 ± 0.04 †	0.02	0.41 ± 0.12 *	0.23 ± 0.07	0.03
TNF- α (0.5 ng/ml)	0.97 ± 0.05 *	0.85 ± 0.03 †	0.02	0.48 ± 0.14 *	0.32 ± 0.06 †	0.04
IL-1 β + TNF- α	1.01 ± 0.08 *	0.86 ± 0.07	0.04	0.41 ± 0.08 *	0.29 ± 0.08 †	0.04

Blots were scanned and analyzed for their band surface areas, using the same sized section of the blot for each scan. Results were expressed as arbitrary densitometric units (mean ± S.D.) with ImageJ software from RA (n = 5) and OA (n = 5) cultured synoviocytes.

Data were analyzed using the Mann-Whitney test and Wilcoxon test. * p < 0.05 versus. RA control; † p < 0.05 versus OA control.

Table 2. Trx80 protein expression in synoviocytes and culture supernatants from five RA patients, as evaluated by quantitative densitometric analysis.

	RA cell lysates	RA supernatants	% of released form*
Control	0.36 ± 0.09	-	-
IL-1 β (1 ng/ml)	0.41 ± 0.07 [†]	0.15 ± 0.02	26.1 ± 5.2
TNF- α (0.5 ng/ml)	0.55 ± 0.04 [†]	0.22 ± 0.03	28.5 ± 6.1
IL-1 β + TNF- α	0.47 ± 0.05 [†]	0.18 ± 0.04	28.1 ± 5.3

Data were expressed as arbitrary densitometric units (mean ± S.D.) (ImageJ software). Blots were scanned and analyzed for their band surface areas, using the same sized section of the blot for each scan, and analyzed using nonparametric Wilcoxon (paired data) tests, [†] $p < 0.05$ versus RA cell lysate control. * The percentage of secreted Trx80 was calculated relative to the total Trx80 produced, using the formula: % secreted Trx80 = supernatant Trx80 / (lysates Trx80 + supernatant Trx80) x 100.

Figure legends

Figure 1: Production and release of Trx80 after pro-inflammatory stimulation in RA and OA synoviocytes.

Cell lysates, and their respective supernatants, were immunoprecipitated with a goat polyclonal antibody Trx_(N14) after IL-1 β or/and TNF- α treatment over a 24h period. (A) Human recombinant full-length Trx and Trx80 were used as controls. Gels are representative data from experiments with RA patients (n = 5) and OA patients (n = 5) of immunoprecipitation with a goat IgG Ab against Trx/Trx80 for cell lysates (B), and for supernatants (C) were shown.

Figure 2: Full length Trx but not Trx80 was induced by H₂O₂ in synoviocytes.

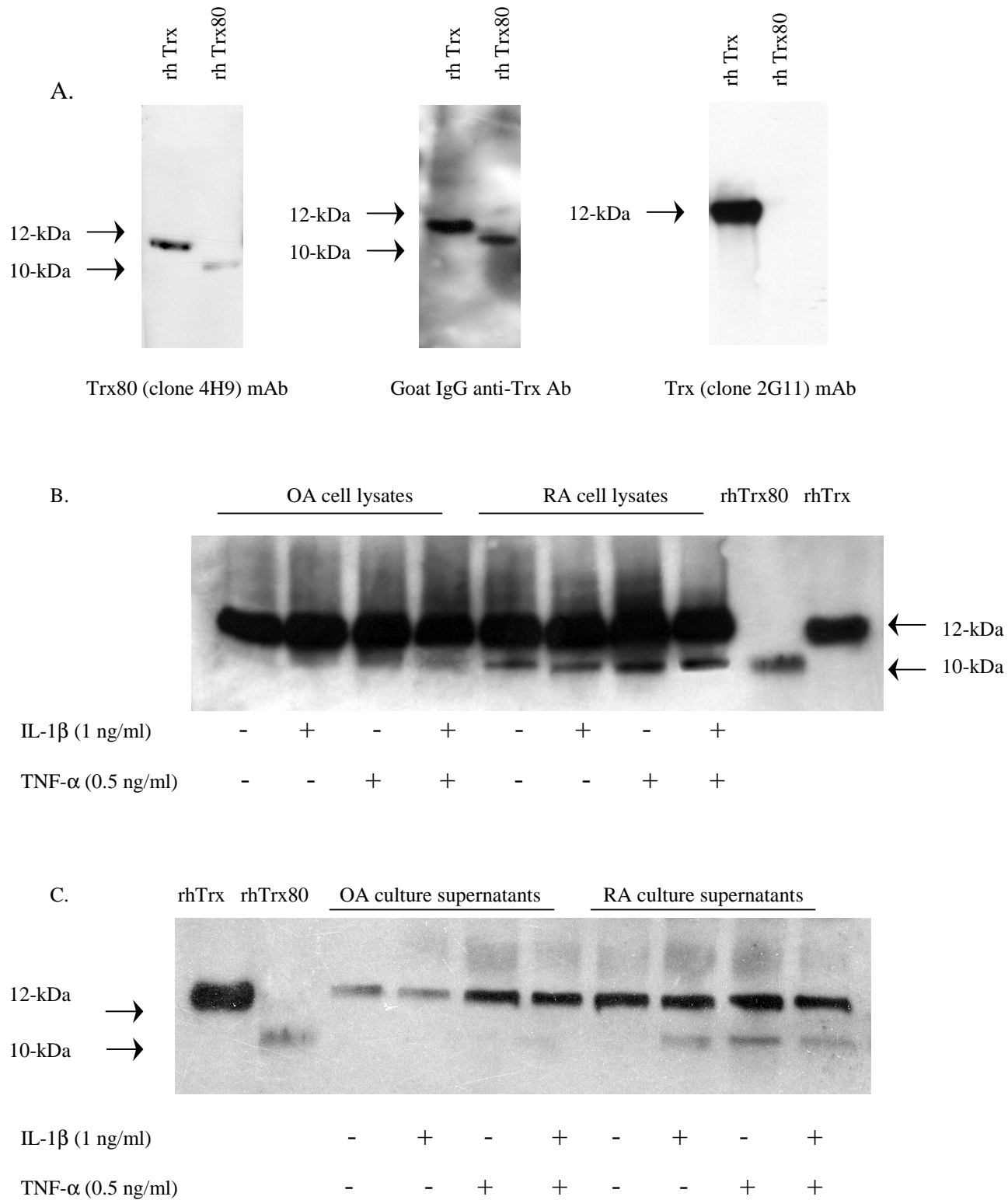
Synoviocytes from RA patients (n = 5) and OA patients (n = 5) were incubated with various concentrations of H₂O₂ for 24h. Immunoblotting experiments were performed and visualized with enhanced chemiluminescence ECL after longer exposure of the blot to X-ray film (>10 min). (A) H₂O₂ increased levels of full-length Trx but did not induced Trx80 protein in cell lysates. (B) Similar results were observed in the respective culture supernatants.

Figure 3: Effects of Trx, and Trx80 on [³H]-thymidine incorporation in cultured synoviocytes.

Synoviocytes from RA patients (n = 5) and OA patients (n = 5) were stimulated with various concentrations of human recombinant Trx and Trx80 for 54 hours and labeled with 0.5 μ Ci/well [³H]-thymidine for 18 hours. Synoviocyte proliferation was expressed as [³H]-thymidine incorporation in counts per minute \pm standard deviation of triplicate wells. The mean effects of Trx80 were shown in RA cells (open bars), and in OA cells (black bar). * p < 0.05 versus. untreated RA cells; † p < 0.05 versus. untreated OA cells.

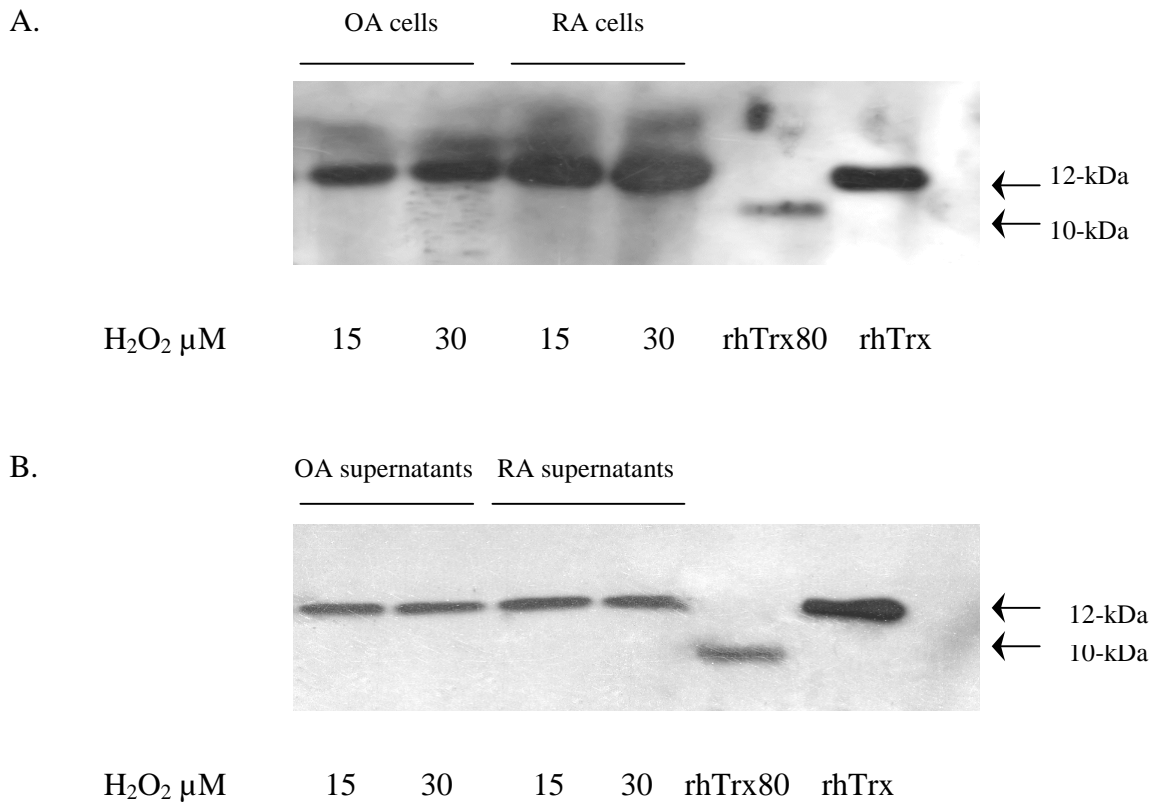
Truncated thioredoxin in RA synoviocytes

Figure 1



Truncated thioredoxin in RA synoviocytes

Figure 2



Truncated thioredoxin in RA synoviocytes

Figure 3

