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The functional CD40 antigen of fibroblasts may contribute to the proliferation of rheumatoid synovium

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SUMMARY

This paper demonstrates that CD40 is expressed on rheumatoid synovial pannus and primary fibroblast cell lines established from rheumatoid and osteoarthritic synovium as well as normal skin. Among various tested cytokines, interferon-gamma (IFN-γ) and to a lower extent, tumour necrosis factor-alpha (TNF-α) were found to upregulate CD40 expression on fibroblasts. Synovial and skin fibroblasts cultured over CD40 Ligand transfected L cells (L-CD40 L) demonstrate a CD40 specific increase of DNA synthesis as measured by tritiated thymidine incorporation. Cell-cycle analysis and enumeration of viable cells further show that CD40 induced fibroblast proliferation. Costimulation with L-CD40 L and IFN-γ resulted in maximal proliferation. Engagement of fibroblasts CD40 increased the IL-1-induced production of granulocyte macrophage-colony stimulating factor and macrophage inflammatory protein-1α MIP-1α. CD40 L activated fibroblasts showed decreased levels of CD40, but only marginal alterations of other cell-surface antigens. Taken together, the present results indicate that fibroblasts express functional CD40 and suggest a possible role of CD40 L expressing cells, such as activated T cells and mast cells, in the development of synovium hyperplasia.

Keywords CD40 rheumatoid synovium fibroblast proliferation

INTRODUCTION

CD40 is a 45–50 kD membrane glycoprotein of 277 amino acids that belongs to the tumour necrosis factor receptor superfamily ([1,2] for review, [3–5]). CD40 expression has been shown on haematopoietic progenitors, mature B cells, monocytes, dendritic cells, follicular dendritic cells, thymic epithelium, endothelial cells, carcinoma cells and HTLV-1 transformed T cell lines. CD40 is the receptor for a ligand (CD40 L) which is a $\approx$35 kD glycoprotein of 261 amino acids that is a member of the tumour necrosis factor (TNF) superfamily [6,7]. While expression of CD40 L is well documented on activated T cells, recent studies also suggest its expression on basophils, eosinophils and activated B cells [8,10].

Studies *in vitro* and *in vivo* have demonstrated that cross-linking of CD40 plays a crucial role in T cell-dependent B cell immunopoiesis. In particular, interrupting CD40–CD40 L interactions *in vivo* prevents the generation of memory B cells. The importance of the CD40–CD40 L interaction has been demonstrated by the molecular analysis of the CD40 ligand defect observed in the hyper-IgM syndrome. Its critical role is not limited to B cell differentiation and induction of memory B cells, since the occurrence of neutropenia and infections indicates a broader pathological consequence of this T cell defect [11].

In addition to mature B lymphocytes, *in vitro* studies have demonstrated the presence of functional CD40 on thymic epithelium [12], progenitor B cells [13], dendritic cells [14] and endothelial cells [15,16].

While analysing the expression of CD40 on various normal and pathological tissue samples by immunohistology, an important staining was observed on rheumatoid synovium. The rheumatoid arthritis (RA) synovium is characterized by the close interaction between resident cells, i.e. synovial fibroblasts with bone-marrow-derived mononuclear cells, i.e. monocytes, T cells, B cells, mast cells, which leads to synovium hyperplasia. Subsequently, CD40 expression could be detected on primary cell lines from rheumatoid synovium but also skin fibroblasts. *In vitro* studies with CD40 L demonstrated the fibroblast CD40 to be functional.

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MATERIALS AND METHODS

Reagents

Purified human cytokines from the following sources were used at the following concentrations, unless otherwise indicated: r-granulocyte-macrophage colony stimulating factor (rGM-CSF) (100 ng/ml), rIL-4 (100 U/ml), rIL-6 (30 ng/ml) were obtained from the Schering-Plough Research Institute (Kenilworth, NJ); rbFGF (10 ng/ml) and platelet-derived growth factor (PDGF) (2·5 U/ml) from Collaborative Research (Bedford, MA); rIL-2 (10 U/ml) from Amgen Biologicals (Thousand Oaks, CA); rIL-1β (100 U/ml) and rTNF-α (0·01–100 ng/ml) from Genzyme Corporation (Cambridge, MA); rIFN-γ (0·01–100 ng/ml) from Amersham (Aylesbury, UK). The MoAbs against CD40 (MoAb 89), CD40 L (LL2) and their control (30N) were generated in the laboratory. Cytokines and antibodies to be tested were added at the onset of the culture at indicated concentrations.

Cells

Synoviocyte isolation. Synovial membranes were obtained from patients with rheumatoid arthritis (RA) or osteoarthritis (OA) undergoing total or partial knee or wrist replacement surgery. Synovial fibroblasts were isolated as previously described [17]. After surgery, the resulting fragments of synovium were finely minced into small pieces and digested with 2 mg/ml collagenase (Worthington, Freehold, NJ) for 2–3 h at 37°C. After centrifugation, cells were resuspended in complete medium made of alpha-minimum essential medium (α-MEM) (GIBCO) with 2 mM L-glutamine, 100 U/ml penicillin, 50 μg/ml gentamycin, 20 mM HEPES buffer, and 10% fetal calf serum (FCS). Cells were cultured in 100 mm Petri dishes in humidified 5% CO_2 atmosphere. After adherence for 48 h, non-adherent cells were removed. Adherent cells were cultured in complete medium and at confluence were passaged in a 150 cm^2 culture flask after trypsin treatment. Synovial fibroblasts were used in passages 3–8. They were negative for the expression of HLA-DR, CD14, CD2, CD19, CD45 and positive for the expression of CD10, CD13, CD29, CD44, CD49a/VLA-1, intercellular adhesion molecule-1 (ICAM-1)/CD54, LFA-3/CD58, carboxypeptidase M and CD95/Fas.

Primary skin fibroblast cell lines were kindly provided by Dr J. F. Nicolas (Lyon).

L cells. Murine fibroblast L cells were stably transfected with human CD40 Ligand (L-CD40 L) according to the experimental procedures described previously for FcγRII (L-CD32) which were used as controls [18]. L cells were irradiated at 75 Gy before use.

Co-culture system. Synovial fibroblasts were cultured in the presence of L-CD40 L or L-CD32 cells at a 1 : 1 ratio in RPMI 1640 (GIBCO BRL, Gaithersburg, MD) supplemented with 10% (v/v) FCS (complete medium), 10 mM HEPES, 2 mM L-glutamine, 100 U/ml gentamycine (Schering-Plough).

Immunohistochemistry

Serial 7 μm sections of frozen rheumatoid synovial tissue were stained using the streptavidin–biotin (LSAB kit) peroxidase method as described by the manufacturer (Dako, Glostrup, Denmark). Briefly, cryostat sections were air dried for 5 min, and acetone fixed for a further 5 min. Endogenous peroxidase activity was quenched for 5 min using a 3% hydrogen peroxide in PBS solution, after which slides were rinsed three times in PBS. A 30% solution of AB human serum in PBS was applied to block non-specific protein uptake, followed by three washes in PBS.

The CD40-specific MoAb 89 and its IgG1 control were diluted at 15 μg/ml in PBS, deposited on the section and incubated for 15 min at room temperature in a moist chamber. After three washes with PBS, biotinylated rabbit anti-mouse antibody was applied for 30 min at room temperature, then slides were washed in PBS. Optimum colour development was achieved with 3-amino-9 ethyl carbazole (AEC) as a chromogen, resulting in a red colour precipitate at the antigen site. The sections were counterstained with haematoxylin, dehydrated, and then mounted.

Culture studies

Proliferation. Synovial fibroblasts ($2{\cdot}5 \times 10^3$/well) were seeded in 96-well microtitre plate (Nunc, Roskilde, Denmark) with 250 μl of complete medium. The cytokines or antibodies to be tested were added at the onset of the culture at the concentrations indicated. Cells were incubated for the culture time shown and pulsed for the last 18 h with 1 μCi tritiated thymidine, ^{3}H-TdR, per well (specific activity 25 Ci/mmol: CEA, Saclay, France). After medium removal, cells were trypsinized with trypsin-EDTA (GIBCO) before harvesting and ^{3}H-TdR uptake was measured by standard liquid scintillation counting techniques. Counts of ^{3}H-TdR incorporation are expressed as means of triplicate determinations.

Cell counting. Synovial fibroblasts were seeded in six-well culture plates (Nunc) at 5×10^4 cells/well in complete medium in the presence of L-CD40 L or L-CD32 as a control. After 3 and 7 days of culture, trypsinized cells were suspended in 500 μl of complete medium, and enumerated using a haemocytometer. Since mouse-derived L cells and human fibroblasts are the same size, these two populations are distinguished according to the human CD44 specifically expressed on fibroblasts. The cell suspension was thus sustained with anti-CD44-FITC and then analysed with a FACScan (Becton Dickinson, Sunnyvale, CA). The number of synovial fibroblasts in co-culture was calculated according to the percentage of $CD44^+$ cells.

Cell cycle studies. Synovial fibroblasts were plated in 75 cm^2 culture flasks ($3{\cdot}5 \times 10^5$ cells/flask) in the presence of L-CD40 L (or L-CD32 cells for negative control), as previously described [17]. Cells were incubated for either 2 days or 7 days. In some experiments, colcemid (0·1 μg/ml; GIBCO) was added for the last 17 h of culture. DNA staining was performed with Hoechst 33342 (10 μg: Calbiochem, La Jolla, CA) which was added for 1 h at 37°C before trypsin treatment. Flow cytometry was performed with a FACStar Plus (Becton Dickinson) fitted with two argon-ion lasers at 488 nm to exclude dead cells with propidium iodide staining and at 351 nm for cell analysis. Fluorescent beads were used for internal calibration before each analysis. Debris were excluded by conventional scatter gating. Thirty thousand cells were analysed using a flow rate of 100 events/s.

FACS analysis

For phenotypic expression, synovial fibroblasts (3×10^4/ml per well) were cultured in 24-well culture plates (Linbro, Flow Laboratories, McLean, VA) for 48 h in complete medium. Cells were harvested by brief trypsinization and stained. The brief trypsinization was shown not to affect the expression of the selected antigens.

Antibodies used for phenotypic studies were: HLA-DR, LFA-3/CD58 (Becton Dickinson Monoclonal Center, Mountain View, CA); ICAM-1/CD54, CD10, CD38, CD13, CD49a/VLA-1, CD95/Fas clone UB2 (Immunotech, Marseille, France); CD44 (Sigma, St

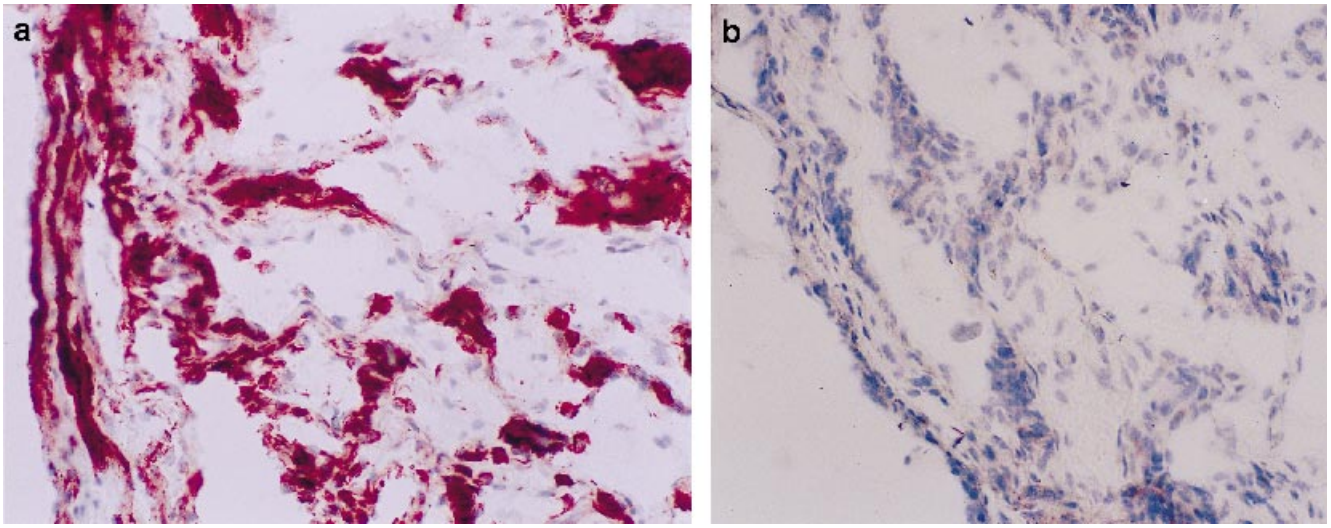

Fig. 1. CD40 is strongly expressed on rheumatoid arthritis synovial membranes. Frozen sections were successively incubated with either (a) the anti-CD40 MoAb 89 or (b) the IgG1 control MoAb, then with a biotinylated rabbit anti-mouse Ig, then streptavidin–biotin, then the chromogen. Sections were counterstained with haematoxylin. Magnification ×200.

Louis, MO); B7-2/CD86 (PharMingen, San Diego, CA). B7-1/CD80 (MoAb 104) was a generous gift of Dr Lanier (DNAX, Palo Alto, CA). Single-colour surface immunofluorescence was performed according to standard techniques using 10 μg/ml of murine MoAb directly labelled with FITC, or a non-related antibody IgG1 as a negative control (Becton Dickinson). Double-colour membrane fluorescence was carried out by sequential incubation of the cells with unconjugated MoAbs, PE-conjugated anti-rabbit Ig (Dako) and MoAbs directly labelled with FITC. For expression of CD40, cells were stained with a biotinylated CD40 antibody (MoAb 89), followed by streptavidin-PE (Becton Dickinson). A biotinylated unrelated antibody was used as control (Caltag, San Francisco, CA). Samples were analysed with a FACScan flow cytometer (Becton Dickinson). Results are presented as mean fluorescence intensity channel (MFC) of total cells.

Cytokine production

For quantification of cytokine secretion, synovial fibroblasts transfected in the presence of L cells were plated in 24-well plates at $1{\cdot}5 \times 10^4$ cells/well in 1 ml of complete medium. Cell-free supernatants were harvested after the indicated time and stored at -20°C until cytokine detection. Levels of IL-6 and IL-10 were measured by specific ELISAs whose antibodies were kindly provided by Dr J. S. Abrams (DNAX) [19]. ELISA kits for GM-CSF, TNF-α, and IL-8 were from Medgenix Diagnostics (Brussels, Belgium), that for IL-1α and IL-1β was from Immunotech and that for MIP-α from R&D Systems, (Minneapolis, MN). Collagens I and III were detected by RIA (Institut Pasteur, Lyon, France). The detection limits of these assays were 10 pg/ml for TNF-α, 10 pg/ml for GM-CSF, 250 pg/ml for IL-6, 15 pg/ml for IL-1α and IL-1β, 0·7 pg/ml for IL-8, 2 pg/ml for MIP-1α and 50 pg/ml for IL-10.

RESULTS

Rheumatoid synovial fibroblasts express CD40 in vivo *and* ex vivo

Immunostaining of biopsies of RA synovium with the anti-CD40 MoAb 89 revealed an intense expression of CD40 in synovial

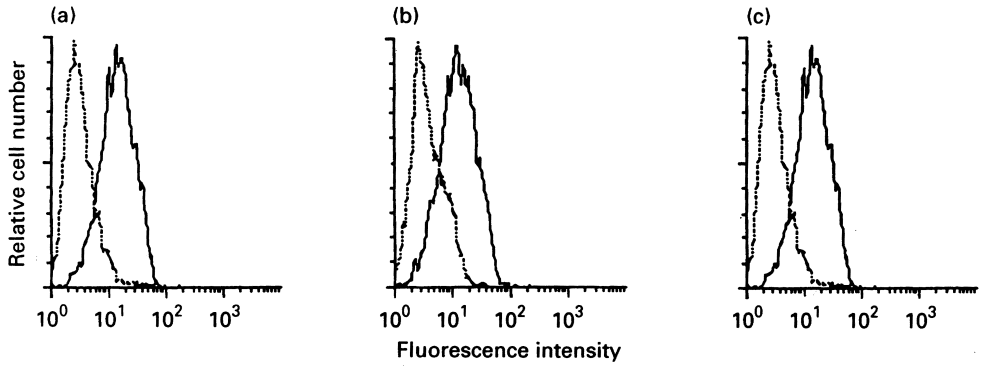

Fig. 2. Primary fibroblast cell lines express CD40. Primary fibroblast lines (less than 10 passages) were established from either (a) rheumatoid arthritis, and (b) osteoarthritis synovial membranes or (c) skin. 48 h after split, cells are slightly trypsinized, resuspended and stained with biotinylated MoAb 89 followed by streptavidin-PE. Basal fluorescence levels are estimated after staining with a biotinylated IgG1 (dotted histogram). The data are representative of one out of 15 experiments for RA cells and one out of three experiments for other cells.

Table 1. Regulation of CD40 expression on synovial fibroblasts by cytokines

Culture conditions	MFI ± s.d.	Number of experiments
Medium	15·6 ± 5·9	12
IL-1β	18·6 ± 7·7	5
IL-4	19·5 ± 0·7	3
IL-6	15·8 ± 2·1	2
GM-CSF	13·2 ± 4·2	3
TNF-α	25 ± 5·7	5
IFN-γ	71 ± 15·5	6
bFGF	15·2 ± 2	2

Synovial fibroblasts (1×10^5 cells/well in six-well plates) were cultured in complete medium, alone or in the presence of cytokines at indicated concentrations. After 2 days cells were trypsinized and staining for CD40 expression by MoAb 89 (10 μg/ml). A non-related antibody IgG1 was used for control at the same concentration. GM-CSF, granulocyte-macrophage colony-stimulating factor; TNF-α, tumour necrosis factor-alpha; IFN-γ, interferon-gamma; bFGF, fibroblast growth factor b.

tissues, with a particularly strong staining of the lining cell layer (Fig. 1a). The staining was specific because a control IgG does not bind (Fig. 1b), and preincubation of the MoAb 89 with a recombinant form of the extracellular CD40 domain abolished the staining (not shown).

Since the synovium hyperplasia represents the proliferation of synovial fibroblasts, the expression of CD40 was further analysed on primary cell lines that were generated after collagenase treatment of rheumatoid synovial tissue. Flow cytometry analysis, after staining with biotinylated MoAb 89 and PE-labelled streptavidin, demonstrates that rheumatoid synovial fibroblasts homogeneously express surface CD40 (Fig. 2a). mRNA analysis demonstrated the presence, in those cultured rheumatoid synovial fibroblasts, of a typical 1·5 kb CD40 mRNA.

CD40 expression was not specific to RA synovial fibroblasts as it was also detected on synovial fibroblasts from patients with osteoarthritis, as well as skin fibroblasts in primary cultures (Fig. 2b,c). It is important to stress that levels of CD40 antigen on fibroblasts were significantly lower than those on B lymphocytes or dendritic cells which, in contrast to fibroblasts, can be easily stained with FITC conjugates (not shown).

IFN-γ and TNF-α upregulate CD40 expression on fibroblasts

The presence of mononuclear cells in inflammatory synovium led us to question whether the cell–cell interactions and action of cytokines produced locally may contribute to an upregulation of CD40 expression on synovial fibroblasts. Thus we set up culture conditions to reproduce *in vitro* the effect of local secretion of cytokines and growth factors known to be involved in the pathogeny of the disease. Therefore, synovial fibroblasts were cultured for 48 h in the presence of an optimal concentration of cytokines and then analysed for CD40 expression using flow cytometry. A composite of several independent experiments (Table 1) shows that IFN-γ is the most powerful inducer of CD40 expression on fibroblasts (as expressed by mean fluorescence intensity levels), while TNF-α reproducibly appears as a weak inducer of CD40 expression. Typical histograms of CD40 expression on synovial fibroblasts cultured with IFN-γ or TNF-α are shown in Fig. 3a. Note that other cytokines that have been shown to affect synovial fibroblast growth or cytokine secretion, such as IL-1β and IL-4, were irregularly weak inducers of CD40, the responsiveness being heterogeneous between synovial samples; IL-2, IL-6, GM-CSF and bFGF were unable to upregulate CD40 expression on fibroblasts. Dose-titration curves (Fig. 3b) showed that 1 ng/ml of TNF-α and 10 ng/ml of IFN-γ induced maximal CD40 expression. A time kinetic analysis further showed that increased CD40 expression reached a maximum after 48 h and remained stable for at least 7 days (data not shown).

Synovial fibroblasts proliferate upon CD40 engagement

As the uncontrolled proliferation of the synovial fibroblasts represents a critical feature of rheumatoid synovitis [20,21] we wondered whether the high expression of CD40 on synovial fibroblasts could be related to this hyperproliferation inasmuch as CD40 engagement has been shown to increase proliferation of mature

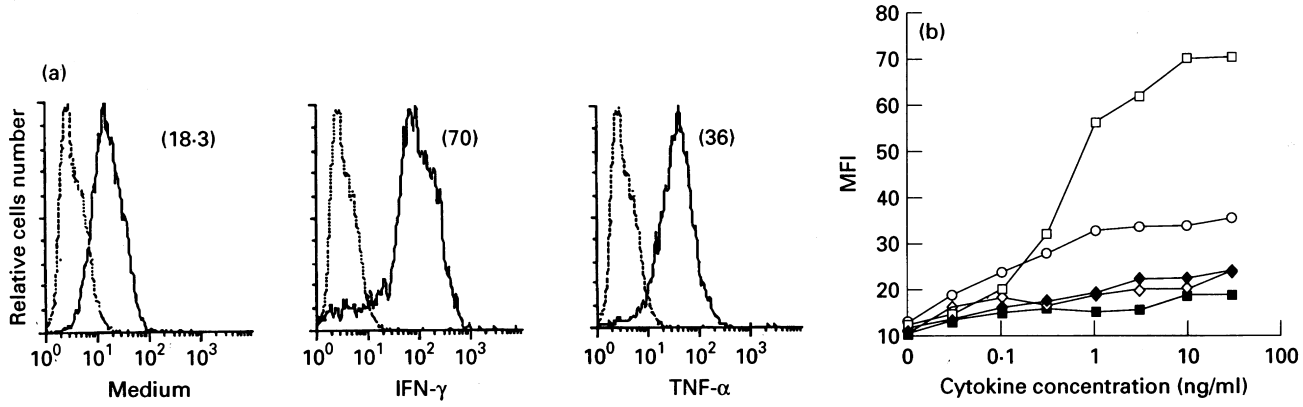

Fig. 3. IFN-γ and, to a lower extent TNF-α, enhance CD40 expression on fibroblasts. Synovial fibroblasts (3×10^4) were cultured in 24-well plates for 48 h and analysed for CD40 expression flow cytometry. MFI, mean fluorescence intensity. (a) Flow cytometry histograms (MFI value in parentheses). (b) Dose-dependence analysis. □, IFN-γ; ○, tumour necrosis factor-alpha; ◆, IL-1β; ◇, IL-4; ■, granulocyte-macrophage colony-stimulating factor.

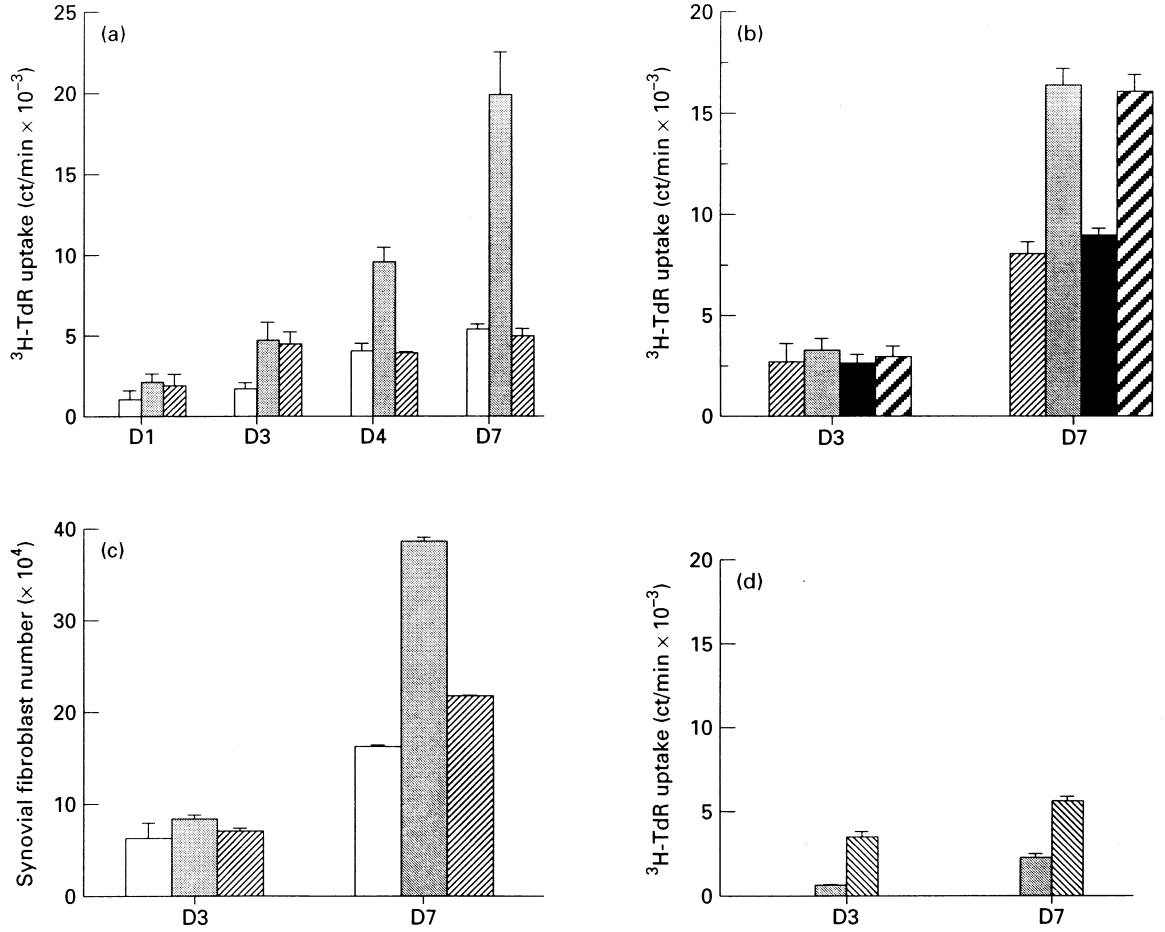

Fig. 4. Engagement of CD40 specifically enhances fibroblast proliferation. (a) Kinetics of rheumatoid arthritis synovial fibroblast DNA synthesis. Synovial fibroblasts were seeded at $2{\cdot}5 \times 10^3$ cells/well in 96-well plates. Irradiated murine fibroblast L cells that had been transfected with human CD40 L (L-CD40 L) (▨) or FcγRII (L-CD32) (▨) as a control were added at a 1 : 1 ratio (medium, □). Cells were incubated for the number of days shown and pulsed with ^{3}H-TdR. Data are presented as ct/min incorporated over the final 17 h of the culture period. (b) The anti-CD40 L MoAb LL2 specifically blocks CD40-induced fibroblast DNA synthesis: cultures as in (a); LL2 and 30 N control antibody were ascites used at 1/1000. ▨, L-CD40 L; ▨, L-CD32; ■, L-CD40 L + LL2; ▨, L-CD40 L + 30N. (c) CD40 ligation results in increased numbers of viable fibroblasts: 5×10^4 synoviocytes were seeded in six-well plates in the presence or absence of irradiated L-CD40 L or L-CD32 (negative control). At day 3 and day 7, cells were trypsinized and counted with a haemocytometer in the presence of trypan blue. Aliquots of cells were stained with CD44-FITC and the percentage of synovial fibroblasts was determined by flow cytometry. In co-culture system, the exact number of cells is calculated following the percentage of CD44$^+$ cells (symbols as (a)). (d) CD40 ligation enhances skin fibroblast DNA synthesis. ▨, skin fibroblast + L-CD32; ▨, skin fibroblast + L-CD40 L.

and progenitor B cells [2,13]. Thus to reconstitute *in vitro* the CD40–CD40 L interaction occurring *in vivo*, synovial fibroblasts were cultured over a layer of irradiated CD40 L transfected L cells (L-CD40 L), and ^{3}H-TdT uptake was measured. As shown in Fig. 4a, synovial fibroblasts cultured over L-CD40 L cells showed increased DNA synthesis both at day 4 and day 7. The observed stimulatory effect was specific to CD40 triggering since: (i) L-CD32 cells did not enhance synovial fibroblast proliferation (Fig. 4a), (ii) addition to co-cultures of MoAb LL2, a blocking MoAb to CD40 L [22], totally inhibited the stimulatory effect of the L-CD40 L (Fig. 4b). Similarly, the blocking anti-CD40 antibody MoAb 89 [23] also blocked CD40 L induced synovial fibroblast proliferation (not shown). The increment of viable fibroblast numbers observed after 7 days of culture on L-CD40 L cells (Fig. 4c) demonstrated that DNA synthesis as measured by ^{3}H-TdR incorporation was indeed an appropriate measure of cell proliferation. While the number of viable fibroblasts increased spontaneously twofold over 7 days, the further engagement of CD40 resulted in a fivefold expansion of the number of cells. The CD40-induced proliferation of rheumatoid synovial fibroblasts was also observed with skin fibroblasts (Fig. 4d), therefore establishing CD40-induced cell growth as a general property of fibroblasts.

To assess further the effect of CD40 ligation on synovial fibroblast proliferation, cell-cycle studies were performed by flow cytometry analysis after DNA staining with the Hoechst 33342. For the last 17 h of culture, colcemid was added to block cell mitosis, so leading to an accumulation of cells in the M phase. The addition of L-CD40 L to cultures of rheumatoid synovial fibroblasts increased, at day 2, the percentage of cells in S/G_2M from 30% to 44%. The L-CD40 L effect was even more striking at day 7, where the proportion of cells in S/G_2M increased from 9% to 26% (Fig. 5).

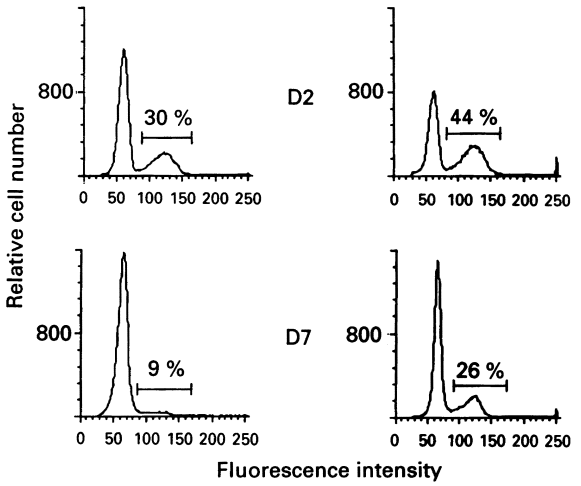

Fig. 5. CD40 ligation accelerates the fibroblast cell cycle. 3×10^5 synovial fibroblasts were cultured in the absence or presence of irradiated L-CD40 L (ratio 1 : 1) for 2 and 7 days of culture, in 75 cm^2 culture flasks. Colcemid (0·1 μg/ml) was added for the last 17 h of culture. After DNA staining with Hoechst 33342 for 1 h, cells were trypsinized, stained with MoAb CD44-FITC and flow cytometric analysis was performed on a FACStar plus. DNA histograms of synovial fibroblasts are shown and the percentage of cells in the S/G2-M phases are indicated. Results are representative of three experiments. Left, synoviocytes + L cells CD32; right, synoviocytes+ *L* cells CD40 L.

Table 2. IFN-γ synergizes with CD40 ligation to induce proliferation of synovial fibroblasts: ct/min $\times 10^{-3}$

	L-CD32	L-CD40 L
Medium	3·1 ± 0·4	6·0 ± 0·8
IL-1β	3·3 ± 0·2	8·5 ± 1·0
IL-2	3·7 ± 0·1	6·0 ± 0·03
IL-4	2·6 ± 0·3	6·7 ± 0·05
IL-6	3·6 ± 0·1	6·5 ± 1·5
TNF-α	3·8 ± 0·06	8·5 ± 0·7
GM-CSF	3·4 ± 0·7	7·5 ± 0·8
bFGF	13·5 ± 0·6	10·5 ± 0·1
IFN-γ	4·6 ± 0·6	15·0 ± 0·5
PDGF	6·5 ± 0·6	7·4 ± 0·06

Synovial fibroblasts ($2{\cdot}5 \times 10^3$ cells/well) were cultured in presence of L-CD32 or L-CD40 L, either in medium or with cytokines at indicated concentrations. ^{3}H-TdR incorporation was measured at 5 days as indicated in Materials and Methods. The counts for L cells alone are below 450 ct/min. Results are expressed as mean ± s.d. of culture triplicate. TNF-α, tumour necrosis factor-alpha; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-gamma; PDGF, platelet-derived growth factor.

IFN-γ and CD40 ligand co-operate to induce the proliferation of synovial fibroblasts

Inasmuch as IFN-γ was found to upregulate CD40 expression, we wondered whether the increase of CD40 expression induced by IFN-γ would result in enhanced CD40-dependent cell proliferation and whether CD40 L-dependent proliferation would act in concert with that induced by fibroblast growth factors. As shown in Table 2, bFGF, PDGF and CD40 L when used alone significantly enhanced the proliferation of synovial fibroblasts. Interestingly IL-1β, TNF-α, IFN-γ enhanced CD40-induced proliferation. The most important proliferation is that obtained with the combination of IFN-γ and CD40 L which even surpasses that induced by bFGF alone.

Engagement of fibroblast CD40 enhances IL-1 induced secretion of GM-CSF and MIP-1α

As CD40 ligation has been shown to enhance cytokine production by B cells [24], epithelial cells [12], monocytes [25] and dendritic cells [14], supernatants of CD40-activated synovial fibroblasts were tested for their levels of IL-1α, IL-1β, IL-6, IL-8, IL-10, TNF-α, GM-CSF and MIP-1α using specific ELISAs. Cytokine levels were tested at multiple time points between 24 h and 6 days after establishment of the cultures. Cytokine level supernatants were only reported at 48 h as their levels are not altered subsequently. As shown in Fig. 6, only IL-6 (2–4 ng/ml) according to the tested cell line and IL-8 (1–2 ng/ml) could be detected in the supernatants of non-activated cells. CD40 engagement did not increase the spontaneous production of these two cytokines and did not turn on the production of the others.

The effect of CD40 triggering on the production of cytokines was subsequently tested in cultures of synovial fibroblasts in the presence of either IFN-γ, which strongly upregulates CD40 expression, or IL-1β, a known inducer of cytokine secretion by fibroblasts, that is present in large amounts in inflammatory synovium [26]. IFN-γ itself displayed minimal effects on the secretion of cytokines by fibroblasts. Dual triggering of cells with IFN-γ and CD40 L was of minimal or no effect on the secretion of IL-6, IL-8 and GM-CSF, but resulted in the production of significant levels of MIP-1α (30–100 pg/ml in three independent experiments). Addition of IL-1β to cultures of fibroblasts increased about fivefold the levels of IL-6 and IL-8 detected in 48 h supernatants. These supernatants also contained detectable levels of GM-CSF (50–150 pg/ml) and MIP-1α (10–30 pg/ml) and further triggering of CD40 resulted in a 3–4-fold increase of GM-CSF and MIP-1α levels. TNF-α, IL-1α, IL-1β, IL-10 levels were below the ELISA detection limit (TNF-$\alpha \leqslant 10$ pg/ml, IL-1$\alpha/\beta \leqslant 15$ pg/ml, IL-10 $\leqslant 100$ pg/ml). Finally, the basal production of PGE_2 and collagen I and III were not altered after CD40 ligation (data not shown).

Thus, in the presence of IFN-γ or IL-1β, CD40 engagement of fibroblasts turns on or enhances the production of GM-CSF and MIP-1α.

CD40 engagement minimally alters fibroblast phenotype

Synovial fibroblasts in primary cultures express CD44, CD40, CD13, CD29, carboxypeptidase M, CD54, CD10, CD58, Fas and VLA-1 (Fig. 7). Addition of IFN-γ resulted in the strong upregulation of several antigens including CD40, HLA-DR, CD54, while others were non-homogeneously affected (CD38).

Because of the different species origin, murine L-CD40 L cells and human synovial fibroblasts could be distinguished according to expression of human CD44. Accordingly, CD40 L activated fibroblasts showed decreased expression of CD40, while other antigens were only minimally affected. Note that neither B7-1/CD80 nor B7-2/CD86 were upregulated, while CD40 triggering

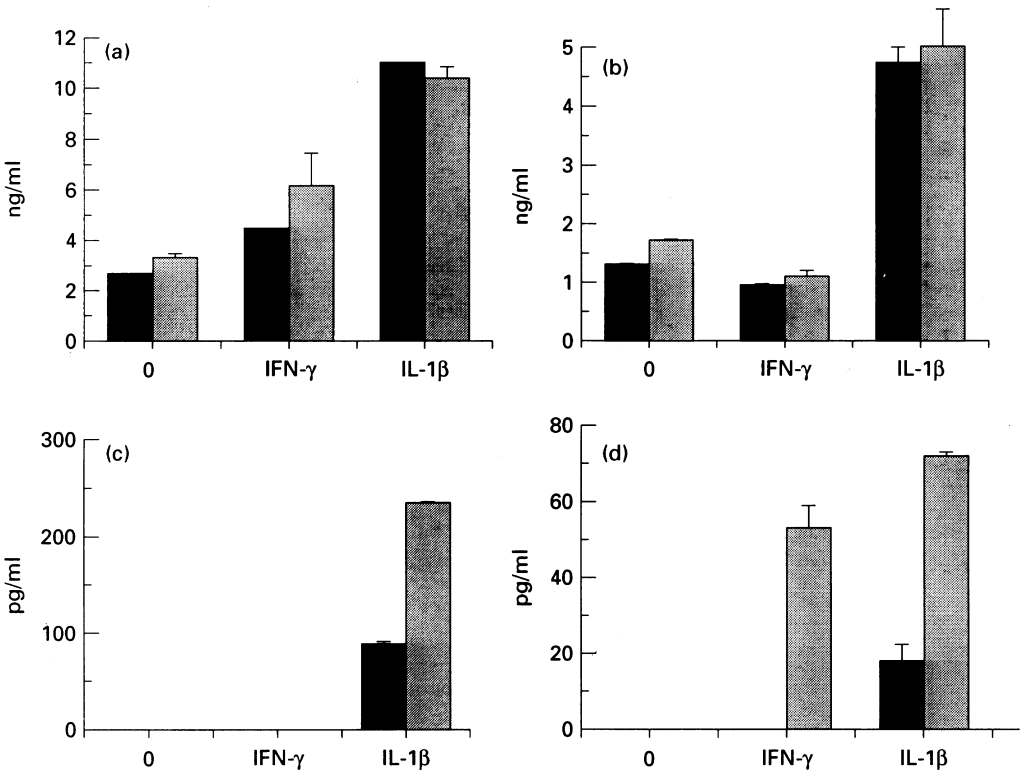

Fig. 6. CD40 triggering enhances cytokine-induced secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-8. Rheumatoid synovial fibroblasts were seeded at $1{\cdot}5 \times 10^4$/well in 24-well plates without or with IFN-γ (10 ng/ml), or IL-1β (100 U/ml), with either irradiated L-CD32 (■) or L-CD40 L (▨) cells ($1{\cdot}5 \times 10^4$/well). After 48 h, supernatants were harvested and tested for levels of (a) IL-6, (b) IL-8, (c) GM-CSF and (d) MIP-1α using specific ELISA assays. Results are expressed as the mean ± s.d. of triplicates. This experiment represents one of three.

strongly upregulates their expression on B cells [27], dendritic cells [14] and monocytes (our unpublished observations). The combination of CD40 L and IFN-γ, that results in strong fibroblast growth, yielded a phenotype which was a composite of that obtained with each individual activator: level of CD40 alone was decreased when compared with untreated cells. Thus CD40 triggering appears to minimally alter fibroblast phenotype.

DISCUSSION

The present study demonstrates that CD40 is expressed on fibroblasts both *in vivo* as shown on sections of synovial tissue and *in vitro* on primary fibroblast cell lines that were established from synovial tissue and skin. The present finding is therefore in accordance with the previously described expression of CD40 on lines of follicular dendritic cells [28–30], stromal elements of primary and secondary follicules which have been shown to display features characteristic of myofibroblasts [31]. Furthermore, a recently published study also described the presence of CD40 on human fibroblasts of different tissular origin [32]. The CD40 expressed by fibroblasts appears to be the bona fide CD40, inasmuch as several anti-CD40 molecules recognizing different epitopes of the CD40 molecules were shown to stain the fibroblasts (data not shown). Thus adherent cells such as epithelial cells, endothelial cells and fibroblasts all express CD40, although it is important to stress that the level of CD40 expression on these cells is lower than that observed on B lymphocytes or interdigitating dendritic cells. Yet CD40 expression can be upregulated by cytokines such as IFN-γ and to a lesser extent TNF-α. Thus identical cytokines upregulate CD40 expression on fibroblasts, epithelial cells and monocytes. At variance with monocytes, GM-CSF (which is present in synovium) does not affect fibroblast CD40 expression and, at variance with B cells, fibroblast CD40 is virtually not altered in response to IL-4 that is able to upregulate CD54 [33]. This contrasts with B lymphocytes and monocytes whose CD40 expression is upregulated by IL-4 and GM-CSF, respectively [23,25]. Upon ligation with immobilized CD40 L, CD40 expression on fibroblasts is downregulated, while such triggering results in increased CD40 expression on dendritic cells [14]. The decreased CD40 expression is likely to result from ligand-induced receptor endocytosis inasmuch as CD40 mRNA levels are comparable (as demonstrated by Northern blot analysis) in cells that are cultured without or with L-CD40 L (data not shown). The presence of IFN-γ-producing cells [34,35] within the synovium and the CD40-inducing capacity of IFN-γ may explain why CD40 expression on rheumatoid synovial pannus was so easily detected.

As observed with B cells, monocytes, dendritic cells, epithelial cells and endothelial cells, and haemopoietic progenitor cells [13], fibroblast CD40 appeared to be functional inasmuch as CD40 L-transfected cells enhance, in a specific fashion, fibroblast proliferation. In line with the present observation, a fibroblast-like follicular dendritic cell line was found to proliferate, in response to activated T cells, in a CD40-dependent fashion [29]. While triggering CD40 considerably alters the phenotype of cells of the

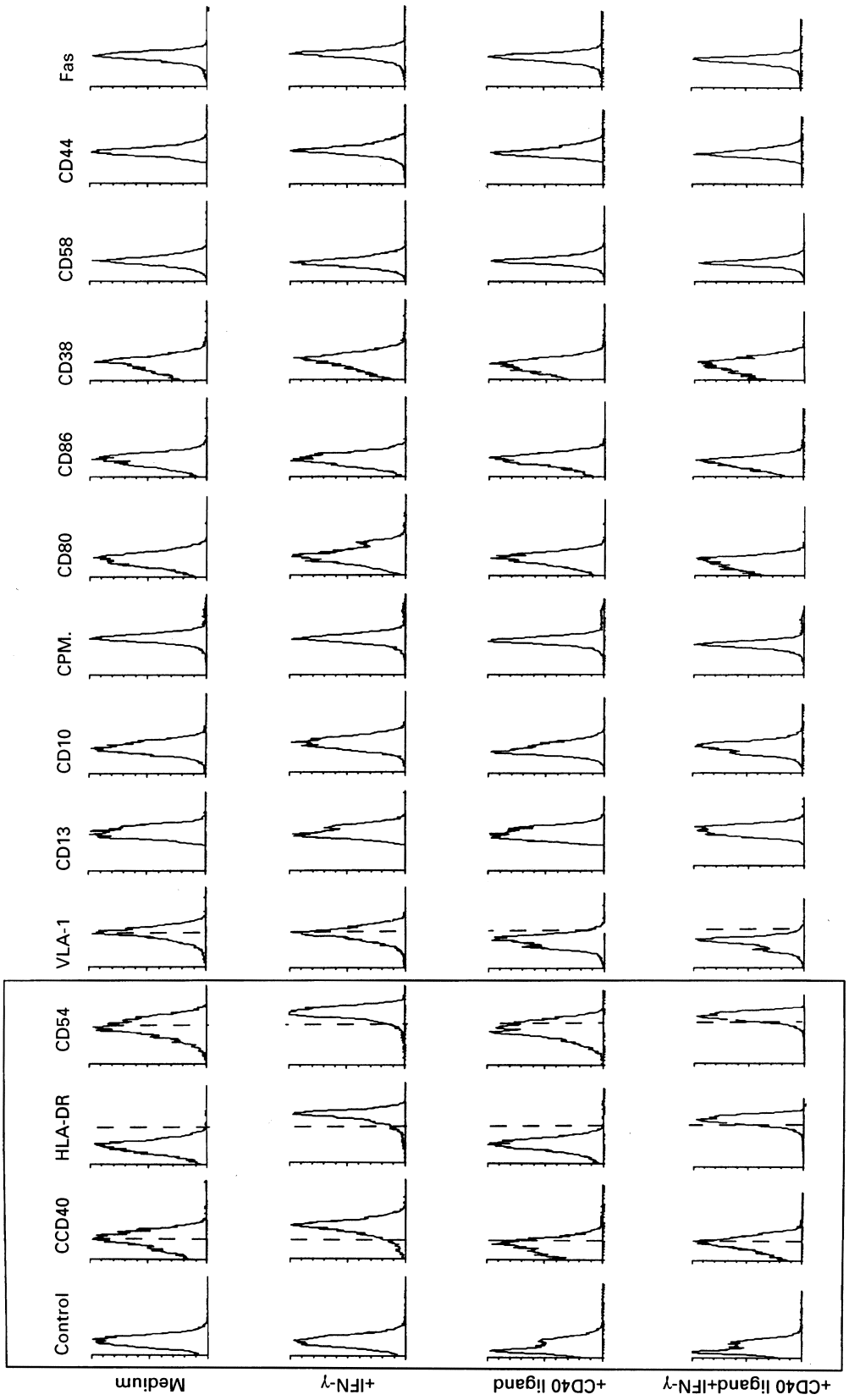

Fig. 7. CD40 ligation moderately alters fibroblast phenotype. Rheumatoid synovial fibroblasts were incubated for 48 h without or with IFN-γ (10 ng/ml) with or without L-CD40 L cells. Staining was performed with the indicated antibodies after brief trypsinization of cells. Vertical lines corresponding to the peak channel of fluorescence intensity of a given antigen, on synovial fibroblast control, have been drawn to allow the comparative analysis of antigen expression of fibroblast in co-culture.

haematopoietic system (B cells and dendritic cells), resulting in upregulation of HLA-class II antigens, CD23, CD80, CD86, these antigens are not upregulated on fibroblasts. The lack of induction of major costimulatory molecules on fibroblasts following ligation of CD40 suggests that CD40, on these cells, may not be associated to antigen presentation as it is for B cells and dendritic cells. In keeping with this, the limited cytokine production by CD40-activated fibroblasts also contrasts with that observed with B cells and dendritic cells.

The actual functional role of CD40 on fibroblasts remains to be established. Clearly patients with altered CD40 L or animals with inactivated CD40 L do not display gross abnormalities in the development of their fibroblast compartment (nor of their epithelial and endothelial cell compartments). This indicates either a subtle, thus undetected, alteration of these cells, or a dispensable role of CD40 on these cells or the existence of an alternative ligand. In fact, a second ligand for CD40 has been identified on lymphoma B cells [36], although it has not been molecularly characterized yet. In this context, several members of the TNF receptor superfamily have been demonstrated to bind several ligands including the receptors for TNF which bind TNF-α as well as TNF-β and the 4-1-B-B molecule which binds a TNF-like molecule [37,38] as well as proteins of the extracellular matrix [39]. By analogy, CD40 expressed on these stromal cells may represent a receptor for such extracellular matrix proteins rather than for the TNF-like CD40 L. In fact, the encounter of a cell expressing such a TNF-like CD40 L, e.g. an activated T cell or a mast cell, may result in a pathological situation. In this context, the expanded rheumatoid synovium pannus may be the consequence of an activated T cell/mast cell infiltration. It will thus be of interest to determine eventually whether the blocking of *in vivo* collagen-induced arthritis by anti-CD40 L [40] may partly be explained by an effect on synovial fibroblast proliferation in addition to a blocking of collagen-induced immune responses. The present observation makes it worth analysing whether other inflammatory disorders may result in increased CD40 expression on fibroblasts and whether fibroblast hyperproliferation (as in psoriasis and granulomatous responses) may be the result of CD40 engagement in the presence of the fibrogenic cytokines [41].

In summary, the present study has demonstrated the expres--sion of functional CD40 on fibroblasts, thereby confirming and further extending the results of a comparable study by Yellin *et al*. [42].

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REFERENCES

1 Stamenkovic I, Clark EA, Seed B. A B-lymphocyte activation molecule related to the nerve growth factor receptor and induced by cytokines in carcinomas. EMBO J. 1989; **8**:1403–10.
2 Banchereau J, Bazan F, Blanchard D *et al*. The CD40 antigen and its Ligand. Annu Rev Immunol 1994; **12**:881–922.
3 Callard RE, Armitage RJ, Fanslow WC, Spriggs MK. CD40 ligand and its role in X-linked hyper-IgM syndrome. Immunol Today 1993; **14**:559–64.
4 Durie FH, Foy TM, Masters SR, Laman JD, Noelle RJ. The role of CD40 in the regulation of humoral and cell-mediated immunity. Immunol Today 1994; **15**:406–10.
5 Spriggs MK, Fanslow WC, Armitage RJ, Belmont J. The biology of the human ligand for CD40. J Clin Immunol 1993; **13**:373–80.
6 Armitage RJ, Fanslow WC, Strockbine L *et al*. Molecular and biological characterization of a murine ligand for CD40. Nature 1992; **357**:80–2.
7 Graf D, Korthäuer U, Mages HW, Senger G, Kroczek RA. Cloning of TRAP, a ligand for CD40 on human T cells. Eur J Immunol 1992; **22**:3191–4.
8 Gauchat J-F, Aubry J-P, Mazzei G *et al*. Human CD40-ligand: molecular cloning, cellular distribution and regulation of expression by factors controlling IgE production. FEBS Lett 1993; **3**:259–66.
9 Gauchat JF, Henchoz S, Fattah D *et al*. CD40 ligand is functionally expressed on human eosinophils. Eur J Immunol 1995; **25**:863–5.
10 Grammer AC, Bergman MC, Miura Y, Fujita K, Davis LS, Lipsky PE. The CD40 ligand expressed by human B cells costimulates B cells responses. J Immunol 1995; **154**:4996–5010.
11 Notarangelo LD, Duse M, Ugazaio AG. Immunodeficiency with hyper-IgM (HIM). Immunodef Rev 1992; **3**:101–22.
12 Galy AHM, Spits H. CD40 is functionally expressed on human thymic epithelial cells. J Immunol 1992; **149**:775–82.
13 Saeland S, Duvert V, Moreau I, Banchereau J. Human B cell precursors proliferate and express CD23 after CD40 ligation. J Exp Med 1993; **178**:113–20.
14 Caux C, Massacrier C, Vanbervliet B *et al*. Activation of human dendritic cells through CD40 cross-linking. J Exp Med 1994; **180**:1263–72.
15 Hollenbaugh D, Mischel-Petty N, Edwards C *et al*. Expression of functional CD40 by vascular endothelial cells. J Exp Med 1995; **182**:33–40.
16 Karmann K, Hughes CCW, Schechner J, Fanslow WC, Pober JS. CD40 on human endothelial cells: inducibility by cytokines and functional regulation of adhesion molecule expression. Proc Natl Acad Sci USA 1995; **92**:4342–6.
17 Dechanet J, Briolay J, Rissoan MC *et al*. Interleukin 4 inhibits growth factor-stimulated rheumatoid synoviocyte proliferation by blocking the early phases of the cell cycle. J Immunol 1993; **151**:4908–17.
18 Peltz GA, Trounstine ML, Moore KW. Cloned and expressed human Fc receptor for IgG mediates anti-CD3 dependent lymphoproliferation. J Immunol 1988; **141**:1891–6.
19 Abrams JS, Roncarolo M-G, Yssel H, Andersson U, Gleich GJ, Silver JE. Strategies of anti-cytokine monoclonal antibody development: immunoassay of IL-10 and IL-5 in clinical samples. Immunol Rev 1992; **127**:5–24.
20 Henderson B, Pettipher ER. The synovial lining cell: biology and pathobiology. Semin. Arthritis Rheum 1985; **15**:1–32.
21 Firestein GS, Zvaifler NJ. How important are T cells in chronic rheumatoid synovitis? Arthritis Rheum 1990; **33**:768–72.
22 van Kooten C, Gaillard C, Galizzi JP *et al*. B cells regulate expression of CD40-ligand on activated T cells by lowering the mRNA level and through the release of soluble CD40. Eur J Immunol 1994; **24**:787–92.
23 Vallé A, Zuber CE, Defrance T, Djossou O, de Rie M, Banchereau J. Activation of human B lymphocytes through CD40 and interleukin 4. Eur J Immunol 1989; **19**:1463–7.
24 Burdin N, van Kooten K, Galibert L *et al*. Endogenous IL-6 and IL-10 contribute to the differentiation of CD40-activated human B lymphocytes. J Immunol 1995; **154**:2533–44.
25 Alderson MR, Armitage RJ, Tough TW, Strockbine L, Fanslow WC, Spriggs MK. CD40 expression by human monocytes: regulation by cytokines and activation of monocytes by the ligand for CD40. J Exp Med 1993; **178**:669–74.
26 Firestein GS, Alvaro-Gracia J, Maki R. Quantitative analysis of cytokine gene expression in rheumatoid arthritis. J Immunol 1990; **144**:3347–53.

27 Ranheim EA, Kipps TJ. Activated T cells induce expression of B7/BB1 on normal or leukemic B cells through a CD40-dependent signal. J Exp Med 1993; **177**:925–35.
28 Clark EA, Grabstein KH, Shu GL. Cultured human follicular dendritic cells. Growth characteristics and interactions with B lymphocytes. J Immunol 1992; **148**:3327–35.
29 Kim H-S, Zhang X, Choi YS. Activation and proliferation of follicular dendritic cell-like cells by activated T lymphocytes. J Immunol 1994; **153**:2951–61.
30 Lindhout E, Lakeman A, Mevissen MLCM, de Groot C. Functionally active Epstein–Barr virus-transformed follicular dendritic cell-like cell lines. J Exp Med 1994; **179**:1173–84.
31 Rademakers LHPM. Follicular dendritic cells in germinal centre development. Res Immunol 1991; **142**:257–60.
32 Fries KM, Sempowski GD, Gaspari AA, Blieden T, Looney RJ, Phipps RP. CD40 expression by human fibroblasts. Clin Immunol Immunopathol 1995; **77**:42–51.
33 Piela-Smith TH, Broketa G, Hand A, Korn JH. Regulation of ICAM-1 expression and function in human dermal fibroblasts by IL-4. J Immunol 1992; **148**:1375–81.
34 Buchan G, Barrett K, Fujita T, Taniguchi T, Maini R, Feldmann M. Detection of activated T cell products in the rheumatoid joint using cDNA probes to interleukin-2 (IL-2) IL-2 receptor and IFN-γ. Clin Exp Immunol 1988; **71**:295–301.
35 Simon AK, Seipelt E, Sieper J. Divergent T-cell cytokine patterns in inflammatory arthritis. Proc Natl Acad Sci USA 1994; **91**:8562–6.
36 Howard MC, Heath AW, Ishida H, Moore KW. Biological roles of IL-10 and the CD40 receptor. In: Gergely J, ed. Progress in immunology, Vol VIII. Budapest: Springer-Verlag, 1992:327–37.
37 Alderson, MR, Smith CA, Tough TW *et al*. Molecular and biological characterization of human 4-1BB and its ligand. Eur J Immunol 1994; **24**:2219–27.
38 Goodwin RG, Din WS, Davis-Smith T *et al*. Molecular cloning of a ligand for the inducible T cell gene 4-1BB: a member of an emerging family of cytokines with homology to tumor necrosis factor. Eur J Immunol 1993; **23**:2631–41.
39 Chalupny NJ, Peach R, Hollenbaugh D, Ledbetter JA, Farr AG, Aruffo A. T-cell activation molecule 4-1BB binds to extracellular matrix proteins. Proc Natl Acad Sci USA 1992; **89**:10360–4.
40 Durie FH, Fava RA, Foy TM, Aruffo A, Ledbetter JA, Noelle RJ. Prevention of collagen-induced arthritis with an antibody to gp39, the ligand for CD40. Science 1993; **261**:1328–30.
41 Kovacs EJ, DiPietro LA. Fibrogenic cytokines and connective tissue production. FASEB J 1994; **8**:854–61.
42 Yellin MJ, Winikoff S, Fortune SM *et al*. Ligation of CD40 on fibroblasts induces CD54 (ICAM-1) and CD106 (VCAM-1) up-regulation and IL-6 production and proliferation. J Leukoc Biol 1995; **58**:209–16.