

Genistein induces adipogenesis but inhibits leptin induction in human synovial fibroblasts

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It was shown recently that synovial fibroblast transformation into adipocytes reduced the expression of interleukin-6 (IL-6) and IL-8. However, the synovial fibroblast adipogenesis was inhibited in inflammatory conditions induced by the tumor necrosis factor- α (TNF- α). Furthermore, adipogenesis is often accompanied by leptin production, a proinflammatory adipokine in rheumatic diseases. In this study, we tested the phytohormone genistein for adipogenic and anti-inflammatory properties on human synovial fibroblasts. Results showed that genistein was able to transform synovial fibroblasts into adipocytes that expressed perilipin-A and produced adiponectin, but not leptin. Furthermore, genistein enhanced glucocorticoid-mediated synovial fibroblast adipogenesis and, in parallel, downregulated glucocorticoid-induced leptin and leptin receptor. Endogenous and TNF- α -induced expressions of IL-6, IL-8, p38, p65 and C/EBP- β were also downregulated by genistein, showing its anti-inflammatory properties. Peroxisome proliferator-activated receptor- γ (PPAR- γ) agonist, rosiglitazone, had a synergic effect on genistein-induced adipogenesis, whereas the non-active tyrosine kinase inhibitor, daidzein, had a significantly inferior adipogenic activity than genistein. The Janus kinase-2 tyrosine kinase inhibitor, AG 490, mimicked the anti-leptin effect of genistein. These results showed that genistein-induced adipogenesis involves PPAR- γ induction and tyrosine kinase inhibition. In conclusion, genistein, alone or coupled with glucocorticoids, have both adipogenic and anti-inflammatory effects on synovial fibroblasts.

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KEYWORDS: adipogenesis; genistein; leptin; peroxisome proliferator-activated receptor- γ ; rheumatoid arthritis; synovial fibroblasts

In rheumatic diseases, such as osteoarthritis (OA) and rheumatoid arthritis (RA), synovial tissue proliferates and becomes the site of inflammation. Synovial proliferation and inflammation lead to chondrocyte apoptosis, cartilage damage and joint dysfunction.¹ Indeed, cadherin-11 k/o mice that form defective synovium are more resistant to experimentally induced arthritis.² Recently, synovial fibroblasts were recognized as multipotent cells that can differentiate into osteocytes, chondrocytes and adipocytes.^{3–6} In addition, synovial fibroblasts were shown to express specific markers of bone marrow-derived multipotent stem cells (MSCs), such as CD-105, CD-90 and CD-73.^{3,7} Among the different sources of MSCs, such as bone marrow, skeletal muscle and adipose tissue, synovium was recognized as a potent source.⁸ Furthermore, synovial MSCs were shown capable of rabbit cartilage repair.⁹ These results indicated that synovium re-

presents a precious source of MSCs, and also opened new approaches for combating synovial inflammation, by interfering with signaling pathways involved in MSC differentiation.

Synovial fibroblast transformation into adipocyte-like cells was shown *in vitro* in the presence of the synthetic peroxisome proliferator-activated receptor- γ (PPAR- γ) agonist, troglitazone.⁶ These adipocyte-like cultures expressed less interleukin-6 (IL-6) and IL-8 than control cells, but adipogenesis was inhibited by the tumor necrosis factor- α (TNF- α), suggesting that in inflammatory conditions, cells may continue to produce high levels of IL-6 and IL-8. In addition, adipogenesis is associated with the production of leptin, an adipokine thought to be proinflammatory in both OA and RA.^{10–13} To that purpose, we searched for the PPAR- γ agonist(s) having anti-inflammatory properties in the

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presence of TNF- α . Three PPAR- γ agonists were tested, namely the synthetic rosiglitazone, the precursor of natural PPAR- γ agonist 15d-PGJ2, prostaglandin D2 (PGD2) and the plant hormone, genistein.^{14,15} The results showed that TNF- α -stimulated synovial fibroblast cultures expressed high levels of IL-6 and IL-8, treated with PGD2 or not. However, we showed that genistein downregulated both endogenous and TNF- α -induced IL-6 and IL-8 in synovial fibroblasts. Rosiglitazone was also able to downregulate TNF- α -induced IL-6, but significantly less than genistein.

We also showed that genistein, alone or in concerted action with rosiglitazone or glucocorticoids, induced synovial fibroblast adipogenesis. Furthermore, we observed that despite their ability to downregulate TNF- α -induced IL-6 and IL-8, glucocorticoids induced marked leptin and leptin receptor expression in synovial fibroblasts. Genistein downregulated glucocorticoid-induced leptin and leptin receptor expression. As leptin has been recognized as proinflammatory for rheumatic diseases,^{10,11} these results suggest that concomitant application of genistein and glucocorticoids may promote their anti-inflammatory properties.

MATERIALS AND METHODS

Synovial Fibroblast Isolation, Cell Culturing and Adipogenesis Induction

The synovial tissue was obtained from OA, RA and traumatic patients not having rheumatoid diseases, during joint replacement. Informed consents were obtained, and experiments were approved by the ethics committee of our academic hospital (CHU, Liège, Belgium). Synovial fibroblasts were isolated as explained earlier.¹⁶ Cells were cultured in the DMEM medium (Cambrex Bio Science, Walkersville, MD, USA), with L-glutamine (2 mM), streptomycin (100 mg/ml) and penicillin (100 U/ml) (BioWhittaker, Walkersville, MD, USA), and supplemented with 10% fetal bovine serum (Lonza, Basel, Switzerland) for at least three passages before being used. Experiments were carried out in the DMEM medium lacking phenol red (Cambrex Bio Science), supplemented with 10% charcoal-stripped fetal bovine serum. Cells (5×10^4 /0.5 ml of medium) were seeded in 24-well plates (BD Biosciences, San Jose, CA, USA) in triplicate. On the second day of culture, cells were stimulated with rosiglitazone, genistein, SB 203580, tyrphostin AG 490 (Alexis Corp., San Diego, CA, USA), PGD2 (Cayman Chemical, Ann Arbor, MI, USA) and/or dexamethasone, prednisolone, cortisone, 17- β -estradiol, N-acetyl cysteine (NAC) or daidzein (Sigma-Aldrich, St Louis, MI, USA), in the presence or absence of TNF- α . The medium was changed and cells were re-stimulated every 6 days. Cells were harvested after 1–4 weeks. The synovial fibroblast origins used for experiments were Figure 1a: OA patient, Figure 1b: traumatic patient, Figure 2a–d: RA patient, Figure 2e: traumatic patient, Figure 2f: OA patient, Figure 3a: RA patient, Figure 3b: Patient 1: traumatic, Patient 2: OA, Patient 3: RA, Patient 4: OA. Figures 4 and 5:

OA patients, Figure 6a–d: OA patient, Figure 6e: OA patient, Figure 6f: RA patient, Figures 7–9: OA patients.

Oil Red Staining

Cells were fixed in 4% paraformaldehyde in PBS for 15 min and then washed with PBS. For staining, an oil red stock solution (5 g/l of isopropanol) was diluted with distilled water in a 4:6 ratio. Staining was performed for at least 1 h at room temperature. Before microscopy, cells were washed with distilled water.

Microscopy

Photos were captured using a digital sight camera (2 MV) under the inverted microscope Eclipse TS100 (Nikon Instruments Inc., Melville, NY, USA), using NIS-Elements Basic Research software (Nikon Instruments Inc.) and objectives with phase contrast.

ELISA

Protein concentrations were determined in the cell supernatant using commercial kits IL-6, IL-8 (BioSource, Fleurus, Belgium), leptin and adiponectin (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Western Blot

Cells were collected, lysed and total proteins separated by SDS-PAGE as explained earlier.¹⁶ Membranes were incubated with perilipin-A (Sigma-Aldrich), PPAR- γ (E-8), ERK1 (K-23), p-ERK (E-4), C/EBP- β (D198), Ob-R (B-3) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), p38, p38 Thr80/Tyr82 (Cell Signaling, Beverly, MA, USA) and NF- κ B p65 (Upstate Biology, Lake Placid, NY, USA) antibodies for 1–3 h at room temperature. Western blots were shown with 1:2000 diluted anti-mouse or anti-rabbit (DAKO A/S, Glostrup, Denmark) antibodies and ECL chemiluminescent reagents (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

Flow Cytometry Analyses

Simple staining was performed with fluorescent-conjugated antibodies. The following monoclonal, PE-conjugated antibodies were used: anti-CD-73, anti-CD-90, anti-105, anti-HLA-DR, anti-CD-80 and anti-CD-45 (BD Biosciences). The phenotype was performed on $(1-5) \times 10^5$ synovial fibroblasts, cultured earlier for three passages. The fluorescence intensity was analyzed on FACSvantage with Cell Quest software (BD Biosciences).

Chip (Chromatin Immunoprecipitation) Assay

Chip (chromatin immunoprecipitation) assay was performed on the basis of Q²ChIP protocol and The Low Cell ChIP Kit (Diagenode, Liege, Belgium),¹⁷ with a few modifications. Synovial fibroblasts were seeded 24 h before the experiment in 10-cm dishes (1.5×10^6 /5 ml). The next day, cells were treated with genistein for 3 h, followed by 15 min of TNF- α stimulation. Protein DNA cross-linking was performed

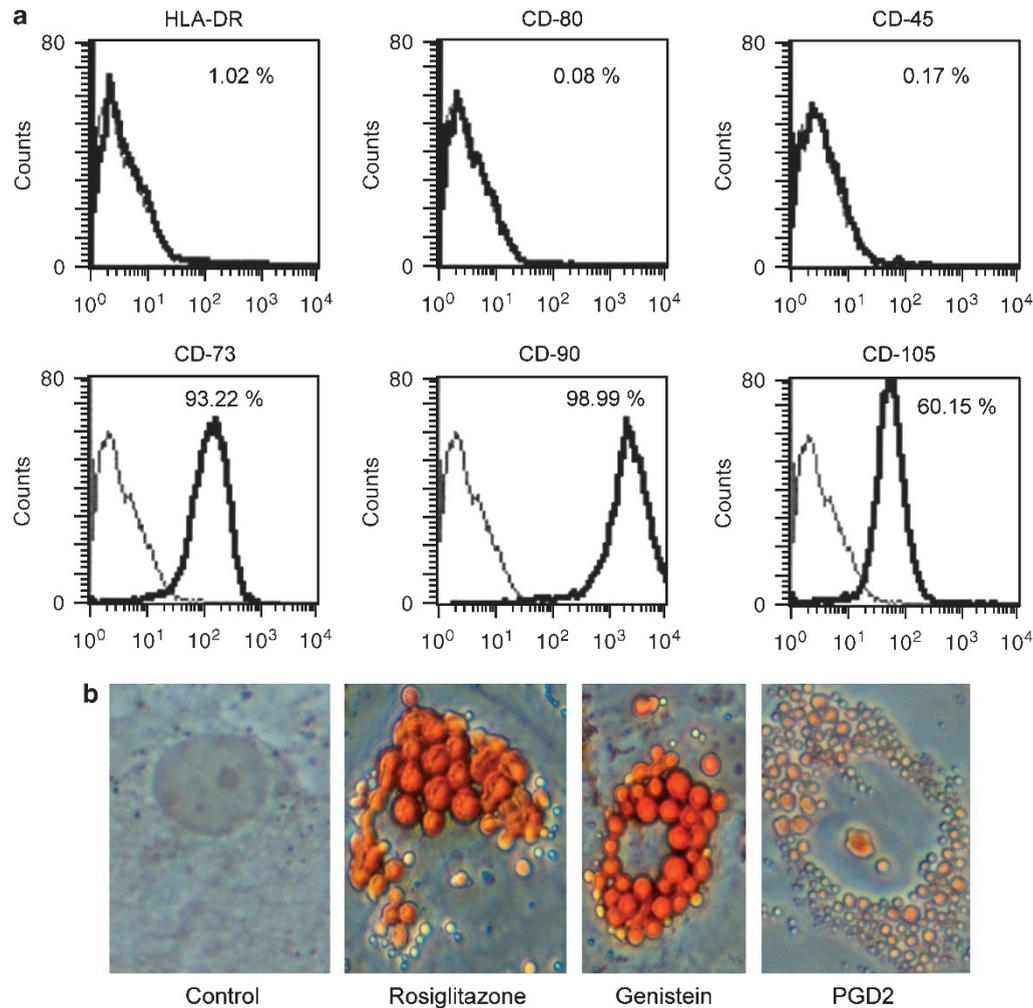


Figure 1 Genistein, rosiglitazone and PGD2 induce synovial fibroblasts adipogenesis. (a) Flow cytometry diagrams showing the expression profile of synovial fibroblasts harvested after three passages and stained for surface molecules as indicated. The percentage of positive cells was calculated and shown in each diagram. (b) Oil-red-stained adipocyte-like single cell in the 3-week-old synovial fibroblast cultures treated with different PPAR- γ agonists only as indicated. Photos were taken under an inverse microscope at magnification $\times 40$.

directly in the culture medium with 1% formaldehyde, at 37°C, for 10 min. After being washed thrice with cold PBS containing complete protease inhibitor cocktail (Roche, Mannheim, Germany), cells were scraped on ice, recuperated by centrifugation (1500 r.p.m., 10 min, 4°C) and dissolved in 130 μ l of lysis buffer (50 mM TRIS-HCl, pH8, 10 mM EDTA, 1% SDS, 1 mM PMSF and protease inhibitor cocktail). Sonication was performed for 3 min, with 30-s pauses, using the maximum power of Bioruptor (Diagenode). Chromatin was diluted with 870 μ l RIPA buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, Na-deoxycholate, 100 mM NaCl), centrifuged for 5 min at 12 000 r.p.m. and supernatant recuperated. For immunoprecipitation, 100 μ l of diluted chromatin was incubated overnight (4°C, at 20 r.p.m.) in 0.2-ml tubes with 10 μ l of magnetic protein A beads (Dynabeads, Invitrogen, Carlsbad, CA, USA) that had

been pre-covered for at least 2 h with NF- κ B p65 (A) X (Santa Cruz) or C/EBP- β (LAP) (Cell Signaling) antibodies. IgG antibodies were used for the negative control. Beads were washed thrice with RIPA buffer, dissolved in TE buffer (10 mM Tris-HCl, pH8, 10 mM EDTA) and transferred into Eppendorf tubes. TE was discarded and DNA was purified by boiling the beads for 10 min with 100 μ l of 10% Chelex-100 slurry (Bio Rad, Hercules, CA, USA), 1 h of proteinase-K treatment (200 μ g/ml) at 55°C, followed by 10 min of sample boiling. DNA containing supernatant was recuperated and used for PCR analyses. Input DNA was purified in the same way from the 100 μ l of starting material used for immunoprecipitation. Standard and real-time PCR were performed using the following IL-8 promoter primer pairs: 5'-GGCCATCAGTTGCAAATCG-3' (forward) and anti-sense 5'-AGTGCTCCGGTGGCTTTTT-3' (reverse). Real-time PCR

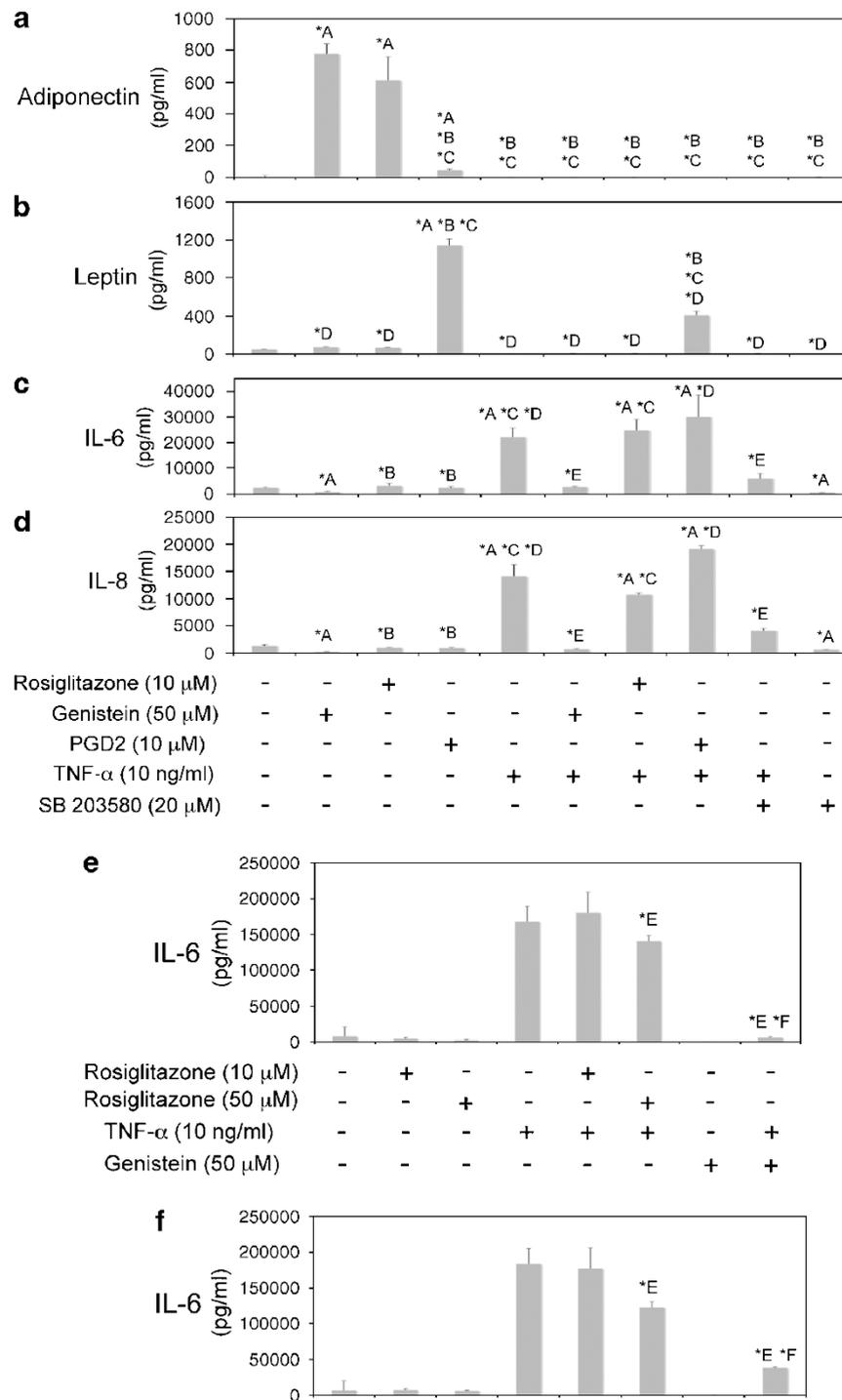


Figure 2 Genistein inhibits TNF- α -induced IL-6 and IL-8. (a–f) ELISA measured adiponectin (panel a), leptin (panel b), IL-6 (panels c, e and f) and IL-8 (panel d) production in cell supernatant. Synovial fibroblasts, obtained from RA (panels a–d), traumatic (panel e) or OA (panel f) patient, were treated for 3 weeks with different PPAR- γ agonists or p38 inhibitor, SB 203580, in the presence or absence of TNF- α , as indicated. *A, statistically different from control cells; *B, statistically different from genistein-treated cells; *C, statistically different from rosiglitazone-treated cells; *D, statistically different from PGD2-treated cells; *E, statistically different from TNF- α -treated cells; *F, statistically different from TNF- α - and rosiglitazone-treated cells.

was performed in triplicate using the SYBR green PCR mix (Applied Biosystems) and the ABI Prism 7000 Sequence Detection System (ABI Prism, Foster City, CA, USA).

Statistics

P-values were obtained using the Mann–Whitney U-test and were considered as significant when <0.05.

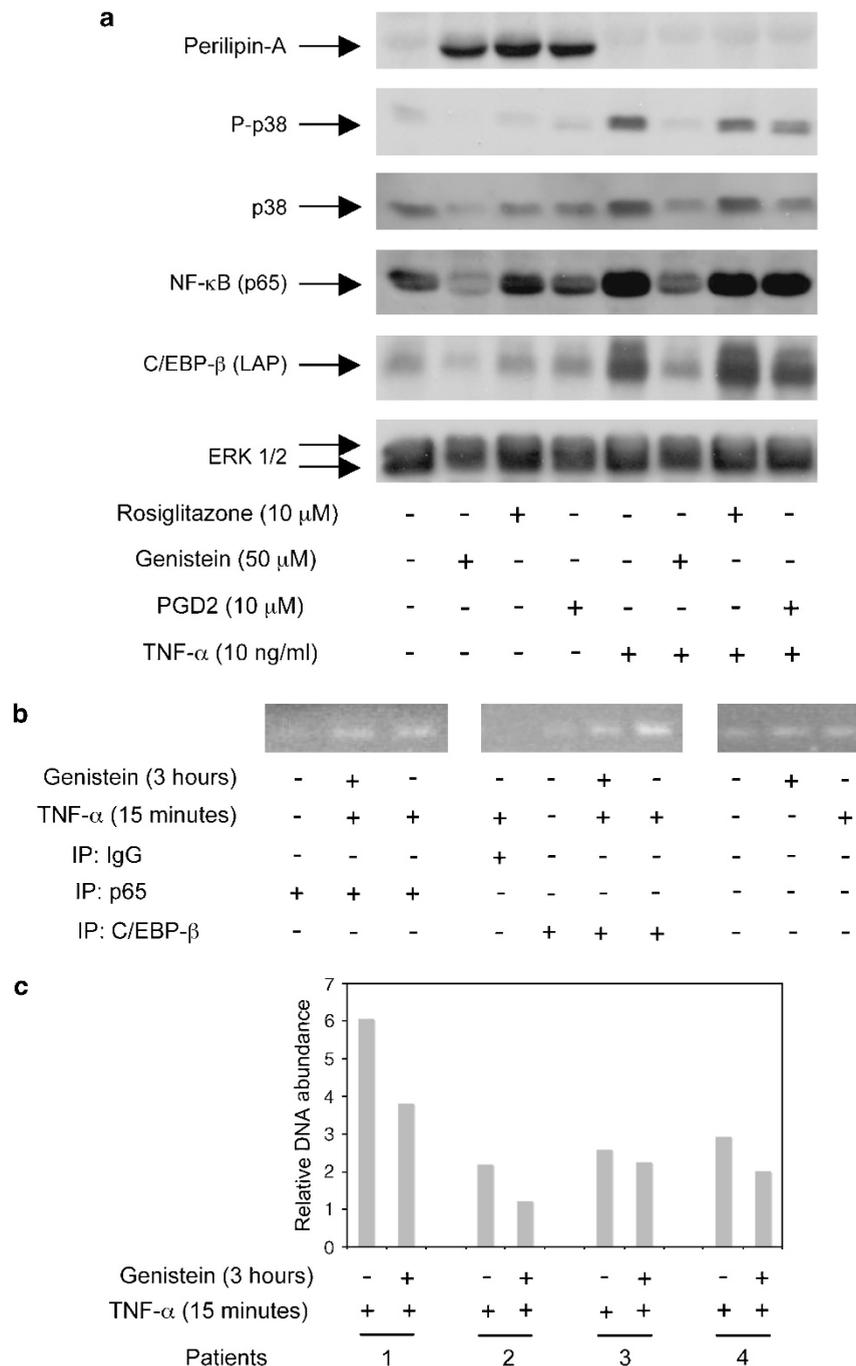


Figure 3 Genistein inhibits p38, C/EBP and NF-κB pathways. (a) Western blots showing expression of adipocyte marker, perilipin-A, phosphorylated p38 (P-p38), p38, p65 units of NF-κB and LAP form of C/EBP-β in synovial fibroblasts extracts. Cells were treated for 4 weeks with different PPAR-γ agonists, in the presence or absence of TNF-α, as indicated. ERK 1/2 expression was used as control. (b and c) ChIP analyses performed with p65 or C/EBP-β antibodies on the synovial fibroblast pre-treated with genistein or not (50 μM), then 15 min of TNF-α (10 ng/ml) treatment. DNA was amplified by standard (panel b) or real-time PCR (panel c) with IL-8 promoter primers. Input DNA was diluted in a ratio of 1:10. IgG antibodies were used as negative control.

RESULTS
Genistein Induces Synovial Fibroblast Adipogenesis and Downregulates both Endogenous and TNF-α-Induced IL-6 and IL-8

Isolated human synovial fibroblasts that were cultured earlier for at least three passages were used. FACS analyses con-

firmed that synovial fibroblasts expressed mesenchymal stem cell markers, CD-73, CD-90 and CD-105 (Figure 1a). Cells were negative for HLA-DR, CD-80, CD-45 (Figure 1a), as well as for CD-3 and CD-31 (data not shown). Synovial fibroblasts were stimulated with genistein, rosiglitazone or PGD2 in the presence or absence of TNF-α for 3–4 weeks.

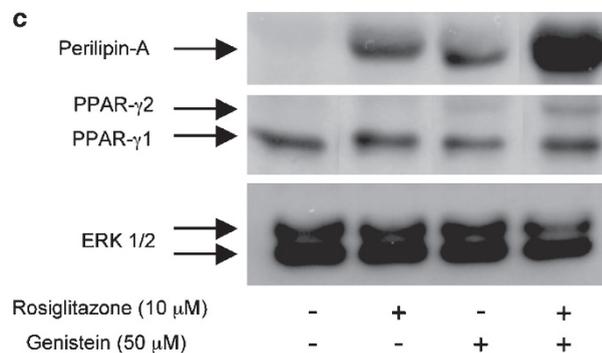
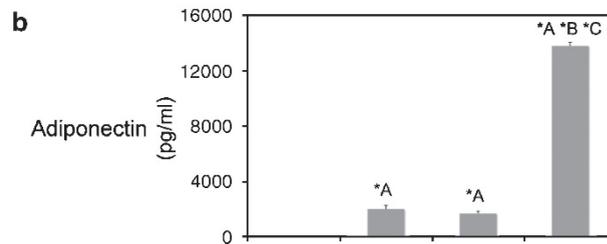
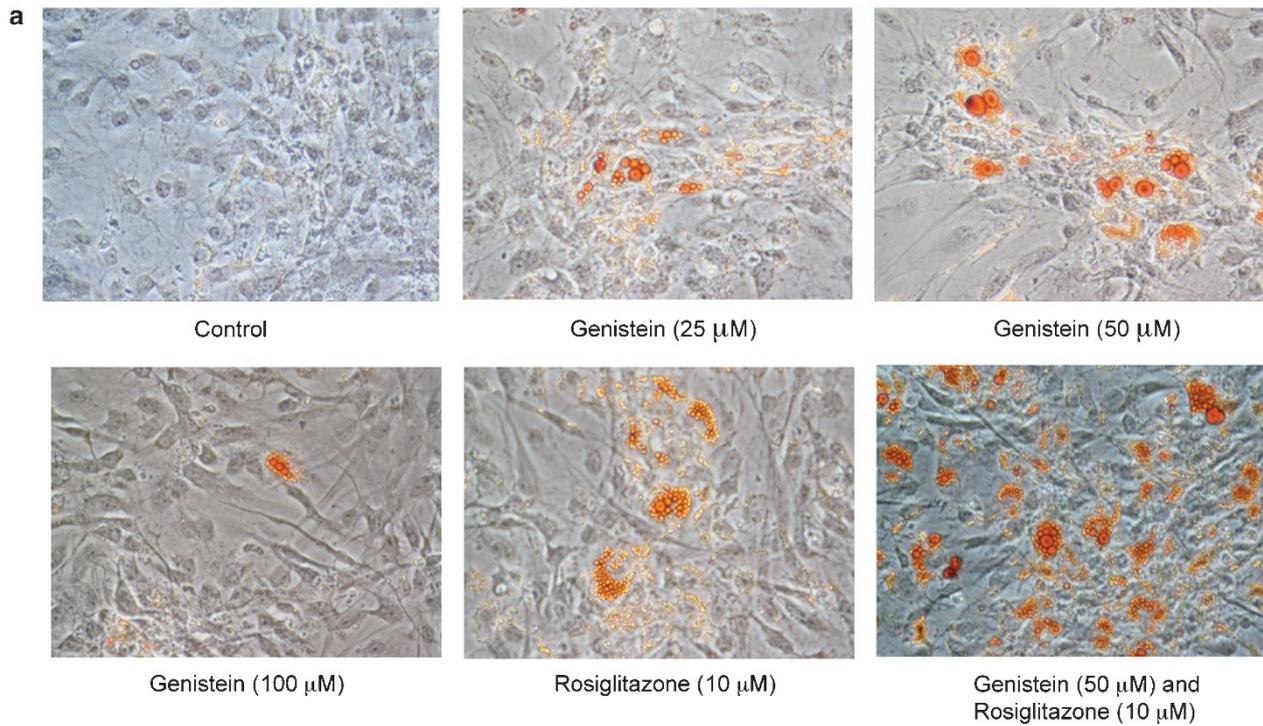


Figure 4 Genistein and rosiglitazone induce adipogenesis in synergism. (a–c) Synovial fibroblasts were cultured with genistein or rosiglitazone only, or both substances simultaneously, at indicated concentrations, for 4 weeks. (Panel a) Oil-red-stained cells. Photos were taken under an inverse microscope at magnification $\times 20$. (Panel b) ELISA measured adiponectin production in cell supernatant. *A, statistically different from control cells; *B, statistically different from genistein-treated cells; *C, statistically different from rosiglitazone-treated cells. (Panel c) Western blots showing perilipin-A and PPAR- γ expressions. ERK 1/2 expression was used as control.

Adipocyte-like cells (Figures 1b and 4a) that produced adiponectin (Figure 2a) emerged in cultures treated with genistein or rosiglitazone. These fat droplets stained positively with oil red (Figures 1b and 4a) and did not produce leptin (Figure 2b). PGD2 induced mainly sebum-like droplets

(Figure 1b) and small amounts of adiponectin (Figure 2a) and leptin (Figure 2b). Both kinds of droplets expressed perilipin-A, inhibited by TNF- α (Figure 3). TNF- α also inhibited genistein- and rosiglitazone-induced adiponectin production, as well as PGD2-induced leptin production

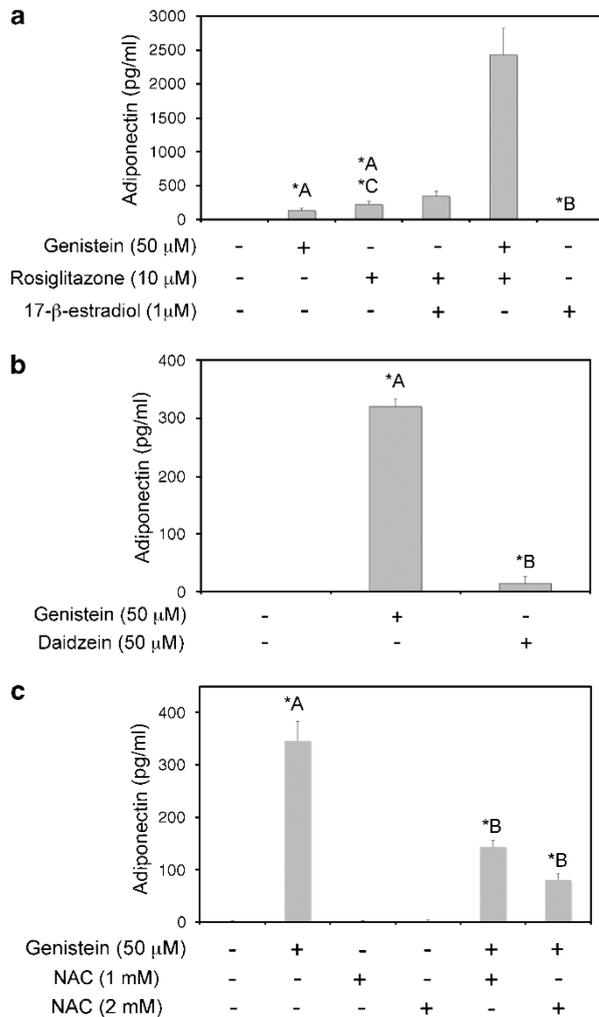


Figure 5 Effects of 17- β -estrogen, daidzein and N-acetyl cysteine (NAC) on synovial fibroblast adipogenesis. (a–c) ELISA measured adiponectin production in cell supernatant. (Panel a) Synovial fibroblasts were treated for 4 weeks with genistein, rosiglitazone or simultaneously with rosiglitazone and 17- β -estrogen, or rosiglitazone and genistein, at indicated concentrations. *A, statistically different from control cells; *B, statistically different from genistein-treated cells; *C, statistically different from rosiglitazone- and genistein-treated cells. (Panel b) Synovial fibroblasts were treated for 4 weeks with genistein or daidzein. *A, statistically different from control cells; *B, statistically different from genistein-treated cells. (Panel c) Synovial fibroblasts were treated for 3 weeks with genistein, NAC or simultaneously with genistein and NAC. *A, Statistically different from control cells; *B, statistically different from genistein-treated cells.

(Figure 2a and b). Genistein, but not rosiglitazone or PGD2, at commonly used concentrations (10 μ M), downregulated both endogenous and TNF- α -induced production of IL-6 and IL-8 (Figure 2c and d). However, at high concentrations, rosiglitazone (50 μ M) also downregulated TNF- α -induced IL-6 production, but its effect was statistically inferior compared with that of genistein (Figure 2e and f). High concentrations of PGD2 could not be tested because they induced synovial fibroblast death (results not shown). The genistein effect was mimicked partially by p38 inhibitor, SB

203580, suggesting that genistein effect may involve p38. Similar results were obtained with synovial fibroblasts originating from the synovium of RA (Figure 2a–d), OA (Figure 2f) or traumatic patients (Figure 2e). These results showed that genistein has both adipogenic and anti-inflammatory effects on synovial fibroblasts.

Genistein Inhibits Endogenous and TNF- α -Induced p38, C/EBP and NF- κ B Pathways

As both genistein and p38 inhibitor, SB203580, inhibited endogenous and TNF- α -induced synovial fibroblasts IL-6 and IL-8 production (Figure 2c and d), and because promoters of IL-6 and IL-8 have binding sites for both C/EBP- β and NF- κ B,^{18,19} we have tested the effect of TNF- α and PPAR- γ agonists on the expression of these proteins in synovial fibroblasts. Western blots showed that TNF- α induced phosphorylation of p38, and expression of p38, C/EBP- β and p65 unit of NF- κ B (Figure 3). Genistein, but not the commonly used concentrations (10 μ M) of rosiglitazone or PGD2, downregulated TNF- α -induced p38 phosphorylation and both endogenous and TNF- α -induced p38, C/EBP- β and p65 (Figure 3).

Genistein has been shown earlier not to change TNF- α -induced p65/DNA binding but to inhibit NF- κ B transactivation.²⁰ Our results, obtained by the ChIP assay, also showed that genistein did not prevent TNF- α -induced binding of p65 to IL-8 (Figure 3b). However, genistein prevented C/EBP- β binding to IL-8 (Figure 3b) and IL-6 promoters (results not shown). These results suggested that genistein inhibition of TNF- α -induced IL-6 and IL-8 production involves C/EBP- β transcription factor.

Genistein and Rosiglitazone Induce Synovial Fibroblast Adipogenesis in Synergism

Genistein was shown to be a PPAR- γ ¹⁴ and estrogen receptor (ER)¹⁵ inducer, as well as an inhibitor of protein tyrosine kinases.²¹ To test the involvement of PPAR- γ in genistein-induced adipogenesis, cells were treated simultaneously with genistein and synthetic PPAR- γ agonist, rosiglitazone. Compared with cultures treated with genistein and rosiglitazone alone, the numbers of oil-red-stained droplets in combined cultures were increased markedly (Figure 4a). Moreover, ELISA and western blots showed that the expression of adipocyte-specific markers, adiponectin and perilipin-A, were both markedly increased when genistein and rosiglitazone were applied simultaneously (Figure 4b and c). The expression of PPAR- γ 2 also increased when both agonists were applied simultaneously (Figure 4c). These results suggested that genistein and rosiglitazone act in synergism on synovial adipogenesis through the PPAR- γ pathway.

To test the involvement of ERs on synovial fibroblast adipogenesis, cells were treated with 17- β -estradiol, which did not induce synovial fibroblast adipogenesis and did not significantly affect rosiglitazone-induced adipogenesis (Figure 5a).

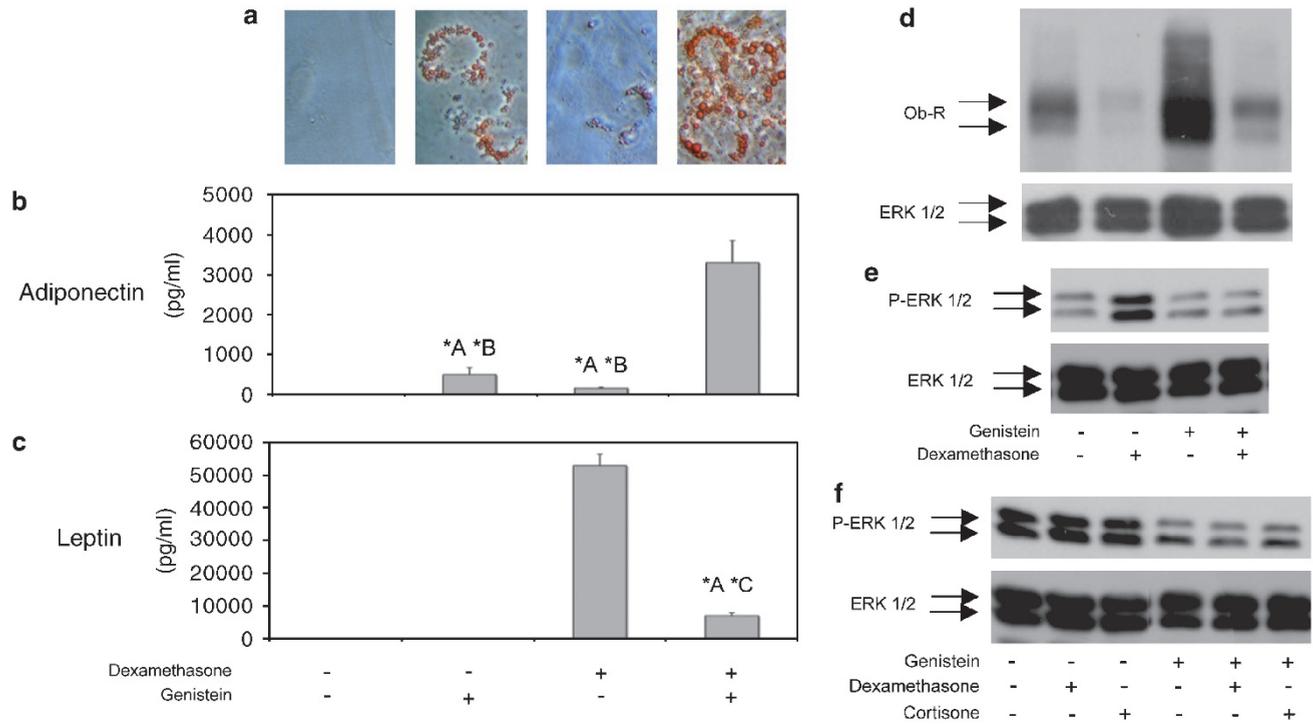


Figure 6 Genistein downregulates dexamethasone-induced leptin and its receptor and ERK1/2 phosphorylation. (a–d) Synovial fibroblasts were cultured in the presence of genistein (50 μ M) or dexamethasone (1 μ M) or both substances simultaneously, for 4 weeks. (Panel a) Oil-red-stained cells. Photos were taken under an inverse microscope at magnification \times 40. (Panel b) ELISA measured adiponectin production in cell supernatant. (Panel c) ELISA measured leptin production in cell supernatant. *A, statistically different from control cells; *B, statistically different from genistein- and dexamethasone-treated cells; *C, statistically different from dexamethasone-treated cells. (Panel d) Western blot showing leptin receptor (Ob-R) expression. ERK 1/2 expression was used as control. (e and f) Synovial fibroblasts were cultured in the presence of genistein (50 μ M), or glucocorticoid (1 μ M) or both substances simultaneously, for 4 weeks. Synovial fibroblast were provided by OA (panel e) and RA (panel f) patients. Western blots are showing ERK1/2 phosphorylation. ERK 1/2 expression was used as control.

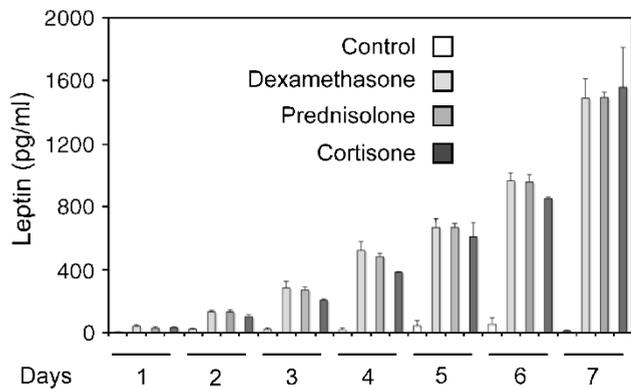


Figure 7 Glucocorticoids induce rapid leptin production in synovial fibroblasts. ELISA measured leptin production in cell supernatant. Synovial fibroblasts were cultured in the presence of dexamethasone (1 μ M), prednisolone (1 μ M) or cortisone (1 μ M) for the time indicated.

The involvement of protein tyrosine kinase inhibition on genistein-induced adipogenesis was tested using the genistein non-active analog, daidzein. Although daidzein was able to induce some fat droplets in synovial fibroblasts (results not

shown), its effect on adiponectin production was negligible compared with genistein (Figure 5b). These results suggested that, in addition to PPAR- γ , genistein-induced adipogenesis involves tyrosine kinase inhibition.

The involvement of anti-oxidant effects on genistein-induced adipogenesis was tested using NAC, which when applied alone, did not have an adipogenic effect on synovial fibroblasts (Figure 5c). Furthermore, it significantly inhibited genistein-induced adipogenesis (Figure 5c), suggesting that genistein-induced adipogenesis does not involve its anti-oxidant properties.

Genistein Downregulates Glucocorticoid-Induced Leptin Production

Glucocorticoids are widely used for the treatment of rheumatic diseases and are also known to be adipogenic *in vivo* and *in vitro*. Surprisingly, dexamethasone alone induced adipogenic transformation of only a few synovial fibroblasts and small amount of adiponectin (Figure 6a and b, respectively) but markedly induced leptin production (Figure 6c) and leptin receptor (Ob-R) expression (Figure 6d). Leptin production was already detected after 48 h of dexamethasone

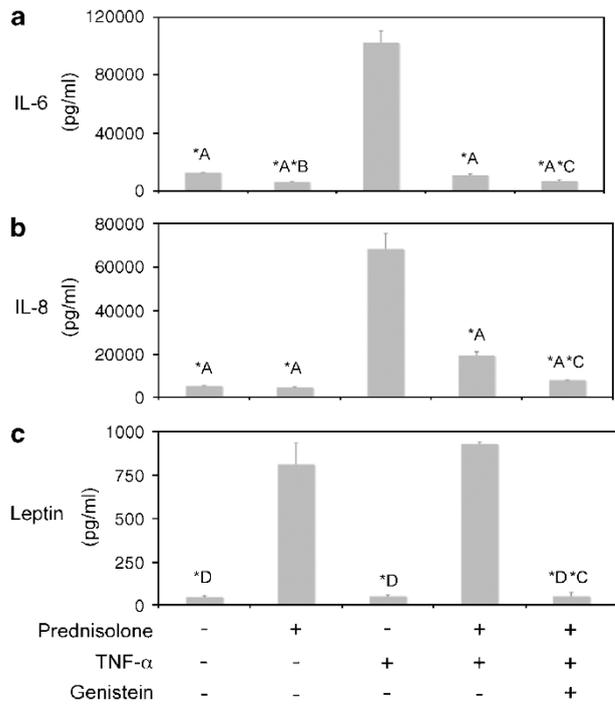


Figure 8 Genistein downregulates glucocorticoid-induced leptin in the presence of TNF- α . (a–c) Synovial fibroblasts were stimulated with prednisolone (1 μ M) in the presence or absence of TNF- α (10 ng/ml) or TNF- α and genistein (50 μ M), for 1 week. ELISA measured (panel a) IL-6, (panel b) IL-8 and (panel c) leptin production in cell supernatant. *A, statistically different from TNF- α -treated cells; *B, statistically different from control cells; *C, statistically different from TNF- α - and prednisolone-treated cells; *D, statistically different from prednisolone-treated cells.

treatment and increased daily (Figure 7). Similar results were obtained with prednisolone and cortisone (Figure 7). Genistein markedly enhanced dexamethasone-induced adipogenesis (Figure 6a and b). Of interest, in parallel, genistein downregulated dexamethasone-induced leptin production (Figure 6c) and leptin receptor expression (Figure 6d). Furthermore, genistein was able to downregulate glucocorticoid-induced leptin in the presence of TNF- α (Figure 8c). In addition, genistein combined with prednisolone further inhibited TNF- α -induced IL-6 and IL-8 compared with prednisolone alone (Figure 8a and b, respectively). Genistein also downregulated both endogenous and glucocorticoid-induced ERK1/2 phosphorylation (Figure 6e and f). Inhibitor of the Janus kinase-2 (JAK-2) tyrosine kinase, tyrphostin AG 490, but not the commonly used concentration of PPAR- γ agonist, rosiglitazone (10 μ M), (results not shown), inhibited prednisolone-induced leptin (Figure 9a). Moreover, western blot showed an increased tyrosine phosphorylation in the extract of dexamethasone- and prednisolone-treated synovial fibroblasts (Figure 9b). Genistein inhibited dexamethasone- and prednisolone-induced tyrosine phosphorylation (Figure 9b). These results suggested that genistein anti-leptin effect mainly involves tyrosine kinase inhibition.

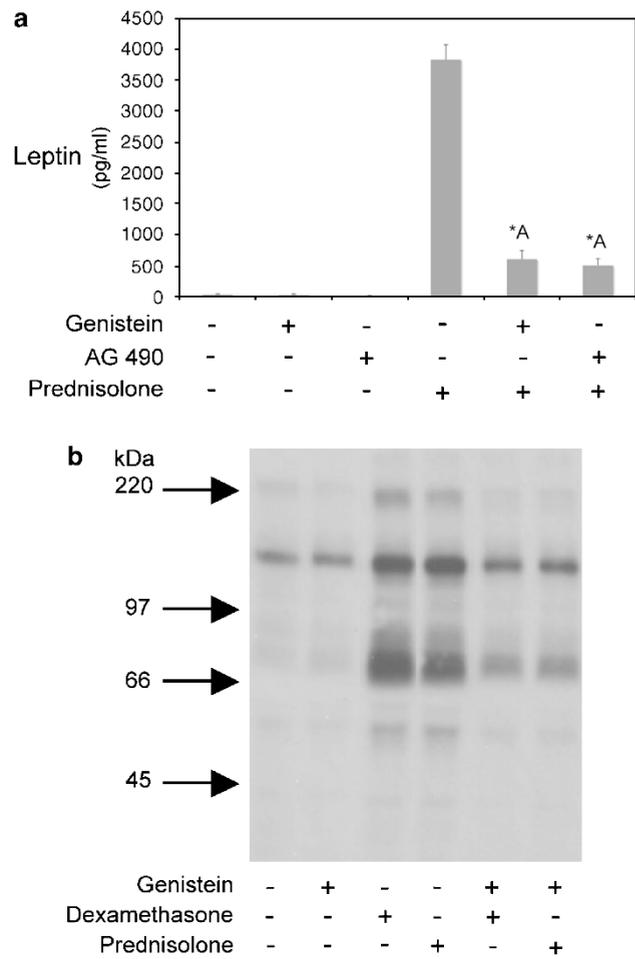


Figure 9 JAK-2 tyrosine kinase inhibitor mimics the anti-leptin effect of genistein and genistein inhibits prednisolone-induced tyrosine phosphorylation. (a) Synovial fibroblasts were stimulated with genistein (50 μ M) or JAK-2 inhibitor tyrphostin AG 490 (50 μ M) in the presence or absence of prednisolone (1 μ M) for 1 week. ELISA measured leptin production in cell supernatant. *A, statistically different from prednisolone-treated cells. (b) Synovial fibroblasts were stimulated with genistein (50 μ M), dexamethasone (1 μ M), prednisolone (1 μ M) or with genistein and each glucocorticoid simultaneously for 3 weeks. Western blot shows tyrosine phosphorylation in total synovial fibroblast extracts. ERK 1/2 expression was used as control.

DISCUSSION

Very recently, synovial fibroblasts were recognized as multipotent mesenchymal stem cells that express stem cell markers^{3,7} and can differentiate into chondrocytes, osteoblasts and adipocytes.^{3–6} Usually, synovial fibroblast adipogenesis was induced using a mixture of substances, including methyl-isobutylxanthine and indomethacin⁵ or insulin, dexamethasone and troglitazone.^{4,6} These adipocyte-like cultures^{4,6} expressed to a lesser degree the proinflammatory cytokines, IL-6 and IL-8, and the adipogenesis of synovial fibroblasts has been proposed as an anti-inflammatory tool for combating rheumatoid diseases.⁶ However, the same work showed that TNF- α inhibited synovial fibroblast adipogenesis,

suggesting limiting effects of adipogenic substances in inflammatory conditions. Therefore, we tested the effects of PPAR- γ agonists on both synovial fibroblast adipogenesis and TNF- α -induced IL-6 and IL-8 production.

Our results showed that in the limited conditions (DMEM medium supplemented with charcoal-stripped FBS and not by any mixture of adipocyte inducing substances), genistein and rosiglitazone were able to transform synovial fibroblasts into adipocytes characterized by oil-red-stained droplets and adiponectin production. In contrast, the endogenous PPAR- γ agonist precursor, PGD₂, induced the transformation of synovial fibroblasts into adipocytes characterized by sebum-like droplets and leptin production. These results suggested that different PPAR- γ agonists may differently induce PPAR- γ .²² Furthermore, we showed that genistein, but not PGD₂, downregulated endogenous and TNF- α -induced IL-6 and IL-8. Rosiglitazone, when applied at high concentrations (50 μ M), had a significant but an inferior inhibitory effect on TNF- α -induced IL-6 production compared with genistein, which also inhibited both endogenous and TNF- α -induced C/EBP- β , a transcription factor involved in inflammation²³ and adipocyte differentiation,²⁴ as well as p65 unit of NF- κ B. Promoters of IL-6 and IL-8 have binding sites for both C/EBP- β and NF- κ B,^{18,19} and functional and physical associations between NF- κ B and C/EBP have been shown.²⁵ The inhibitory effect of genistein on NF- κ B-mediated transcription of IL-6 has been shown²⁰ and we have presented, in this study, that genistein inhibits the binding of C/EBP- β on the IL-8 promoter. These results suggest that genistein inhibits TNF- α -induced expression of IL-6 and IL-8 through the inhibition of NF- κ B and C/EBP- β transcription factors. The inhibitory effect of genistein on IL-17-induced IL-8 had already been observed in synovial fibroblasts.²⁶ Consequently, genistein has been suggested as a substance that has anti-inflammatory potential in the treatment of rheumatic diseases.^{26,27} However, although genistein downregulates TNF- α inflammatory effects, it still cannot induce adipogenesis in the presence of TNF- α . These results suggest that TNF- α -induced inflammatory and anti-adipogenic pathways are at least partially distinct.

Although genistein enhanced the adipogenesis of osteoprogenitor KS483 cells and mouse bone marrow cells,^{14,28} it is also described as an inhibitor of adipocyte differentiation of mouse 3T3-L1^{29–31} cells. Furthermore, genistein decreased the adipogenic differentiation and maturation of bone marrow stromal cells, and stimulated their differentiation into osteoblasts.³² These differences have been explained by the genistein differential effects on ER and PPAR- γ pathways.^{14,28} Indeed, the genistein-enhanced osteogenesis of osteoprogenitor KS483 cells is ER-mediated, whereas the genistein-enhanced adipogenesis of osteoprogenitor KS483 cells and mouse-bone marrow cells is PPAR- γ -mediated.^{14,28} Our results showed that genistein induced adipogenesis in synergy with synthetic PPAR- γ agonist, rosiglitazone. This synergy was observed in the number of adipocytes generated, peri-

pin-A production and PPAR- γ 2 expression. Furthermore, genistein inhibited ERK1/2 phosphorylation, which may consequently inhibit PPAR- γ 2 phosphorylation, and enhanced adipogenesis, as shown earlier.³³ In contrast to genistein, 17- β -estradiol had no significant effect on synovial fibroblast adipogenesis, whereas genistein analog and non-active inhibitor of protein tyrosine kinase, daidzein, had negligible adipogenic activities. These results suggest that genistein-induced adipogenesis in synovial fibroblasts involves PPAR- γ induction and protein tyrosine kinase inhibition.

Our results have shown that glucocorticoids stimulated leptin production in synovial fibroblasts. It was shown recently *in vivo*, in animals and in humans, that glucocorticoid-induced osteoporosis has been linked to leptin production.^{34,35} In view of the known properties of leptin on bone metabolism, it is tempting to speculate that corticosteroid exposure, leptin production and demineralization are linked events. Accordingly, the blockade of the leptin production would be favorable. We have observed that genistein adipogenesis was enhanced markedly by dexamethasone, but genistein downregulated glucocorticoid-induced leptin production in synovial fibroblasts. Furthermore, our results suggest that glucocorticoid-induced leptin production involves the induction of tyrosine phosphorylation, and that genistein downregulated leptin production by its ability to inhibit protein tyrosine kinases. In addition, JAK-2 protein tyrosine kinase inhibitor mimicked the anti-leptin effect of genistein, suggesting that leptin production in synovial fibroblasts involves JAK-2. In rheumatic diseases, leptin is usually considered as a proinflammatory adipokine. In fact, leptin-deficient mice are protected from experimentally induced arthritis,¹⁰ and protection may include leptin effects on T cells.³⁶ Leptin was shown to be an inducer of nitric oxide in chondrocytes¹² and of IL-8 in synovial fibroblasts.¹³ In addition, leptin is produced by chondrocytes in OA.¹¹ Finally, an anti-leptin-based approach was proposed for the treatment of inflammatory and autoimmune diseases.³⁷ These results suggest that genistein might be beneficial to rheumatic diseases by downregulating leptin.

Although the protective effect of adiponectin on chondrocytes was shown recently,³⁸ it was also described that adiponectin enhanced IL-6 production in synovial fibroblasts, involving p38 and NF- κ B pathways.³⁹ Our results showed that genistein induced adiponectin production but downregulated p38, p65 and IL-6. These results suggest that genistein may downregulate the negative effects adiponectin by inhibiting p38 and NF- κ B pathways.

In vivo, PPAR- γ ligands have been shown to be effective in several mouse models of inflammation, including arthritis.⁴⁰ Rosiglitazone improved both collagen-induced arthritis⁴¹ and adjuvant-induced arthritis in mice.⁴² Genistein-treated and collagen II immunized mice showed milder inflammation and joint destruction.⁴³ *In vitro*, we have shown that, unlike rosiglitazone and PGD₂, genistein had both adipogenic and

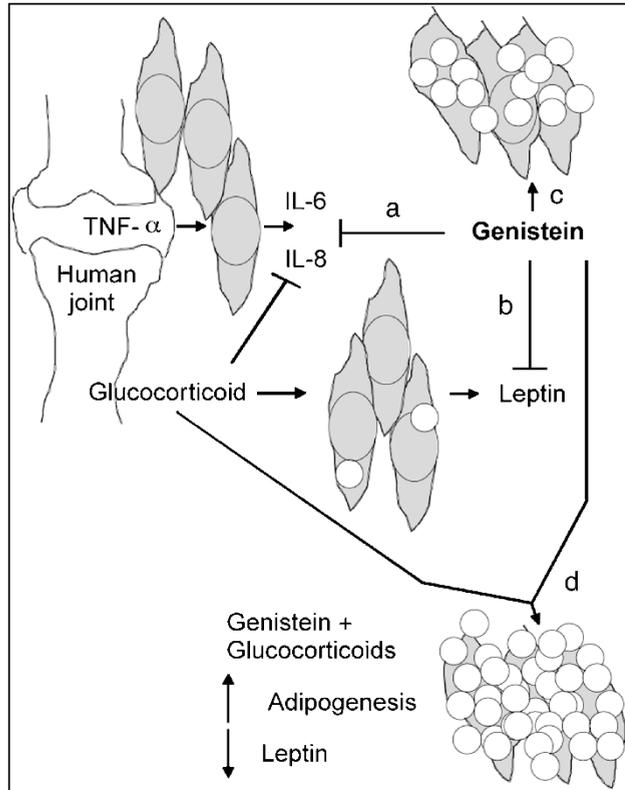


Figure 10 Summary scheme of genistein effects on synovial fibroblasts. In summary, genistein may have several anti-inflammatory effects on synovial fibroblasts: (a) through inhibition of TNF- α -induced IL-6 and IL-8 (Figure 2c and d); (b) through leptin inhibition (Figure 8c); and (c) through the induction of adipogenesis that may *per se* speed down synovial fibroblast proliferation (Figures 1b and 4a). (d) In addition, in concomitant treatment of synovial fibroblasts with glucocorticoid, genistein enhanced adipogenesis (Figure 4a), and downregulated leptin (Figure 6c), whereas TNF- α -induced IL-6 and IL-8 are further downregulated compared with glucocorticoid effect without genistein (Figure 6a and b).

anti-inflammatory properties on synovial fibroblasts. Furthermore, we have shown that genistein downregulated glucocorticoid-induced leptin production and leptin receptor expression in synovial fibroblasts while having additive anti-inflammatory properties. For these reasons, genistein may have a potential for arthritis treatment in association with glucocorticoids or not. A schematic illustration of multiple anti-inflammatory effects of genistein on synovial fibroblasts is shown in Figure 10.

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