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Interleukin-18 induces apoptosis in human articular chondrocytes

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Summary. Elevated levels of the pro-inflammatory cytokine, interleukin-18 (IL-18) have recently been demonstrated in osteoarthritic cartilage. However, the effects of IL-18 on chondrocyte signalling and matrix biosynthesis are poorly understood. Therefore, the present study was undertaken to further characterize the impact of IL-18 on human articular chondrocyte *in vitro*.

Human articular chondrocytes were stimulated with various concentrations of recombinant human IL-18 (1, 10, 100 ng/ml) for 0, 4, 8, 12, 24, 48, 72 h *in vitro*. The effects of IL-18 on the cartilage-specific matrix protein collagen type II, the cytoskeletal protein vinculin, the cell matrix signal transduction receptor ß-integrin, key signalling proteins of the MAPKinase pathway (such as SHC (Sarc Homology Collagen) and activated MAPKinase [ERK-1/-2]), the pro-inflammatory enzyme cyclo-oxygenase-2 (COX-2) and the apoptosis marker activated caspase-3 were evaluated by Western blot analysis and immunofluorescence labelling. Morphological features of IL-18 stimulated chondrocytes were estimated by transmission electron microscopy.

IL-18 lead to inhibition of collagen type II-deposition, decreased ß-integrin receptor and vinculin synthesis, SHC and MAPKinase activation, increased COX-2 synthesis and activation of caspase-3 in chondrocytes in a time- and dose-dependent manner. Furthermore, chondrocytes treated with IL-18 exhibited typical morphological features of apoptosis as revealed by transmission electron microscopy.

Taken together, the results of the present study underline key catabolic events mediated by IL-18 signalling in chondrocytes such as loss of cartilage-specific matrix and apoptosis. Inhibition of MAPKinase signalling is hypothesized to contribute to these features. Future therapeutics targeting IL-18 signalling pathways may be beneficial in rheumatoid arthritis and osteoarthritis therapy.

Key words: IL-18, Apoptosis, Chondrocyte, COX-2, MAPKinase, Matrix proteins, Osteoarthritis

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Introduction

A wide variety of predisposing factors have been implicated in the pathogenesis of osteoarthritis (OA). A non exclusive list of risks factors includes 1) systemic, metabolic and endocrinological factors such as obesity and nutritional imbalance; 2) genetic factors; 3) mechanical factors including joint injury (acute or chronic mechanical trauma), joint overload, muscle weakness and tendon injuries leading to joint instability; 4) increasing age and normal wear and tear (Jackson et al., 2001; Dieppe and Lohmander, 2005). Many of these conditions and traumatic cartilage injuries lead to cell stress and activation of inflammatory pathways which provoke excessive production and secretion of pro-inflammatory cytokines such as TNF-α and IL-1ß (Van de Loo et al., 1995; Van der Kraan and van den Berg, 2003; Goldring and Goldring, 2004). The disturbance in cartilage homeostasis induced by these cytokines results in the release and activation of degradative proteases such as matrix metalloproteinases which mediate loss of matrix macromolecules, secretion of pro-inflammatory mediators such as COX-2 and nitric oxide (NO) leading to further joint inflammation and pain (Lotz, 2001; Sandell and Aigner, 2001). Furthermore, chondrocyte stress and excessive pro-inflammatory cytokine release initiate enhanced chondrocyte apoptosis which has been suggested to contribute to the pathogenesis of OA

(Aizawa et al., 2001; Kühn et al., 2004). In contrast Aigner et al. could show that apoptotic cell death is not a widespread phenomenon in aging or OA knee cartilage (Aigner et al., 2001).

Recent studies have implicated the pro-inflammatory cytokine interleukin-18 (IL-18) in OA and other inflammatory joint diseases (Bessis and Boissier, 2001; Kasiwamura et al., 2002; Vervoordeldonk and Tak, 2002; Matsui et al., 2003). IL-18 appears to be involved in various other inflammatory conditions such as Crohn's disease, Sjögren's syndrome, graft-rejection and abruptio placentae (Kasiwamura et al., 2002). IL-18 is a member of the IL-1 family proteins that exert pro-inflammatory and catabolic effects (Akira, 2000). It was originally identified as IFN-α inducing factor acting synergistically with IL-12 to increase the production of IFN-γ (Joosten et al., 2000, 2004; Kasiwamura et al., 2002). Many cell types such as endothelial cells, epidermal cells, osteoblasts, chondrocytes and synoviocytes have been shown to release IL-18 (Dinarello et al., 1998; Olee et al., 1999; Matsui et al., 2003). Although IL-18 secretion has been demonstrated in normal cartilage, it is significantly enhanced in osteoarthritic cartilage (Boileau et al., 2002). IL-18 is produced as a biologically inactive precursor of 24 kDa that requires enzymatic processing by Interleukin Converting Enzyme (ICE or caspase-1, the same enzyme that activates IL-1ß) to the mature 18 kDa form (Akita et al., 1997; Boileau et al., 2002; Kasiwamura et al., 2002). Cytokine activity is also regulated by the endogenous inhibitor of IL-18, the IL-18 binding protein (Banda et al., 2003; Smeets et al., 2003). Both, IL-18 and IL-1ß consist of similar ß-chains and bind to receptors of the same family, the interleukin-1 receptor/toll-like receptor superfamily. However, there are also some differences between these cytokines: their genes are located on different chromosomes and their biological activities may differ because their receptors are differently expressed and distributed in various tissues (Kasiwamura et al., 2002; Dunne and, O`Neill, 2003). Nevertheless, both cytokines employ a common signal-transduction pathway leading to activation of the transcription factor NF-κB (Kasiwamura et al., 2002).

In a recently published study by Dai et al. the authors investigated the synthesis of IL-18 and IL-18R in chondrocytes as well as the effect of chondrocytes stimulated with IL-18α. There data suggested an expression of IL18Rα which was upregulated after stimulation with IL-1ß. However IL18ß was only detectable by RT-PCR and its expression could not be enhanced by stimulation of the chondrocyte culture with IL-1ß prior to the detection (Dai et al., 2005).

There are conflicting reports in the literature regarding the mitogenic effects of IL-18; Cornish and co-workers have shown that IL-18 act as a mitogen on chondrogenic cells (Cornish et al., 2003). However, Olee et al. (1999) reported that IL-18 inhibits the TGF-ß induced proliferation of chondrocytes. The specific inhibition of IL-18 leads to decreased joint swelling, attentuation of proteoglycan inhibition and decreased secretion of the pro-inflammatory cytokines IL-18, TNF-α and IL-1ß (Plater-Zyberk et al., 2001). Similar results have been found in IFN deficient mice suggesting that these effects are IFN independent (Joosten et al., 2000). Furthermore, IL-18 leads to increased expression of catabolic and pro-inflammatory mediators and enzymes such as matrix metalloproteinase-3, IL-6, COX and iNOS (Olee et al., 1999).

The question of whether IL-18 also affect chondrocyte apoptosis has not yet been addressed. Chondrocyte loss by apoptosis is a common feature in the pathogenesis of OA, induced by pro-inflammatory cytokines such as TNF-α (Heraud et al., 2000; Aizawa et al., 2001; Blanco et al., 2004; D'Lima et al., 2004; Park et al., 2005). Various apoptotic pathways are involved in chondrocyte apoptosis (Kühn et al., 2004). In other cell systems IL-18 has been shown to cause apoptosis (Chandrasekar et al., 2004), but the effects of IL-18 on apoptosis of articular chondrocytes have not been investigated. Accordingly, the present study was undertaken to test the hypothesis that IL-18 signalling in chondrocytes may affect MAPKinase signal transduction and may contribute to chondrocyte apoptosis.

Materials and Methods

Antibodies

Polyclonal anti-collagen type-II antibody (AB746), alkaline phosphatase linked sheep anti-mouse antibody (AP326A), and sheep anti-rabbit antibody (AP304A) were purchased from Chemicon International (Temecula, CA, USA). Polyclonal anti-SHC antibody (S14630) and monoclonal anti-ßl-integrin antibody (I41720) were obtained from Transduction Laboratories (Hamburg, Germany). The monoclonal anti-phospho P42/44 Erk 1/2 (M37520/M12320) antibody was purchased from Transduction Laboratories (Hamburg, Germany). Goat-anti-rabbit immunoglobulin conjugated with FITC derived from Dianova (Hamburg, Germany). Monoclonal COX-2 (160112) antibody was obtained from Cayman Chemical (Ann Arbor, MI, USA). R&D System, Inc., (Heidelberg, Germany) provided the polyclonal active caspase-3 (AF835) antibody. The monoclonal antibody against vinculin (MAB 9131) was purchased from Sigma (München, Germany).

Growth medium and chemicals

Growth medium (Ham's F-12/Dulbecco's modified Eagle's medium (50/50) containing 10% fetal calf serum. 25 µg/ml ascorbic acid, 50 IU/ml streptomycin, 50 IU/ml penicillin, 2.5 µg/ml amphotericin B, essential amino acids and L-glutamine derived from Seromed (Munich, Germany). Alginate, collagenase, OCT compound embedding medium and trypsin/EDTA (EC 3.4.21.4) were obtained from Sigma (Munich, Germany). Pronase was from Roche (Mannheim, Germany). Epon was obtained from Plano (Marburg, Germany). Recombinant human IL-18 derived from Biomol (Hamburg, Germany).

Cell culture

Cartilage from the femoral head was obtained in patient without the history of joint disease who had femoral neck fracture and underwent surgery. Primary human chondrocytes were isolated enzymatically from articular cartilage as previously described (Shakibaei and de Souza, 1997): Non-arthritic human femoral head articular cartilage (obtained during joint replacement surgery for femoral neck fractures from 3 patients, mean age 64 years, range 61-65) was cut into small slices. Slices were rinsed several times with fresh Ham's F-12 medium and digested primarily with 1% pronase (v/v) for 1.5 h at 37°C and subsequently with 0.2% (v/v) collagenase for 2 h at 37°C. Cells were resuspended in growth medium and cultured at a concentration of $2x10^6$ cells/ml in alginate beads as previously described (Shakibaei and de Souza, 1997; Schulze-Tanzil et al., 2004). After a few days of culture, the chondrocytes migrated from alginate and adhered on Petri dishes forming a monolayer (Schulze-Tanzil et al., 2004). These cells were cultured at $0.1x10^6$ cells/ml in Petri dishes in monolayer culture and on glass plates for a period of 24 h at 37°C with 5% CO^2. Serum-starved human articular chondrocytes were exposed to various concentrations of recombinant human IL-18 (1, 10, 100 ng/ml) for 0, 4, 8, 12, 24, 48, and 72 h.

Immunofluorescence microscopy

Chondrocytes were seeded on glass coverslips and serum-starved before they were stimulated with 10 or 100 ng/ml IL-18 for 4, 24, 48 h or remained untreated (control). Glass coverslips seeded with chondrocytes were rinsed three-times in Hanks solution before fixed with methanol for 10 min at ambient temperature (AT). Cells were rinsed with PBS, overlaid with protease-free bovine serum albumin (1% diluted in PBS) for 10 min at AT, rinsed again and incubated with primary antibodies (anti-collagen type II, anti-fibronectin, 1:50 in PBS) in a humid chamber overnight at 4°C. They were gently washed with PBS before incubation with secondary antibody (goat-anti-rabbit immunoglobulin conjugated with FITC [GAR--FITC], diluted 1:50 in PBS) for 1 h at AT followed. Finally, cells were washed several times with PBS, covered with fluoromount mounting medium, and examined under a light microscope (Axiophot 100, Zeiss, Germany).

Transmission electron microscopy

Cells were fixed in Karnovsky-fixative for 30 min and post-fixed in an 1% OsO_4 solution (in 0.1 M phosphate buffer). Chondrocytes were dehydrated in an ascending alcohol series before they were embedded in Epon, and cut at a Reichert Ultracut. 2% uranyl acetate/lead citrate was used for contrasting these ultrathin sections. For examination a transmission electron microscope (TEM 10, Zeiss, Germany) was used.

For statistical analysis, ultrathin sections of the samples were prepared and evaluated with an electron microscope (Zeiss EM 10). The number of cells with morphological features of apoptotic cell death was determined by scoring 250 cells from 30 different microscopic fields.

Western blot analysis

Chondrocyte cultures were washed three times with Hanks solution and cell proteins were extracted by incubation with lysis buffer (50 mM Tris/HCl, pH 7.2, 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium pyrophosphate, 100 mM sodium fluoride, 0.01% (v/v) aprotinin, 4 μg/ml pepstatin A, 10 μg/ml leupeptin, 1 mM PMSF) on ice for 30 min. Cell debris was removed by centrifugation and supernatants containing the cell proteins were stored at -70°C until use. Total protein concentration of protein extracts was determined using the bicinchoninic acid system (Uptima, Interchim, Montlucon, France) using BSA as a standard. Total protein concentrations were adjusted and proteins were separated by SDS-PAGE (5, 7.5 and 10% gels) under reducing conditions. The separated proteins were transferred onto nitrocellulose membranes. Membranes were pre-incubated in blocking buffer (5% (w/v) skimmed milk powder in PBS/0.1% Tween 20) for 30 min, and incubated with primary antibodies (1 h, AT). Membranes were washed three times with blocking buffer, and incubated with alkaline phosphatase conjugated secondary antibodies for 30 min. They were finally washed three times in 0.1 M Tris pH 9.5 containing 0.05 M $MgCl_2$ and 0.1 M NaCl. Nitro blue tetrazolium and 5-bromo-4-chloro-3-indoyl-phosphate (p-toluidine Salt; Pierce, Rockford, IL, USA) were used as substrates to reveal alkaline phosphatase conjugated specific antigen-antibody complexes. Specific binding was quantified by densitometry using "quantity one" (Bio-Rad Laboratories Inc. CA, USA).

Statistical methods

The significance of difference between group means was determined by a Student's t test in the program Stat View.

Results

Decrease in collagen type II and up-regulation of fibronectin deposition in chondrocytes by IL-18 as revealed by immunofluorescence microscopy

Monolayer chondrocytes seeded on glass plates were incubated with primary antibodies against collagen type

II and fibronectin and FITC-labeled secondary antibodies. The fluorescence intensity was estimated to reveal the content of these extracellular matrix proteins in the chondrocytes matrix and therewith to characterize the cells condition. As shown in (Fig. 1) the content of collagen type II surrounding the untreated chondrocytes constantly increased over a period of 48 h. Furthermore, the quantity of collagen type II decreased by IL-18 treatment in a concentration- and time-dependent manner. After treating the cells with 100 ng/mL IL-18 for 48 h collagen type II was barely detectable. Conversely, fibronectin synthesis (Fig. 2) increased in a time- and dose-dependent manner. After 4 h of incubation with 100 ng/mL IL-18 a clear increase in fibronectin expression was be observed.

Demonstration of IL-18 induced apoptosis in chondrocytes by transmission electron microscopy

Monolayer chondrocytes treated with IL-18 or untreated (control) were fixed and embedded for transmission electron microscopy. The untreated cells appeared as viable chondrocytes exhibiting characteristic morphological features: they exhibited round to oval shapes with prominently large and mostly euchromatic cell nuclei and a well structured cytoplasm (Fig. 3 A1,

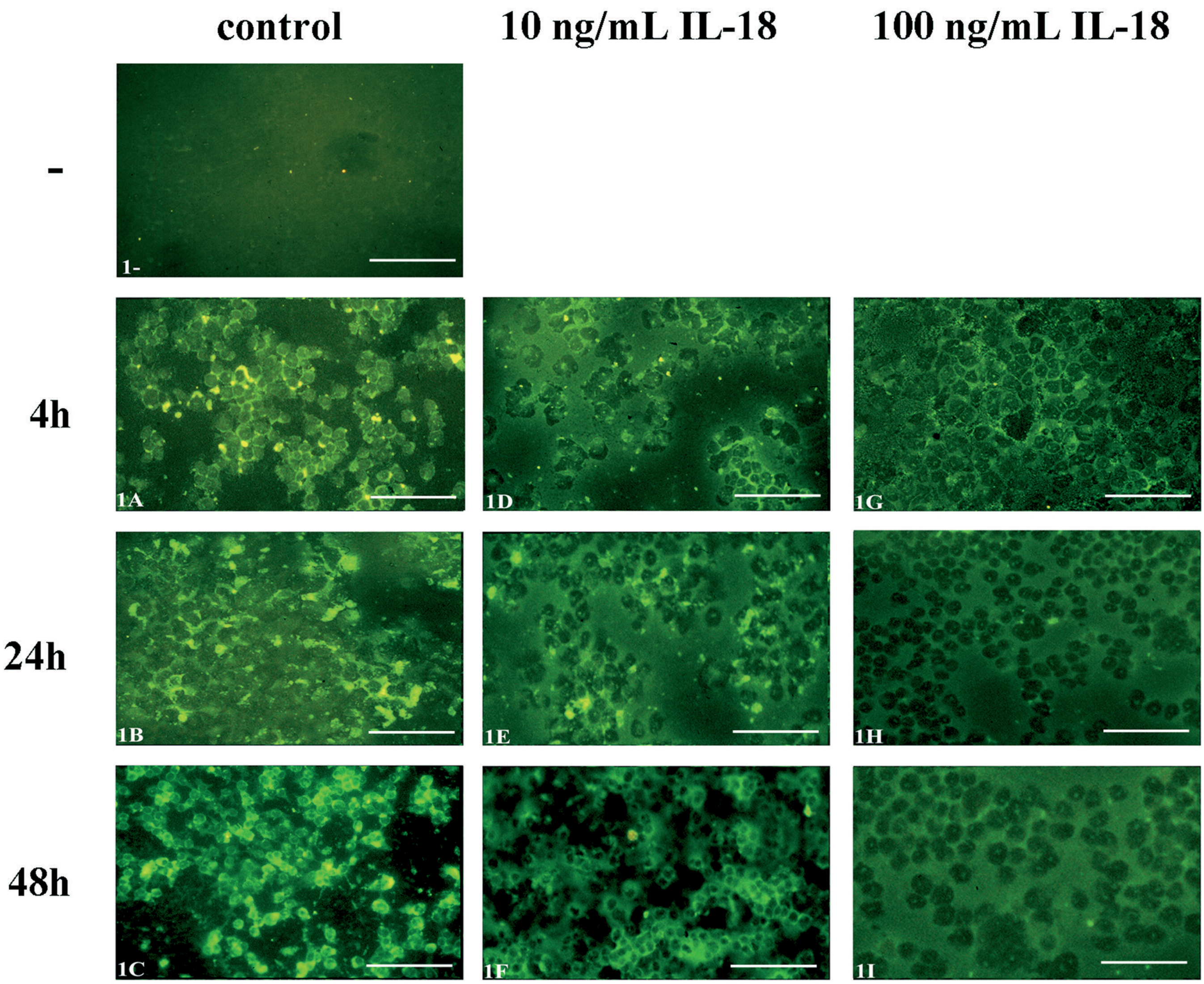

Fig. 1. Collagen type II expression on human articular chondrocytes treated with IL-18 as revealed by immunofluorescence microscopy. Chondrocytes were serum-starved and treated with 10 (**D, E, F**) or 100 (**G, H, I**) ng/ml IL-18 for 4, 24 or 48 h or remained untreated (**-, A, B, C**) for the same time points. In response to IL-18 chondrocyte collagen type II deposition decreased in a dose-dependent manner. (1-: control, chondrocytes were labelled with secondary antibodies without incubation with primary antibodies). Magnification x 200. Bar (1cm = 50 μm).

B1, C1). The IL-18 treated cells showed characteristic signs of degeneration, organelle destruction and programmed cell death: increased amounts of condensed heterochromatin in the nuclei, anular chromatin condensation at the nuclear envelope, budding of the nuclear envelope, clusters of swollen mitochondria, multiple vacuoles, multiple protrusions of the cell membrane and fragmentation of the cell into apoptotic bodies. Furthermore, IL-18 mediated cell degeneration and apoptosis increased in a time- and dose-dependent manner (Fig. 3 A2, B2, C2; A3, B3, C3).

Scoring the frequency of apoptotic cells and statistical evaluation of the data clearly highlighted changes in the number of apoptotic and viable cells before and after IL-18-treatment. The frequency and total number of apoptotic cells increased in Il-18 treated cultures in a dose and time dependent manner. The data confirm the findings observed by immunofluorescence and electron microscopy (Fig. 4).

Effects of IL-18 on collagen type II and vinculin synthesis as revealed by Western Blot analysis

Immunoblotting was performed to semi-quantitatively determine the effects of IL-18 treatment on protein synthesis in human chondrocytes. Serum-

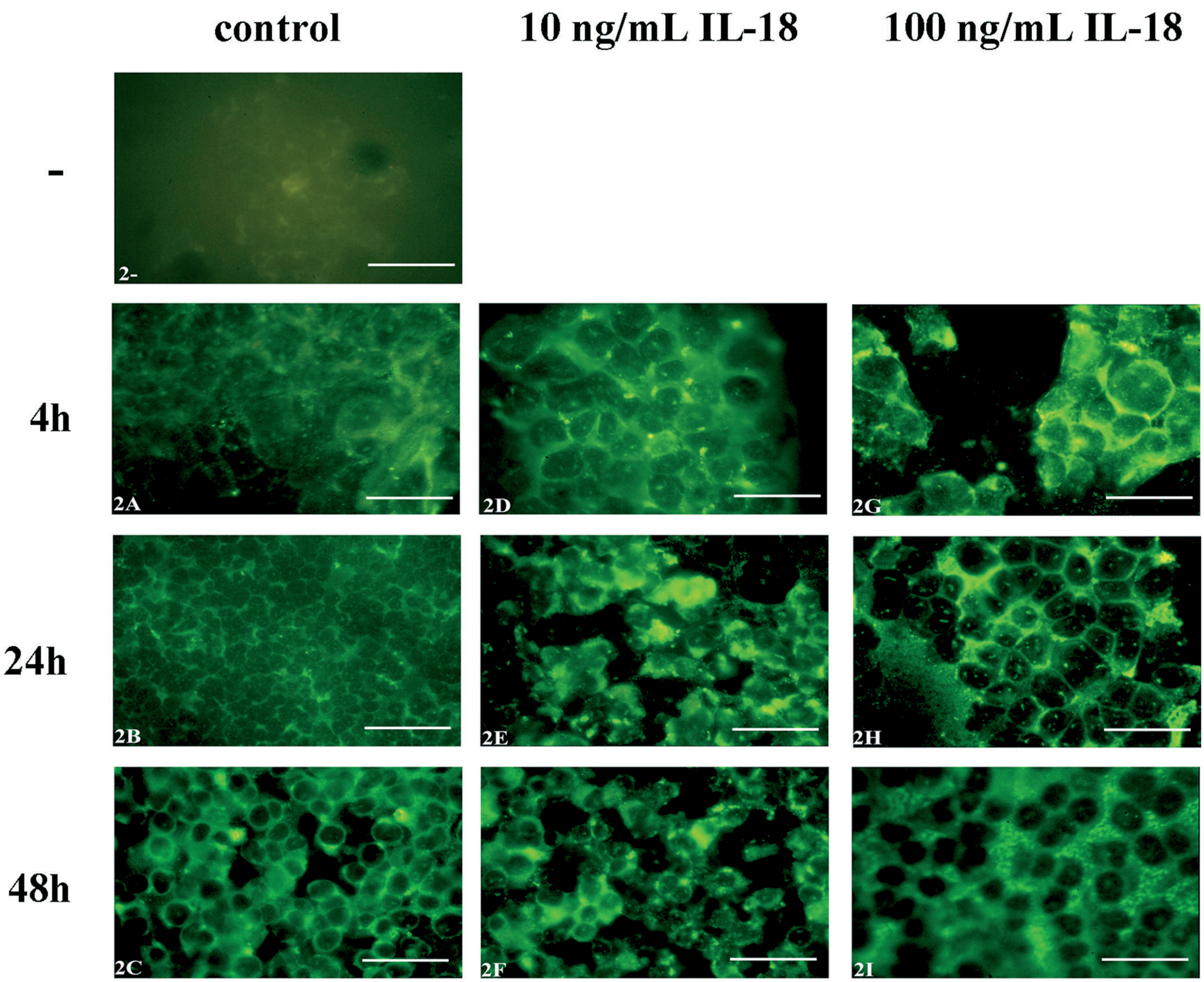

Fig 2. Fibronectin expression on human articular chondrocytes in response to IL-18 as estimated by immunofluorescence microscopy. Serum-starved chondrocytes were treated with 10 (**D, E, F**) or 100 (**G, H, I**) ng/mL IL-18 for 4, 24 or 48 h or remained untreated (**-, A, B, C)** for the same time periods. In response to IL-18 chondrocyte fibronectin deposition around the chondrocytes increased in a dose-dependent manner. (2-: control, chondrocytes were labelled with secondary antibodies without incubation with primary antibodies). Magnification x 400. Bar (1cm = 25µm).

starved chondrocytes were stimulated with 1, 10 or 100 ng/mL recombinant human IL-18 for 0, 4, 8, 12, 24, 48 and 72 h. In agreement with the immunohistochemical investigations detailed above, the quantity of the cartilage-specific collagen type II was already reduced after 4 h of treatment with 1 ng/mL IL-18 (Fig. 5a). This trend continued over 8, 12, and 24 h, until the quantity of collagen type II was significantly decreased after 48 and 72 h of incubation. Furthermore, this effect was dramatically intensified by the higher concentrations of IL-18: 10 ng and 100 ng (Fig. 5b,c). The cytoskeletal associated protein vinculin migrating at 116 kDa decreased in response to IL-18 in a time- and dose-dependent manner (Fig. 6 a-c).

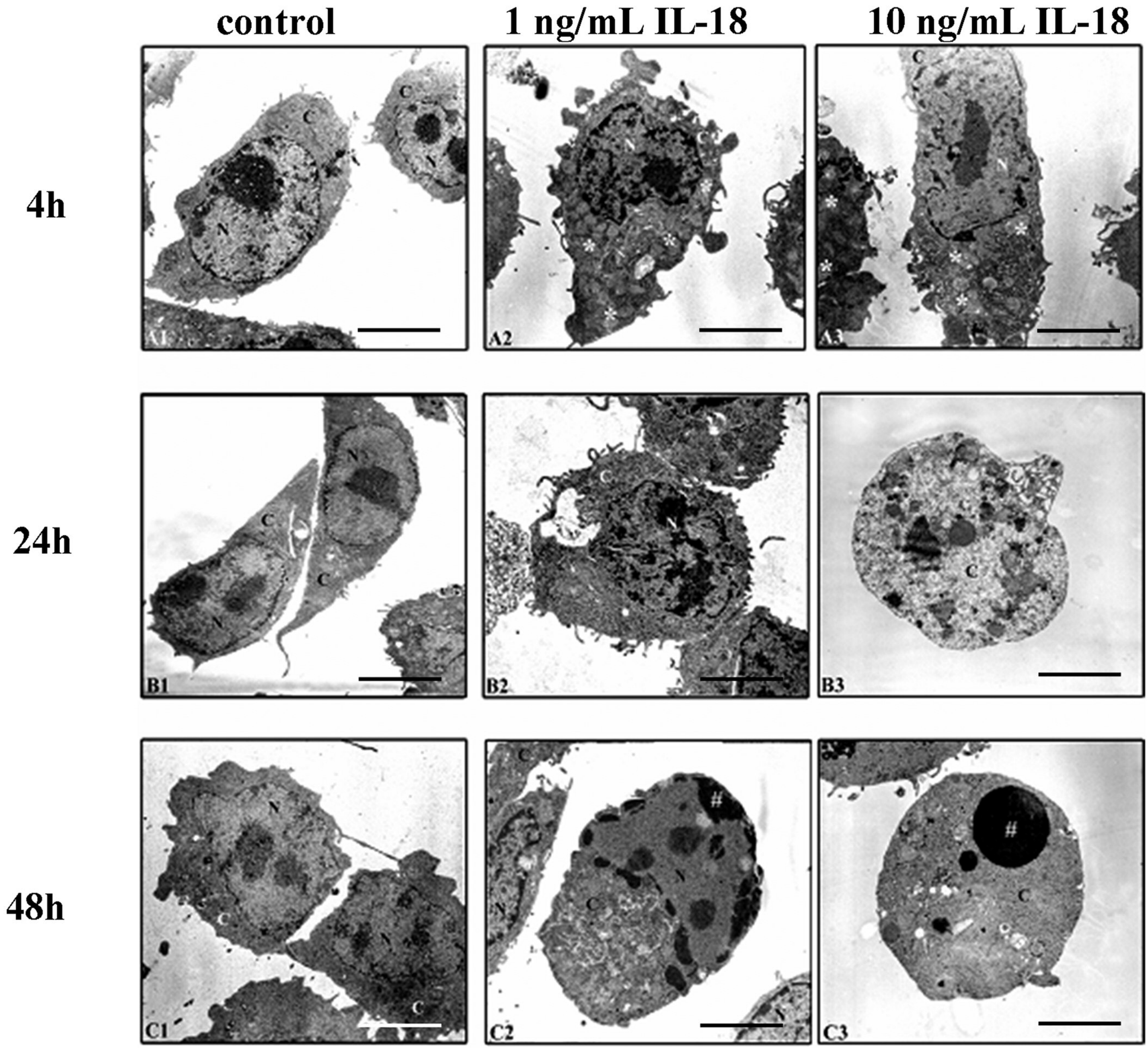

Fig. 3. Ultrastructural morphological alterations of chondrocytes in response to IL-18 treatment as revealed by transmission electron microscopy. Monolayer chondrocytes were treated with 10 (**A2, B2, C2**) or 100 (**A3, B3, C3**) ng/mL IL-18 or remained untreated (**control, A1, B1, C1**) for 4 (**A**), 24 (**B**), 48 (**C**) h and immediately fixed and embedded for transmission electron microscopy. The control cells exhibited flattened, round or oval shapes. These cells had large euchromatic nuclei and a well structured cytoplasm. In response to IL-18 treatment the cells exhibited characteristic features of degeneration and apoptosis such as enlarged areas of condensed heterochromatin in the nuclei, budding of the nuclear envelope, clusters of swollen mitochondria, multiple vacuoles and apoptotic bodies. These changes occurred following IL-18 treatment in a time- and dose-dependent manner. Magnification x 2000. Bar (1cm = 5µm)

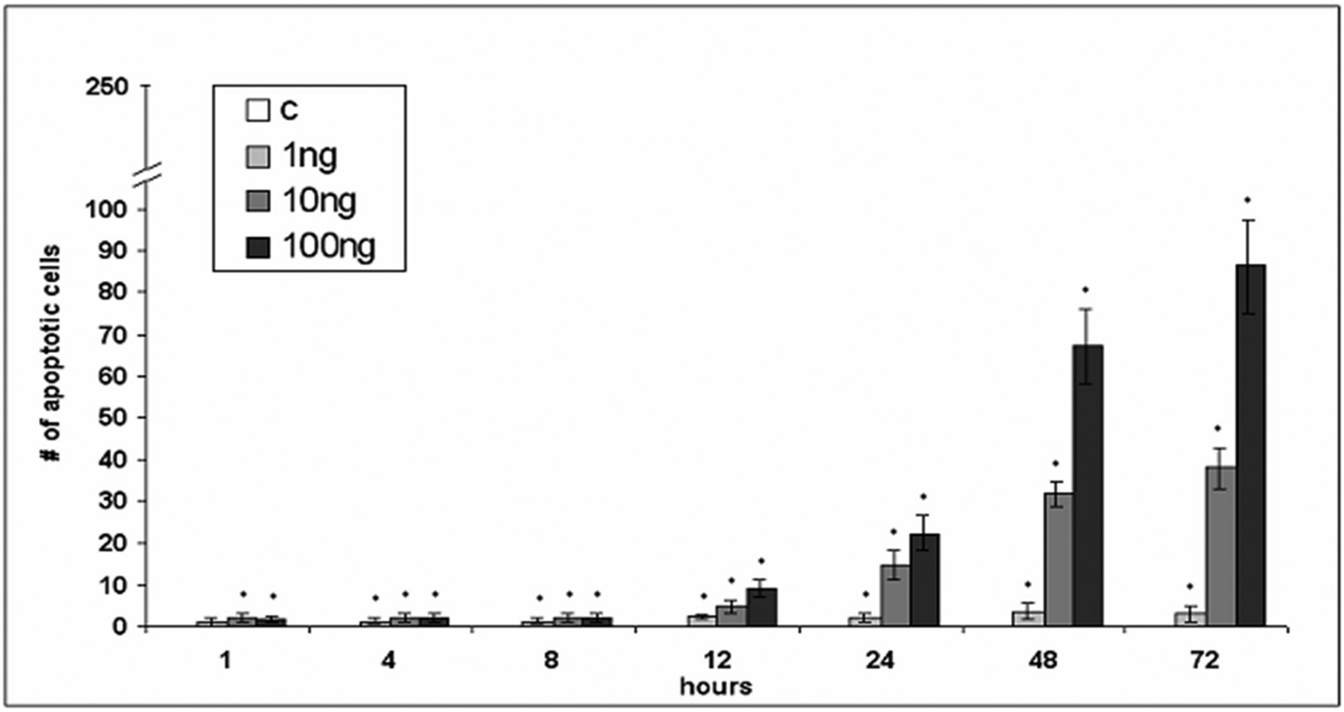

Fig. 4. Statistical evaluation of electron microscopic data. The frequency of apoptosis in control and IL-18-treated cultures was evaluated by scoring 250 cells from 30 different microscopic fields in each situation. The graph highlights the number of pathologically altered cells in IL-18 cells compared to control, untreated cells; mean values are shown with standard deviations (n = 3 independent experiments).

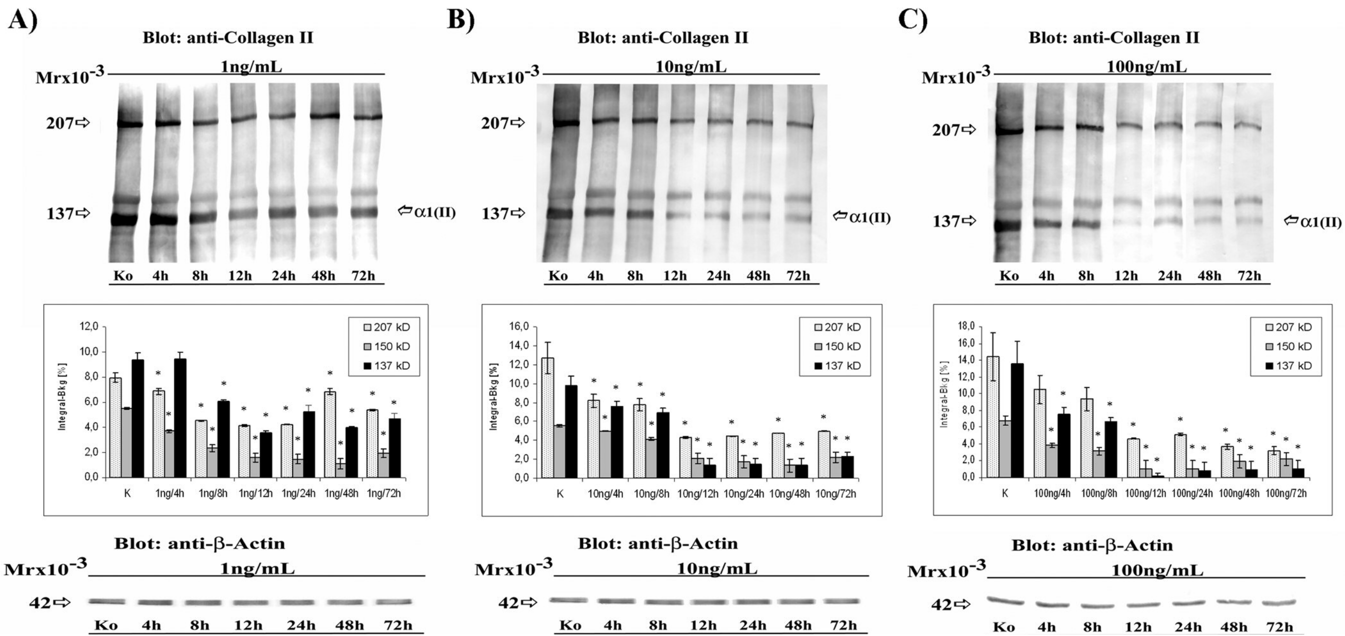

Fig. 5. Effect of IL-18 on cartilage-specific collagen type II expression by chondrocytes. Serum starved monolayer chondrocytes were treated with 1 (**a**), 10 (**b**) or 100 (**c**) ng/mL IL-18 or remained untreated (Ko) for 4, 8, 12, 24, 48 and 72 h and were investigated by Western blot analysis. Collagen type II expression was inhibited in a time- and dose-dependent manner as also revealed by densitometric evaluation. The arrow in the right margin indicates the relative position of the collagen type II α-chain [α1(II)]. Expression of the house-keeping gene ß-actin was not affected by control and treatment with IL-18.

Synthesis of key signalling proteins of the MAPKinase pathway and MAPKinase activation are affected by IL-18 as demonstrated by Western Blot analysis

Expression of the transmembrane signal transduction receptor ß1-integrin was inhibited in response to IL-18 (Fig. 7a-c) and the expression of all three isoforms of the cytoplasmatic signal adaptor protein SHC were downregulated by IL-18 in a time- and dose-dependent manner as also shown by densitometry (Fig. 8a-c). Activation of MAPKinase was determined using an antibody directed against the tyrosine-phosphorylated

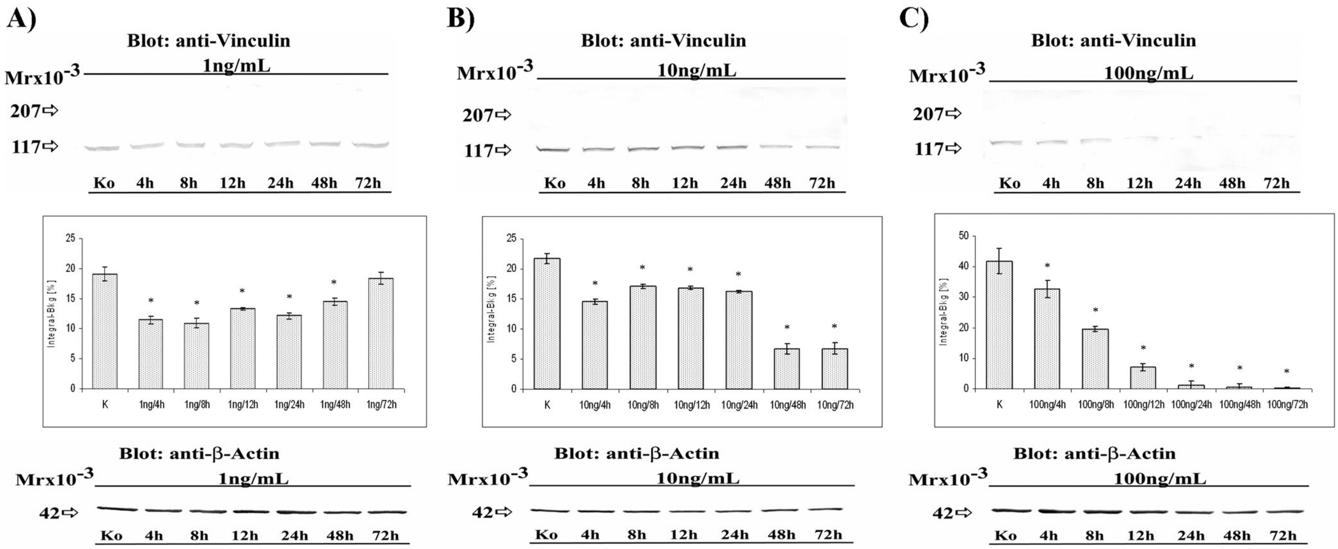

Fig. 6. Effect of IL-18 on vinculin expression by chondrocytes. Monolayer chondrocytes were treated with 1 (**a**), 10 (**b**) or 100 (**c**) ng/mL IL-18 or remained untreated (Ko) for 4, 8, 12, 24, 48 and 72 h and were investigated by Western blot analysis. Vinculin expression was inhibited in a time- and dose-dependent manner. Expression of the house-keeping gene ß-actin was not affected by control and treatment with IL-18.

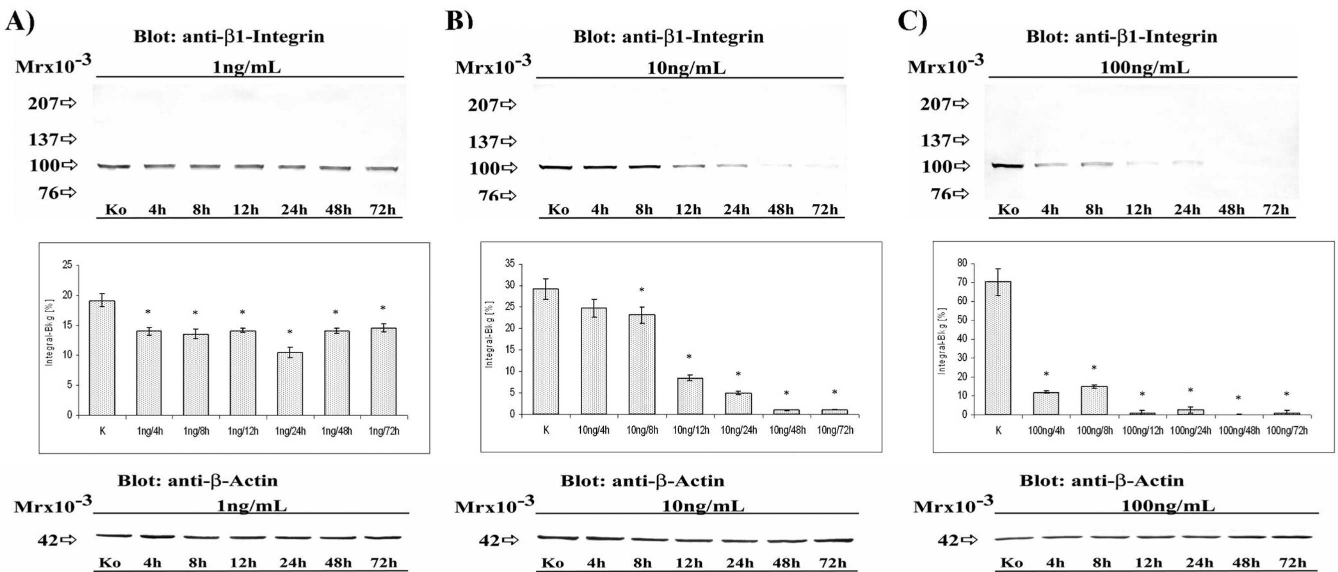

Fig. 7. ß1-integrin expression on chondrocytes in response to IL-18. Protein lysates of monolayer chondrocytes treated with 1 (**a**), 10 (**b**) or 100 (**c**) ng/mL IL-18 or untreated (Ko) for 4, 8, 12, 24, 48 and 72 h and were analysed by Western blot analysis using anti-ß1-integrin antibodies. ß1-integrin expression was inhibited in a time- and dose-dependent manner. Expression of the house-keeping gene ß-actin was not affected by control and treatment with IL-18.

protein ERK 1/2. Many proteins in the MAPKinase cascade are activated by phosphorylation of their tyrosine residues. Several other tyrosine-phosphorylated proteins with apparent molecular weights of 130 and 52 kDa could be detected, which also decreased with the dose and incubation time of IL-18. A significant decrease in activated MAPKinase could be observed suggesting an inhibition of MAPKinase signalling in response to IL-18 in a dose- and time-dependent manner (Fig. 9a-c). The phosphorylated protein bands migrating at 42 and 44 kDa represent activated ERK-1 and -2.

Evidence of increased synthesis of COX-2 and caspase-3 activation in response to IL-18 demonstrated by Western Blot analysis

The proinflammatory enzyme COX-2 was induced in chondrocytes by IL-18 treatment (Fig. 10a-c). Finally, in order to highlight the morphological apoptotic features, ultrastructural studies and Western blot analyses were carried out for the apoptosis marker activated caspase-3. Activation of caspase-3 was evident as revealed by increase of the 17 kDa active form.

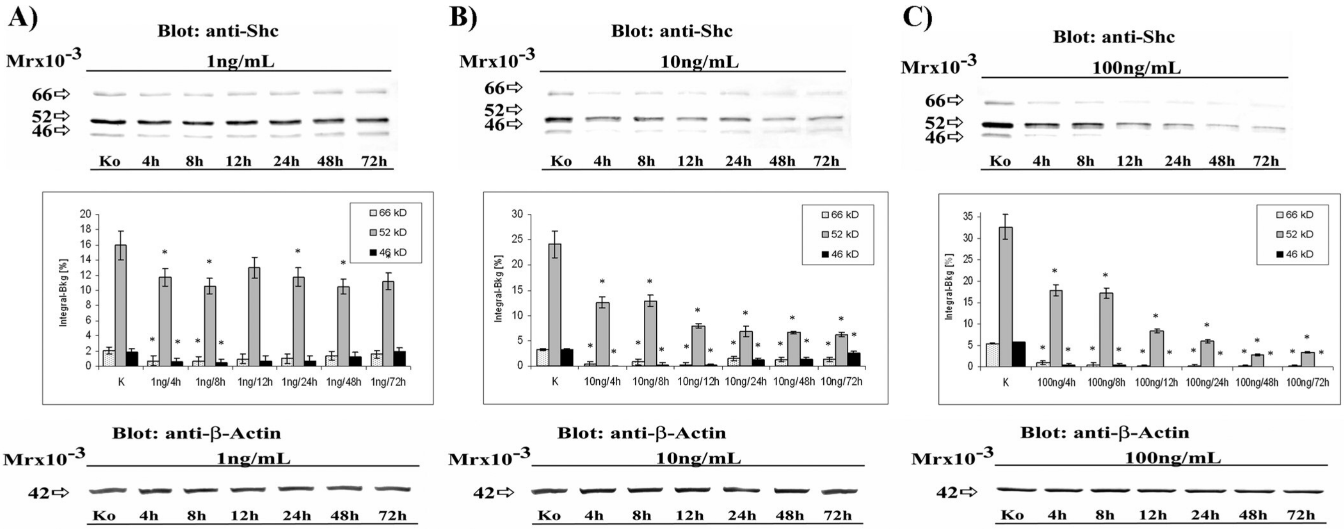

Fig. 8. Western blot analysis for SHC expression by chondrocytes treated with IL-18. Monolayer chondrocytes were treated with 1 (**a**), 10 (**b**) or 100 (**c**) ng/mL IL-18 or remained untreated for 4, 8, 12, 24, 48 and 72 h and were investigated by Western blot analysis. SHC expression was inhibited in a time- and dose-dependent manner. Expression of the house-keeping gene ß-actin was not affected by control and treatment with IL-18.

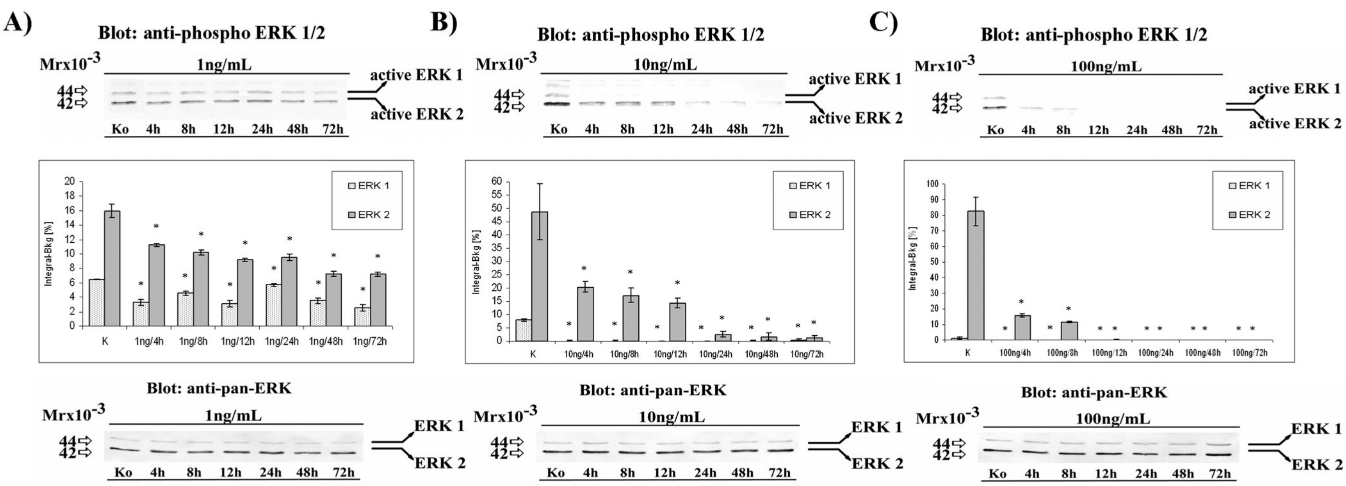

Fig. 9. Effect of IL-18 on anti-phospho Erk1/2 by chondrocytes. Monolayer chondrocytes were treated with 1 (**a**), 10 (**b**) or 100 (**c**) ng/mL IL-18 or remained untreated for 4, 8, 12, 24, 48 and 72 h and were investigated by Western blot analysis. Erk 1/2 expression was inhibited in a time- and dose-dependent manner. Expression of the house-keeping gene ß-actin was not affected by control and treatment with IL-18.

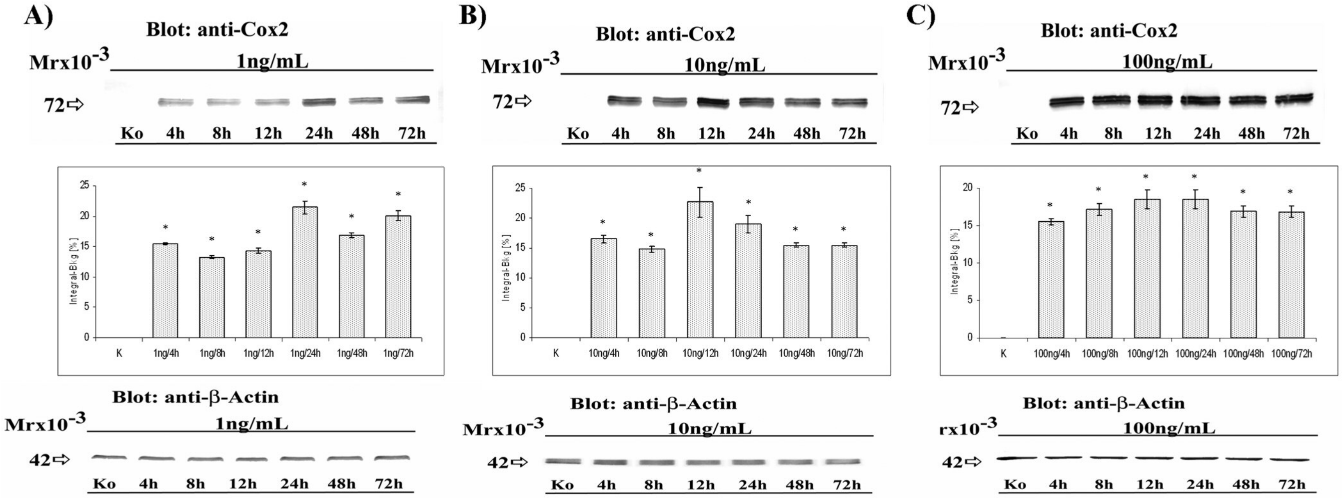

Fig. 10. Effect of IL-18 on COX-2 expression by chondrocytes. Monolayer chondrocytes were treated with 1 **(a),** 10 **(b)** or 100 **(c)** ng/mL IL-18 or remained untreated for 4, 8, 12, 24, 48 and 72 h and were investigated by Western blot analysis. COX-2 expression increased in a time- and dose-dependent manner. Expression of the house-keeping gene ß-actin was not affected by control and treatment with IL-18.

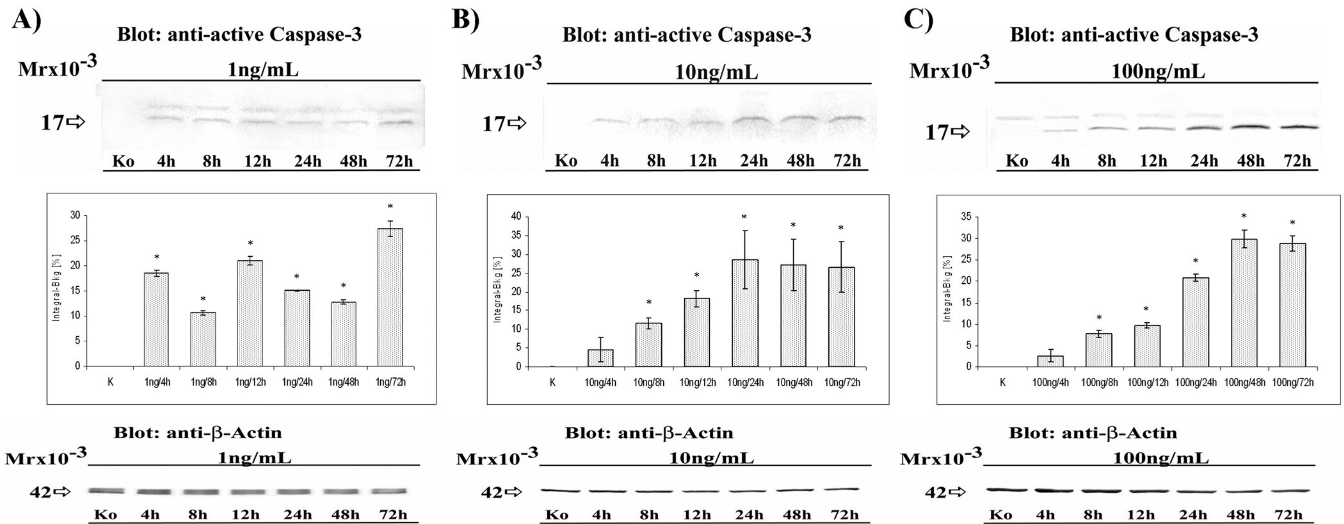

Fig. 11. Effect of IL-18 on activated caspase-3 by chondrocytes. Monolayer chondrocyte lysates were treated with 1 **(a)**, 10 **(b)** or 100 **(c)** ng/mL IL-18 or remained untreated for 4, 8, 12, 24, 48 and 72 h and were investigated by Western blot analysis. In response to IL-18 treatment high levels of the apoptosis marker activated caspase-3 were detected. Expression of the house-keeping gene ß-actin was not affected by control and treatment with IL-18.

Caspase 3 was induced by IL-18 in a time- and dose-dependent manner (Fig. 11a-c). In the untreated cultures caspase activation was not observed.

Discussion

Pro-inflammatory cytokines such as TNF-α and IL-1ß, are known to play a pivotal role in the pathogenesis and progression of OA (Goldring, 2000; Lotz, 2001; Sandell and Aigner, 2002; Dai et al., 2005). In recent years the pro-inflammatory cytokine IL-18 has also been implicated in the pathogenesis of osteoarthritis and rheumatoid arthritis (Bessis and Boissier, 2001; Vervoordeldonk and Tak, 2002). Recent investigations have revealed that IL-18 also leads to joint inflammation and joint destruction (Matsui et al., 2003; Joosten et al., 2004). Adenoviral IL-18 gene transfer induces joint inflammation and cartilage proteoglycan loss *in vivo* suggesting a direct role for IL-18 in the development of arthritis (Joosten et al., 2004). The particular effects of

IL-18 on chondrocyte signalling and synthesis program remain poorly understood.

The present *in vitro* study was undertaken to test the hypothesis that IL-18 signalling in chondrocytes is adversely affected by IL-18 and that this pro-inflammatory cytokine may contribute to chondrocyte apoptosis. The results presented support the idea that IL-18 exerts detrimental effects on human articular chondrocytes *in vitro*. IL-18 inhibited the deposition of collagen type II. In addition, IL-18 suppressed ß1-integrin synthesis on the chondrocytes and lead to a decrease in the expression of SHC and vinculin. Furthermore, activation of the MAPKinase ERK-1/2 was clearly suppressed by IL-18 treatment. In contrast the production of the pro-inflammatory enzyme COX-2 was induced by IL-18. Moreover, chondrocytes treated with IL-18 exhibited activation of caspase-3 and typical morphological features of apoptosis as revealed by transmission electron microscopy. These results highlight the pro-apoptotic and catabolic capacity of IL-18 on human chondrocytes *in vitro*.

IL-18 suppresses expression of cartilage matrix components while upregulating fibronectin

The pro-inflammatory cytokines TNF-α and IL-1ß have been shown previously to suppress the expression of cartilage-specific extracellular matrix (ECM) proteins such as aggrecan and collagen type II (Murakami et al., 2000; Robbins et al., 2000; Kolettas et al., 2001; Schulze-Tanzil et al., 2004). In response to these mediators chondrocytes decrease synthesis of collagen type II and aggrecan, but express the collagen types I, III and V (Sandell and Aigner, 2001). In the present study we have demonstrated a similar dose- and time-dependent suppressive effect for IL-18 on collagen type II deposition in chondrocytes. Expression of the cytoskeletal protein vinculin was also affected by IL-18 stimulation in chondrocytes. Vinculin is localized at the cytoplasmic faces of cell-extracellular adhesion plaques. This cytoskeletal protein represents a key element in the transmembrane linkage of the extracellular matrix to the cytoplasmatic microfilament system (Shakibaei et al., 1999). It is therefore likely that similarities exist in the cell signalling mechanism(s) employed by transcription factors directly activated by these three pro-inflammatory cytokines. Our present understanding of the mechanisms responsible for the negative regulation of cartilage-specific matrix components in chondrocytes stimulated by these pro-inflammatory cytokines is very limited. While the expression of certain matrix components decreases in osteoarthritic cartilage, fibronectin is upregulated (Homandberg, 1999). Proteolytic degradation of fibronectin in osteoarthritic cartilage has been shown to produce fibronectin fragments with enhanced capacity to induce further cartilage degradation which may exacerbate the existing pathology and contribute further to the osteoarthritic processes (Homandberg, 1999). IL-18 may therefore upregulate fibronectin expression in cartilage and contribute to the catabolic pathway mediated by its proteolytic fragmentation. The immunofluorescence data presented in the present study confirms this possibility.

IL-18 inhibits expression of proteins involved in the MAPkinase signalling pathway

It is well known that the close interaction between cartilage-specific matrix components and chondrocytes via cell surface signalling receptors such as integrins is an essential prerequisite for chondrocyte survival and maintenance of their highly differentiated phenotype (Meredith et al., 1993; Hirsh et al., 1997; Cao et al., 1999). In healthy cartilage chondrocytes are embedded in an extracellular matrix whose turnover is tightly regulated. Cell-matrix interactions are mediated by integrins. Inhibition of cell matrix interaction is known to lead to apoptosis (Hirsh et al., 1997; Yang et al., 1999). Work from our laboratory has demonstrated that activation of the MAPKinase signalling pathway is essential for maintaining chondrocyte survival (Shakibaei et al., 2001). The MAPKinase pathway is known to be activated by ß1-integrins in response to stimulation by cartilage-specific matrix components (Shakibaei et al., 1999). The adaptor protein SHC is activated by integrin stimulation and transduces these signals to the MAPKinase cascade leading to ERK activation (Barberis et al., 2000). In the present study we observed downregulation of ß1-integrin, SHC, and ERK1/2. These proteins represent key signalling proteins in signal transduction of the MAPKinase pathway (Barberis et al., 2000). Since the MAPKinase pathway is important for survival and differentiation of chondrocytes (Shakibaei et al., 1999, 2001), inhibition of expression of its key components leads to chondrocyte apoptosis (Shakibaei et al., 2001). Therefore, we can conclude that IL-18 may interfere with MAPKinase signalling in human articular chondrocytes.

IL-18 stimulates COX-2 in chondrocytes

To investigate the pro-inflammatory capacity of IL-18 in chondrocytes the expression level of COX-2 was evaluated in the presence and absence of IL-18. COX-2 is a key enzyme in the prostaglandin E2 synthesis pathway. Increased prostaglandin E2 expression coincides with the upregulation of the inducible COX isoform COX-2 in response to pro-inflammatory cytokines (Kojima et al., 2004). IL-18 has been reported to induce COX-2 expression in articular chondrocytes (Olee et al., 1999). In contrast, Lee et al., (2004) were not able to demonstrate any significant COX-2 upregulation in IL-18 transduced epithelial cells (Lee et al., 2004) suggesting that IL-18 effects on COX-2 may also depend on the cell type or other experimental conditions. Further catabolic and pro-inflammatory effects of IL-18 have been documented such as increased matrix metalloproteinase synthesis, iNOS induction and GAG release (Olee et al., 1999).

IL-18 contributes to chondrocyte apoptosis

It is well accepted that apoptosis induced by IL-1ß and TNF-α plays a significant role in the pathogenesis of osteoarthritis (Aizawa et al., 2001; Aigner and Kim, 2002). The contribution of IL-18 to this process in chondrocytes has not yet been intensive studied or conclusively demonstrated. Some reports exist that describe pro-apoptotic properties of IL-18 in other cell types: pro-apoptotic signalling of IL-18 has been demonstrated in human cardiac endothelial cells acompanied by decrease of anti-apoptotic factors such as Bcl-2, Bcl-x, upregulation of Fas, FasL and various caspases (Caspase-3, -8, -9, BID), and cytochrome C release (Marino and Cardier, 2003; Chandrasekar et al., 2004). Interestingly, we have also demonstrated that IL-18 is able to induce chondrocyte apoptosis as revealed by caspase-3 activation. Caspase-3 is a protease and a downstream mediator of the apoptotic signalling cascade. Activated caspases play a central role in the execution phase of apoptosis and caspases are also known to be associated with many morphological features of this form of cell death (Cohen, 1997).

The apoptotic pathways activated by IL-18 are poorly studied. It has been assumed previously that chondrocyte death may also be caused by loss of cell matrix interaction via integrins (Hirsh et al., 1997; Goggs et al., 2003; Kühn et al., 2004). Collagen type II is particularly important for chondrocyte survival *in vivo* (Yang et al., 1997). Therefore, IL-18 induced apoptosis may also result from loss of cell-matrix interactions and inhibition of MAPKinase pathway. Effects of IL-18 on cell death in other cell types have been shown to be direct and not mediated by intermediaries such as TNF-α and IL-1ß. In addition to its role in apoptosis, caspase-3 has a regulatory effect on IL-18: it cleaves both the precursor and mature forms of IL-18 into biologically inactive degraded products and may constitute a potential down-regulator of IL-18 (Akita et al., 1997).

The *in vitro* data presented in this study indicate that IL-18 may be implicated in the catabolic and pro-inflammatory events that characterize the pathogenesis of osteoarthritis. Furthermore, our experimental evidence suggests that IL-18 may contribute directly to chondrocyte apoptosis a process closely correlated with inhibition of MAPKinase signalling. Much work has yet to be done to further define the particular role of IL-18 in the pathogenesis of OA and to determine whether inhibition of IL-18 signalling may be a suitable approach in OA therapy.

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