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PKC-ζ Expression Is Lower in Osteoblasts From Arthritic Patients: IL1-β and TNF-α Induce a Similar Decrease in Non-Arthritic Human Osteoblasts

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Abstract Protein kinase C (PKC) is a family of enzymes detected in a diverse range of cell types where they regulate various cellular functions such as proliferation, differentiation, cytoskeletal remodelling, cytokine production, and receptor-mediated signal transduction. In this study we have analyzed the expression of 11 PKC isoforms (-α, -β_I, -β_{II}, -γ, -δ, -η, -θ, -ε, -ζ, -ι/λ, and -μ) in osteoblasts from patients with osteoarthritis (OA) and rheumatoid arthritis (RA) in comparison with osteoblasts from post-traumatic (PT) patients. By Western blotting analysis, nine isoforms, -α, -β_I, -β_{II}, -δ, -θ, - ε, -ζ, - ι/λ, and -μ, were detected in osteoblasts. In RA and OA patients, PKC -θ and -μ were greater expressed whereas PKC-ε and -ζ decreased when compared with normal cells. The subcellular distribution and quantitative differences were confirmed by immuno-electron microscopy. Furthermore, we demonstrated that treatment with the proinflammatory cytokines, IL-1β and TNF-α, significantly decreased PKC-ζ expression in PT osteoblasts. This suggests that proinflammatory cytokines can modulate the expression of this PKC isoform in osteoblasts in a way which is similar to changes detected in arthritic patients. J. Cell. Biochem. 103: 547–555, 2008.

Key words: protein kinase C; osteoblast; rheumatoid arthritis; osteoarthritis

Protein Kinase C (PKC), a serine/threonine protein kinase family, consists of at least eleven isozymes. They have been classified into three main groups which share a common requirement of phospholipid for their activity, although they differ in structure and dependency on other activators.

Conventional PKCs (α, β_I, β_{II}, γ) require phosphatidylserine (PS), diacylglycerol (DAG), generated by phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis mediated by phospholipase C (PLC) or phorbol esters, and Ca^{2+} [Nishizuka, 1995]. Novel PKCs (δ, ε, η, θ) need PS and DAG but not Ca^{2+}. Atypical PKCs (ζ, ι/λ) are insensitive to both DAG and Ca^{2+} and dependent on PS. Finally, PKC-μ (human) and its murine analog, PHD1, have been characterized and together with PKD2 and PKCν are grouped into the PKD family [Van Lint et al., 2002; Poole et al., 2003].

PKC isoforms are monomeric proteins ranging from 72 to 115 kDa in mass, and display an amino-terminal regulatory domain and a conserved carboxy-terminal catalytic domain. Each isoform possesses specific sequences which are

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localized in the variable regions of the enzyme and are well conserved among different species [Hug and Sarre, 1993]. This characteristic has permitted the production of isoform-specific antisera [Wetsel et al., 1992].

The biological functions of PKC have mostly been linked with events occurring at the plasma membrane level and, or in the cytoplasm, because PKC isotypes are thought to be associated with the cytoskeleton in an inactive state and, after maturation, that is phosphorylation, they translocate to the plasma membrane or the membrane of cytoplasmic organelles, to become fully activated in the presence of specific cofactors [Hug and Sarre, 1993; Newton, 1997]. Moreover, in the last few years, many reports have indicated that PKC isozymes can translocate to the nucleus or are resident within this organelle.

This suggests that they could represent a pathway to communicate signals generated at the plasma membrane to the nucleus [Olson et al., 1993; Buchner, 1995; Martelli et al., 2006].

PKCs play a central role in phosphoinositide-mediated signal transduction networks which modulate various physiological cell responses, such as cell proliferation, differentiation, neoplastic transformation, apoptosis, release of hormones and neurotransmitters, as well as cytokine production [Hug and Sarre, 1993; Nishizuka, 1995; Dempsey et al., 2000; Musashi et al., 2000]. Alteration of these pathways is common to many disease states, including chronic inflammation, diabetes, cardiovascular disease, and cancer [Prestwich et al., 2002; Pendaries et al., 2003].

Several reports have described the basal expression of some PKC isoforms in osteoblast cell lines (animal and human origin) [Sanders and Stern, 1996; Lampasso et al., 2006] and in primary human osteoblast cultures [Lampasso et al., 2001]. PKC isoforms have also been demonstrated to be involved in the early phase of mechanotransduction of osteoblastic cell lines through the activation of cytoskeletal molecules [Geng et al., 2001]. In chondro-osseous tissues, PKC has been reported to play an important role in cartilage metabolism, and it has been proposed that it modulates proteoglycan metabolism [Arner and Pratta, 1991]. Moreover, a re-distribution of some PKC isoforms has been described in chondrocytes of osteoarthritic articular cartilage [Satsuma et al., 1996] and synovial fibroblasts from patients with rheumatoid arthritis (RA) [Mino et al., 1998].

Several reports indicate that progression of joint cartilage degeneration in osteoarthritis (OA) and in RA is associated with subchondral bone modification [Woolley and Tetlow, 1997; Lajeunesse and Reboul, 2003; Buckwalter and Brown, 2004]. It has also been reported that bone cells are able to influence cartilage metabolism in OA and in RA [Westacott et al., 1997; Bogoch and Moran, 1998; Mansell and Bailey, 1998], suggesting that subchondral bone changes may precede cartilage deterioration.

To add new insight in this field, we evaluated PKC isoforms expression in primary human osteoblast cultures obtained from OA and RA patients with respect to osteoblasts from post-traumatic (PT) patients, by means of Western blot and quantitative immuno-electron microscopy.

Moreover, in order to determine whether the different expression of PKC isoforms could depend on cytokine action, cultures of PT osteoblasts were also analyzed after treatment with IL-1β and TNF-α.

MATERIALS AND METHODS

Specimens

Human subchondral femoral heads were obtained from 10 RA, 10 OA, and 10 PT patients undergoing selective total hip replacement. As shown in Table I, diagnosis of OA and RA was based on clinical, laboratory, and radiologic evaluation. Patients with OA and RA were selected according to the American College of Rheumatology criteria [Altman et al., 1986; Arnett et al., 1988], and the samples were obtained with informed consent, according to the Declaration of Helsinki principles. The study was approved by the local ethics committee of Istituti Ortopedici Rizzoli.

Human Osteoblast Cultures

Primary osteoblast cell cultures (10 from patients affected by RA, 10 from patients with OA, and 10 from patients with PT) were obtained from trabecular bone of femoral heads using a modification of the methods described by Robey and Termine [1985]. Briefly, femoral heads were collected and bone chips minced in a sterile glass tube (Pierce React-vials,

TABLE I. Baseline Characteristics of Patients

Characteristics	RA	OA	PT
No. (male/female)	10 (3/7)	10 (3/7)	10 (4/6)
Age mean ± SD	63 ± 4	65 ± 8	61 ± 7
Elapsed time from first symptoms (years ± SD)	11 ± 4	5 ± 3	—
CRP	4.10 ± 2.22	0.40 ± 0.23	ND
ESR	57 ± 33	27 ± 15	35 ± 25
Drug treatment	NSAIDs and corticosteroids (3 of 10 patients, mean dose ≤ 10 mg/day)	NSAIDs	—

RA, rheumatoid arthritis; OA, osteoarthritis; PT, post-traumatic after fall; CRP, C-reactive protein (normal value 0–0.5 mg/dl); ESR, erythrocyte sedimentation rate (normal value 2–20 mm/h).

Rockford, IL) containing serum-free 1:1 mixture DMEM/Ham's F12K without Ca^{2+} (Life Technologies Ltd, Paisley U.K.) supplemented with 100 U/ml penicillin, 25 μg/ml ascorbic acid (Sigma, St. Louis, MO) and 2mM Ca^{2+} (Sigma). The minced bone chips were digested using 1 mg/ml collagenase P (Boehringher Mannheim Corporation, Indianapolis, IN) at 37°C for 2 h with rotation. Then, they were washed and placed in 35 mm dishes (approximately 0.05 ml condensed chips/dish) in the same medium containing 10% heat inactivated fetal bovine serum (Life Technologies Ltd) without Ca^{2+} (complete medium). Complete medium of cultures was changed twice weekly. After 2 weeks they were removed and osteoblasts were allowed to grow until confluent. As already reported by our group [Lisignoli et al., 1999], cultured cells expressed an osteoblastic phenotype (alkaline phosphatase and osteocalcin positive) and contamination of osteoblasts by hematopoietic cells was less than 0.5%.

Western Blotting

Osteoblasts (5×10^6) isolated from RA, OA, and PT patients as detailed above, were seeded in T150 flasks and allowed to adhere overnight. The cells were lysed in Laemmli's sample buffer, boiled and electrophoresed on SDS–8% polyacrylamide gels. Gels were electroblotted onto nitrocellulose membranes and blocked with 3% bovine serum albumin (BSA), and incubated overnight at 4°C with the appropriate anti-PKC antibodies (diluted 1:500) followed by peroxidase conjugated-secondary antibody (Amersham Biosciences, UK) (diluted 1:3000) in PBS-buffered saline containing 0.1% Tween-20 for 20 min at room temperature. Bound antibody was visualized using a chemiluminescence kit (Amersham Biosciences). Polyclonal (rabbit) antibodies against PKC-α, -β_I, -β_{II}, -γ, -δ, -ε, -η, -θ, -ζ, -ι/λ and -μ (Santa Cruz Biotechnology, Santa Cruz, CA) were used as primary antibodies. To verify equal loading, blots were always probed with a mouse monoclonal antibody to β-actin (from Sigma-Aldrich), diluted 1:1000.

Western blotting was also performed on osteoblasts, isolated from PT patients incubated at 37°C as follows: untreated or activated with IL-1β (100 U/ml) (specific activity 10^7 U/mg) + TNF-α (500 U/ml) (specific activity 10^8 U/ml) for 24 h.

Electron Microscopy Immunocytochemistry

Osteoblasts (6×10^5) obtained from subchondral femoral heads of RA, OA, and PT patients were fixed 24 h after seeding with 1% glutaraldehyde in 0.1 M phosphate buffer for 40 min at 4°C, dehydrated up to 70% ethanol, and embedded in London Resin White (LR White) at 0°C. Thin sections were incubated for 10 min at room temperature in 0.05 M Tris-Cl, pH 7.6, 0.14 M NaCl, and 0.1% BSA (TBS I), preincubated for 30 min at room temperature with 5% normal goat serum (NGS) in TBS I, and then incubated overnight at 4°C with the following primary antibodies: anti-PKC-ε, -θ, -ζ, and -μ polyclonal antibodies (Santa Cruz Biotechnology), diluted 1:40, 1:30, 1:40, 1:20, respectively, in TBS I. After several washes in TBS I, the grids were washed in 0.02 M Tris-Cl, pH 8.2, 0.14 M NaCl, and 0.1% BSA (TBS II) and incubated with a goat anti-rabbit (GAR) conjugated with 15 nm colloidal gold particles (Amersham Biosciences) diluted 1:10 in TBS II for 1 h at room temperature. After several washes in TBS II and in distilled water, the gold particles were amplified with Silver Enhancer Kit (Amersham Biosciences).

Controls consisted of samples not incubated with the primary antibody: no gold particles were detected (data not shown). Before observation with a Zeiss EM 109 electron microscope (EM), thin sections were stained with aqueous uranyl acetate and lead citrate.

Immunocytochemistry was also performed on osteoblasts isolated from PT patients treated at 37°C as follows: untreated or activated with IL-1β (100 U/ml) (specific activity 10^7 U/mg) + TNF-α (500 U/ml) (specific activity 10^8 U/ml) for 24 h.

Quantitative Evaluation

At least five micrographs at the same magnification were obtained for each sample. The labelling density (mean of gold particles number/$\mu m^2 \pm$ SE) was determined. The Kruskal–Wallis non-parametric test followed by Dunn's test for post-hoc multiple comparison were used to compare the labelling in the nucleus or in the cytoplasm of osteoblasts from PT, RA, and OA patients.

Comparison between controls and in vitro-treated PT cells were performed using the Mann–Whitney test. P values less than 0.05 were considered significant.

RESULTS

Western Blot Analysis

Western blots of total homogenates of osteoblasts isolated from PT, RA, and OA patients were performed using antibodies against all the PKC isoforms. To rule out that changes in the expression pattern of PKC isoforms were not due to proliferation/differentiation, we employed differentiated osteoblasts which were cultured for only 24 h after seeding. Indeed, our previous results have shown that under these conditions, changes in the proliferation rates were detected only after 72 h [Lisignoli et al., 1999]. Nine isoforms were detected: -α, -β_I, -β_{II}, -δ, -θ, (80 kDa), -ζ (72 kDa), -ι/λ (74 kDa), -ε (95 kDa), and -μ (115 kDa) (Fig. 1). The other two PKC isoforms were undetectable (data not shown). In RA and OA four isoforms presented differences with respect to PT cells; in fact, PKC-θ and -μ were increased whereas PKC-ε and -ζ decreased. Anti-β-actin antibody demonstrated equal loading. Therefore, quantitative differences in PKC isoform subcellular distribution were investigated by immuno-electron microscopy.

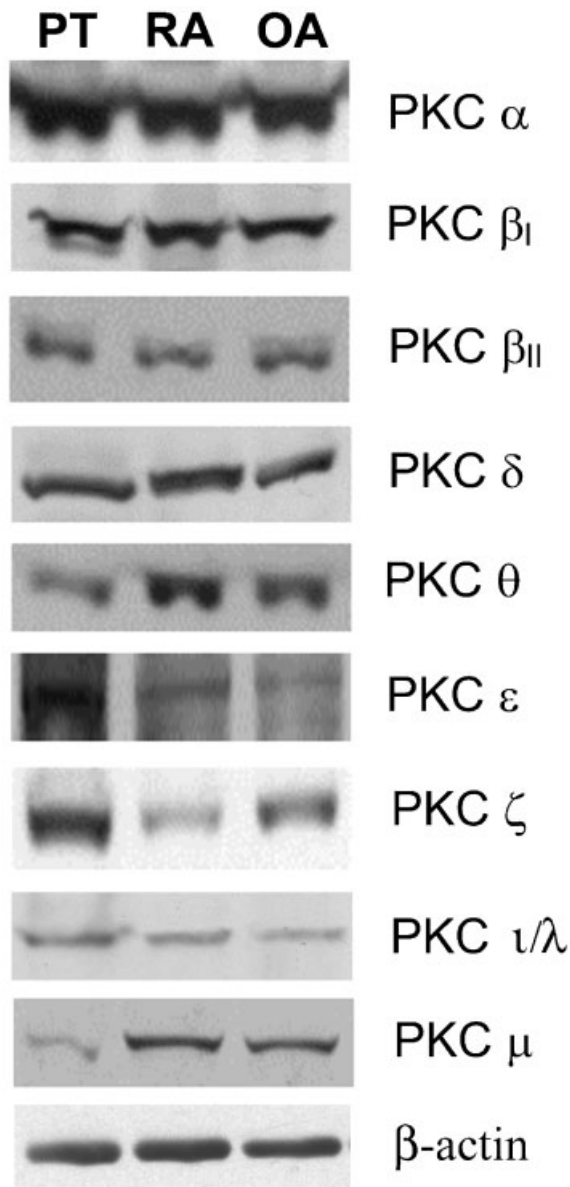

Fig. 1. Western blot analysis of PKC isoforms expressed in PT, RA, and OA osteoblasts. The immunoblots are representative of three different experiments. Anti-β-actin antibody demonstrated equal loading of protein in each lane. PT, post-traumatic after fall; RA, rheumatoid arthritis; OA, osteoarthritis.

PKC -θ, -μ, -ε and -ζ Quantitative Immuno-Electron Microscopy

Immunolabelling at the EM level was performed on thin sections of specimens partially dehydrated and embedded in LR White at low temperature. LR White has been demonstrated to allow a better antigen detection with respect to epoxy resin. Due to the absence of osmium fixation and to the characteristics of

the hydrophilic resin, membranes were not electron-dense.

The labellings with anti-PKC-θ, -μ, -ε, and -ζ appeared to be localized within the cytoplasm, diffused throughout cytosol, and in the interchromatin domain of the nucleus, whereas heterochromatin and nucleolus were scantly labelled (Figs. 2a–c, e–g, 3a–c, e–g).

The quantitative analysis of PKC-θ and -μ label density showed an increase in RA and OA osteoblasts, when compared with PT samples (Fig. 2d,h), whereas PKC-ε and -ζ decreased (Fig. 3d,h). In particular, PKC-θ label density analysis showed a significant increase in the cytoplasm of RA and OA cells ($P < 0.05$), with respect to PT cells; no significative variation occurred in the nucleus (Fig. 2d). A significantly increased PKC-μ was observed in both nucleus and cytoplasm of RA and OA samples ($P < 0.05$), with respect to PT cells (Fig. 2h). Whereas, PKC-ε labelling showed a significative decrease in both nucleus and cytoplasm of RA and OA osteoblasts ($P < 0.05$) when compared to PT cells (Fig. 3d). Quantitative analysis of PKC-ζ label density showed that labelling decreased in both RA and OA samples, and a significant difference with respect to PT samples was observed in the cytoplasm of RA osteoblasts ($P < 0.05$), whereas no significant variation occurred in the nucleus (Fig. 3h).

Effect of IL-1β and TNF-α on PKC-θ, -μ, -ε, and -ζ immunodetection in PT Osteoblast Culture

The variation of PKC-θ, -μ, -ε, and -ζ were evaluated by Western blot and electron microscopy immunocytochemistry (EMI) in PT osteoblasts cultured in vitro after 24 h of treatment with IL-1β and TNF-α. Difference with respect to PT untreated osteoblasts was obtained only for the PKC-ζ isoform, both in Western blot (Fig. 4d) and in immuno-electron microscopy (Fig. 4a,b). The EMI showed that the decrease evidenced by Western blot analysis in treated samples occurred both in the cytoplasm and nucleus. Quantitative evaluation of immunogold distribution indicated a significant decrease in the nucleus ($P < 0.05$) and cytoplasm

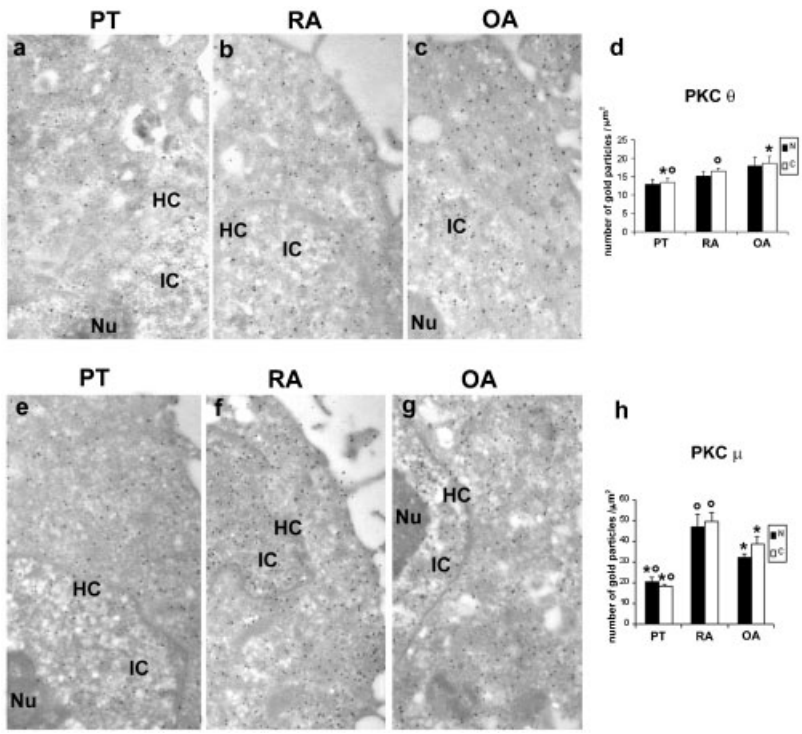

Fig. 2. PKC-θ and PKC-μ electron microscopy immunocytochemistry in PT, RA, and OA osteoblast cultures. **a–d**: PKC-θ immunolocalization and quantitative evaluation of the labelling density in the nucleus (N) and in the cytoplasm (C); values are the means of total examined cases ± SE. Significant differences ($P < 0.05$): *PT versus OA cytoplasm; °PT versus RA cytoplasm. **e–h**: PKC-μ immunolocalization and quantitative evaluation of the labelling density; values are the means of total examined cases ± SE. Significant differences ($P < 0.05$): *PT versus OA nucleus and cytoplasm; °PT versus RA nucleus and cytoplasm. HC, heterochromatin; IC, interchromatin; Nu, nucleolus. a–c, e–g × 16,000.

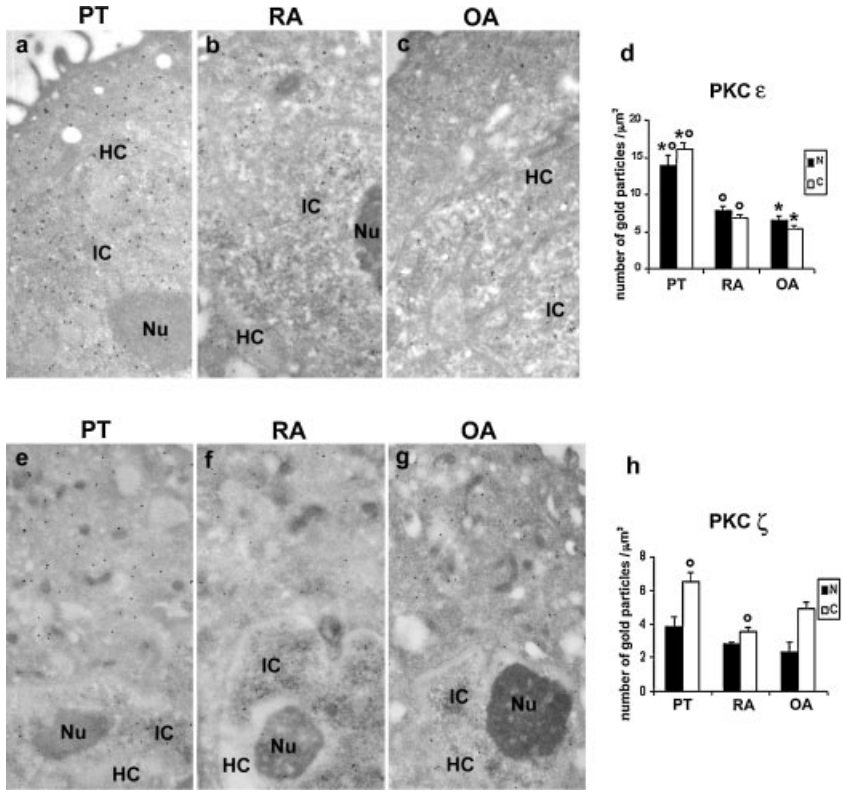

Fig. 3. PKC-ε and PKC-ζ electron microscopy immunocytochemistry in PT, RA, and OA osteoblast cultures. **a**–**d**: PKC-ε immunolocalization and quantitative evaluation of the labelling density; values are the means of total examined cases ± SE. Significant differences ($P < 0.05$): *PT versus OA nucleus and cytoplasm; °PT versus RA nucleus and cytoplasm. **e**–**h**: PKC-ζ immunolocalization and quantitative evaluation of the labelling density; values are the means of total examined cases ± SE. Significant differences ($P < 0.05$): °PT versus RA cytoplasm. a–c, e–g × 16,000.

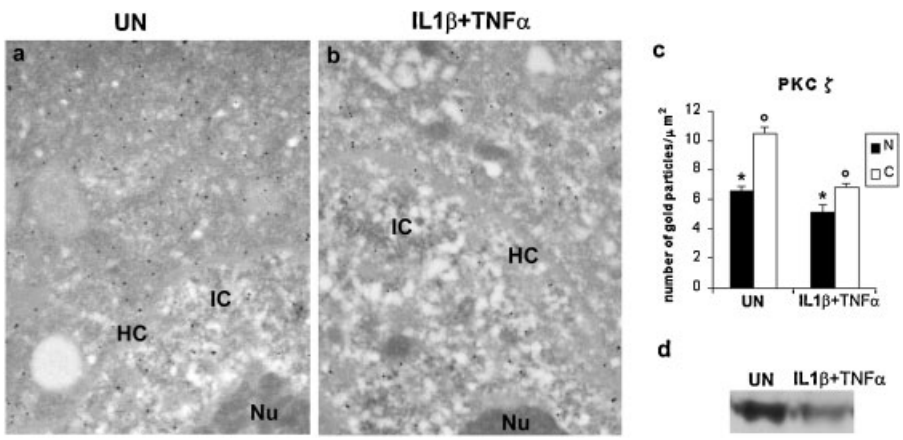

Fig. 4. PKC-ζ detection in PT osteoblast cultures in the absence (UN) or in the presence of IL-1β and TNF-α. **a**–**b**: Electron microscopy immunocytochemistry, **c**: Quantitative evaluation of the labelling density; values are the means of three experiments ± SE. Significant differences: *UN versus treated cell nucleus, ($P < 0.05$); °UN versus treated cell cytoplasm, ($P < 0.005$). **d**: Western blot. a,b × 16,000.

($P < 0.005$), with respect to untreated cells (Fig. 4c).

DISCUSSION

RA and OA differ in their pathogenesis, although both diseases are characterized, at least in the late phase, by significant alteration of cartilage which is usually associated with bone remodeling.

Several reports have addressed the issue of PKC isoform expression in normal bone, while scarce evidence is available regarding changed in expression of PKC isozymes during pathological conditions. It has been reported that PKC-α plays a very important role in primary human osteoblast proliferation [Lampasso et al., 2002], whereas PKC-μ expression changes during murine preosteoblast cell line MCT3-E1 differentiation [Cheung et al., 2006]. Moreover, changes in some PKC isoform distribution have been linked with osteoblast mechanical stress in UMR-108 cells [Geng et al., 2001].

In this study, we demonstrated by Western blot that human osteoblasts, isolated from subchondral bone of OA, RA, and PT patients, expressed nine, -α, -β_I, -β_{II}, -δ, -θ, -ε, -ζ, -ι/λ, and -μ, of the 11 PKC isoforms evaluated. We did not observe the presence of -γ and -η isoforms. Sanders and Stern [1996] also reported the absence of -γ isoform in normal mouse osteoblasts and in osteoblastic cell lines (rat and human), and the presence of -η isoform in only selected cell lines. Furthermore, these two isoforms were also not detected in normal human osteoblasts obtained from bone explants retrieved during oral surgery [Lampasso et al., 2001].

In accordance with our results on non-arthritic and arthritic osteoblasts, Lampasso et al. [2002] demonstrated that human osteoblasts obtained from mandibular bone explant expressed PKC-α, -δ, -ι. Moreover, they evidenced a direct involvement of PKC-α in the proliferation of primary human osteoblasts. This modulation was not observed in our samples even though we performed the experiments using low-proliferating osteoblasts cultured in the presence of serum.

Among the isoforms detected in our study, only PKC-θ, -μ, -ε, and -ζ presented differences in RA and OA with respect to PT osteoblasts. Quantitative immuno-gold labelling confirmed that in pathological conditions PKC-θ and -μ were greater expressed, whereas PKC-ε and -ζ decreased.

Interestingly, in articular chondrocytes of rabbit affected by OA, a redistribution of some PKC isoforms has been described [Satsuma et al., 1996] and decreased expression of PKC-ε was evident during progression of the disease, in agreement with our data on arthritic osteoblasts [Tanaka et al., 1998].

The decrease of PKC-ζ was already demonstrated in our previous report and it was found to be associated with decreased expression of PLC γ_1, phosphatidylinositol-3-kinase, and cell proliferation [Zini et al., 2005].

On the other hand, we demonstrated an increase of both PKC-θ and -μ in arthritic osteoblasts. It has been reported that prolonged activation of PKC pathways is sufficient to induce osteoblastic differentiation in the DMSO treated murine preosteoblast cell line MC3T3-E1, associated with increased PKC-μ [Cheung et al., 2006]. Activation of PKC-μ in turn requires upstream PKC-θ signaling, as previously suggested by Yuan et al. [2002] who demonstrated that presence of a constitutively activated PKC-θ induced PKC-μ activation in COS-7 cells and T lymphocytes. Cheung et al. [2006] did not observe changed PKC-θ expression in the murine preosteoblast cell line activated with DMSO, while Lampasso et al. [2006] using the same cell type activated by other factors found a decrease of this isoform. These data suggest that expression of this isoform is diversly modulated by different factors.

Our results showing an increased PKC-θ expression in arthritic osteoblasts could be due to the effect of factors, such as cytokines and chemokines, highly represented in the local milieu of OA and RA patients.

On the other hand, PT osteoblasts treated with proinflammatory cytokines, such as IL-1β and TNF-α, presented a decrease of the -ζ isoform with respect to untreated samples. By contrast, we did not found any modification of PKC-θ, -μ, and -ε suggesting that their modulation might not depend on IL-1β and TNF-α.

Therefore, the previously described decreased proliferation and reduced expression of PKC-ζ in osteoblasts of OA and RA, with respect to PT cells [Zini et al., 2005], could depend on the influence of these cytokines.

In Saos-2 cells, which display some osteoblastic characteristics [Rodan et al., 1990] and

present receptors for IL-1α and TNF-α, it was described that treatment with IL-1α induced variation in the amount and localization of some key elements of signal transduction mediated by polyphosphoinositides [Marmiroli et al., 1994; Zini et al., 1996]. Moreover, IL-1β and TNF-α have been reported to induce changes in the expression of PLC β_1 and phoshatidylinositol 4,5-bisphosphate, two key elements of the polyphosphoinositide signal transduction system, in normal osteoblasts, similar to those occurring in patients with RA [Zini et al., 2003].

In conclusion, this study provides a comprehensive description of PKC isoforms expressed in osteoblasts of non-arthritic and arthritic patients. The finding that the quantity of specific PKC isoforms changes in OA and RA osteoblasts when compared to PT patients and that the amount of one of them (-ζ) might depend on cytokine interactions, indicates that this family of protein kinases could be a potential target for therapeutic agents.

Studies involving PKC isoform overexpression and silencing are currently underway to better understand the signaling pathways controlled by this family of protein kinases during the evolution of arthritic disorders.

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