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PHENOTYPIC MODULATION OF HUMAN ARTICULAR CHONDROCYTES BY BISTRATENE A

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Abstract

Chondrocytes undergo phenotypic alterations following extended periods in monolayer culture, i.e., they become bipolar and flattened, proliferate, and synthesise type I as opposed to type II collagen. This process has been termed chondrocyte dedifferentiation. Bistratene A is a macrolide polyether that specifically activates the delta isoform of protein kinase C (PKCδ) in some cell types. Here, we show that dedifferentiated human articular chondrocytes became rounded and underwent cell growth arrest after treatment with bistratene A. In addition, bistratene A-treated chondrocytes became more immunopositive for type II collagen, but less immunopositive for type I collagen. These phenotypic changes were associated with a prior and extensive disruption of actin microfilaments and translocation of PKCδ to the nuclear membrane. Concurrent treatments of chondrocytes with a specific inhibitor of PKCδ, rottlerin, partially blocked the morphological effects of bistratene A.

Key Words: Bistratene A, protein kinase C, articular chondrocyte, cell shape and differentiation.

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Introduction

In vivo, most human articular chondrocytes display a spherical or oval morphology, are normally non-proliferative and characteristically express type II collagen. Following isolation and culture as monolayers, however, these cells adopt a flattened and elongated "fibroblast-like" shape, they enter cell cycle, proliferate, and cease synthesising type II collagen in favour of type I (von der Mark *et al.*, 1977; Benya *et al.*, 1978). Such behaviour, termed dedifferentiation, has been of use to clinicians and tissue engineers in that it provides an increased cell population during autologous chondrocyte transplantation procedures that promote the repair or replacement of damaged cartilage (Richardson *et al.*, 1999). However, in order to produce a hyaline-like cartilage, that consists of appropriate matrix molecules including type II but not type I collagen, the re-expression of a mature chondrocytic phenotype is required. Previous studies have shown that returning flattened chondrocytes to a spherical (rounded) cell shape induces their renewed maturation or redifferentiation, i.e., the cells become less proliferative and reexpress type II collagen (Benya and Shaffer, 1982). Mature chondrocyte growth has thus been encouraged by utilising alternative *in vitro* culture conditions, such as embedding cells within gels or through culture with fibrous materials too fine to permit the cells to flatten and spread (Benya and Shaffer, 1982; Freed *et al.*, 1994).

Despite its importance to cartilaginous tissue engineering, the mechanisms that regulate chondrocyte shape and differentiation *in vitro* remain relatively poorly understood. Several workers have suggested that the organisation of actin microfilaments within cultured chondrocytes is intrinsically linked to their shape and/or collagen synthesis (Benya *et al.*, 1988; Brown and Benya, 1988). In general, the formation of actin stress fibres in cells of the chondrogenic lineage appears to inhibit the expression of differentiated chondrocyte markers (Benya and Shaffer, 1982; Zanetti and Solursh, 1984; Mallein-Gerin *et al.*, 1991). However, the intracellular regulatory molecules that determine the structure of the actin cytoskeleton and chondrocyte shape are largely unknown. A number of studies have suggested that the delta isoform of protein kinase C (PKCδ) represents a likely target in the regulation of cell morphology. For example, over-expression of PKCδ in NIH 3T3 fibroblasts results in their rounding, growth inhibition and reduced adherance (Mischak *et al.*, 1993). In addition, both the focal adhesion kinase pp125FAK and PKCδ are recruited, along with the

cytoskeletal proteins talin, paxillin, and vinculin, to newly formed focal adhesions in fibroblasts in response to serum (Barry and Critchley, 1994). Furthermore, IL-3 withdrawal from Baf3 (B cell lymphoma) cells disrupts membrane ruffling and induces cell rounding, but overexpression of a dominant negative PKCδ (but not of other PKC isoforms) inhibits these effects, i.e., activation of PKCδ is required for the disruption of membrane ruffles (Romanova *et al.*, 1999).

Bistratene A (bis A) is a macrolide polyether toxin that specifically activates PKCδ in a variety of cell types (Watters *et al.*, 1996; Griffiths *et al.*, 1996; Watters *et al.*, 1998). In fibroblasts, bis A treatment induces reorganisation of the actin cytoskeleton, a rounded morphology and detachment from tissue culture substrates (Watters *et al.*, 1996), whilst in cultured melanoma cells treatment with bis A similarly alters cell morphology and induces onset of melanocyte differentiation (Watters *et al.*, 1998). Here, we describe the effects of bis A on the shape, growth and phenotype of cultured human articular chondrocytes.

Materials and Methods

Human articular chondrocytes were isolated from donor biopsies of macroscopically normal cartilage. These biopsies were obtained from patients (2 male, 5 female, ages 25-73 years) with osteochondral defects or ostearthritic lesions of other regions of the knee or hip. Tissue samples were finely dissected and chondrocytes obtained following enzymic digestion in type XI collagenase (C 9407, Sigma, Poole, UK) at a concentration of 0.8mg/ml in DMEM/F12 culture medium (Life Technologies Ltd, Paisley, Scotland) and DNAse (D4263 Sigma, 333U/ml in culture medium) for a period of 18 hours at 37°C. Digested samples were filtered through a 70μm mesh and obtained cells washed twice by centrifuging and resuspending in DMEM/F12, before seeding into flasks in DMEM/F12 medium (Life Technologies) supplemented with 20% foetal bovine serum (FBS, Life Technologies), L-ascorbic acid (50mg/ml, AnalaR, Merck Ltd, Lutterworth, UK) and antibiotics (50mg/ml Gentamicin; 2.5mg/ml Amphotericin). After 5 days of culture, any non-adherent cells were discarded, whilst adherent cells were cultured as monolayers in medium containing 10% FBS for a further 2-4 passages, at which time all cells exhibited a "fibroblast-like" morphology. Monolayered cells were passaged at 70% confluence using trypsin/EDTA solution (45300-019; Gibco, Life Technologies).

For each experiment, $5x10^3$ cells/cm^2 were seeded onto tissue culture slides (Nunc LabTek, Life Technologies) or in T25cm^2 tissue culture flasks (Life Technologies), permitted to adhere and spread for 24 hours, and then treated with medium containing either 100ng/ml bis A or, as control cultures, with carrier alone (DMSO dissolved 1/10 000 in DMEM/F12). Parallel flask cultures were treated with medium supplemented with the PKCδ-specific inhibitor, 5 mM rottlerin (Gschwendt *et al.*, 1994), alone and in addition to 100ng/ml bis A. Medium was completely replaced with fresh bis A-treated or control medium, as appropriate, after 3 days. Viable cell number was determined using trypan blue exclusion of trypsinised cultures and culture viability further assessed using incorporation of propidium iodide (PI, Sigma) and fluorescein diacetate (F-7378; Sigma) into unfixed cells, adapting methods previously described (Jones and Senft, 1985). Cells harvested by trypsinisation were used for cytocentrifuge preparations (cytospins) which were later analysed in some immunolocalisation studies (see below).

The appearance of cells in culture was recorded using phase contrast microscopy with an image capture system or by Jenner and Giemsa staining of harvested culture slides. Immunolocalisation of parallel culture slides was performed for PKCδ (1/500 polyclonal anti-human PKCδ, Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) using a secondary sheep anti-rabbit antibody conjugated with fluorescein (Vector Laboratories, Peterborough, UK). Filamentous actin was visualised following labelling of culture slides with fluorescein-conjugated phalloidin (Sigma). Cytospins of cells harvested from culture flasks, and day 5 culture slides, were used for immunolocalisation of the proliferation-associated Ki-67 antigen (1/50 Clone MM1, Novacastra, Newcastle-upon-Tyne, UK), type I collagen (1/100 Clone I-8H5, ICN, Basingstoke, UK) and type II collagen (1/10 monoclonal CIICI, Developmental Studies Hybridoma Bank, USA). Immunopositivity for these proteins was revealed using commercial secondary labelling systems (Vector Laboratories) combined with 3.3' diaminobenzidine (DAB, for the Ki-67 antigen, and types I and II collagen in day 5 cultures slides) or fluorescein (day 5 cytospins for types I and II collagen). All immunolocalisations using fluorescein for visualisation of immunopositivity were counterstained with a commercial mountant containing PI (Vector Laboratories), whilst those using DAB were counterstained with Meyer's haematoxylin. Control immunolabelling of parallel cultures and cytospins using isotype-matched antisera was negative. A minimum of 200 cells/sample were scored, using conventional light and fluorescent microscopy, to determine the proportions of cells in cytospins expressing the Ki-67 antigen, type I collagen or type II collagen. The relative pixel intensity of cells immunolabelled for types I and II collagen (where immunopositivity was revealed by fluorescein) was determined using a confocal microscope and associated software (LSM510, Zeiss, Welwyn Garden City, UK). All data given represent means ±standard errors of at least 3 independent experiments, i.e., using chondrocytes derived from at least 3 donors.

Results

Control articular chondrocytes exhibited a "fibroblast-like" morphology in monolayer, appearing flattened and bipolar throughout a 5-day time course. By contrast, following the addition of bis A-supplemented medium, flattened chondrocytes began retracting their extended cell processes and adopted a rounded morphology. Cell process retraction was observed 20-30 minutes after the addition of bis A to the culture medium and by 60 minutes, all bis A-treated chondrocytes had become rounded but

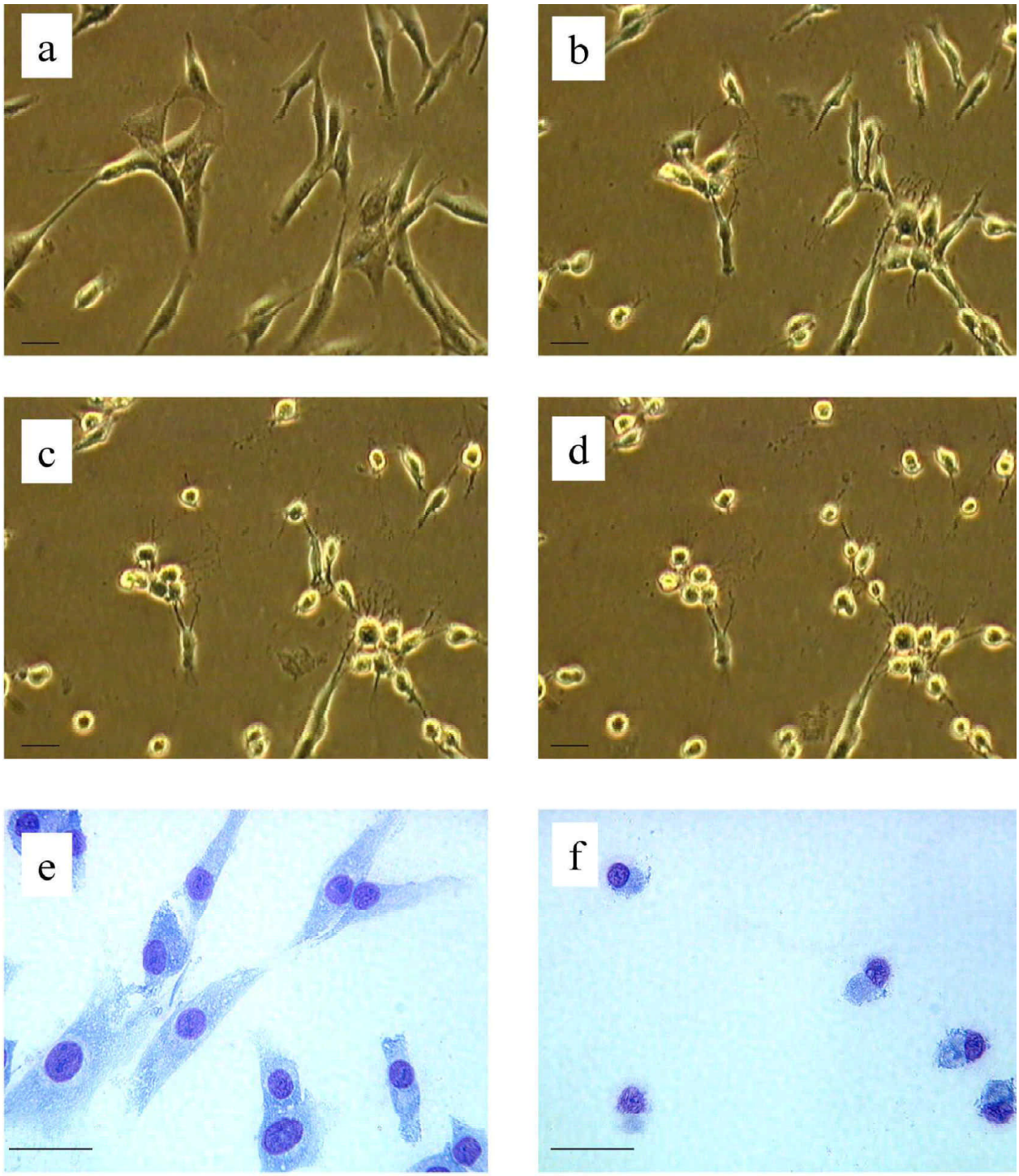

Figure 1. Bis A induces a rounded morphology in monolayered chondrocytes. Representative images of the appearance of chondrocytes prior to bis A treatment (a) and following 20 (b), 40 (c) and 60 (d) minutes of treatment with 100ng/ml bis A. Jenner/Giemsa staining of control cells (e) and bis A-treated cells (f) at day 5. Bar = 20µm.

remained attached to culture substrates (Fig. 1a-d). This rounded cell shape was maintained in bis A-treated cultures thereafter (Fig. 1e-f). Control chondrocytes proliferated, increasing in cell number from a seeding density of $5x10^3$ cells/cm^2 to $27.7\pm3.3x10^3$ cells/cm^2 by day 5. However, there was no marked increase in chondrocyte confluency or cell number in bis A-treated cultures. Thus, cultures established at a seeding density of $5x10^3$ cells/cm^2 were still only at $7.4\pm0.5x10^3$ cells/ cm^2 by day 5 after the addition of bis A (Fig. 2a). Immunolabelling of cytospins for the proliferation-associated Ki-67 antigen further demonstrated that whilst a large proportion of control cells were proliferative, bis A-treated cells soon exited from cell cycle (Fig. 2b-d). At day 1, 57±3 % of

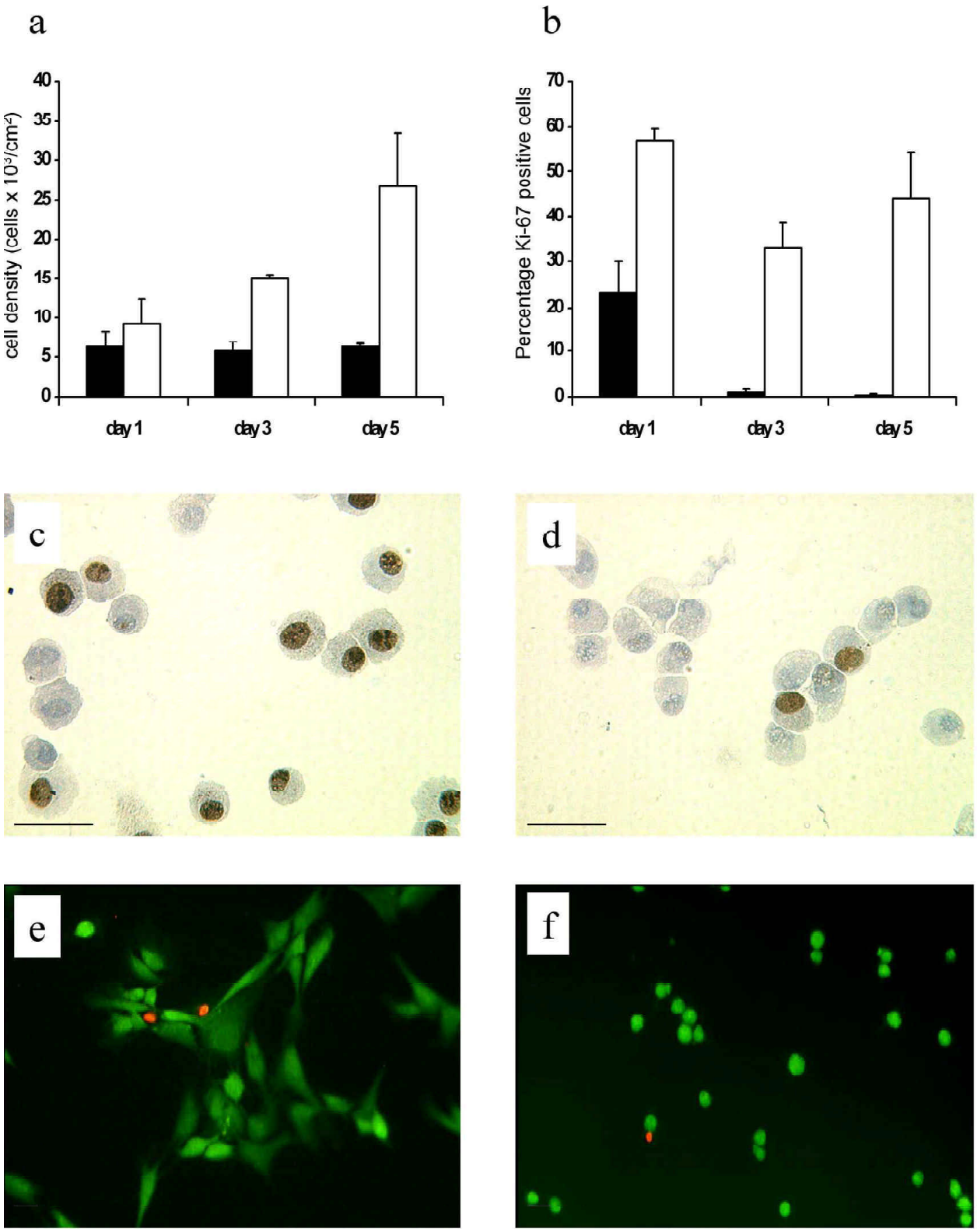

Figure 2. Bis A-induced chondrocyte growth arrest. (a). the density of viable chondrocytes following treatment with carrier alone (control, open bars) or 100ng/ml bis A (treated, closed bars) for the times indicated. (b). the proportion of control and treated chondrocytes immunopositive for the Ki-67 antigen at times indicated. Data shown are means±standard errors of at least 3 independent experiments. Representative images Ki-67 immunolabelled cytospins of control (c) and bis A-treated chondrocytes (d) at day 1 are also shown. FDA/PI incorporation in chondrocytes demonstrated that the majority of cells in both control (e) and bis A-treated (f) cultures remained viable at day 5 (viable cells appear green, non-viable cells appear red). Bar = 20µm

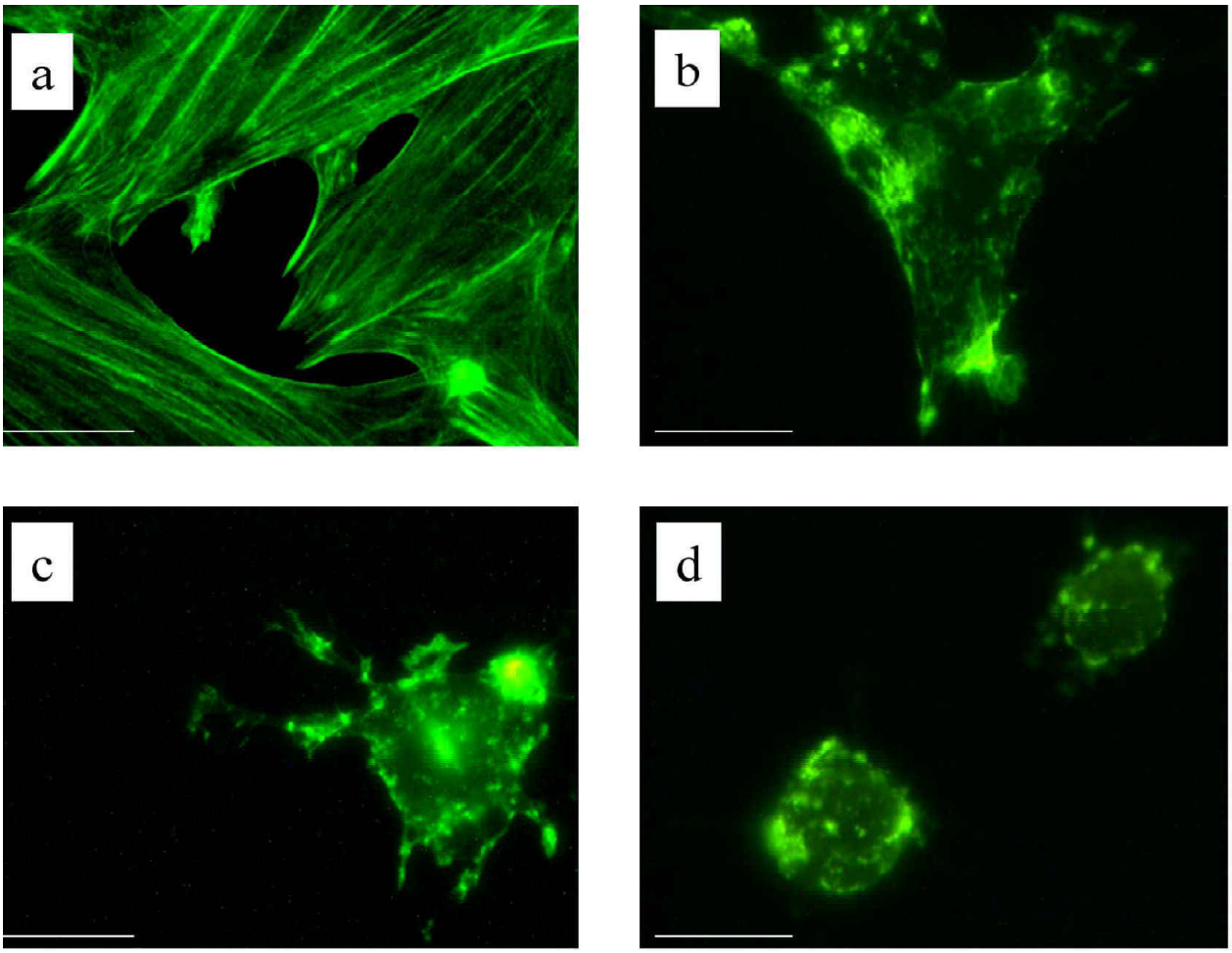

Figure 3. Actin microfilaments are profoundly disrupted by bis A treatment prior to cell rounding. Representative images are shown of the appearance of actin microfilaments within control chondrocytes (a) and in 100ng/ml bis A-treated chondrocytes after 10 (b), 20 (c) and 60 (d) minutes. Bar = 20μm

control cells were Ki-67-immunopositive, compared with 27±7 % of bis A-treated cells and by day 3, virtually all bis A-treated cells were immunonegative for the Ki-67 antigen. No cells were detected in culture medium throughout the 5-day time course, indicating that all control and bis A-treated cells remained adherent. Furthermore, cell viability, as determined by trypan blue exclusion and labeling with FDA/PI, remained greater than 95% in all cultures throughout the 5-day time course (Fig. 2e-f). Thereafter, the cell viability of bis A–treated cultures decreased below 90%. Thus, the initial response of human articular chondrocytes to bis A-treatment was to adopt a rounded morphology and undergo cell growth arrest.

In control cultures, chondrocytes exhibited an F-actin cytoskeleton consisting of numerous, pronounced stress fibres running throughout the cell, parallel to each other or to the cell membrane of extended processes (Fig. 3a). By contrast, after treatment with bis A for only 10 minutes, at a time when the treated chondrocytes were still flattened and spread, these stress fibres were no longer clearly apparent and a more diffuse patch-like pattern of F-actin positivity was observed (Fig. 3b). By 20 minutes, the bis A-treated cells had begun retracting processes and punctate F-actin staining was seen in foci throughout the cell (Figure 3c). These foci remained, apparently in association with the cell membrane, in rounded cells following 60 minutes of bis A treatment (Fig. 3d). It appeared, therefore, that the bis A-induced changes in chondrocyte morphology were associated with a prior and extensive disruption of the F-actin cytoskeleton.

Activation of PKCδ following bis A treatment has previously been shown to result in rapid translocation of the enzyme from the cytoplasm to the nuclear membrane (Watters *et al.*, 1996; Griffiths *et al.*, 1996). Immunolocalisation of harvested culture slides demonstrated that both control and bis A-treated chondrocytes were immunopositive for PKCδ. In control cells, a diffuse pattern of immunoposivity was observed throughout the cytoplasm. However, increased PKCδ immunopositivity at the nuclear membrane of treated cells was seen within 60 minutes of the addition of bis A-supplemented medium (Fig. 4a-b). Concurrent treatment of chondrocytes with 100ng/ml bis A and concentrations of rottlerin (5mM) that specifically inhibit PKCδ, partially blocked the retraction of cell processes and cell rounding, resulting in cells that remained elongated and bipolar after 60 minutes of treatment (Fig. 4c-d), whilst treatment

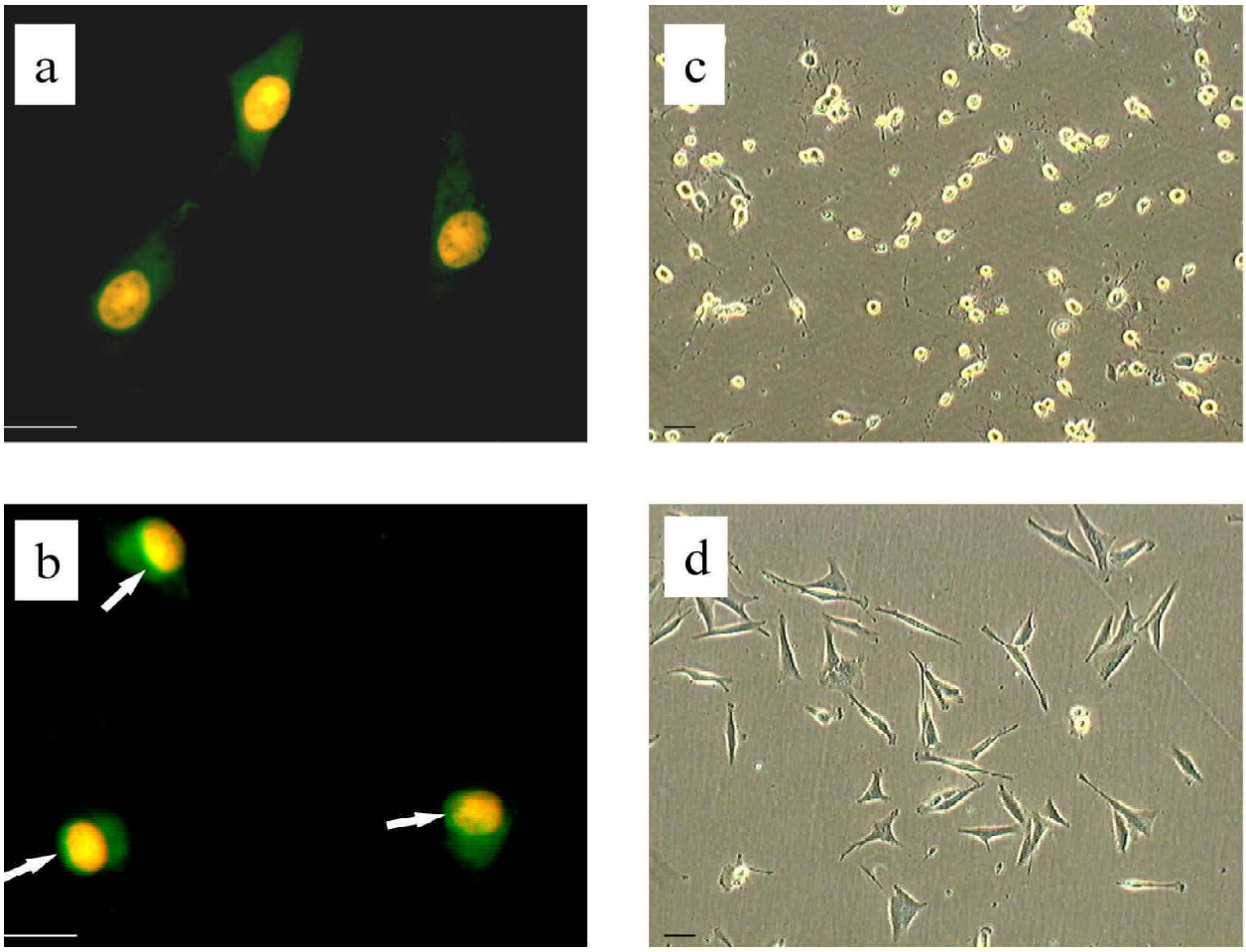

Figure 4. Evidence for PKCδ activation in bis A-induced chondrocyte rounding. Representative images of PKCδ immunostaining in control (a) and bis A-treated (b) chondrocytes at 60 minutes. Increased immunopositivity is evident at the nuclear membrane (arrowed) in bis A-treated cells. Also shown are representative images of the appearance of chondrocytes following 60 minutes of treatment with 100ng/ml bis A (c) or following concurrent treatment with 100ng/ml bis A and 5mM rottlerin (d). Bar = 20μm

of flattened chondrocytes with rottlerin alone (at 5mM) had no effects on morphology.

After 5 days of culture, cytospins of harvested control chondrocytes were either immunonegative or weakly positive for type II collagen. By contrast, type II collagen immunopositivity was observed in day 5 cytospins of all cultures of bis A-treated cells, with a marked increase in pixel intensity, as determined using confocal microscopy of these flattened cell preparations, evident in some of the fluorescein-immunolabelled cells (Fig. 5a-d). The proportions of such strongly immunopositive cells in bis A-treated and control cultures at this time point were 16±5.6 % and 1.7±0.4%, respectively. Immunopositivity for type II collagen in day 5 culture slides, revealed with DAB, appeared to be intracellular, distributed diffusely within the cytoplasm, and thus was likely to be for type II procollagen. There was no indication of extracellular type II collagen deposition (Fig. 5e-f). Conversely, immunopositivity for type I collagen was most marked and prevalent in cytospins of control chondrocytes at day 5, compared with cytospins of bis A-treated cells. Similarly, the relative pixel intensity of type I collagen immunolabelled cells was seen to decrease in harvested bis A-treated cells in comparison with control cells (Fig. 6a-d). Whilst 76.3±10.5 % of control cells were strongly immunopositive for type I collagen at day 5, only 13.2±3.1% of bis A-treated cells were similarly immunopositive. Type I collagen immunopositivity of day 5 culture slides also appeared to be intracellular, distributed in a punctate pattern throughout the cytoplasm, and thus was most likely to be for type I procollagen (Fig. 6e-f). There were no apparent differences in any of the reported behaviour of the cultured chondrocytes in response to bis A treatment that related to biopsy location, donor age, sex or pathology.

Discussion

To further our understanding of regulatory mechanisms that affect the differentiation status of human articular chondrocytes, we have examined the effects of treating cultured cells with a specific activator of PKCδ, bistratene

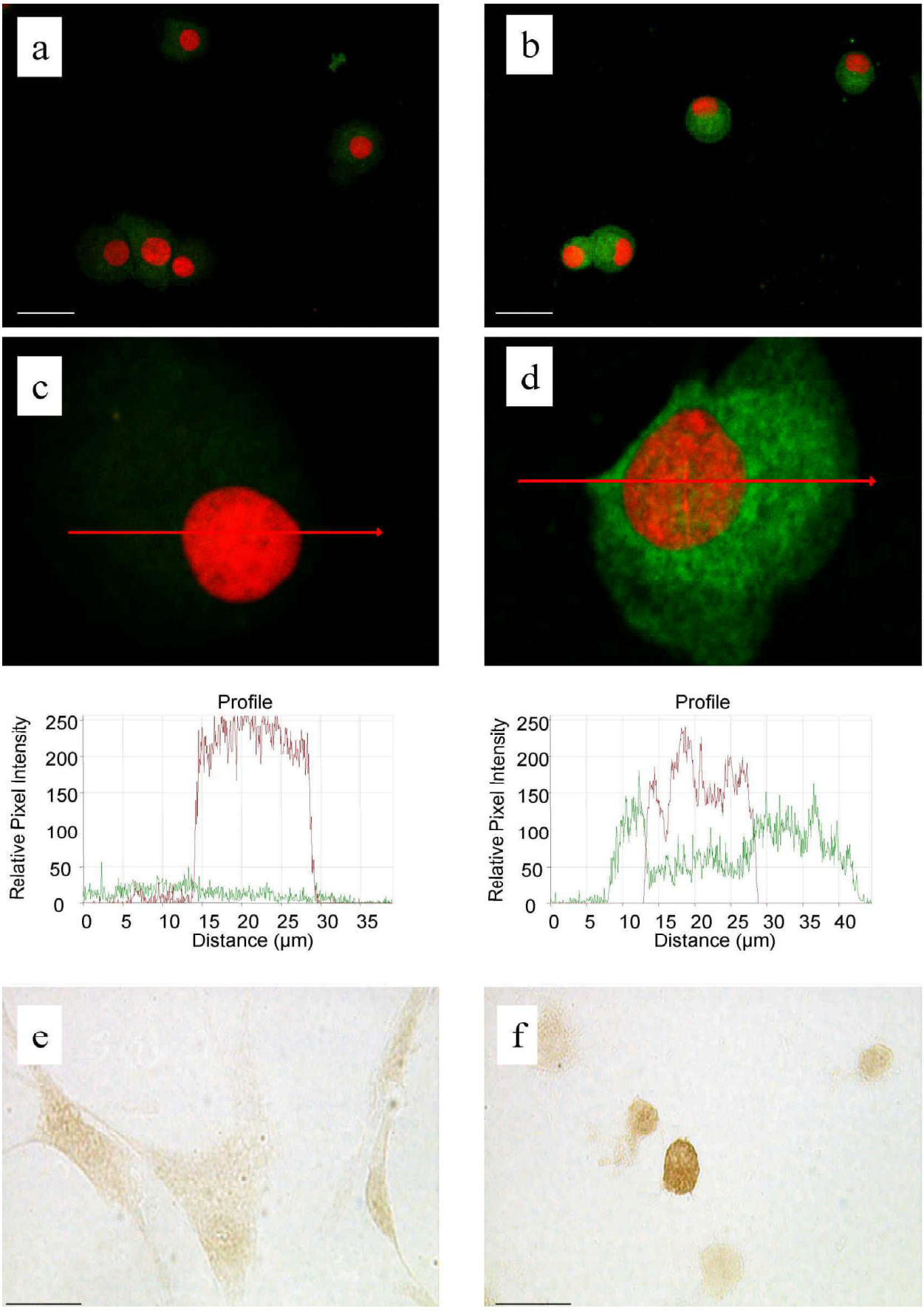

Figure 5. Type II collagen immunopositivity increases following bis A treatment. Control (a and c) and 100ng/ml bis A-treated (b and d) chondrocytes following type II collagen immunolocalisation of day 5 cytospins: the relative pixel intensity through the transecting lines of representative fluorescein-immunolabelled cells are shown in the bottom panels of c and d, where green is fluorescein-label and red is PI. The intracellular immunolocalisation apparent in day 5 control (e) and bis A-treated (f) culture slides suggests that type II procollagen was detected. Bar = 30µm

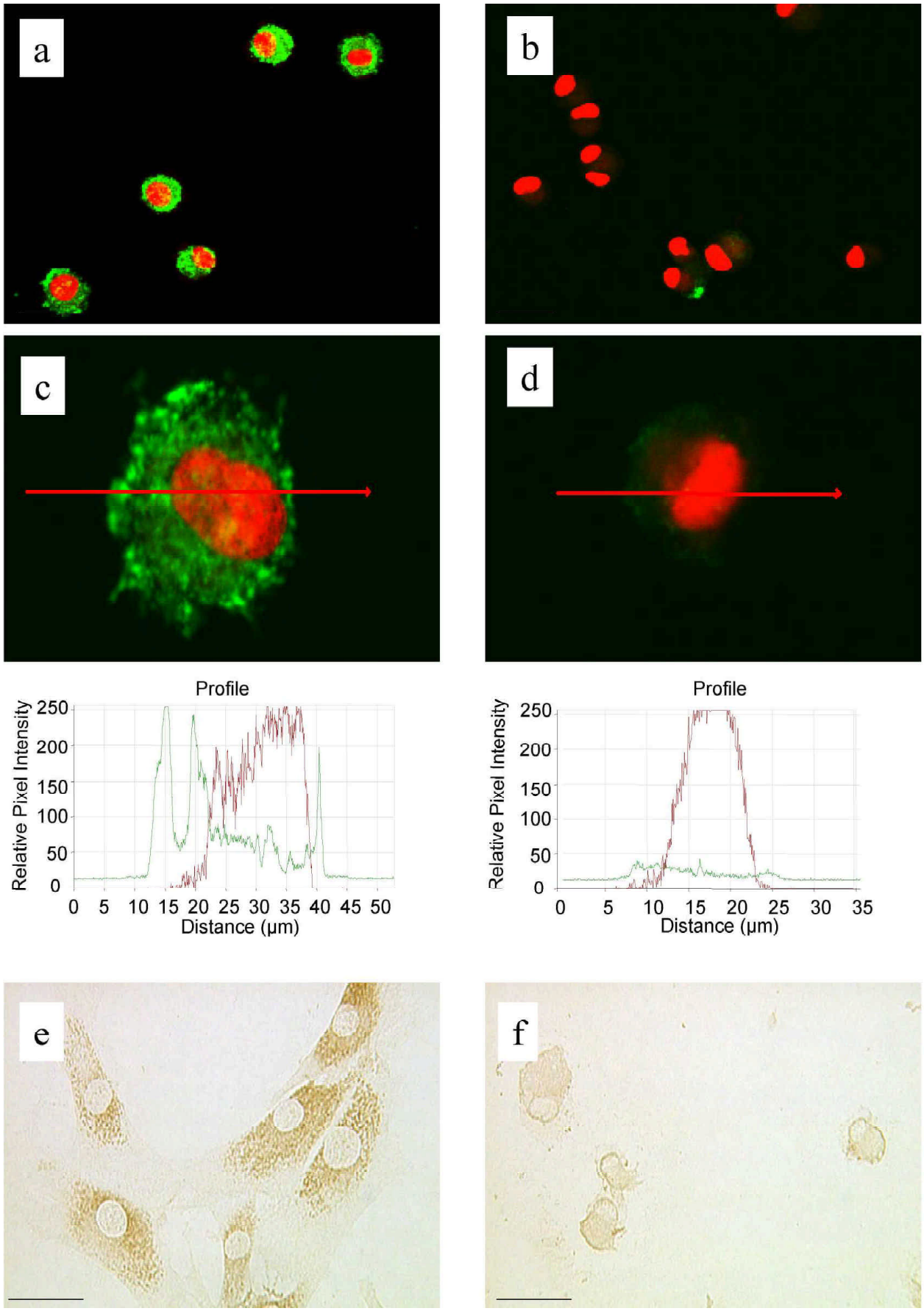

Figure 6. Type I collagen immunopositivity decreases following bis A treatment. Control (a and c) and 100ng/ml bis A-treated (b and d) chondrocytes following type I collagen immunolocalisation of day 5 cytospins: the relative pixel intensity through the transecting lines of representative fluorescein-immunolabelled cells are shown in the bottom panels of c and d, where green is fluorescein-label and red is PI. The intracellular immunolocalisation apparent in day 5 control (e) and bis A-treated (f) culture slides suggests that type I procollagen was detected. Bar = 30µm

A (bis A). The responses of these cells in monolayer culture to such treatment were very similar to those reported for primary human fibroblasts (Watters *et al.*, 1996), with a loss of actin stress fibres and cell rounding. In contrast to this previous study, however, human chondrocytes remained firmly adherent when rounded. The bis A-induced morphological changes were associated with chondrocyte growth arrest and an increase in immunopositivity for the differentiated chondrocyte marker, type II collagen. Conversely, type I collagen immunopositivity was markedly less intense (or absent) in bis A-treated cells in comparison to control cells. Immunopositivity for both of these markers appeared to be intracellular and therefore was most likely to represent antibody binding to the procollagen forms. In addition, we have shown that bis A-treatment was associated with a translocation of PKCδ to the nuclear membrane. Identical translocations of the activated enzyme have previously been shown in human fibroblasts and leukemic cells following bis A treatment (Watters *et al.*, 1996; Griffiths *et al.*, 1996). Furthermore, the morphological effects of bis A on chondrocytes were abrogated by concurrent treatment with rottlerin, at doses that specifically inhibit the delta isoform of the PKC family (Gschwendt *et al.*, 1994). Taken together, these observations suggest that the morphological effects of bis A on cultured chondrocytes were associated with PKCδ activation.

To our knowledge, this study is the first to identify PKCδ as having a regulatory role in determining chondrocyte shape; however, further studies are required to determine the relationship between PKCδ activation and the subsequent chondrocyte growth arrest and alterations in collagen expression. Not least, PKCδ is an important regulator of apoptosis in other cell types, notably neutrophils (Pongracz *et al*., 1999; Webb *et al*., 2000; Cross *et al*., 2000). In the present study, chondrocyte viability remained greater than 95% thoughout the experimental time course, but subsequently decreased in bis A-treated cultures. The growth arrest and alterations in collagen expression of bis A-treated chondrocytes may therefore represent part of a "default" response of these cells that is associated with inductions of cell death, rather than being part of any physiological, maturation pathway. Conversely, as PKCδ activation has also been associated with increased cell maturation in a number of systems (Denning *et al*., 2000; Watters and Parsons, 1999; Watters *et al*., 1998), the decreased viability of chondrocytes following bis A-treatment may occur independently or even as a consequence of this maturation. To some extent, the findings are thereby limited by the use of pharmacological agents, where pleiotropic effects may operate. Nonetheless, this study implicates PKCδ activation in the regulation of chondrocyte gene expression.

In mesenchymal limb bud cultures, activation of different PKC isoforms, notably the alpha isoenzyme (PKCα), is both associated with and required for chondrocyte maturation (Yang *et al.*, 1998; Choi *et al.*, 1995). Treatments of developing mesenchyme with PKC inhibitors or with doses of phorbol ester that down-regulate PKCα inhibit chondrogenesis, and these effects are mediated through increased Erk-1 activity and are associated with increased and prolonged expression of alpha5beta1 integrins (Chang *et al.*, 1998). Chondrogenesis of cultured chick limb mesenchymal cells, delineated by collagen II upregulation and cell rounding, has also previously been demonstrated following treatments with the actin microfilament disrupting agent, dihydrocytochalasin B (Zanetti and Solursh, 1984). Interestingly, it has recently been shown that such treatment results in PKCα upregulation and activation, Erk-1 inhibition and then type II collagen expression (Lim *et al.*, 2000). An intriguing report has also recently shown that ectopic overexpression of PKCα in Baf3 cells results in increased endogenous synthesis of PKCδ, but has no effect on expression of the epsilon, eta or mu isoforms (Romanova *et al.*, 1998). This study thereby demonstrates specific cross-talk between the alpha and delta isoforms of PKC that may regulate cell shape. The bis A-induced alterations in chondrocyte behaviour reported here may, therefore, provide a new model system to study an as yet unresolved pathway, potentially involving cytoskeletal and signalling components, that regulates chondrocyte differentiation in vitro.

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