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Increased apoptosis in human knee osteoarthritis cartilage related to the expression of protein kinase B and protein kinase Cα in chondrocytes

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Abstract: Protein kinase B (Akt) and protein kinase Cα (PKCα) play important roles in the regulation of cell apoptosis. The aim of this study was to investigate the expression of Akt and PKCα in chondrocytes of human knee osteoarthritic (OA) cartilage, further evaluating their role in chondrocyte apoptosis during OA progression. Human knee OA cartilages were obtained from 38 patients undergoing knee arthroplasty, which is the medium-late stage of OA. Healthy knee cartilages were obtained from 11 amputees. The samples taken from the condyle of femur were collected routinely for morphological, immunohistochemical and Western blot detection, respectively. Light microscopy and laser-scanning confocal microscopy were used for morphological observation. The optical density with computer image analysis evaluated the intensity of immunohistochemical reaction of Akt and PKCα in OA cartilage. Western blot detected the protein expression levels. The results indicated that Akt and PKCα were involved in OA progression, along with the increase of cell apoptosis. In OA cartilage, Akt decreased ($p < 0.05$) and PKCα increased ($p < 0.05$). There was a negative correlation and interaction between Akt and PKCα ($r = –0.8$). These results demonstrated that both Akt and PKCα are related to increased chondrocyte apoptosis in human OA cartilage. The correlation between human OA progression, the role of Akt and PKCα, and chondrocyte apoptosis allows for new therapeutic strategies to be considered. *(Folia Histochemica et Cytobiologica 2012, Vol. 50, No. 1, 137–143)*

Key words: Akt, PKCα, correlation, chondrocytes, human OA cartilage

Introduction

Osteoarthritis (OA) is characterized by a slow focal destruction of articular cartilage, causing a roughening and thinning of the weight-bearing regions of the articular surface, resulting in progressive immobility and pain. Chondrocytes, as only a single cell type in articular cartilage, is responsible for the homeostasis of the tissue by synthesizing extracellular matrix that surrounds the cells and provides the important biophysical characteristics of the tissue [1]. Recently, several studies have provided evidence that chondrocyte apoptosis and dedifferentiation play an important role in OA [1, 2]. In addition, apoptosis has been positively correlated with the severity of cartilage destruction and with matrix depletion in human specimens [3]. However, the intrinsic signaling pathways regulating cell apoptosis are not fully understood.

Protein kinase C (PKC) is a subfamily of serine/threonine kinases involved in the regulation of various cell responses, such as cell proliferation, differentiation, and apoptosis. PKCα, one of 12 types of PKC isoforms, mediates a number of cellular fates in some mammal cell lines, including normal articular chondrocytes [4]. In addition, Akt (also known as protein kinase B), another subfamily of serine/threonine

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kinases, has emerged as a crucial regulator of diverse cellular processes, such as apoptosis, survival, proliferation, differentiation and metabolism [5–7]. For instance, activated by a variety of stimuli through a phosphorylation mechanism, Akt is known to be a potent inhibitory signal for apoptosis in several kinds of cells [5–8]. Especially, Akt1 in osteoblasts and osteoclasts controls bone remodeling [8] and the actin cytoskeleton mediates NO-induced regulatory effects in chondrocytes by modulating the down-regulation of PI3K-Akt [9]. Taken together, both PKCα and Akt are involved in the regulation of cellular fates. However, it is not clear whether PKCα and Akt also regulate the cell responses of chondrocytes in OA cartilage such as cell apoptosis, which is one of the crucial elements in OA progression.

The present study was undertaken to investigate whether PKCα and Akt signal pathways play roles in OA progression, with chondrocyte apoptosis occurring in OA cartilage, and, if so, further to investigate the correlation between them.

Our results indicated differing roles for Akt and PKCα, along with an increase of chondrocyte apoptosis in OA cartilage, and there was a negative correlation and interaction between Akt and PKCα. This study might provide a molecular basis for studying the regulatory mechanism of chondrocyte apoptosis in human OA cartilage, and a novel target for the treatment of OA.

Materials and methods

Human articular cartilage. After approval by the Ethical Committee of Zhongshan hospital, Xiamen University, human OA cartilage was obtained from 38 patients (55–67 years, average age 63.2 years) undergoing knee arthroplasty, which is the medium-late stage of OA (Mankin score 8–12, average value 10.3) [10]. Healthy knee cartilage was obtained from 11 amputees (31–48 years, average age 43.1 years), of whom four had been amputated because of bone tumor, and seven had been amputated due to trauma. All samples were taken from the condyle of femur. Each sample was divided into two parts: one was fixed in paraformaldehyde and embedded for observation using a light microscope and a laser-scanning confocal microscope, and the other was preserved in the freezer (at –80°C) for protein detection.

Histomorphology. The sample was fixed in 4% paraformaldehyde for 48 hr, decalcified in 15% EDTA (pH 7.0, 37°C) for two weeks, and then paraffin-embedded for further routine histological preparation. Serial frontal 4 μm sections were observed under a light microscope in order to evaluate matrix abundance and cellularity after hematoxylin and eosin staining. In normal articular cartilage, the zones are easy to delineate, and their cells are clearly distinguishable, including four zones: superficial zone, transitional zone, radical zone, and calcification. Since the superficial cartilage zone had been substantially destroyed in the OA group, only the chondrocytes in the transitional zone and the radical zone were observed in this study.

Immunohistochemistry. As described in the manufacturer's instructions (MAIXIN.BIO China), the sections were incubated overnight at 4°C with primary antibody: Akt (1:100 dilutions, Cell Signaling Technology, Danvers, MA, USA) or PKCα (1:150 dilutions, Cell Signaling Technology, Danvers, MA, USA) and subsequently, with secondary antibody (1:400) for 60 min, respectively. Diaminobenzidine (DAB) was used to visualize the immunohistochemical reaction, followed by counterstaining with hematoxylin. Finally, dark brown cells were considered to be positive under a light microscope and were counted throughout the microscopically magnified fields (× 400) of each articular cartilage section. According to previous studies [11–13], the average optical density (OD) of cells is the symbol of protein expression, so it was detected by ImagePro Plus6.0 system in each cartilage zone, followed by statistical analysis.

TUNEL. For *in situ* visualization of apoptotic cells, the TUNEL method was performed according to the manufacturer's instructions with in Situ Cell Death Detection Kit, POD (Roche Diagnostics). In TUNEL staining, dark brown cells were considered to be positive, and were counted throughout microscopically magnified fields (× 400) of each articular cartilage section. The percentage of positive cells was analyzed by SPSS v.15.0 for Windows.

Laser-scanning confocal microscopy. Articular cartilage was fixed in 4% paraformaldehyde. For staining endogenous Akt and PKCa proteins, sections were incubated with anti-Akt or anti-PKCa antibody (1:100 or 1:150 dilutions, Cell Signaling Technology, Danvers, MA, USA) followed by FITC or Texas Red conjugated secondary antibodies (1:200 dilutions, Santa Cruz Biotechnology, Inc. CA, USA). Cells were stained by 4',6-diamidino-2-phenylindole (DAPI, 50 mg/ml, Sigma) to visualize the nuclei simultaneously [14]. The stained cells were finally visualized under laser-scanning confocal microscopy (Leica Tcs Sp2 SE).

Western blot and immunoprecipitation. Articular cartilage was lysed in RIPA buffer (10 mmol/L Tris pH7.4, 150 mmol/L NaCl, 1%TrionX-100, 1% deoxycholic acid, 0.1%SDS, 5 mmol/L EDTA pH 8.8, 1 mmol/L PMSF, 10% Cocktail (Roche), 1% dithiothreitol) for 30 min at 4°C [14]. Lysates were centrifuged at 12,000 × g for 30 min at 4°C. Protein concentration was determined using the Bio-Rad protein assay system according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). Protein extracts were electrophorezed on 8% or 10% denaturing gel and electroblot-

ted onto a nitrocellulose membrane. The membranes were incubated with different antibodies as required at 4°C overnight, followed by the addition of a corresponding secondary antibody at room temperature for 1 hr. An ECL kit (Pierce, Thermo Scientific, Rockford, IL, USA) was used to detect the antibody reactivity.

For immunoprecipitation [14], protein extracts were incubated with the appropriate antibody for 1 hr, and were subsequently incubated with protein A-sepharose beads (Sigma) for 1 hr. The protein-antibody complexes that were recovered on beads were subjected to Western blot analysis as described above.

Statistical analysis. Data was expressed as mean ± standard deviation. Statistical analyses were done using SPSS v.15.0 for Windows. Independent two-sample *t*-test and paired two-sample *t*-test were used for the comparison of protein expression between OA group and normal group. P values of less than or equal to 0.05 were considered significant. Pearson correlation test was used for the correlation between Akt and PKCα.

Results

Chondrocyte apoptosis increases in human OA cartilage

The histological differences between the human OA group and the normal group were initially observed, and it was found that the zones in the articular cartilage of the OA group were irregular compared to the normal group because of the cellular changes that took place (Figure 1A). These changes included the articular cartilage becoming thinner, and the superficial zone being virtually destroyed. Moreover, the arrangement of chondrocytes throughout transitional and radical zones were irregular, following the decrease of the chondrocyte number and the rupture of the tidemark, which is at the junction of the articular hyaline cartilage and adjacent subchondral bone (Figure 1A).

The decrease of chondrocyte number is crucial to the loss of articular cartilage in OA. To confirm whether chondrocyte apoptosis was present in human OA cartilage, we performed the TUNEL method in the OA and the normal group. TUNEL is a common method for detecting DNA fragmentation that results from apoptotic signaling cascades. The assay relies on the presence of nicks in the DNA which can be identified by terminal deoxynucleotidyl transferase, an enzyme that will catalyze the addition of dUTPs that are secondarily labeled with a marker [15]. Although TUNEL staining cells were present in all zones in the normal and in the OA group, there was a significant increase in the number of TUNEL staining cells in the OA group compared to the normal group (Figure 1B, Table 1, $p < 0.05$). Moreover, the number of TUNEL staining cells in the transitional zone was significantly higher than that in the radical zone (Figure 1B, Table 1, $p < 0.01$).

Akt and PKCα are involved in human OA progression

The result of immunohistochemistry showed that the average OD of the heavy staining cells for Akt in the OA group was significantly lower than that of the normal group (Figure 2A, Table 1, $p < 0.05$). Like the TUNEL staining result, the average OD of the cells for Akt in the transitional zone was significantly higher than that in the radical zone, whether in the OA or the normal group (Figure 2A, Table 1, $p < 0.01$).

Furthermore, Western blot results showed that the expression level of total Akt in the OA group decreased compared to that in the normal group, while the P-Akt increased in the OA group (Figure 3). These findings confirmed that Akt and its phosphorylation are involved in OA progression. Nevertheless, the result of immunohistochemistry showed that the average OD for PKCα in the OA group is significantly higher than in the normal group (Figure 2B, Table 1, $p < 0.05$). In addition, the expression of PKCα is more prominent in the transitional zone than the radical zone, whether in the OA or the normal group (Figure 2B, Table 1, $p < 0.01$). The result of the Western blot analysis also showed that the expression level of PKCα increased in the OA group compared to that in the normal group (Figure 3).

Correlation between Akt and PKCα in human OA progression

The distribution of Akt and PKCα in chondrocytes was initally observed under laser-scanning confocal microscopy. Both Akt and PKCα mainly localized in the cytoplasm of chondrocyte in both the OA group and the normal group (Figures 4A, C for Akt, Figures 4B, D, for PKCα).The distribution of Akt and PKCα did not alter in OA progression. Then, as shown in Figure 5, Akt bound to PKCα, but the degree of their interaction in the OA group was similar to that in the normal group (Figure 5). Furthermore, there exists a negative correlation between Akt and PKCα by analyzing the average OD of Akt and PKCα (Table 1, $r = -0.8$).

Discussion

Chondrocyte apoptosis has been implicated as one of the etiological features in articular cartilage degradation during OA development [1–3]. In this study, Akt and PKCα participate in human OA progression,

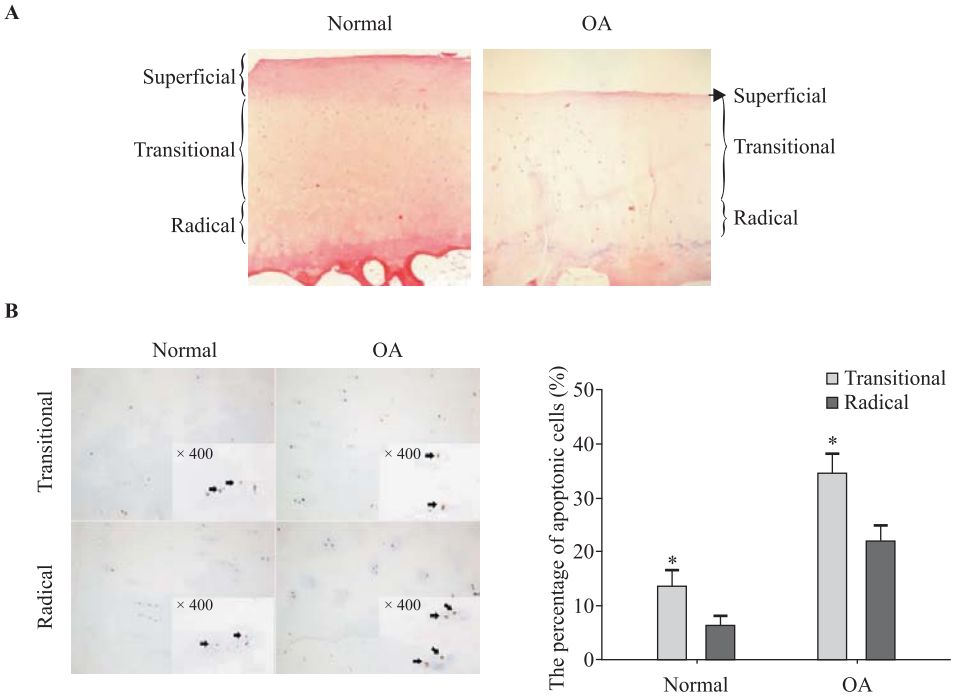

Figure 1. Comparison of histological and chondrocyte apoptosis in normal and OA group. (**A**) Histological changes in normal and OA group. Left: normal group, Right: OA group (magnification × 50). (**B**) Apoptotic chondrocytes in normal and OA group. Left: TUNEL staining chondrocytes (brown) (magnification × 200, × 400). Right: percentage of apoptotic chondrocytes in normal and OA group (*p < 0.01)

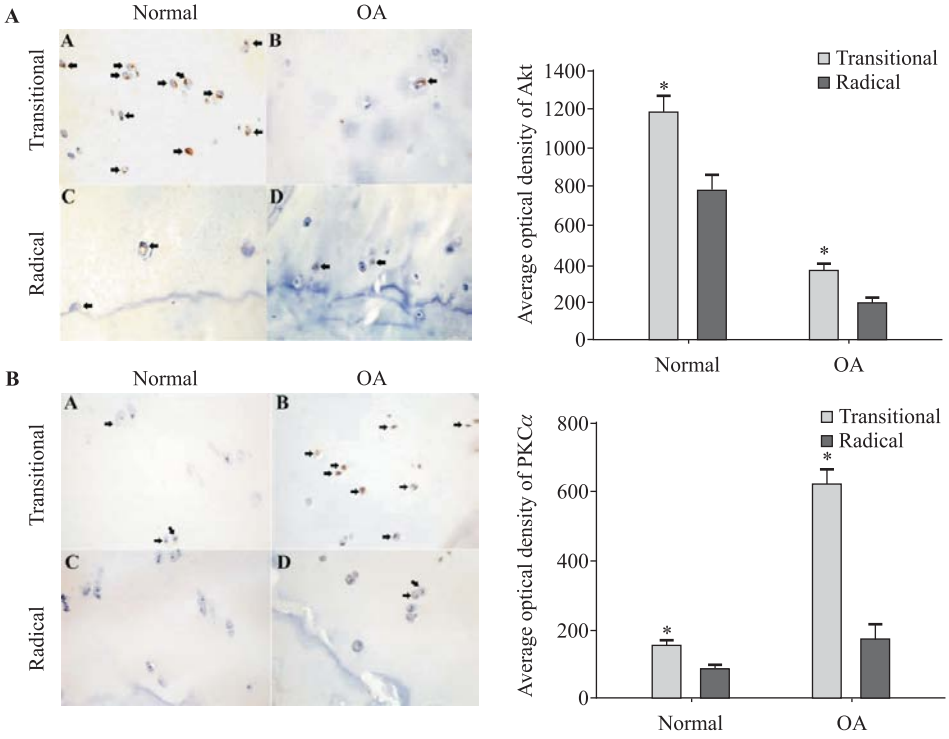

Figure 2. Expression of Akt and PKCα by immunohistochemical staining in normal and OA group. (**A**) Expression of Akt in normal and OA group. Left: Akt positive chondrocytes (magnification × 400). Right: Histogram of the average OD of chondrocytes (*p < 0.01). (**B**) Expression of PKCα in normal and OA group. Left: PKCα positive chondrocytes (magnification × 400). Right: Histogram of the average OD of chondrocytes (*p < 0.01)

Table 1. Expression of Akt, PKCa and chondrocyte apoptosis in normal and OA group

	Akt		PKCα		TUNEL	
	Normal	**OA**	**Normal**	**OA**	**Normal**	**OA**
Transitional	*1176.74 ± 135.39	*356.5 ± 65.87	*155.97 ± 17.56	*621.47 ± 103.19	*13.4 ± 3.2%	*34.5 ± 3.7%
Radical	775.33 ± 122.58	18620 ± 40.93	88.26 ± 26.29	175.71 ± 114.26	6.2 ± 1.8%	21.8 ± 2.8%
Total	#1076.03 ± 140.54	271.18 ± 102.22	126.16 ± 41.46	#398.59 ± 39.57	10.7 ± 2.6%	#28.4 ± 3.6%

*$p < 0.01$, #$p < 0.05$, R = –0.802

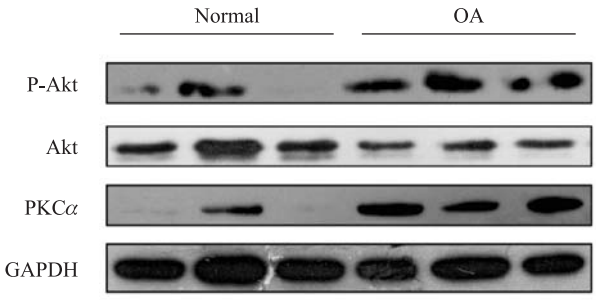

Figure 3. Expression level of Akt, p-Akt and PKCα in normal and OA group. Protein extracts from articular cartilages were prepared as described in the 'Materials and methods' section, and then analyzed by Western blot analysis with an antibody against Akt or P-Akt or PKCα. The blots were normalized to an endogenous protein (GAPDH). The data is representative of ten independent experiments, each yielding similar results

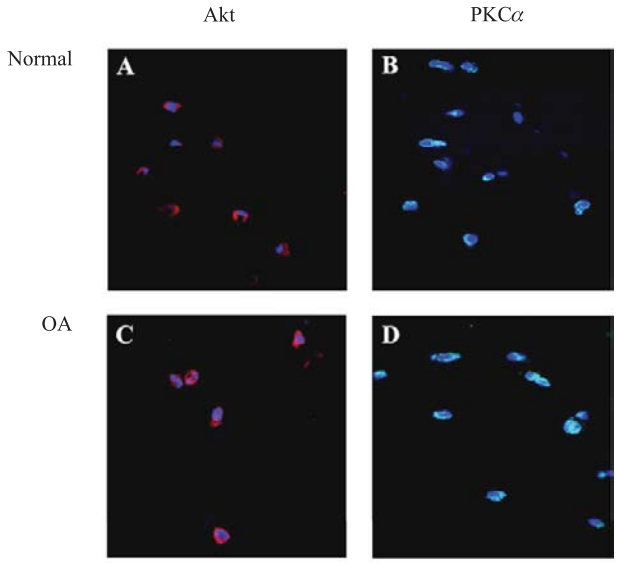

Figure 4. Subcellular distribution of Akt and PKCα in the chondrocytes of normal and OA group. As described in the 'Materials and methods' section, the cells were immunostained with Akt or PKCα-antibody, followed by Texas-conjugated secondary antibody (**A** & **C** for Akt) and FITC--conjugated secondary antibody (**B** & **D**, for PKCα), respectively. The nuclei were simultaneously indicated by DAPI staining. The fluorescent images were observed under a laser-scanning confocal microscope. The data is representative of 15 independent experiments, each yielding similar results

along with the increase of chondrocyte apoptosis. There exists a negative correlation and interaction between Akt and PKCα.

Firstly, apoptosis is significantly higher in OA cartilage than in normal cartilage (Figure 1B, Table 1, $p < 0.05$), consistent with the belief that chondrocyte apoptosis is an indication of OA cartilage [1–3, 16]. Furthermore, we found that the increase of chondrocyte apoptosis in the transitional zone was higher than that in the radical zone in the OA cartilage, something also found in other authors' reports [17].

This increase of chondrocyte apoptosis would thus explain the failure of maintenance of the cartilage matrix and the loss of articular cartilage in OA. In 2004, Roach et al. [18] suggested the term 'chondroptosis' to define the type of cell death present in articular cartilage, which included the presence of both apoptotic (late stage) and autophagic (early stage) processes [19]. Here, the chondrocytes obtained are most likely undergoing chondrocyte apoptosis of 'chondroptosis', due to the fact that they were obtained from patients in the medium-late stage for undergoing knee arthroscopy (Mankin score 8–12, average value 10.3).

The activation of the chondrocyte by various extracellular factors resulted in an imbalance between the synthesis and degradation of extracellular matrix in OA progression. All these factors act through specific receptors that transmit the signals to the nucleus to activate the transcription of matrix metalloproteinases and inflammatory genes. Targeting these intrinsic signaling pathways in osteoarthritis is a novel approach to modulate this imbalance. In 2008, Keisuke et al. [20] reported that the activation of Akt in embryonic chondrogenesis enhanced chondrocyte proliferation. Other articles also have demonstrated enhanced apoptosis in osteoblasts, testis cells, thymocytes, and embryo fibroblasts in Akt1-/- mice [8].

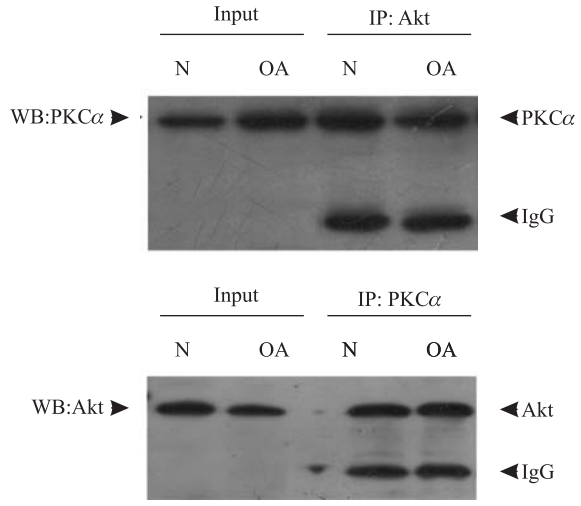

Figure 5. Interaction between Akt and PKCα in normal and OA group. As described in the 'Materials and methods' section, tissue lysates were immunoprecipitated with Akt or PKCa antibody, and then subjected to Western blotting with PKCa or Akt antibody. IgG expression served as a control for indicating similar proteins in each lane. The same lysates were applied to ascertain the position and expression of PKCa and Akt by Western blotting (Input). The data is representative of five independent experiments, each yielding similar results

In this study, Akt not only declined in OA cartilage, but also its decrease was higher in the transitional zone, consistent with the increase of chondrocyte apoptosis in the transitional zone (Figure 2A, Table 1). In addition, Western blot analysis also indicated the decrease of Akt level in the OA cartilage (Figure 3).

Taken together, we suggest that Akt decline accompanied by chondrocyte apoptosis hastens human OA progression. However, the high expression level of phosphorylation of Akt(P-Akt) in the OA group (Figure 3) might act as a compensatory role to protect chondrocytes from the apoptosis in the OA cartilage. The latest paper by García S et al. [21] also reported that the endogenous phosphorylation of Akt protects rheumatoid arthritis (RA) fibroblast such as synovial cells against the apoptosis induced by Fas. Elevated production of TRB3, an inhibitor of Akt activation, in osteoarthritic chondrocytes, would increase chondrocyte death in OA cartilage [22]. Therefore, the decrease of Akt level makes for the increase of chondrocyte apoptosis in the OA group, and Akt may be a novel target for new therapeutic strategies in OA.

Recently, growing evidence has indicated that PKCα not only plays an essential role in chondrocyte apoptosis [5, 9], but is also involved in the early stage of OA progression [23, 24]. Ca^{2+}influx-dependent PKCα-MAPK signaling cascades have been implied to be activated in the experimental dog OA model system, indicating that the PKCα pathway may mediate development and progression of OA [23]. PKCα appears in chondrocytes in the early stage of OA progression [24]. Here, the increase of chondrocyte apoptosis in the late stage of OA (Figures 2B, 3, Table 1) and the high expression level of PKCα, showed that the increase of PKCα might promote the chondrocyte apoptosis in OA cartilage, suggesting its involvement in the total progression of OA.

Interaction between Akt and PKCα has been reported [9, 11, 14]. Previous studies, both in our lab and others, have suggested that Akt could be regulated by PKCα negatively in cancer cells [14, 25]. On the other hand, the overexpression of PKC also could stimulate Akt activity and suppress cytokine-dependent apoptosis [26]. Thus, PKCα might be a potential regulator, positively or negatively, for Akt. In the current case, the co-localization of Akt and PKCα in the cytoplasm of chondrocyte (Figure 4) provided a clue to the next step: a study on the interaction between them. Unfortunately, the alternation of the bind between them in OA progression could not be observed in this study (Figure 5). Combined with the analysis in Table 1, we suggest that Akt might be associated with PKCα in OA chondrocyte, and PKCα may regulate Akt negatively. It is necessary to study their phosphorylation in future in order to elucidate their interaction in OA progression. It also implied that it is crucial to pay attention to the antagonism between them in therapy of OA and interesting to delineate further the correlation between Akt with PKCα in OA cartilage.

However, there exists some limitation in this study because of the difficulty in obtaining human OA cartilage. It is regrettable not to be able to pay more attention to studying the relationship between the range age differences and the expression of Akt or PKCα in the normal and the OA group.

In summary, our study demonstrated that both Akt and PKCα related to the increased chondrocyte apoptosis in human OA cartilage. This is a very significant finding, indicating that Akt and PKCα play differing roles in human OA progression. It may provide a molecular basis for molecular diagnosis and therapeutic strategies. For instance, the change of the ratio of Akt/PKCα might provide reference points for doctors in diagnosing the degree of OA or determining therapeutic strategies.

Thus, the correlation between human OA progression, the role of Akt and PKCα, and chondrocyte apoptosis allows for the possibility of new therapeutic strategies.

Acknowledgements

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