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Radiation Sensitization of Keloid Firboblasts by Quercetin through PI3K/Akt Pathway Dependent Regulation of HIF-1α

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Abstract

Objective: Keloid is a benign tumor in the derma, which is generally treated by surgical excision and radiation in clinic. However, recurrence has been an intricate problem in treatment of keloid. Therefore, it is imperative to develop novel interfering means to increase the sensitivity of keloid to radiation. Quercetin is a natural compound, which has been shown to sensitize some tumors to radiation. We tested the potential of quercetin in sensitizing keloid fibroblast to radiation and identified its downstream pathways.

Methods: Keloid fibroblast cells were challenged by radiation with or without the presence of quercetin. Cellular response was detected by flow cytometry, western blot, qRT-PCR and immunofluorescence staining. HIF-1α was down-regulated by siRNA transfection.

Results: In this study, we firstly demonstrate that keloid fibroblasts acquire resistance after IR treatment, and this can be relieved by treating with quercetin. Further, we showed that hypoxia-inducible factor 1 (HIF-1), a prognostic marker used in clinical practice after radiation therapy, was associated with stronger radioresistance in keloid fibroblast, which was downregulated after quercetin treatment. The inhibition on HIF-1 expression by quercetin was found to depend on phosphatidylinositol-3-kinase (PI3K)/Akt pathway and quercetin also reduce phosphorylation of Akt.

Conclusion: Taken together, we revealed a mechanism underlying the suppression on radioresistance by quercetin, which involves the regulation of HIF-1α by PI3K/Akt pathway. Our study provides molecular interpretation for the application quercetin in sensitizing radiation in keloid treatment.

Keywords: Keloid; Quercetin; Radio resistance; Hypoxia-inducible factor 1; phosphatidylinositol-3-kinase/Akt pathway

Introduction

Keloid is a benign tumor in the derma, which is characterized by infiltration into adjacent normal tissues. In Asia population, it occurs at a frequency of 4%-16% [1], most of which are young adults. Keloids appear as red, hard, and tickling raised irregular scar tissues that could overtrude out of area of injury [2]. Prolonged existent keloid could cause necrosis, recurrent hemorrhage and suppuration. Histopathologic sections showed overabundance of fibroblasts during active mitotic division within keloid, accompanied by dense collagen fibers and abnormal increase of myxoid stroma [3]. The etiology of keloid is reminiscent of tumorigenesis, dysfunction of tissues, abnormally excessive extracellular matrix, and dyregulation of apoptosis etc, [4-7]. However, there is still a lack of definite mechanisms that could explain keloid pathogenesis. Recurrence has been an intricate problem in treatment of keloid. Berman and Bieley et al. comprehensively compared the efficacy of different therapies, and showed that combining surgical excision and radiation could minimized the recurrence, yet some patients still suffered from radioresistance [8]. Therefore, it is imperative to develop novel interfering means to increase the sensitivity of keloid to radiation, which is expected to reduce secondary insults.

Adjuvant therapy emerged as an effective means to reduce resistance. Quercetin, a flavonoid that can be extracted from plants, such as vegetables, bark roots, flowers and grains, which were found beneficial to health [9]. Natural as it was, it has drawn extensive research interest to invesitgate the pharmacological effect, which offers substantial amount of evidence for potential therapeutic uses in a variety of conditions, including allergies, asthma, anti-bacteria activity and cancer [10-15]. Among them, cancer is a similar condition with keloid. Indeed, the effective inhibition on radiation-induced Protein kinase C (PKC) activity, one of the means conferring radioresistance to tumor cells, has been achieved by quercetin treatment [16]. However, the role of quercetin in treatment of keloid and the feasibility of its clinical application remain elusive.

In this study, we firstly compared the radio resistance of keloid fibroblasts, the majority constituent cells of keloid, with that of normal fibroblasts, and showed that keloid fibroblasts had less severe apoptosis, which can be deteriorated by treatment of quercetin. Subsequently, we found that the expression level of Hypoxia-Inducible Factor-1 (HIF-1), an indicator of radioresistance, is correlated with consentration of quercetin, and associated with degree of

radioresistance. We further employed phosphorylation inhibitor and agonist to examine whether quercetin inhibits HIF-1α through PI3K/Akt pathway which has been highly implicated in tumorigenesis, tumor development and escape from apoptosis [17]. Quercetin was found to reduce protein level of p-Akt and the Akt phosphorylation, indicating the involvement of PI3K/Akt in suppression of radioresistance by quercetin and the existence of another potential inhibitory pathway.

Materials and Methods

Source of keloid fibroblasts

The keloid fibroblasts used in this study were sampled from disposed skin keloid tissues of five patients who received surgery in plastic surgery department of our hospital, and the normal fibroblasts derive from normal skin tissues. None of patients had been administrated with any therapy prior to sampling and had complicating disease that affects wound healing. All keloid tissue specimens were examined and confirmed pathologically, and the full-thickness biopsy specimens were used in subsequent experiments. The keloid fibroblasts from different patients were used as biological replicates. Normal fibroblasts were sampled from surgical excised normal skin from nonkeloid people. Written informed consent of all patients was obtained and this study was approved by Ethic Committee of our hospital.

Cell culture and treatment

Tissues were obtained from central parts of each keloid lesion, which was then dissected into small pieces (<1 mm in diameter) cultured in DMEM supplemented with 10% FBS in a 5% CO_2 humidified atmosphere at 37. The edge of explanted tissue at 8-10 days post primary culture were passaged, and the cells harvested from the central of the keloid clonies in passages 3-6 were used for subsequent experiments. Cells were divided into Normal Fibroblast Group (Norm) and Keloid Fibroblasts Group (KLD). For radiation treatment, cells were cultured in DMEM supplemented with FBS, and were subjected to 24 h or 48 h of radiation, at 15 Gray, 20 Gray and 25 Gray, respectively. Each treatment has 5 replicates. For quercetin treatment, different quantities of quercetin was added to DMEM supplemented with FBS to obtain a concentration of 20 μmol/l, 40 μmol/l and 80 μmol/l, and cells were cultured in these modified medium for a period of time depending on experiment.

Apoptosis assessment by flow cytometry

Annexin V and propidium iodide (PI) flow cytometry was used to measure apoptosis in fibroblasts. Fibroblasts were plated in 6-well plates at a density of 5×10^4 cell/cm^2. After 24 h or 48 h of exposure to ionizing radiation or treatment with quercetin for 24 h, 100 μL cells were suspended in 1 × Annexin-binding buffer and then incubated with 5 μL Fluorescein isothiocyanate (FITC) annexin V and 5 μL PI (Invitrogen, US) for 15 min at room temperature in darkness. After adding 400 μL to annexin-binding buffer each replicate, the viability and apoptosis were analyzed by flow cytometry under 530 nm and >575 nm, respectively (BD Bioscience).

Western blot

Total proteins were extracted from cells using RIPA. Equivalent quantities of extraction of total proteins were subject to 10% sodium dodecylsulfate-polyaclylamidegel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes. Blocking was done by 5% skimmed milk and 0.1% Tween 20 resolved in PBS (pH 7.4), which were then incubated with primary antibodies for 60 min at 37. The following antibodies were used: mouse-anti-human HIF-1α (1:250, Abcam), p-Akt (1:1000, Abcam), Akt (1:1000, Abcam), and β-actin (1:1000, Abcam). Horseradish peroxidase (HRP) conjugated secondary antibody (diluted in 0.01 M PBS) was added followed by 4 times of washes by 0.01 M PBS. Enhanced chemiluminescence solution was used to visualize antigens, and Image J was employed to assess the signal intensities.

Quantitative RT-PCR

Total RNA was extracted using Trizol. Revert Aid M-MulV Reverse Transcriptase was used to synthesize cDNA which served as template for PCR. The primers used were: β-actin forward: 5'-GAGACCTTCAACACCCCAGCC-3'; β-actin reverse: 5'-AATGTCACGCACGATTTCCC-3'; HIF-1α forward: 5'-ACAAGTCACCACAGGACAG-3'; HIF-1α: 5'-AGGGAGAAAATCAAGTCG-3'. SYBR mix (Roche Applied Science) was added to PCR mixture as per the manufacturer's instructions. Each sample contained 3 replicates.

Immunohistochemistry staining

Tissues were fixed in 10% formaldehyde solution, and then rehydrated through gradually decreasing concentration of ethanol, followed by immersion in EDTA (pH 8.0) at 270 to recover antigens. Repair of peroxidase was done by adding 50 μl 3% H_2O_2. Goat serum was used to block uninterested epitopes. After removal of serum, the specimens were incubated with the mouse anti human HIF-1α monoclonal antibody (1:100), followed by 3 times of washes with PBS. Horseradish peroxidase labelled goat anti-mouse antibody was added and the mixture was incubated for 30 min at room temperature. Newly prepared DAB was added to visualize antigens.

siRNA transfection

siRNA of HIF-1α was purchased from Santa Cruz (Santa Cruz, CA, USA), and negative control siRNAs were purchased from QIAGEN. Cells were transfected with HIF-1α siRNAs or negative control siRNAs (QIAGEN) using Lipofectamine 2000 Transfection Reagent when grew to 70-90% confluence. Two days after incubation at 37, cells were harvested for subsequent assays.

Immunofluorescence staining

Cells were fixed in 4% paraformadehyde in PBS for 15 min at room temperature, and then permeated with 0.5% Triton at 4 for 10 min. After blocking with 2% BSA at 37 for 30 min, primary mouse anti human HIF-1α was diluted in 3% BSA and then added to the plate. After incubation overnight, secondary antibody (1:100) was added, and incubation was performed in the darkness for 1 h. Hochest 33342 was diluted at 1:800 with PBS and added to the cells. Samples were visualized under fluorescence microscope.

Statistical analysis

Each biological replicate has three technical replicates to calculate the average value of each parameter. Data are reported as mean ± SD.

Student's t-test was used to test the significance of differences, and $p<0.05$ was considered significant.

Results

Keloid fibroblasts are more resistant to ionizing radiation than normal fibroblasts

We treated keloid fibroblasts and normal fibroblasts with different doses of Ionizing Radiation (IR). After 24 h, we observed no significant difference in percentage of necrosis between normal fibroblasts and keloid fibroblasts (Figures 1A and 1C). The percentage of apoptosis cells in normal group showed no significant difference among different doses ($p>0.05$). In keloid group, the 20 Gray caused least severe apoptosis, and the apoptosis was less severe than the parallel contrast in normal group ($p<0.05$), suggesting that keloid fibroblasts possessed resistance to ionizing radiation. Particularly, normal group and keloid group exhibited largest difference under 20 Gray. Thus, we chose 20 Gray as an optimal radiation dose in subsequent experiments.

After 48 h, normal and keloid group showed no significant difference in percentage of necrosis, while at difference doses, keloid group exhibited consistently less severe degree of apoptosis, suggesting keloid maintained the resistance to radiation (Figures 1B and 1D). The percentage of apoptosis was not dose-dependent, since under 20 Gray, both groups showed least severe degree of apoptosis, while under 15 Gray the apoptosis was higher. Compared with 24 h post-radiation, we observed some degree of recovery in the apoptosis at 48 h post-radiation. Therefore, the detection of apoptosis and radioresistance was then performed every other 24 h.

Quercetin sensitize keloid fibroblasts to ionizing radiation at a dose-dependent manner

We used 0, 20, 40, and 80 μmol/L quercetin to treat keloid fibroblasts, with and without ionizing radiation treatment, respectively. As expected, there is no significant difference in necrosis between different doses. At 24 h post-radiation, the degree of apoptosis increased with doses applied (Figure 2A), and the radiation-treated group showed higher degree of apoptosis than their parallel untreated contrast (Figures 2A and 2B). This indicates that combination of quercetin and radiation may be conducive to the removal of keloid after excision. Under 80 μmol/L, both group demonstrated largest percentage of apoptosis (Figure 2C). It is obvious that, the degree of apoptosis between cells with and without radiation treatment differed to the largest extent less than 40 μmol/L. At this level, the quercetin treated cells remarkably sensitized the radiation treated cells (Ra +40Qu *vs.* Ra+0Qu) by 3 folds (69.7% *vs.* 17.6%).

Knockdown of HIF-1α promotes apoptosis of keloid fibroblasts exposed to IR

We detected elevated expression of HIF-1α in IR-treated keloid fibroblasts, and quercetin was found to reduce HIF-1α, suggesting quercetin might function through targeting HIF-1α. To address this, we knocked down HIF-1α in keloid fibroblasts and treated them with IR. The knockdown efficiency was confirmed by western blotting (Figures 3A-E and 4A). Corresponding percentage of apoptosis and necrosis were measured by flow cytometry. The HIF-1α deficient cells showed substantially higher degree of apoptosis than their non-deficient counterparts at 24 h and 48 h post IR (Figures 4B and 4C), which implies that HIF-1α may be implicated in inducing cell apoptosis under IR.

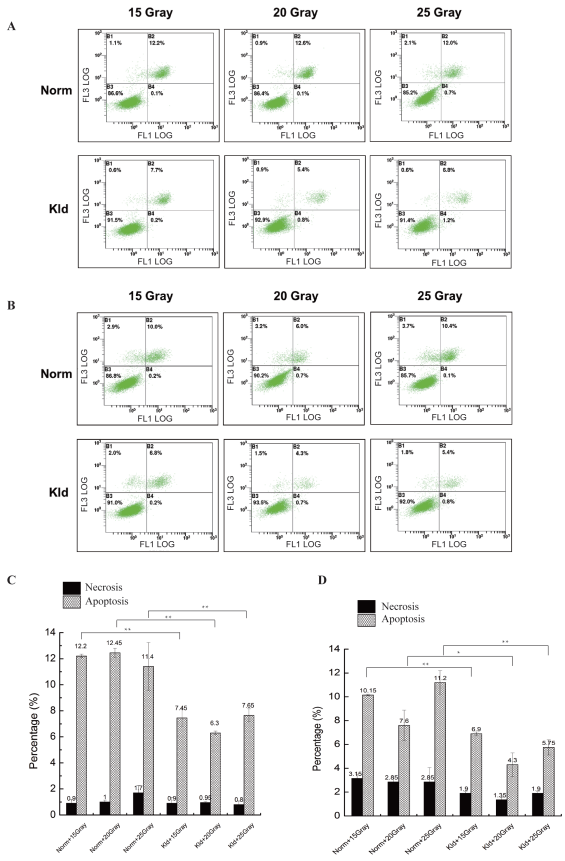

Figure 1: Keloid fibroblasts have stronger resistance at 24 h and 48 h post IR treatment. A and B are representative example of Annexin V flow cytometry analysis at 24 h and 48 hrs post IR treatment, respectively. Y-axis indicates propidium iodide (PI) staining and x-axis shows Annexin V-FITC staining. Upper left quadrant represents necrosis, upper right shows late apoptosis and lower right shows early apoptosis. C and D are histograms of percentage of necrosis and apoptosis; * :$p<0.05$, **: $p<0.01$.

PI3K/Akt pathway is involved in the decrease of HIF-1α in IR treated keloid fibroblasts by quercetin

Previous studies reported that phosphatidylinositol 3'-kinase (PI3K)/Akt signaling pathway is involved in regulation of HIF-1α [18-23]. We further sought to examine the downregulation of HIF-1α by quercetin during IR treatment involves PI3K signaling pathway. We treated cells with quercetin, LY294002 (10 μmol/L), or IGF-1 (30 ng/mL), or their combination. LY294002 inhibited phosphorylation of Akt, which can be seen from the blotting weaker than the blank control (Figure 5A). Of note, treatment with quercetin alone also displayed similar phenomenon, indicating that quercetin may also be an inhibitor of PI3K/Akt pathway. Furthermore, Hif-1α expression decreased along with protein level of p-Akt is treated with LY294002 or quercetin, and IGF-1 was able to attenuate suppression of HIF-1α

(Figures 5B and 5C). Taken together, we surmised that quercetin inhibits HIF-1α expression *via* PI3K/Akt pathway.

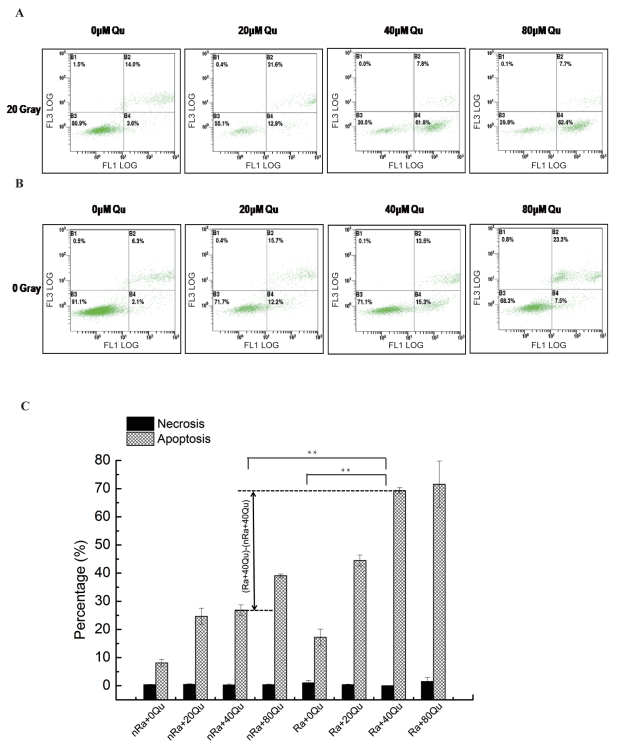

Figure 2: Quercetin increase apoptosis of IR treated keloid fibroblasts. A. Annexin cytometry analysis of keloid fibroblasts treated with different concentration of quercetin and IR; B. Annexin cytometry analysis of keloid fibroblasts treated with quercetin alone; C. Histogram of percentage of necrosis and apoptosis in both experiments; *:p<0.05, **: p<0.01.

Discussion

Histologically, keloid is a class of benign tumor composed of irregular fibroblasts and accumulation of extracellular matrix, such as collagen, annexin, proteoglycan, and elastin, which are prone to confer oncogenic properties to the inflexible scars [24]. Currently, excision followed by radiation therapy has been reported as effective. However, IR exposure not only causes cell death but also induce resistance in the tumor cells, which constitutes another pitfall [25]. In the present study, we observed that the necrosis of normal cells increased with IR doses, while keloid did not. The apoptosis was rescued even more as the treatment continued. These evidences demonstrated that keloid acquired radio resistance after IR treatment.

Radioresistance remains an intractable clinical problem that leads to poor outcome in cancer treatment. Multiple realms of research have unveiled a few signaling pathways that contribute to resistance to IR. Among them, hypoxia pathway was one of the most extensively studied pathways, due to its intimate association with ionizing conditions [26-28]. Here, we demonstrated that quercetin could sensitize keloid fibroblasts to IR treatment, and the effect was associated with expression of HIF-1α, a critical component of radioresistance related hypoxia pathway.

Quercetin is abundant in broccoli, berries, apples and onions. It was frequently reported as an antioxidant, and raised intense attention. Previous studies showed that quercetin exerts protective effect in reperfusion ischemic tissue damage by scavenging free radicals [29]. It is reported that both *in vivo* and *in vitro* low oxygen tension is not related to HIF-1α [22], rather the reoxygenation markedly increased the expression of HIF-1α [30].

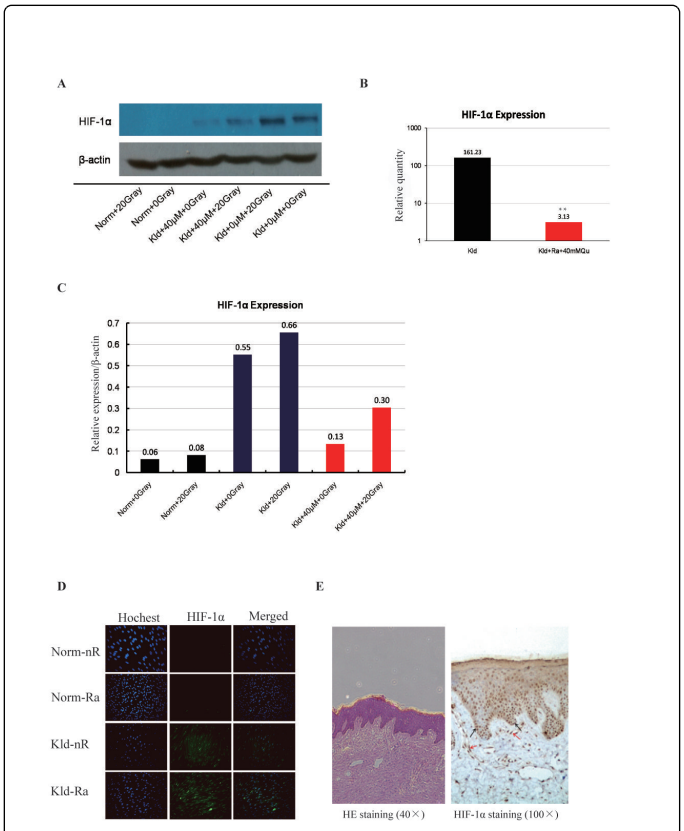

Figure 3: HIF-1α expression in keloid fibroblasts. A. Western blot of HIF-1α of treatment with 20 Gray IR, 40 μM quercetin, or combination of them in normal fibroblasts and keloid fibroblasts; B. Relative quantity of mRNA of HIF-1α measured by RT-PCR; C. Relative expression of HIF-1α corresponding to A; D. Immunofluorescence staining of HIF-1α; E. Immonuhistochemistry staining of HIF-1α.

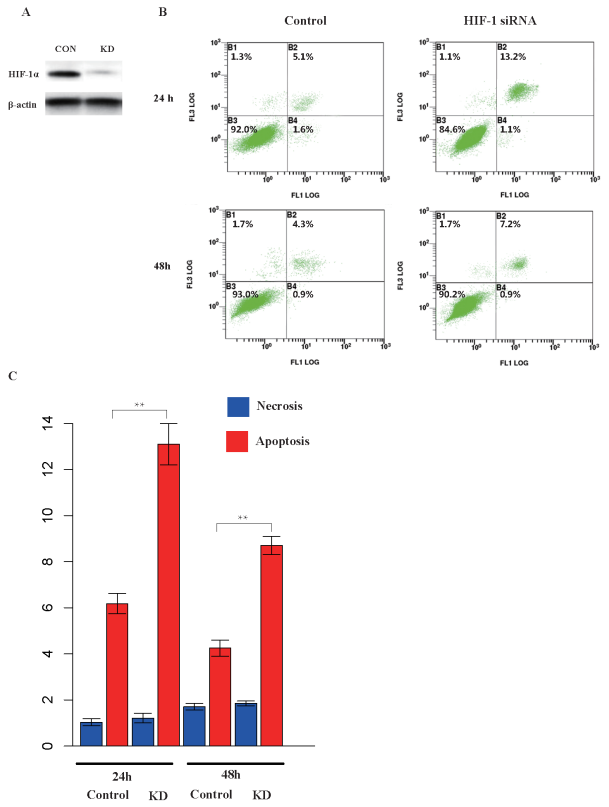

Figure 4: Apoptosis was increase after knock-down of HIF-1α. A. Western blot of HIF-1α of keloid fibroblasts transfected with HIF-1α siRNAs (right) and control (right); B. Annexin-V flow cytometry analysis of HIF-1α deficient keloid fibroblasts and control at 24 h and 48 h post 20 Gray IR; C. Histogram indicating percentage of necrosis and apoptosis of HIF-1α deficient keloid fibroblast; *:p<0.05, **:p<0.01.

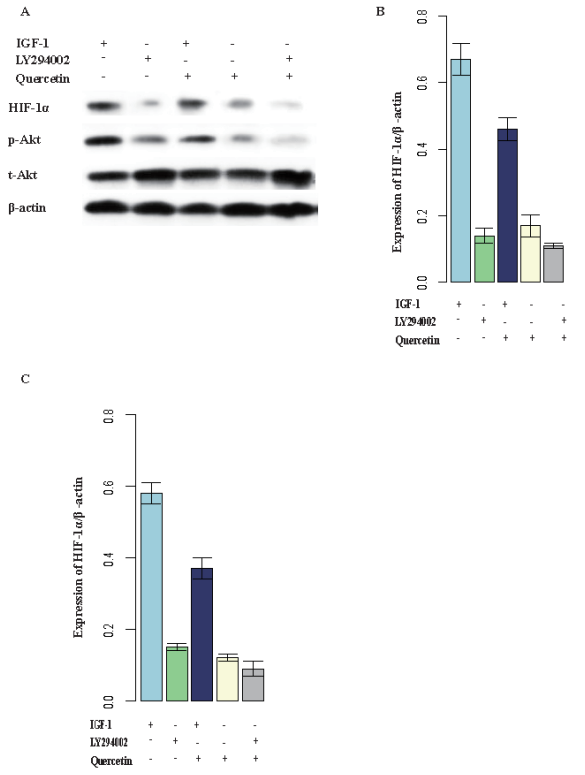

Figure 5: Downregulation of HIF-1α was dependent on PI3K/Akt pathway. A. Western blot of HIF-1α, Total Akt and phosphorylated Akt (p-Akt) after treatment with IGF-1, LY294002, and quercetin; B and C are histogram indicating the relative quantity of protein of HIF-1α and p-Akt; *:p<0.05, **:p<0.01.

The links among proliferation, apoptosis, angiogenesis, hypoxia and radioresistance are well established. PI3K/Akt pathway is highly involved in those biological processes, and has been documented as an oncogenic pathway [31-33]. In the present study, we employed LY294002 to abrogate phosphorylation of Akt, which inhibited PI3K/Akt pathway. Quercetin exerted inhibitory effect on both Akt phosphorylation and HIF-1α accumulation, which suggest that PI3K/Akt pathway might be implicated in suppression of HIF-1α by quercetin. When treated with IGF-1, an agonist of Akt phosphorylation, the inhibition of quercetin on HIF-1α was attenuated, but did not recover completely, suggesting alternative regulator of HIF-1α in addition to p-Akt. Since PI3K/Akt was affected by quercetin, the sensitizing might arise from promoted apoptosis by HIF-1α downregulation as well as diminished proliferation by blockage of PI3K/Akt pathway, which warrants further comprehensive investigation.

In conclusion, this study revealed stronger radioresistance of keloid fibroblasts, characterized by aggravated apoptosis, is associated with HIF-1α downregulation, which can be achieved by quercetin treatment. The proapoptosis of quercetin is PI3K/Akt dependent, which implies the potential inhibition of quercetin on cell proliferation in keloid fibroblasts. These findings provide potent experimental support for the adjuvant therapy after excision and radiation treatment of keloid fibroblasts.

Acknowledgements

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