行政院國家科學委員會專題研究計畫 成果報告

提昇私校研發能量專案計畫-自體免疫疾病中心之建立--子計畫四:類風濕性關節炎的致病機轉的探討：蛋白質脫亞氨化在抗原辨識及抗體產生上的影響與第四型胜肽精胺酸脫亞氨酶的功能及調節的研究(3/3)

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行政院國家科學委員會補助專題研究計畫■成果報告

（計畫名稱）

類風濕性關節炎的致病機轉的探討：
蛋白質脫亞氨化在抗原辨識及抗體產生上的影響與第四型胜肽精胺酸脫亞氨酶的功能及調節的研究

The Pathogenesis of Rheumatoid Arthritis: Protein Deimination in Antigen Recognition and Autoantibody Generation, and the Regulation and Function of Peptidylarginine Deiminase IV

計畫類別：□ 個別型計畫　　■ 整合型計畫

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目錄

中文摘要

胜肽精胺酸脫亞氨酶（Peptidylarginine deiminase; PADI）作用屬於後轉譯反應，可將精胺酸胜肽（peptidylarginine）轉換成瓜胺酸胜肽（citrulline），其在免疫細胞的分化及細胞凋亡扮演一個重要的角色。第四型胜肽精胺酸脫亞氨酶單核苷酸多型性單倍體與造成類風濕性關節炎嚴重程度具有相關性，其酵素活性增加會使得瓜胺酸蛋白量提高，進而增加自體抗體。在我們之前的研究證實了第四型胜肽精胺酸脫亞氨酶會造成免疫細胞的凋亡 (Liu, et al, 2006 Apoptosis)。在這裡，我們研究類風濕性關節炎的危險因子：第四型胜肽精胺酸脫亞氨酶單倍體（SNP PADI4; S55G, A82V and A112G）與其酵素活性的增加是否會誘導細胞凋亡的產生。在Tet-On系統Jurkat T細胞，當只加入Ionomycin（Ion）提高細胞內鈣離子並不會造成細胞凋亡，但加入四環素起始Tet-On系統Jurkat T細胞中的第四型胜肽精胺酸脫亞氨酶基因高度表現會造成細胞生存能力減少，並增加細胞凋亡。在體外及體內胜肽精胺酸脫亞氨酶酵素活性分析，我們證明第四型胜肽精胺酸脫亞氨酶單核苷酸多型性單倍體的酵素活性高於類風濕性關節炎非危險因子第四型胜肽精胺酸脫亞氨酶單倍體（第四型胜肽精胺酸脫亞氨酶野生型）。同時發現第四型胜肽精胺酸脫亞氨酶單核苷酸多型性單倍體優於第四型胜肽精胺酸脫亞氨酶野生型能夠誘發細胞凋亡。另外，第四型胜肽精胺酸脫亞氨酶單核苷酸多型性單倍體給予Ion後所誘發的細胞凋亡更優於給予Ion的第四型胜肽精胺酸脫亞氨酶野生型，並且會造成Bcl-xL蛋白減少與造成Bax蛋白增加，也能夠造成細胞色素C蛋白從粒線體釋放至細胞質。由西方墨點法的結果瞭解在誘發第四型胜肽精胺酸脫亞氨酶單核苷酸多型性單倍體表現與給予Ion之後，引發的程式性細胞死亡過程會增加細胞凋亡體之凋亡蛋白酵素活性。綜合這些研究結果顯示給予Ion的第四型胜肽精胺酸脫亞氨酶單核苷酸多型性單倍體的酵素活性增加，能夠增加經由粒線體的訊息路徑的細胞凋亡，並提供一個可信的解釋在於類風濕性關節炎致病機轉與第四型胜肽精胺酸脫亞氨酶單核苷酸多型性單倍體酵素活性增加之相關性。

蛋白質脫亞氨化現象發生於細胞凋亡過程之中。然而少有研究探討第四型胜肽精胺酸脫亞氨酶在細胞凋亡中所扮演的角色。因此，我們發現第四型胜肽精胺酸脫亞氨酶會引發細胞凋亡及其機制，並已發表於2006, *Apoptosis*（SCI: 4.49）。此外，我們已完成研發新的偵測胜肽精胺酸脫亞氨酶方法（2005, *Anal. Biochem.*）及發現PADI IV SNP（S55G, A82V, A112G）更易促使細胞凋亡的機制，並已發表於2007, *Apoptosis*。

因此第四型胜肽精胺酸脫亞氨酶所造成的蛋白質脫亞氨化在自體抗原的形成、自體抗體的產生及致病機轉上扮演重要角色。在此，我將目標集中在臨床上蛋白質脫亞氨化在自體抗原及抗體的產生的影響和第四型胜肽精胺酸脫亞氨酶對細胞凋亡的作用，期望能對於類風濕性關節炎致病機轉、臨床診斷及治療上有所貢獻。

成果報告壹

Abstract

Peptidylarginine deiminase IV (PADI4) posttranslationally converts peptidylarginine to citrulline. It plays the essential role in immune cell differentiation and apoptosis. A haplotype of single-nucleotide polymorphisms (SNPs) in PADI4 is functionally relevant as a rheumatoid arthritis (RA) gene. It could increase enzyme activity leading to raised levels of citrullinated protein and stimulating autoantibody. Previous our study exposes that inducible PADI4 causes haematopoietic cell death (Liu, et al, 2006 Apoptosis). Herein, we further investigate whether RA risk PADI4 haplotype (SNP PADI4; S55G, A82V and A112G) and the increase of its enzymatic activity induce apoptosis. In the tetracycline (Tet)-On Jurkat T cells, ionomycin (*Ion*) only treatment didn't induce apoptosis however it promoted inducible PADI4-decreased cell viability and –enhanced apoptosis. *In vitro* and *in vivo* PADI enzyme activity assay, we demonstrated PADI4 enzyme activity of SNP PADI4 was higher than RA non-risk PADI4 haplotype (WT PADI4). The effect of SNP PADI4-induced apoptosis was superior to WT PADI4. In addition, both *Ion* and SNP PADI4 synergistically provoked apoptosis compared with both *Ion* and WT PADI4. Concurrently, in the conditionally inducible SNP PADI4 cells of *Ion* treatment-induced apoptosis, not only the expression of Bcl-xL was down-regulated and Bax up-regulated, but also cytochrome *c* released from mitochondria to cytoplasm significantly. Western blotting data showed the increase of apoptosomal caspase activation during the programming cell death in the inducible SNP PADI4 cells subsequent to *Ion* treatment. These data demonstrated that both SNP PADI4 and increasing its enzyme activity could enhance apoptosis through mitochondrial pathway and further provide a conceivable explanation in the pathogenesis of RA following the upregulation of PADI4 activity in its SNPs. The early study revealed that PADI4 was shown to be associated with RA, but PADI2 appeared the feasible role. In addition, we examine whether inducible overexpression of PADI2 enhances apoptotic cell death. PADI2 reduced the viability in a does- and time-dependent manner of Jurkat-Tet-On system cells. The apoptosis-inducing activities were determined by nuclear condensation and DNA fragmentation.

Introduction

Peptidylarginine deiminases (PADIs; protein-arginine deiminases, EC 3.5.3.15) are a family of Ca^{2+}-dependent enzymes that catalyze the conversion of arginyl residues to citrullyl residues in proteins with concomitant production of ammonia. PADIs and citrullinated proteins are associated with the human diseases rheumatoid arthritis (RA), multiple sclerosis, psoriasis, geriatric diseases and Alzheimer's disease [*1–6*]. Five distinct isotypes of PADIs, PADI1-4 and PADI6, have been characterized and they have different tissue-specific distributions [*7–11*]. PADI1 is found in uterus and skin epidermis. PADI2 is seen in various tissues including the brain, skeletal muscle, spinal cord, oligodendrocytes, uterus, pancreas, salivary gland, pituitary gland, sweat gland, spleen, macrophages, bone marrow, and leukocytes. PADI3 is localized in the hair follicles, where formation of the trichohyalin citrullination occurs during hair follicle hardening. PADI4 is expressed mainly in the granulocytes, T and B lymphocytes, endothelial cells, monocytes, and macrophages, as well as PADI6 being specifically expressed in oocytes and embryonic stem cells [*12–14*]. Many proteins citrullinated by PADIs *in vivo* have been identified such as keratin [*15*], filaggrin [*16*], trychohyalin[*17*], vimentin [*18*], myelin basic proteins (MBPs) [*19*], histones [*20*, *21*] fibrinogen [*22*], fibrins [*23*], collagen type I [*24*], alpha-enolase [*25*],

antithrombin [*26*] and translation initiation factor 4G1 (eIF 4G1) [*27*]. However little is known about the specificity of PADIs toward protein substrates.

PADIs play essential roles in cell differentiation [*15*], nervous growth [*19*, *28*], mammalian embryonic development [*29*], cell death [*30*, *31*] and gene regulation [*20*, *21*]. For instance, in the epidermis, PADI1 is thought to citrullinate keratins and filaggrins in skin epidermis during the process of skin keratinization, and it is also involved in the final stages of keratinocyte differentiation to develop several epithelial structures [*15*]. Expression of PADI2 in oligodendrocytes causes citrullination of MBPs that are associated with myelin maturation in the central nervous system during the early stages of growth [*32*]. Human PADI4 could assist monocyte differentiation and regulate histone arginine methylation levels via demethylimination [*20*] or deimination [*21*] to repress hormone (estrogen)-receptor regulated transcription.

Besides, protein deimination occurs during the process of apoptosis. When the death signal is approaching (for example, when treating with calcium ionophore), increased intracellular calcium concentration can activate the enzyme activity of PADI4. In the nucleus, the major substrates are histone H2A, H3 and H4, and nucleophosmin/B23 [*33–37*]. Protein deimination changes the positive charge of arginine to no charge of citrulline, affects the intra-protein and inter-protein connection, and causes protein unfolding. The high arginine content gives the histones a strong net positive charge, which is important for their interaction with the negatively charged DNA backbone. Both the conformation and the net charge of the histone are altered by deimination, potentially causing the nucleosomes to open up, which makes the DNA more susceptible to degradation. PADI might be predicted to play a decision role in DNA fragmentation of apoptosis. During the apoptosis, vimentin was deiminated and then depolymerization occurred in the cytoplasm, which was thought to be associated with the morphologic change of apoptosis. Haematopoietic progenitor cells undergo enhanced apoptosis in patients with RA [*38*]. A functional haplotype of four exonic single-nucleotide polymorphisms (SNPs) in PADI4, was reported to be associated with RA susceptibility in the Japanese and Korean population and was shown to increase the generation of anti-cyclic citrullinated peptide antibodies (anti-CCP) [*2*, *39*]. These studies indicated that the PADI4 haplotype associated with susceptibility to RA increases production of citrullinated proteins acting as autoantigens. Recently, our previous studies showed conditionally inducible PADI4 induced apoptotic cell death in haematopoietic cells [*12*]. However, the role of PADI4 haplotype associated with susceptibility to RA in apoptosis is still uncertain.

In this study, we investigate the effect of RA risk PADI4 haplotype (SNP PADI4; S55G, A82V and A112G) on Jurkat (JK)-tetracycline (Tet)-On system. Conditionally inducible SNP PADI4 decreases cell viability and simultaneously induces the formation of apoptotic bodies, increases the sub-G1 ratio and DNA fragmentation. To be exact, we have found SNP PADI4-induced apoptosis is involved in its enzymatic activity. Furthermore, SNP PADI4 induces apoptosis as a consequence of the mitochondria-mediated pathway.

Materials and methods

Cell culture and chemical materials

The JK-Tet-On-Vector cells, JK-Tet-On-PADI4 cells and JK-Tet-On-SNP PADI4 cells were grown in 90% RPMI 1640 and 10% fetal bovine serum (FBS) obtained from Gibco BRL (Grand Island, NY) at a temperature of 37°C under a humidified and 5% CO_2 atmosphere. Ribonuclease A (RNase A), acridine orange, propidium iodide (PI), rhodamine 123, doxycycline (*Dox*) and ionomycin (*Ion*) were purchased from Sigma (St Louis, MO).

Human *padi4* gene sub-cloning and cell transfection

The entire coding sequence of human PADI4 cDNA was harvested from RZPD Deutsches Ressourcenzentrum für Genomforschung. Briefly, PADI4 cDNA was amplified using a sense primer (PADI4 IV-s: 25-mer) consisting of a 5′ *Bam*HI site (underlined) and a 19-nt sequence (nt 27–45): 5′-GGATCCATGGCCCAGGGGACATTG A-3′, an antisense primer (PADI4-as: 25-mer) consisting of a 5′ *Eco*RI site (underlined) and a 19-nt sequence (nt 2,093–2,075): 5′-GAATTCGAGCTCTTGCTTGCCACAC-3′ and *Taq* DNA polymerase (MDBio, TWN) as previous study [*12*]. The polymerase chain reaction (PCR) product was ligated into the T vector and transformed into *E. coli* strain JM109. The bacteria was grown overnight at 37°C, eluted plasmids and the amplified plasmid was digested with *Bam*HI-*Eco*RI and sub-cloned into the *Bam*HI-*Eco*RI site of pCMV-2b (Novagen, Madison, WI), and named WT-PADI4. WT-PADI4 plasmid was digested with *Bam*HI-*Not*I and sub-cloned into the *Bam*HI-*Not*I site of pTRE2hyg for the Tet-On system (BD Biosciences Clontech, San Jose, CA). pTRE2hyg-PADI4 and pTRE2hyg-vector only were transfected into JK-Tet-On system cells (BD Biosciences, Clontech) using calcium phosphate-mediated transfection for stable transfection selection. Stably transfected cells were selected with the antibiotic hygromycin (400 μg/ml). After approximately 3 weeks, hygromycin-resistant clones were screened for mRNA overexpression, protein expression and enzymatic activity by RT-PCR, immunoblotting and colorimetric assay of PADI enzyme, respectively. *In vitro* promoter induction of JK-Tet-On cell system was accomplished with the addition of *Dox* to the growth medium. As well, Bcl-xL cDNA was sub-cloned and termed pCMV-2a-Bcl-xL (m-Bcl-xL; frame-shift mutant) and pCMV-2b-Bcl-xL (WT-Bcl-xL; overexpressing Bcl-xL), respectively. pCMV-2a-Bcl-xL plasmid expresses transcript (mRNA) but doesn't encode Bcl-xL protein [*40*]. Cells (1×10^6/ml) were seeded into 100-mm Petri dishes and incubated at 37°C. m-Bcl-xL and WT-Bcl-xL plasmids were transiently transfected into the variant JK-Tet-On cells using calcium phosphate-mediated transfection. Following incubation at 37°C for 12 h, the medium was changed and then cell death was observed at various times for apoptotic studies. Through using pcDNA3-β-gal as a normalized reporter, the transfection efficiency was found to consistently be between 45 and 65%, staining with the chromogenic substrate ortho-nitrophenyl-β-galactoside (ONPG).

In vitro site-directed mutagenesis of SNPs in PADI4

To construct variant SNPs (S55G, A82V and A112G) in PADI4, *in vitro* site-directed mutagenesis was performed using *pfu* DNA polymerase (MDBio, TWN) and as described previously [*40*]. PADI4 plasmid and two pairs of synthetic oligonucleotide primers containing the desired mutations (point mutations to glycine at serine 55 [S55G], valine at alanine 245 [A82V] and glycine at alanine [A112G] residues, respectively) were utilized.

Cell viability and acridine-orange staining

Cell numbers were counted using trypan blue exclusion assay. The percentage of cell viability is calculated by the viable cell numbers of experiment groups in contrast with the untreated control group. To identify apoptotic character upon the conditionally inducible PADI4 and SNP PADI4, 5×10^4 cells in 10 μl phosphate-buffered saline (PBS) were mixed with an equal volume of acridine orange solution (10 μg/ml) in

PBS on each slide. Green fluorescence was detected by microscope as being between 500–525 nm (Olympus America, St Huntington, NY).

DNA fragmentation analysis

Cells (5×10^6) were harvested in PBS and lysed overnight in a digestion buffer (0.5% sarkosyl, 0.5 mg/ml proteinase K, 50 mM Tris-HCl, pH 8.0 and 10 mM EDTA) at 55°C. Subsequently, cells were treated with 0.5 μg/ml RNase A for 2 h. The genomic DNA was extracted by phenol-chloroform-isoamyl alcohol extraction and analyzed by gel electrophoresis at 50 volts for 30 min using 2% agarose. Approximately 20 μg of genomic DNA was loaded in each well, visualized under ultraviolet (UV) light and photographed [*12*].

Apoptotic assay by flow cytometer

For apoptotic sub-G1 analysis, 1×10^6 cells were harvested and washed with PBS, resuspended in 0.2 ml of PBS and fixed in 0.8 ml of ice-cold 99% ethanol at −20°C overnight. The cell pellets were collected by centrifugation, resuspended in 1 ml of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 μg/ml RNase A), and incubated at 37°C for 30 min. Then, 1 ml of PI solution (50 μg/ml) was added and the mixture was allowed to stand on ice for 30 min. The nuclei were analyzed in a FACSCAN laser flow cytometer (Becton Dickenson, San Jose, CA).

Detection of the mitochondrial membrane potential ($\Delta\psi_m$)

The $\Delta\psi_m$ was monitored by fluorescence of rhodamine 123. Cells were incubated with 10 μM rhodamine 123 for 10 min. Finally, cells were detached and fluorescence was measured in a flow cytometer. In each study, 10000 events (cells) were counted. Data were acquired and analyzed by using WinMDI software.

Immunoblotting

Mitochondrial and cytosolic proteins were isolated from cells following pretreatment with *Dox* for 6 h and then treatment with *Ion* for 36 h as described previously [*41*]. In order to extract mitochondrial proteins, 5×10^6 cells were washed once with ice-cold PBS and resuspended with 50 μl of mitochondrial buffer (25 mM Tris, pH 6.8, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF and 250 mM sucrose). To purify the total proteins, cells were harvested and lysed in cold lysis buffer (10% v/v glycerol, 1% v/v Triton X-100, 1 mM sodium orthovanadate, 1 mM EGTA, 10 mM NaF, 1 mM sodium pyrophosphate, 20 mM Tris, pH 7.9, 100 μM β-glycerophosphate, 137 mM NaCl, 5 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin), homogenized and centrifuged. The supernatant was boiled in a loading buffer and an aliquot corresponding to 100 μg of protein was separated by SDS-PAGE. After blotting, the membranes were incubated with anti-PADI4 (MDBio) anti-Bid (BD Biosciences, San Jose, CA), anti-Bax, anti-Bcl-2, anti-Bcl-xL anti-cytochrome *c*, anti-caspase 9, anti-caspase 3, anti-PARP and anti-actin antibodies (Santa Cruz, Santa Cruz, CA) for 6 h and the second antibody labeled with horseradish-peroxidase was adjacently incubated for 1 h. The antigen-antibody complexes were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

Assay of PADI enzyme

In vitro assay of PADI enzymatic activity was performed with a continuous spectrophotometric assay method as we described previously [*42*]. Recombinant *padi4* and the variant SNP *padi4* genes were constructed into a prokaryotic pQE expression system. Purified PADI4 or SNP PADI4 was added to the reaction mixture containing 10 mM BAEE, 10 mM $CaCl_2$, 2.5 mM DTT, 8.5 mMα-KG, 0.22 mM NADH, and 3U of GDH in 100 mM Tris-HCl (pH 7.5), the decrease in absorbance at 340 nm was continuously recorded in a Beckman DU 7500 spectrophotometer. An enzyme unit is defined as the amount of enzyme that catalyzes the reduction of 1 μmol of NADH to NAD^+ per min. An absorption coefficient of 6220 $M^{-1}cm^{-1}$ for NADH was used in calculating the enzyme activities.

In vivo assay of PADI enzymatic activity was determined by the colorimetric method [*43*]. Briefly, the reaction mixtures containing 100 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, 2.5 mM DTT, 10 BAEE and an appropriate amount of cell lysates (5 mg/sample) in a final volume of 50 μl were incubated at 37°C for 6 h. The reaction of enzyme activity was stopped by adding 10 μl of 5 M perchloric acid and the perchloric acid-soluble fraction was subjected to 150 μl of carbidino detection reagent, which was assembled freshly from its two components with one of part A containing 0.5% diacetyl monoximine and 0.01% thiosemicarbazide added to two of parts B containing 0.25 mg of $FeCl_3$/ml in 24.5% sulfuric acid (H_2SO_4) and 17% phosphoric acid (H_3PO_4). The reaction was mixed vigorously and heated at 110°C for 5 min. After heating, samples were cooled to room temperature and the absorbance was measured at 535 nm in a Perkin Elmer HTS 7000 Plus Bio Array Reader. Quantization of citrulline was determined as a standard.

Statistical analysis

Statistical analyses for significant differences between the control and experimental groups were evaluated using Student's *t* test.

Results

***Ion* promotes inducible PADI4-induced apoptotic cell death**

Calcium ionophore such as *Ion* and A23187 induces Ca^{2+} influx and can then activate the enzyme activity of PADI4 in the apoptotic processes [*30*, *31*]. The Ca^{2+} concentration of RPMI 1640 medium is approximately 0.5 mM, however the concentration of Ca^{2+} in cytosol only exists on the 0.1~1 μM. Our previous data have indicated that overexpression of PADI4 decreases cell viability and simultaneously induces the formation of apoptotic bodies, increases in sub-G1 ratio and DNA fragmentation [*12*]. To examine whether elevated PADI4 enzymatic activity could promote apoptosis, JK-Tet-On-PADI4 cells were pretreated with or without 50 μM *Dox* for 6 h and then treated with 0, 0.1 and 1 μM of *Ion* for 0, 12, 24 and 36 h (Fig. 1). The data were presented as proportional viability (%) by comparing the time zero in which group, the viability of which was assumed to be 100%. Treatment with *Ion* observed a significant decrease in cell viability in a dose- and time-dependent manner by using trypan blue exclusion assay. The viability of both *Dox* (50 μM) and *Ion* (1 μM)-treated JK-Tet-On-PADI4 cells was more sensitively reduced to 84, 79 and 76% of *Dox*-treated JK-Tet-On-PADI4 cells at 12, 24 and 36 h, respectively. The typical apoptotic morphologic changes were judged by acridine orange and observed by fluorescence-microscope. Both *Dox* and *Ion*-treated JK-Tet-On-PADI4 cells were observed to have significant membrane blebbing and nuclear breakdown (Fig. 2(A)). To confirm further *Ion* promoted PADI4-induced apoptotic cell death, we utilized DNA gel electrophoresis to detect DNA fragmentation (Fig. 2(B)) and flow cytometry with PI staining for the presence of the sub-G1 fraction (Fig. 2(C)). *Ion* only treatment didn't induce apoptosis however, we observed DNA ladder following *Ion* stimulus and the sub-G1 ratio which was time-dependent increase in both *Dox*- and *Ion*-treated JK-Tet-On-PADI4 cells. Simultaneously, we evaluated the effect of PADI4 overexpression on the cell cycle distribution. In Table 1,

both *Dox*- and *Ion*-treated JK-Tet-On-PADI4 cells showed the fraction of cells in the G0/G1 phase slightly increased at 12 h to compare with *Dox*-treated JK-Tet-On-PADI4 cells. These data suggested that *Ion* enhanced PADI4-mediated apoptotic cell death.

Establishment of the stable clones of SNP PADI4 on JK-Tet-On system cells

A functional haplotype of four exonic SNPs in PADI4 associated with susceptibility to RA has three amino acid substitutions: padi4_89, padi4_90 and padi4_92, resulting in S55G, A82V and A112G, respectively (Fig. 3(A)). padi4_104 (349T→C in exon 4) does not involve amino acid substitution (L117L). Therefore, we constructed variant SNPs (triple or double of S55G, A82V and A112G) of PADI4 by site-directed mutagenesis assay, purified recombinant enzymes from the pQE expression system (data not shown) and assayed their enzymatic activity on 0.5 mM Ca^{2+} by a continuous spectrophotometric method *in vitro* [*42*] (Fig. 3(B)). The RA risk PADI4 haplotype (SNP PADI4) of enzymatic activity was more marked than the RA non-risk PADI4 haplotype (WT PADI4) and the other variant SNP PADI4 of double mutations. In addition, we assayed the effect of variant Ca^{2+} concentration on their enzymatic activity by a continuous spectrophotometric method *in vitro* (Fig. 3(C)). It was revealed that SNP PADI4 of triple mutation (SNP PADI4) of enzymatic activity was approximately twofold compared with PADI4 (WT PADI4). The activity of PADI4 was shown to be dependent on Ca^{2+}. This result showed that SNP PADI4 would have high affinity with Ca^{2+}. To explore the effect of SNP PADI4 on apoptotic cell death, we constructed PADI4 [*12*] and SNP PADI4 into Tet-On expression plasmid, and generated cell lines that expressed an inducible form of PADI4 and SNP PADI4 in parental JK-Tet-On cells, named by JK-Tet-On-PADI4 and JK-Tet-On-SNP PADI4 cells. Furthermore, *In vivo* enzyme activity of the *Dox*-treated JK-Tet-On-PADI4 and JK-Tet-On-SNP PADI4 cells expressed approximately 2.7- and 3.1-fold higher than *Dox*-treated JK-Tet-On-Vector cells, respectively (Fig. 3(D)).

Conditional expression of SNP PADI4 induces apoptosis and *Ion* promotes SNP PADI4-induced apoptotic cell death in the JK-Tet-On cell system

To elucidate the role of inducible SNP PADI4 expression in the JK-Tet-On cell system, cells were stimulated with 50 μM of *Dox* for 0, 12, 24 and 36 h. JK-Tet-On-SNP PADI4 cells underwent a significant decrease of cell viability in a time-dependent manner using trypan blue exclusion assay (Fig. 4(A)). The morphologic changes including chromatin condensation, membrane blebbing and shrinkage, and apoptotic body were significantly increased after 50 μM *Dox* treatment for 36 h in JK-Tet-On-SNP PADI4 cells but not JK-Tet-On-PADI4 cells (Fig. 4(B)). Subsequently, we utilized *Ion* to induce intercellular calcium concentration for elevated SNP PADI4 activity. JK-Tet-On-PADI4 and JK-Tet-On-SNP PADI4 cells were pretreated with 50 μM *Dox* for 6 h and then treated with 1 μM *Ion* for 0, 12, 24 and 36 h, respectively. As shown in Fig. 5(A), *Ion*-treated JK-Tet-On-SNP PADI4 cells underwent a significant decrease of cell viability in a time-dependent manner using trypan blue exclusion assay. Furthermore, *Ion*-treated JK-Tet-On-SNP PADI4 cells underwent membrane blebbing and nuclear breakdown (data not shown). To further confirm that *Ion* promoted SNP PADI4-induced apoptotic cell death, we utilized DNA gel electrophoresis to assess DNA fragmentation (Fig. 5(B)) and flow cytometry with PI staining to identify the presence of the sub-G1 fraction, and the results were revealed in Fig. 5(C). Following *Ion* treatment for 36 h, DNA fragmentation of *Dox*-treated JK-Tet-On-SNP PADI4 cells was higher than *Dox*-treated JK-Tet-On-PADI4 cells. Moreover, the ratios of sub-G1 fraction in JK-Tet-On-SNP PADI4 cells were higher than JK-Tet-On-PADI4 cells, as shown in Table 1. These data suggested that the increased enzymatic activity of SNP PADI4 by *Ion* stimulus could induce apoptosis in the conditionally inducible JK-Tet-On system.

***Ion* evokes conditionally inducible SNP PADI4 further reducing Bcl-xL, increasing Bax, releasing cytochrome *c* and activating caspases**

To elucidate the apoptotic pathways of SNP PADI4 overexpression, we examined mitochondrial components, Bid, Bcl-2, Bcl-xL, Bax, and cytochrome *c* translocation as well as caspase activation during overexpression of PADI4 and SNP PADI4 in JK-Tet-On cells. Here, JK-Tet-On-PADI4 cells and JK-Tet-On-SNP PADI4 cells were pretreated with *Dox* for 6 h and then treated with *Ion* for 36 h. Total proteins were extracted for immunoblotting with anti-Bid, Bcl-2, Bcl-xL, Bax and actin antibodies. Bcl-xL expression was down-regulated and Bax expression up-regulated within 36 h of both *Dox* and *Ion* treatment in JK-Tet-On-SNP PADI4 cells to compare with JK-Tet-On-PADI4 cells, but not affect other Bcl-2 family expression (Fig. 6(A)). To observe the effect upon mitochondrial permeabilization, JK-Tet-On cells were treated with *Dox*, *Ion*, or both *Dox* and *Ion* individually and measured the $\Delta\psi_m$ by staining with rhodamine 123. The $\Delta\psi_m$ of JK-Tet-On-SNP PADI4 cells was decreased as compared with JK-Tet-On-PADI4 cells (Fig 6(B)). Besides, cytosol and mitochondrial proteins of *Dox*- and *Ion*-treated cells were detected by immunoblotting with anti-cytochrome *c* antibody. Cytochrome *c* release from mitochondria into cytosol increased on JK-Tet-On-SNP PADI4 cells at 36 h to compare with JK-Tet-On-PADI4 cells (Fig. 6(C)). The release of cytochrome *c* is considered to trigger a series of events leading to the activation of effector caspases. Therefore, we transiently transfected with Bcl-xL plasmid into *Dox*-treated JK-Tet-On-PADI4 and JK-Tet-On-SNP PADI4 cells, and overexpression of Bcl-xL could increase cell viability on these cells (Fig. 6(D)) and prevent cytochrome *c* release (data not shown). This result suggested that overexpression of PADI4 and SNP PADI4 induced apoptosis through the intrinsic mitochondrial pathway. The following experiments defined whether the caspase 9 and 3 were activated by overexpression of SNP PADI4 following *Ion* stimulus. In the JK-Tet-On-SNP PADI4 cells, caspase 9 and 3 were activated as well as PARP cleaved as compared with the JK-Tet-On-PADI4 cells following both *Dox* and *Ion* treatment (Fig. 7). These results indicated that overexpression of SNP PADI4 with *Ion* stimulus promoted the activation of apoptosome and increased the activation of caspase 3 during apoptotic progression in a conditionally inducible system.

Discussion

The classification of cell death discriminates non-programmed cell death and programmed cell death. Programmed cell death comprises necrapoptosis, aponecrosis [*44*], autoschizis [*45*], mitoptosis [*46*], anoikis [*47*], autophagy and apoptosis [*48*, *49*], Apoptosis is defined as a specifically morphological and biochemical form such as chromatin condensation,

membrane blebbing and shrinkage, as well as apoptotic body formation and DNA fragmentation that plays an important role in the embryogenesis of complex multicellular organisms and elimination of cancerous cells, removal of virus infecting cells, and deletion of autoreactive lymphocytes. If the apoptotic function is failing, we might anticipate that the probability of development of autoimmune diseases, persistent viral infection, malignancy, and developmental faults will increase. During the past ten years scientists have paid attention to the actuality that many environmental and intracellular genetic factors known to be involved in the beginning of autoimmunity lead to enhanced apoptosis, and this means that during apoptosis many autoantigens are exclusively modified. The presence of apoptotic cells in synovial tissue has recently become obvious [*14*]. It is possible that environmental or genetic factors (including pathogenic processes or gene polymorphism) may induce abnormal cell death or disturb the clearance of apoptotic cells. *Asaga et al.* [*30*] demonstrated that vimentin is selectively deiminated when mouse peritoneal macrophages undergo apoptosis upon exposure to calcium ionophore. *Mizoguchi et al.* [*31*] subsequently demonstrated that an unidentified 70-kDa nuclear protein is similarly modified in apoptotic rat keratinocyte. Both citrullination of cellular proteins occurs during apoptotic events. These researches and our previous studies [*12*] have reported that PADIs may be specifically activated in the apoptotic process.

Subsequently, citrullinated peptides may be presented to the immune system. In RA synovial tissue, two of five PADI isotypes (PADI2 and PADI4) are known to be expressed, and their expression is regulated at multiple steps: transcription, translation, intracellular localization, and activation/inactivation of PADIs [*18*]. In addition, unique citrullinated epitopes from apoptotic haematopoietic cells might be represented and responded to by the immune system. The resulting autoantibodies recognize citrullinated epitopes on apoptotic cells that express autoantigenic molecules at the cell surface, and such opsonized apoptotic cells will generate further proinflammatory responses. The co-localization of PADI4, citrullinated protein and apoptotic cells in extracellular fibrin deposits suggests that PADI4 is responsible for fibrin citrullination and is involved in apoptosis of RA synovial tissue. In our previous studies, overexpression of PADI4 enhances apoptosis of haematopoietic cells. Although citrullinated peptides are not yet detected in these cells, the enzyme activity appears in high levels in SNP PADI4. A RA-susceptible haplotype in the gene encoding SNP PADI4 is established. However, the susceptible haplotype is more positive for antibodies for citrullinated filaggrins than non-susceptible haplotype in sera from individuals with RA [*2*]. Human PADI4 structural data indicate that Ca^{2+} binding induces conformational changes by generating the active site cleft [*50*]. The present data indicate that the RA risk PADI4 haplotype has a higher affinity with calcium and superior enzymatic activity than RA non-risk PADI4 haplotype. The abnormal enzyme activation of RA risk PADI4 haplotype may be due to the conformational change of the enzyme caused by these amino acid substitutions. Moreover, overexpression of SNP PADI4 and *Ion*-induced PADI4 enzymatic activity promotes further earlier apoptotic cell death than PADI4. Therefore, we postulate that such elevated enzyme activity might be the cause of cell programmed death.

The early study revealed that PADIs converted arginine residues to citrulline residues in desmin, glial filaments and soluble vimentin, in a Ca^{2+}-dependent deimination-induced disassembly of intermediate filaments in cytosol [*51*]. PADI4 was first found in HL-60 cells when cells were induced to differentiate into granulocytes by all-*trans*-retinoic acid (ATRA) and differentiate into monocytes by 1,25-dihydroxyvitamin D3 [*10*]. Deimination of arginine residues in nucleophosmin/B23 (40-kDa), histones H4, H2A, and H3 (14-, 17- and 18-kDa, respectively) sensitizes their nuclear functions by stimulation with calcium ionophore A23187 in HL-60 cells [*36*, 37]. HL-60 cells, human neutrophils, and eosinophils stained with specific antibody for PADI4 exhibited distinct positive signals in the nucleus. PADI4 was also found in peripheral blood CD 15^+ granulocytes, $CD68^+$ monocytes, $CD3^+$ T cells and $CD20^+$ B cells [*14*]. While the citrullinated target proteins are widely distributed over intra- and extra-cellular areas, the events of inducible overexpression PADI4 enhanced chromatin condensation, membrane blebbing and shrinkage. Further apoptotic body formation and DNA fragmentation should occur in the nucleus. Since PADI4 has been identified as a nuclear localization signal (NLS) motif (56-PPAKKKST-63), but not in PADI1, 2, 3 and 6 [*52*].

In our previous experiments, PADI4 overexpression induced apoptosis and was involved with the phosphorylative activity of cyclin-dependent kinases (CDKs) in the G1-S and G2-M phases. It was also involved with the decrease of cyclin-CDK complexes in the G1-S phase [*12*]. Activation leads either to growth arrest at the G1/S or G2/M transitions of the cell cycle by $p21^{Waf\text{-}1/Cip\text{-}1}$ [*53*, *54*]. p53 can stimulate p21 expression and Bax, a dominant-negative inhibitor of Bcl-2. Multiple independent routes have recently been traced by which the signal of nuclear DNA stress can transfer to the mitochondria, tipping the balance in favour of cell death by Bax rather than repair and survival [*55*]. Overexpression of PADI4 [*12*] and SNP PADI4 in this study induces Bax accumulation, Bcl-xL decrease but does not influence Bid and Bcl-2. The Bcl-2 family reveals evidence of cell-autonomous coordination between the opposing pathways of proliferation and cell death. After the decrease of Bcl-xL/Bax ratio, overexpressed SNP PADI4 cells enhanced the release of mitochondrial cytochrome *c* into cytoplasm and proteolytic activation of caspase 9 and 3. Simultaneously, we have shown that overexpression of Bcl-xL can rescue PADI4- and SNP PADI4-mediated growth arrest of these variant cells. The mechanism of SNP PADI4 overexpression-induced apoptotic cell death might be through the mitochondrial death pathway.

Furthermore, in view of the biochemical and physiological functions of haplotype PADI4, we apprehend the feasible roles of SNP PADI4 enzyme activity and protein expression in RA during programmed cell death. First, RA-susceptible haplotype in *padi4* gene might harvest the more stable transcripts and result in overexpression of SNP PADI4 protein as well as possibly encoding the higher enzyme activity of SNP PADI4. Second, *padi4* gene product is Ca^{+2}-dependent enzyme that catalyzes the conversion of arginine to citrulline in peptides. This activity itself suggests that the rheumatoid arthritis susceptibility haplotype of *padi4* gene is associated with higher levels of citrullinated peptide and elevated antibody to citrullinated peptide in sera of individuals with RA [*2*]. Third, PADI4 and SNP PADI4 might deiminate the nuclear histones, which interfere in DNA interaction. Both the conformation and the net charge of the histone altered by deimination cause loose nucleosomes, which might cause DNA damage and p53 accumulation. A previous study revealed that the apoptosis of RA synovial fibroblasts could be a p53-dependent mitochondrial apoptotic pathway [*56*]. Nuclear p53 transcribes its downstream genes and cytosolic p53 directly interacts with members of the Bcl-2 family of

apoptotic regulators [*57*, *58*]. Although Salmon et al. described that the inhibition of T cell apoptosis existed in the rheumatoid synovium, the synovial T cells in RA expressed a susceptible apoptosis phenotype ($CD45RB^{dull}$, $CD45RO^{bright}$, $Bcl\text{-}2^{low}$, Bax^{high} and Fas^{high}) [*59*]. Besides, in microenvironments of our experimental system, there was no assistance by IL-2R γ chain signaling cytokines (which could upregulate Bcl-2 and Bcl-xL) and by interaction with synovial fibroblasts (which could upregulate Bcl-xL, but not Bcl-2). Moreover, SNP PADI4 reduced Bcl-xL expression in our Tet-On cell system. Therefore, both the synovial T cells *in vitro* [*59*] and our inducible SNP PADI4 Jurkat T cells might be susceptible to apoptosis when they themselves exist alone. Fourth, the over deimination of vimentin interferes with its polymerization in the cytoplasm, which is thought to be associated with the morphologic change during apoptosis. Fifth, the elevated enzyme activity of PADI4 dominates cell death in our study, besides immune cell death might interfere with the clearance of cell debris (engulfment) in autoimmune diseases [*60*]. Additionally, the novel targets of SNP PADI4 and mechanisms of apoptotic cell death following SNP PADI4 enzyme activity and protein expression in RA will be further investigated.

Conclusion

In summary, we have demonstrated SNP PADI4 (S55G, A82V and A112G) dominates apoptosis of acute T leukemia Jurkat cells. *In vitro* and *in vivo* PADI enzyme activity assay, the enzymatic activity of SNP PADI4 was higher than PADI4. SNP PADI4-induced apoptosis was superior to PADI4. In addition, both *Ion* and SNP PADI4 synergistically provoked apoptosis compared with both *Ion* and PADI4. SNP PADI4 overexpression induced the decline of Bcl-xL and Bax accumulation which triggered cytochrome *c* release toward the activation of effector caspases and the $\Delta\psi_m$ loss when compared with PADI4 overexpression. Furthermore, overexpression of Bcl-xL could salvage cell viability in these cell models. Consequently these data demonstrated that increasing PADI4 enzyme activity could enhance apoptosis through the mitochondrial pathway and provide a feasible explanation as to the pathogenesis of RA following the upregulation of SNP PADI4 activity.

Figure

Fig. 1 Ionomycin (*Ion*) decreased cell viability on conditionally inducible PADI4 cells. JK-Tet-On-Vector and JK-Tet-On-PADI4 cells were pretreated with or without 50 μM *Dox* for 6 h and then treated with *Ion* at 0, 0.1 and 1 μM for 0, 12, 24 and 36 h. Cell viability was determined by trypan blue exclusion assay. Data were means ± SD of three experiments. * $P < 0.05$ and ** $P < 0.01$

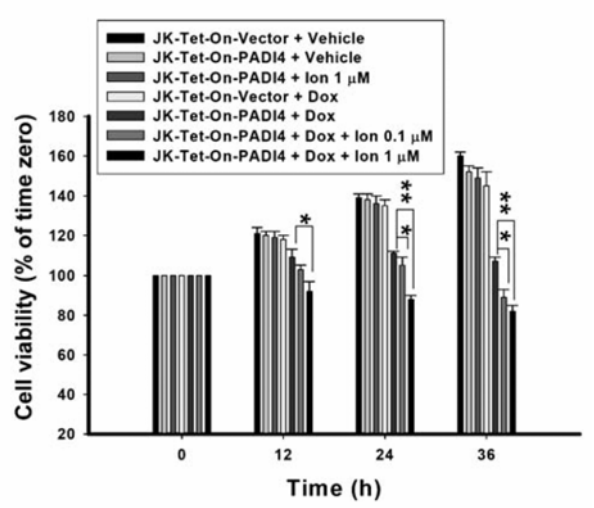
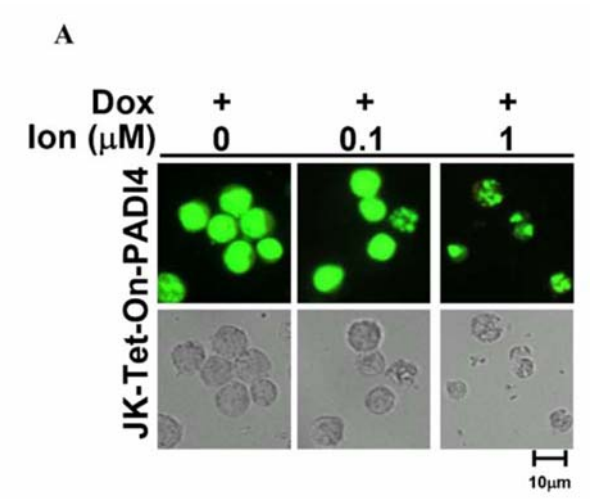


Fig. 2 *Ion* elicited apoptosis on inducible PADI4 cells. (A) JK-Tet-On-PADI4 cells were pretreated with 50 μM *Dox* for 6 h and then treated with *Ion* at 0, 0.1 and 1 μM for 36 h. Cells were stained with acridine orange and then detected by fluorescence-microscope. (B) JK-Tet-On-PADI4 cells were pretreated with or without 50 μM *Dox* for 6 h, treated with or without 1 μM *Ion* for 36 h and then DNA fragmentation was detected by DNA gel electrophoresis. (C) JK-Tet-On-PADI4 cells were pretreated with 50 μM *Dox* for 6 h, treated with 1 μM *Ion* for 0–36 h and then the percentage of sub-G1 was measured by flow cytometry using PI staining. Data are representative of at least three experiments. M, DNA ladder marker



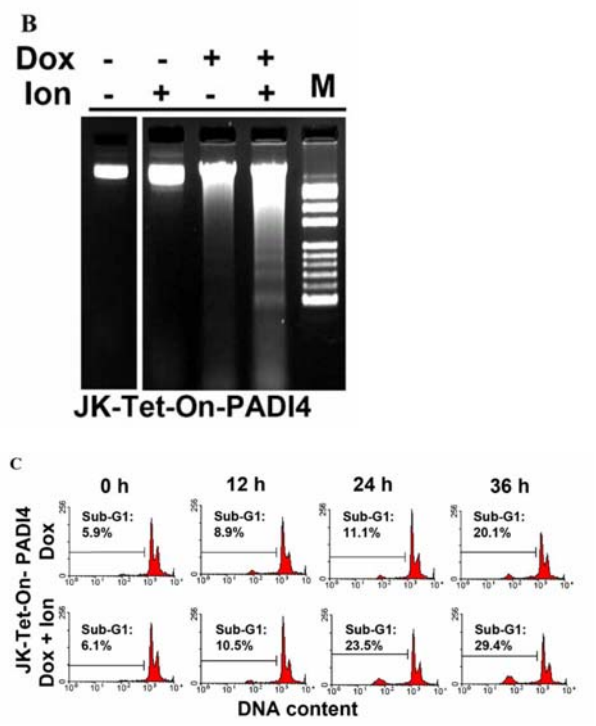
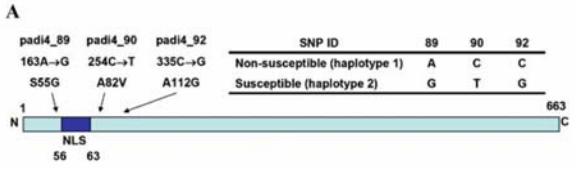


Fig. 3 *In vitro* and *in vivo* enzymatic activity of PADI4. (A) The profile of PADI4 protein. A haplotype of PADI4 associated with susceptibility to RA contains three amino acid substitutions: S55G, A82V and A112G, as well NLS indicates the nuclear translocation signal peptide [2, 52]. (B) PADI4, SNPs (S55G and A82V), SNPs (A82V and A112G), SNPs (S55G and A112G) and SNPs (S55G, A82V and A112G) of PADI4 enzymes were purified and measured the enzyme activity by a continuous spectrophotometric method *in vitro*. The enzyme amount used in each assay was 25 μg [42]. * $P < 0.05$ and ** $P < 0.01$ as compared with WT PADI4. (C) PADI4 and triple SNP PADI4 were assayed the effect of variant Ca^{2+} concentrations on the enzyme activity. (D) JK-Tet-On-PADI4 and JK-Tet-On-SNP PADI4 cells were treated with 50 μM *Dox* for 24 h. Following harvest of the cells, total proteins were extracted and an equal of amount protein was measured the enzyme expression and activity by the immunoblotting and colorimetric method *in vivo*. * $P < 0.05$ and ** $P < 0.01$. Data are representative of at least three experiments



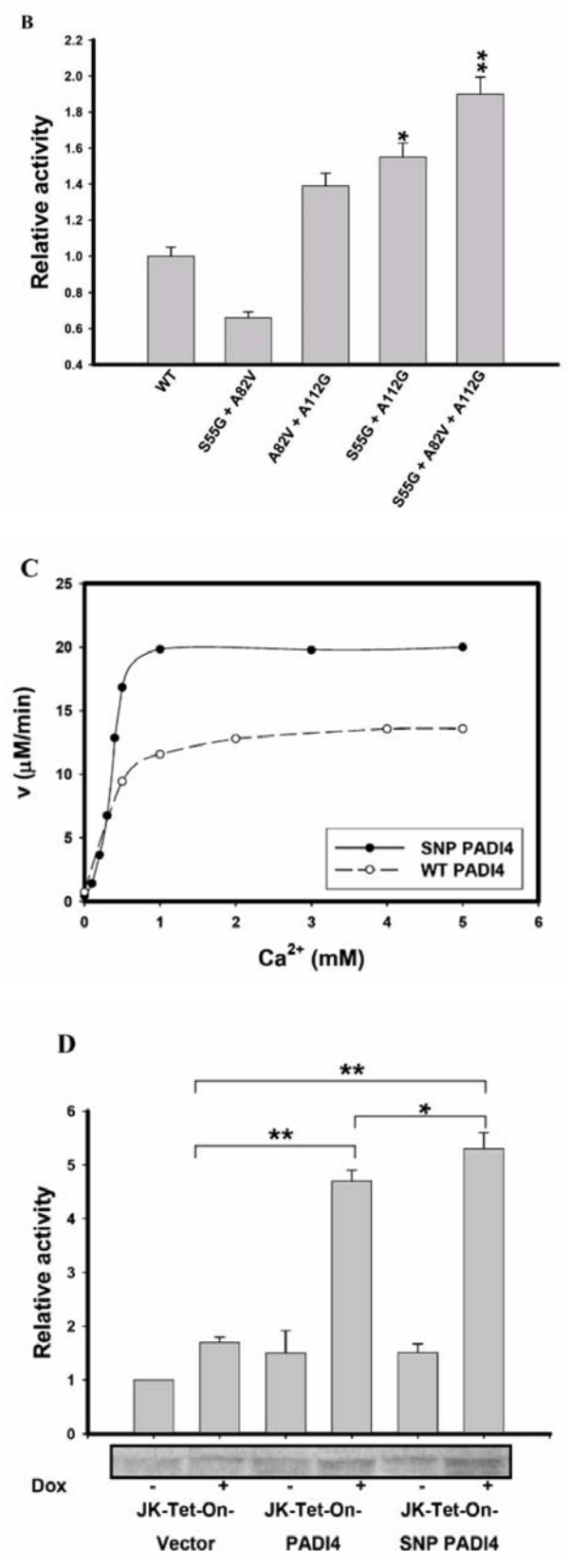


Fig. 4 Conditionally inducible SNP PADI4 diminished cell viability on JK-Tet-On cells. (A) JK-Tet-On-PADI4 and JK-Tet-On-SNP PADI4 cells were treated with 50 μM *Dox* for 0, 12, 24 and 36 h. Cell viability was determined by trypan blue exclusion assay. (B) JK-Tet-On-PADI4 and JK-Tet-On-SNP PADI4 cells were treated with 50 μM *Dox* for 36 h.

Cells were stained with acridine orange and then detected by fluorescence-microscope. Data were means ± SD of three experiments. * $P < 0.05$ and ** $P < 0.01$. Data are representative of at least three experiments

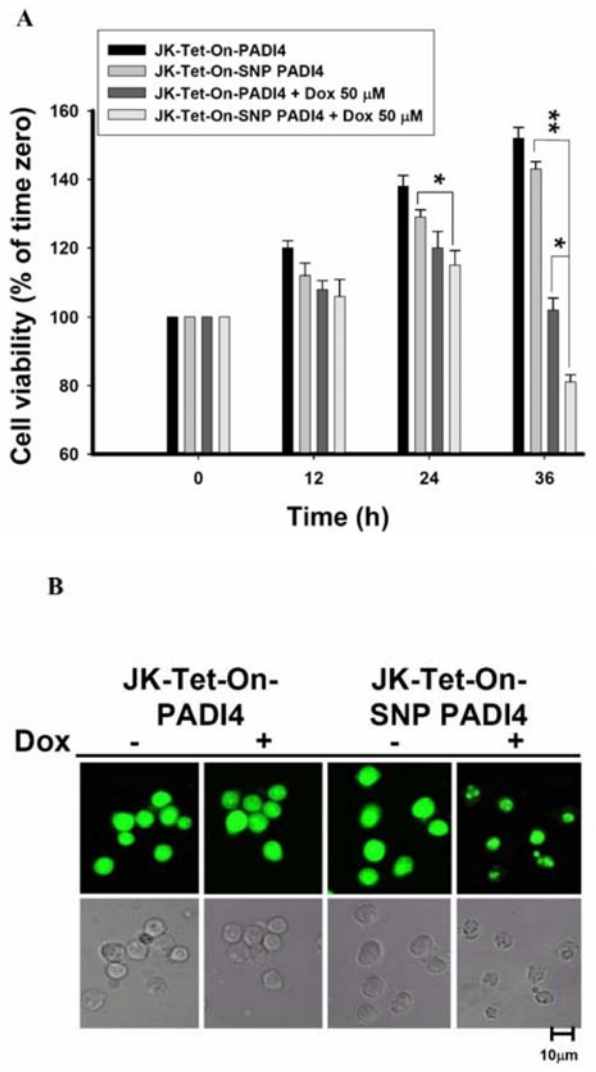


Fig. 5 *Ion* raised the inducible SNP PADI4-stimulated apoptosis when compared with PADI4. (A) JK-Tet-On-PADI4 and JK-Tet-On-SNP PADI4 cells were pretreated with or without 50 μM *Dox* for 6 h, treated with or without 1 μM *Ion* for 0–36 h and then the cell viability was determined by trypan blue exclusion assay. (B) JK-Tet-On-Vector, JK-Tet-On-PADI4 and JK-Tet-On-SNP PADI4 cells were pretreated with or without 50 μM *Dox* for 6 h and then treated with 1 μM *Ion* for 36 h. Genomic DNA was isolated, and then analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. (C) JK-Tet-On-PADI4 and JK-Tet-On-SNP PADI4 cells were pretreated with or without 50 μM *Dox* for 6 h, treated with or without 1 μM *Ion* for 36 h and then apoptotic events were measured by flow cytometry using PI staining. * $P < 0.05$ and ** $P < 0.01$. M, DNA ladder marker. Data are representative of at least three experiments

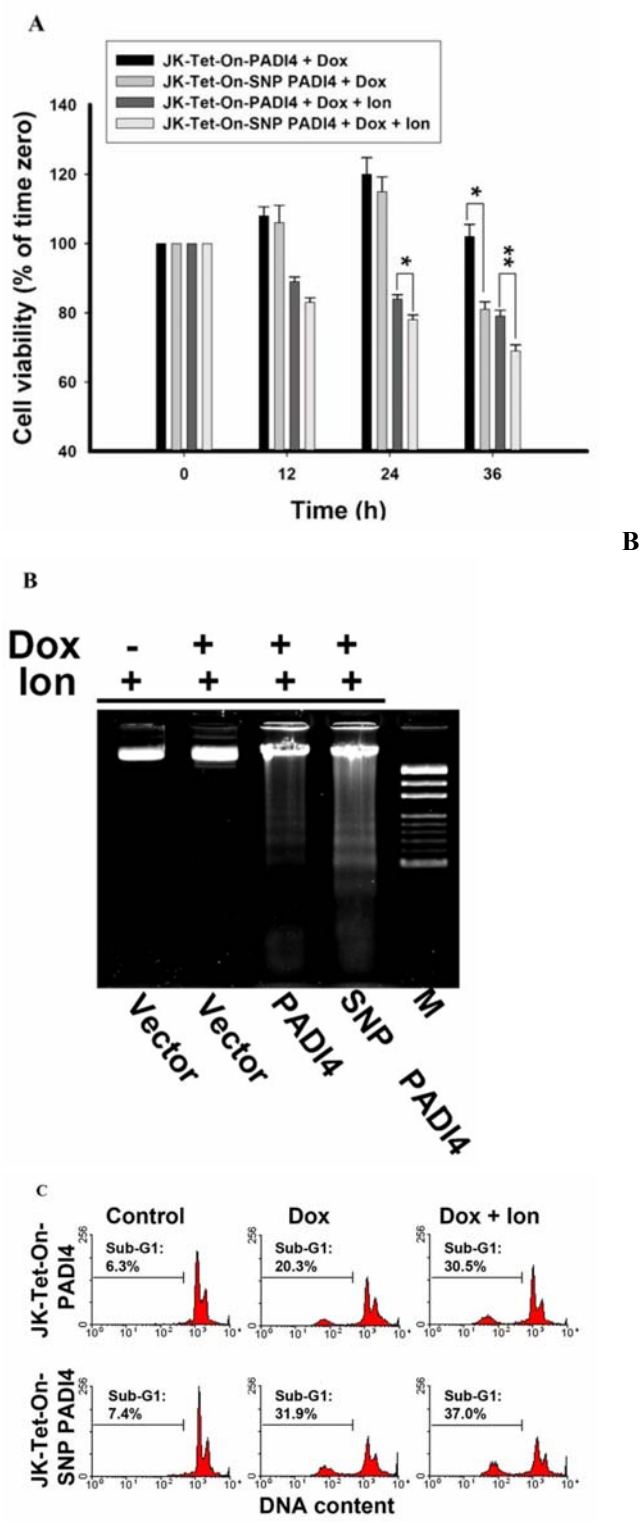


Fig. 6 Conditionally inducible SNP PADI4 overexpression induced the decline of Bcl-xL and Bax accumulation which triggered cytochrome *c* release. JK-Tet-On-PADI4 and JK-Tet-On-SNP PADI4 cells were pretreated with or without 50 μM *Dox* for 6 h and afterward treated with or without 1 μM *Ion* for 36 h. (A) Cells were harvested and total proteins were extracted

for immunoblotting with anti-Bid, Bcl-2, Bcl-xL, Bax and actin antibodies. (B) The $\Delta\psi_m$ was analyzed by flow cytometry using rhodamine 123. (C) Cytoplasmic and mitochondrial proteins were separated and detected by immunoblotting with anti-cytochrome *c* antibody. (D) JK-Tet-On-PADI4 and JK-Tet-On-SNP PADI4 cells were transiently transfected with m-Bcl-xL or WT-Bcl-xL plasmid for 12 h (−12 h), respectively. Afterward, cells were treated with 50 μM *Dox* for the indicated time (0, 24 and 48 h) and then cell viability was determined by trypan blue exclusion assay. * $P < 0.05$ and ** $P < 0.01$. Data are representative of at least three experiments

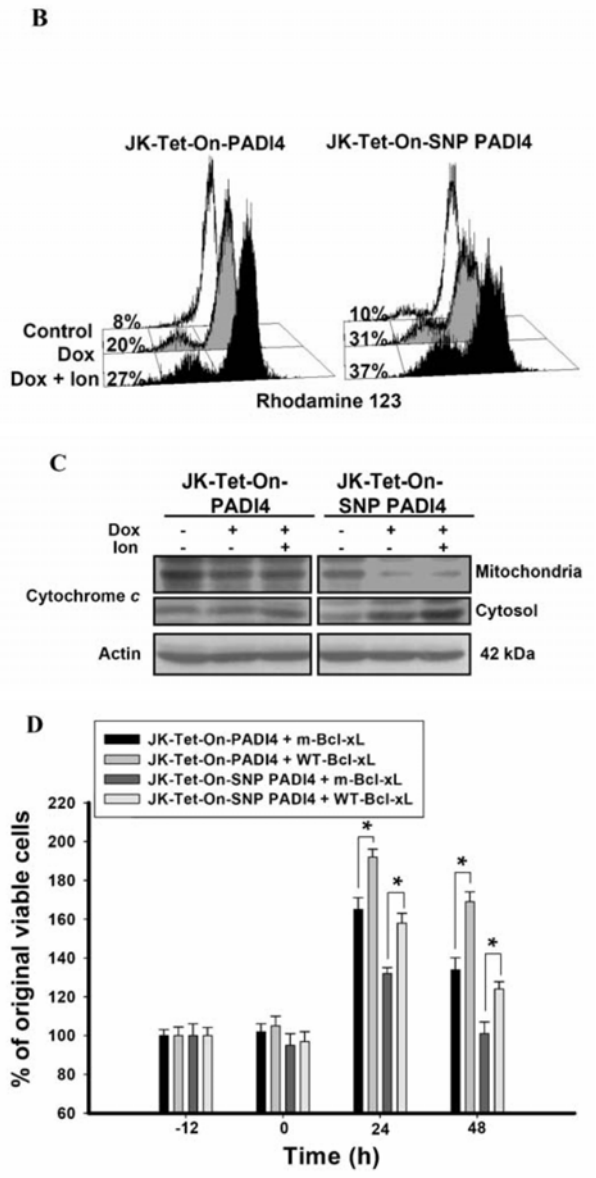


Fig. 7 Overexpression of SNP PADI4 by both *Dox* and *Ion* treatment induced caspase 9 and 3 activation, and the cleavage of PARP. JK-Tet-On-PADI4 and JK-Tet-On-SNP PADI4 cells were pretreated with or without 50 μM *Dox* for 6 h and treated with or without 1 μM *Ion* for 36 h. Cells were harvested and total proteins were extracted for immunoblotting with anti-caspase 9, caspase 3, PARP and actin antibodies. Data are representative of at least three experiments

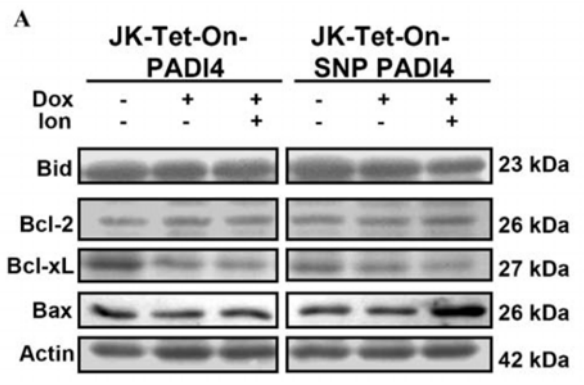


Table 1 Ionomycin (*Ion*) provoked the inducible PADI4- and SNP PADI4-stimulated cell apoptosis in the Jurkat T (JK)-Tet-On cells

Cell line	Time (h)	Cell cycle			
		Sub-G1	G1	S	G2/M
JK-Tet-On-PADI4 + Dox					
	0	5.9 ± 1.5	46.8 ± 2.0	16.7 ± 4.3	27.2 ± 1.3
	12	8.9 ± 1.5	46.6 ± 1.5	16.7 ± 4.6	21.8 ± 1.6
	24	11.1 ± 1.5	49.9 ± 1.4	16.3 ± 3.6	18.6 ± 3.6
	36	20.1 ± 1.6	44.5 ± 2.7	12.6 ± 1.7	19.4 ± 1.2
JK-Tet-On-PADI4 + Dox + Ion					
	0	6.1 ± 1.3	47.5 ± 2.4	15.2 ± 2.2	25.4 ± 2.1
	12	10.5 ± 1.3	50.8 ± 1.3	12.9 ± 2.5	20.4 ± 2.1
	24	*23.5 ± 1.1	42.3 ± 1.4	12.2 ± 1.6	16.8 ± 2.8
	36	**29.4 ± 1.4	41.1 ± 1.4	10.8 ± 1.6	16.5 ± 2.1
JK-Tet-On-SNP PADI4 + Dox					
	0	7.4 ± 1.2	48.8 ± 2.5	15.2 ± 2.1	28.6 ± 1.8
	36	**31.9 ± 1.8	33.4 ± 1.7	14.6 ± 2.3	20.1 ± 2.8
JK-Tet-On-SNP PADI4 + Dox + Ion					
	0	8.0 ± 1.4	47.9 ± 1.7	16.3 ± 1.3	29.6 ± 2.1
	36	*37.0 ± 1.5	33.8 ± 2.2	10.1 ± 1.9	19.1 ± 1.7

*$P < 0.05$ and **$P < 0.01$ as compared with JK-Tet-On-PADI4 + Dox cells.
*$P < 0.05$ as compared with JK-Tet-On-SNP PADI4 + Dox cells.

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成果報告 貳 （已發表於2005, *Anal. Biochem.*）

Abstract

A simple, continuous spectrophotometric assay for peptidylarginine deiminase (PAD) is described. Deimination of peptidylarginine results in the formation of peptidylcitrulline and ammonia. The ammonia released during peptidylarginine hydrolysis is coupled to the glutamate-dehydrogenase-catalyzed reductive amination of α-ketoglutarate to glutamate and reduced nicotinamide adenine dinucleotide (NADH) oxidation. The disappearance of absorbance at 340 nm due to NADH oxidation is continuously measured. The specific activity obtained by this new protocol for highly purified human PAD is comparable to that obtained by a commonly used colorimetric procedure, which measures the ureido group of peptidylcitrulline by coupling with diacetyl monoxime. The present continuous spectrophotometric method is highly sensitive and accurate and is thus suitable for enzyme kinetic analysis of PAD. The Ca2+ concentration for halfmaximal activity of PAD obtained by this method is comparable to that previously obtained by the colorimetric procedure.

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Abstract

Peptidylarginine deiminases (PADIs) convert peptidylarginine into citrulline via posttranslational modification. One member of the family, PADI4, plays an important role in immune cell differentiation and cell death. To elucidate the participation of PADI4 in haematopoietic cell death, we examine whether inducible overexpression of PADI4 enhances the apoptotic cell death. PADI4 reduced the viability in a dose- and time-dependent manner of human leukemia HL-60 cells and human acute T leukemia Jurkat cells. The apoptosis-inducing activities were determined by nuclear condensation, DNA fragmentation, sub-G_1 appearance, loss of mitochondrial membrane potential ($\Delta\psi_m$), release of mitochondrial cytochrome c into cytoplasm and proteolytic activation of caspase 9 and 3. Following PADI4 overexpression, cells arrest in G1 phase significantly before their entrance into apoptotic cell death. PADI4 increases tumor suppressor p53 and its downstream p21 to control cell cycle. In the detections of protein expression and kinase activity, all protein levels of cyclin-dependent kinases (CDKs) and cyclins are not reduced except cyclin D, however, CDK2 (G1 entry S phase) and CDK1 (G2 entry M phase) enzyme activities are inhibited by conditionally inducible PADI4. p53 also expands its other downstream Bax to induce cytochrome c release from mitochondria. According to these data, we suggest that PADI4 induces apoptosis mainly through cell cycle arrest and mitochondria-mediated pathway.

Furthermore, p53 features in PADI4-induced apoptosis by increasing intracellular p21 to control cell cycle and by Bax accumulation to decline Bcl-2 function, destroy $\Delta\psi_m$, release cytochrome c to cytoplasm and activate the caspase cascade.

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