行政院國家科學委員會專題研究計畫　成果報告

胞漿素原活化劑與胞漿素原活化抑制劑在人類正常頰黏膜及口腔黏膜下纖維化症的表現

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一、中文摘要

嚼食檳榔是造成口腔黏膜下纖維化的主要原因，其會造成黏膜上皮萎縮與過量的膠原蛋白堆積在結締組織層中。檳榔素是檳榔中含量最多的植物鹼，其亦被認為與口腔黏膜下纖維化(OSF)關係密切，以往有關此類研究多從膠原蛋白著手，鮮少有研究探討檳榔成分對第一型胞漿素原活化抑制劑的影響。本研究發現 OSF 較人類正常頰黏膜組織有著大量 type I plasminogen activator inhibitors (PAI-1)表現，arecoline 可提高正常頰黏膜纖維母細胞 PAI-1 的表達，且在病理切片以免疫組織化學染色法亦發現 PAI-1 在 OSF 較正常組織有明顯的表現。從本研究結果發現檳榔可能透過此一機轉造成正常黏膜轉變成黏膜下纖維化症。

關鍵詞：檳榔素；口腔黏膜下纖維化症；頰纖維母細胞；第一型胞漿素原活化抑制劑

Abstract

Type I plasminogen activator inhibitor (PAI-1) is a 50 kDa glycoprotein belonging to the serine protease superfamily. PAI-1 is consistently and dramatically upregulated in a variety of fibrotic diseases. The aim of this study was to compare PAI-1 expression in normal human buccal mucosa and oral submucous fibrosis (OSF) specimens and further explore the potential mechanism that may lead to induce PAI-1 expression. Twenty-five OSF specimens and six normal buccal mucosa were examined by immunohistochemistry. The activity of PAI-1 from cells cultured from OSF and normal buccal mucosa were using reverse-transcriptase polymerase chain reaction (RT-PCR) and Western blots. PAI-1 expression was significantly higher in OSF specimens and expressed mainly by fibroblasts, endothelial cells, and inflammatory cells. In addition, OSF exhibits higher PAI-1 expression than normal buccal mucosa fibroblast (BMF) both in mRNA and protein levels. To verify whether arecoline, a major areca nut alkaloid, could affect PAI-1 expression by human BMFs, RT-PCR and Western blots were used. The results demonstrated highly elevated PAI-1 mRNA and protein expression in normal human BMFs stimulated by arecoline. Taken together, these results suggest that PAI-1 expression is significantly upregulated in OSF tissues from areca quid chewers and arecoline may be responsible for the enhanced PAI-1 expression in vivo.

Keywords: Arecoline; Buccal mucosal fibroblasts; Oral submucous fibrosis; Type I plasminogen activator inhibitor

1. Introduction

Oral submucous fibrosis (OSF) is regarded as a precancerous condition [1], and is a chronic insidious disease of oral subepithelial connective tissue resulting in stiffness of the oral mucosa and inability to open the mouth. OSF is histologically characterized by epithelial atrophy and progressive accumulation of collagen fibers in the lamina propria and submucosa of the oral mucosa [2].

Although the etiology of OSF is not completely understood, there is a close epidemiological association with the habit of chewing areca quid [3,4]. Fibroblasts obtained from OSF subjects revealed a higher elevation for collagen synthesis than normal buccal mucosa fibroblasts (BMFs) [5]. Stabilization of collagen and prevention of collagenase degradation in oral mucosa [6,7] and the attendant increase of lysyl oxidase activity [8] also contribute to abnormal deposition of collagen fiber in OSF. Arecoline, a major areca nut alkaloid, was found to stimulate human BMF proliferation [9,10] and collagen synthesis in vitro [9]. Recently, our studies have shown that elevation of vimentim expression [11], upregulation of cyclooxygenase-2 expression [12], increased tissue inhibitor metalloproteinase-1 and decreased matrix metalloproteinase-2 activity [13] may contribute to the extracellular components accumulation in OSF. However, very little is currently known the precise mechanisms about the biochemical/molecular biology of OSF.

Plasminogen activators (PAs) and their inhibitors are thought to be key participants in the balance of proteolytic and antiproteolytic activities that regulates matrix turnover. PAs in normal plasma and in tissue are inactive and complexed to

plasminogen activator inhibitors, of which type I plasminogen activator inhibitor (PAI-1) is believed to be the most important [14,15]. PAI-1 is consistently and dramatically upregulated in a variety of fibrotic diseases, including glomerulosclerosis [16], liver fibrosis induced by carbon tetrachloride [17], and spontaneously occurring [18] or bleomycin-induced pulmonary fibrosis [19]. Importantly, bleomycin-induced fibrosis is more severe in transgenic mice overexpressing PAI-1, and less so in PAI-1 deficient mice [20].

Currently, there is limited information about the regulation of PAI-1 expression in OSF both in vitro and in vivo. The purpose of this study was to test whether PAI-1 expression regulated within OSF specimens and to further explore possible pathogenetic mechanisms that might lead to enhanced expression of PAI-1 in vivo. Moreover, we set out to explore whether expression of PAI-1 triggered in human BMFs by arecoline in vitro.

2. Materials and methods

2.1. Immunohistochemistry

Formalin-fixed, paraffin-embedded specimens of six normal buccal mucosa from non-areca quid chewers, and twenty-five OSF specimens from areca quid chewers, were drawn from the files of the Department of Pathology, Chung Shan Medical University Hospital. Diagnosis was based on histological examination of hematoxylin- and eosin-stained sections. Five μm sections were stained with the monoclonal anti-PAI-1 antibody (Santa Cruz Biotechnology, CA, USA) (1:100 dilution) using a standard avidin-biotin-peroxidase complex method [11]. AEC (DAKO, Carpinteria, USA) was then used as the substrate for localizing the antibody binding. Negative controls included serial sections from which either the primary or secondary antibodies were excluded. The preparations were counterstained with hematoxylin, mounted with Permount (Merck, Darmstadt, Germany) and examined by light microscopy.

2.2. Cell culture

Six healthy individuals without areca quid chewing habits were selected from the Department of Oral Surgery (Chung Shan Medical University Hospital, Taichung, Taiwan) with the informed consent for this study. Biopsy specimens were derived from histologically normal areas of surgical third molar extraction from patients. The

OSF specimens were obtained from ten male patients with areca quid chewing habits during surgical biopsy. Clinical diagnosis was confirmed by histopathological examination of the biopsy specimens. Fibroblasts were cultured using an explant technique as described previously [21,22]. The tissues were minced using sterile techniques and wash twice in phosphate buffer saline (PBS) supplemented with antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml of fungizone). Explants were placed into 60 mm Petri dishes and maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10 % fetal calf serum (FCS) (Gibco Laboratories, Grand Island, NY, USA) and antibiotics as described above. Cell cultures between the third and eighth passages were used in this study.

2.3. Expression of PAI-1 mRNA in OSF and BMF

Total RNA was prepared using TRIzol reagent (Gibco Laboratories, Grand Island, NY, USA) following the manufacturer's instructions. Single-stranded DNA was synthesized from RNA in a 15 µl reaction mixture containing 100 mg random hexamer and 200 units of Moloney murine leukemia virus reverse transcriptase (Gibco Laboratories, Grand Island, NY, USA). The reaction mixture was diluted with 20 µl of water and 3 µl of the diluted reaction mixture was used for the polymerase chain reaction (PCR). PCR reaction mixture contains 10 pmol of forward and reverse

primers and 2 units of Tag DNA polymerase. Amplification was performed at 25 cycles for GAPDH and 30 cycles for PAI-1 in a thermal cycle. Each cycle consisted of 1 min of denaturation at 94°C, 1 min of annealing at 57°C, and 1 min of extension at 72°C. The sequences of primers used were as follows [23]:

A) GAPDH Forward: 5'-TCCTCTGACTTCAACAGCGACACC-3'

Reverse: 5'-TCTCTCTTCCTCTTGTGCTCTTGG-3'

B) PAI-1 Forward: 5'-ATCACCATCTTCCAGGAG-3'

Reverse: 5'-ATCACCATCTTCCAGGAG-3'

The PCR products were analyzed by agarose gel electrophoresis and a 325 bp band for PAI-1 was noted. When the band densities were measured and compared with the density of the band obtained for the housekeeping gene GAPDH, relative proportions of mRNA synthesis could be determined within each experiment. The intensity of each band after normalization with GAPDH mRNA was quantified by the photographed gels with a densitometer (AlphaImager 2000; Alpha Innotech, San Leandro, CA, USA).

2.4. Western blot for PAI-1 in OSF and BMF

Cell extracts from BMF and OSF were solubilized with SDS-solubilization buffer (5 mM EDTA, 1 mM $MgCl_2$, 50 mM Tris-HCl , pH 7.5 and 0.5 % Trition X-100, 2 mM phenylmethysulfonyl fluoride, and 1 mM N-ethylmaleimide) for 30 min on ice.

Then, cell lysates were centrifuged at 12,000 g at 4°C and the protein concentrations determined with Bradford reagent using bovine serum albumin (BSA) as standards. Equivalent amounts of total protein per sample of cell extracts were run on a 10 % SDS-PAGE and immediatelt transferred to nitrocellulose membranes. The membranes were blocked with PBS containing 3 % BSA for 2 h, rinsed, and then incubated with primary antibodies anti-PAI-1 diluted 1:500 in PBS containing 0.05 % Tween 20 for 2 h. After 3 washes with Tween 20 for 10 min, the membranes were incubated for 1 h with biotinylated secondary antibody diluted 1:1000 in the same buffer, washed again as described above and treated with 1:1000 streptavidin-peroxidase solution for 30 min. After a series of washing steps, the reactions were developed using Diaminobenzidine (DAB, Zymed Laboratories, San Francisco, CA, USA). The intensities of the obtained bands were determined using densitometer AlphaImager 2000.

2.5. Effect of arecoline on PAI-1 mRNA and protein in BMF

BMF were seeded 1×10^5 cells per well into 10 cm culture dish and incubated for 24 h. Then the medium was changed to a medium containing 10 % heated activated FCS and various concentrations of arecoline (Sigma, St. Louis, MO, USA) (0 to 160 μg/ml). Total RNA was isolated after 6 h incubation period for RT-PCR as described above. Cell extracts were collected after 24 h incubation period for Western blotting

as described earlier.

2.6. Statistical analysis

Triplicate or more separate experiments were performed throughout this study. The significance of the results obtained from control and treated groups was statistically analyzed by the paired Student *t*-test. A *p*-value of < 0.05 was considered to be statistically significant.

3. Results

The connective tissue from normal buccal mucosa consists of loosely woven collagen bundles in the lamina propria revealing a fine reticular pattern next to the epithelium and a coarser pattern deeper in the lamina propria. Normal human buccal mucosa demonstrated with very faint PAI-1 expression. (Fig. A). All OSF samples exhibited the features of either moderately advanced or advanced submucous fibrosis. PAI-1 expression was observed mainly in the cytoplasm of fibroblasts, endothelial cells, and inflammatory cells (Fig. 1B).

RT-PCR and Western blotting for PAI-1 were used to compare the cells cultured from BMF and OSF. As shown in Fig. 2, OSF specimens exhibited significantly higher PAI-1 mRNA expression than BMFs. From the AlphaImager 2000, the intensity of PAI-1 mRNA from OSF was elevated 3.1 fold as compared with BMFs ($P<0.05$). In addition, OSF demonstrated significantly higher PAI-1 protein expression than BMF by Western blot (Fig. 3). From the AlphaImager 2000, the intensity of PAI-1 protein from OSF was elevated 3.8 fold as compared with BMFs ($P<0.05$).

To examine the effect of arecoline on the PAI-1 expression, human BMFs were treated with arecoline and the levels of mRNA and protein were measured. The effects of arecoline on the PAI-1 gene expression in three different cell strains were similar,

and their intracellular variations were limited.

RT-PCR were used to verify whether arecoline could affect PAI-1 mRNA expression by human BMFs. Figure 4A reveals a dose-dependent change following treatment of BMF with arecoline for 6 h. Arecoline was found to elevate PAI-1 expression in a dose-dependent manner ($P<0.05$). From the AlphaImager 2000 (Fig. 4B), the amount of PAI-1 was elevated about 1.8 and 2.5 fold at concentrations of 20 and 80 μg/ml, respectively, compared with control ($P<0.05$).

The effect of arecoline on PAI-1 protein in BMF during 24 h incubation period is shown in Figure 5A. Arecoline was found to elevate PAI-1 expression in a dose-dependent manner ($P<0.05$). However, the arecoline concentration up to 160 μg/ml, PAI-1 diminished to control level. From the AlphaImager 2000 (Fig. 5B), the amount of PAI-1 was about 4.8 fold at a concentration level of 40 μg/ml compared with control ($P<0.05$).

4. Discussion

The biochemical events involved in the development of the OSF are not well understood at present, but the fibrinolytic pathway is believed to play an important role. It is probably a consequence of disturbances in the homeostatic equilibrium between synthesis and degradation of extracellular matrix (ECM) molecules. One of the key regulators of fibrinolysis is the plasminogen/plasmin system. Plasminogen is activated by tissue type activator (t-PA) and the urokinase type activator (u-PA) to plasmin which promote the degradation of ECM. This process is counterbalanced by PAI-1, which inactivated t-PA and u-PA, resulting in a decreased production of plasmin and hence an accumulation of ECM [14]. Impaired fibrinolysis may result from increased concentrations of the principal inhibitor of the fibrinolytic system, PAI-1.

PAI-1 is a 50 kDa glycoprotein belonging to the serine protease superfamily [24]. PAI-1 is consistently and dramatically upregulated in a variety of fibrotic diseases [16-20]. To the best of our knowledge, we first found that PAI-1 expression is upregulated in OSF specimens compared to normal buccal mucosa. Strong immunostaining for PAI-1 was detected in fibroblasts, endothelial cells and inflammatory cells. In addition, OSF has significantly higher PAI-1 expression than BMF derived from normal buccal mucosa both in mRNA and protein levels. PAI-1

deposition is associated with OSF, suggesting that it could be play an important role in the ECM turnover. This phenomena promoted us to elucidate that OSF may be due to increased synthesis and deposition of ECM proteins, altered fibrolysis or both.

The etiology of OSF is still not quite clear, it is known that an association exists the chewing of areca quid. In this study, we first report upregulation of PAI-1 mRNA and protein expression in normal human BMFs stimulated by arecoline. This suggests that one of the pathogentic mechanisms of OSF may be the synthesis of PAI-1 expression by resident cells in response to areca nut challenge. The accumulation of ECM components in oral mucosal connective tissue may be caused by a simultaneous effect on PAI-1.

The other main extracellular proteolytic system, the matrix metalloproteinases (MMPs) and their inhibitors. Recently, our previous study has demonstrated that arecoline reduces the secretion of gelatinolytic activity of MMP-2 from BMF in cell culture experiments [13]. Plasmin degrades fibrin and several ECM and adhesion proteinases and, by activation of procollagenases, may contribute to collagen degradation [14]. Indeed, the plasmin-dependent pathway is understood to be a significant alternative pathway for the initiation of ECM degradation by MMPs [25]. PAI-1, as a potent inhibitor of u-PA, was demonstrated to inhibit u-PA-induced, MT1-MMP-mediated MMP-2 activation [26]. Thus, PAI-1 regulates plasmin

formation and fibrinolysis and, through several different mechanisms, plays a role in the control of MMP activation. The interaction between MMPs and PAI-1 is worthy of further investigation.

As far as we known, this is the first systematic attempt to evaluate the role of PAI-1 expression in areca quid associated-OSF in human at both in vivo and in vitro levels. We have demonstrated for the first time that PAI-1 is elevated in OSF than normal buccal mucosa. Data from our in vitro experiments showed that arecoline was capable of stimulating PAI-1 mRNA and protein expression in human BMFs. This suggests that one of the pathogenic mechanisms of OSF in vivo may be the synthesis of PAI-1 by resident cells in response to areca nut challenge. However, the genetic and environmental determinants of PAI-1 expression are still incompletely understood. Further research is required, however, including detection of PAI-1 gene trancripts, specifically whether OSF evolves solely as a result of increased/altered *de novo* synthesis and deposition of PAI-1 by areca nut constitutes.

Acknowledgments

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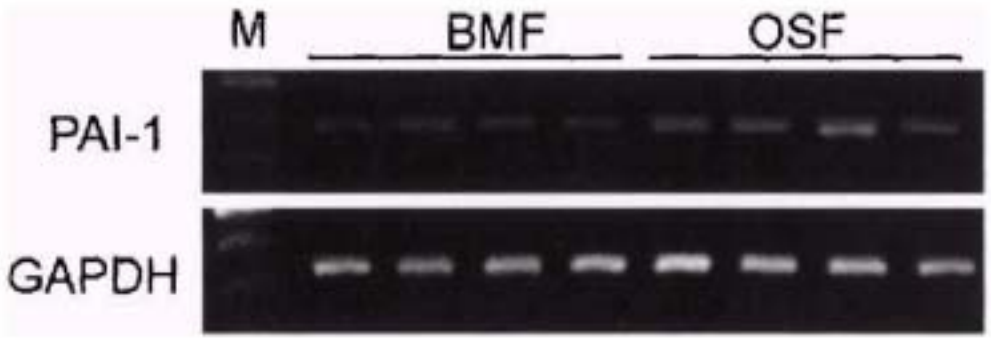

Fig. 2. Comparison of the PAI-1 mRNA level from BMFs and OSFs using RT-PCR assay. GAPDH gene was performed in order to monitor equal RNA loading. OSF specimens are significantly upgegulated PAI-1 mRNA expression than BMFs.

Fig. 3. Comparison of the PAI-1 protein expression from BMFs and OSFs using Western blot assay. α-Tubulin was performed in order to monitor equal protein loading. OSF specimens exhibit significantly higher PAI-1 expression than BMFs.

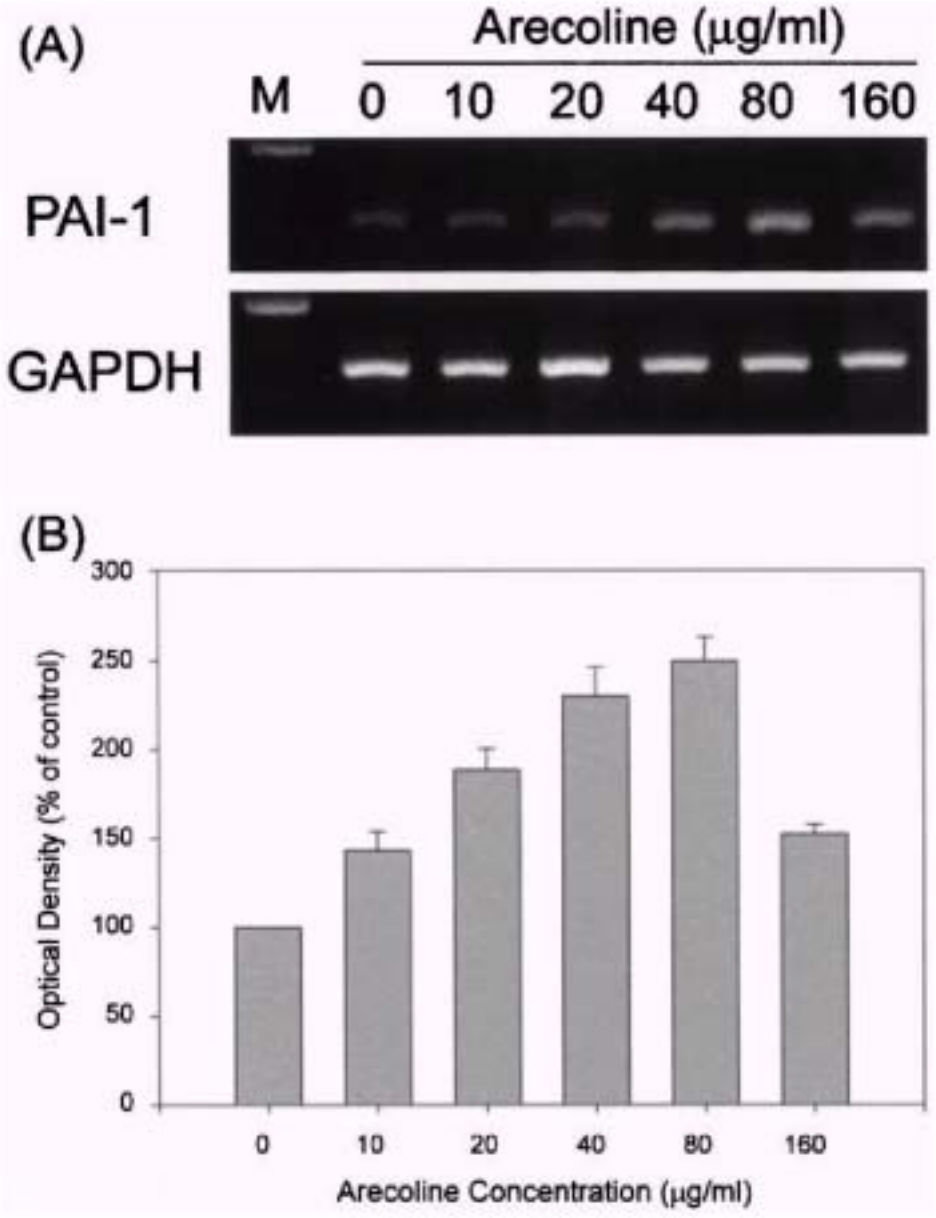

Fig. 4. (A) Expression of PAI-1 mRNA gene in arecoline-treated human BMFs by RT-PCR assays. M = DNA molecular size marker. (B) Densitometric analysis of the PAI-1 bands was calculated from their mRNA activity. Optical density values represent the means of three different BMF ± standard deviations.

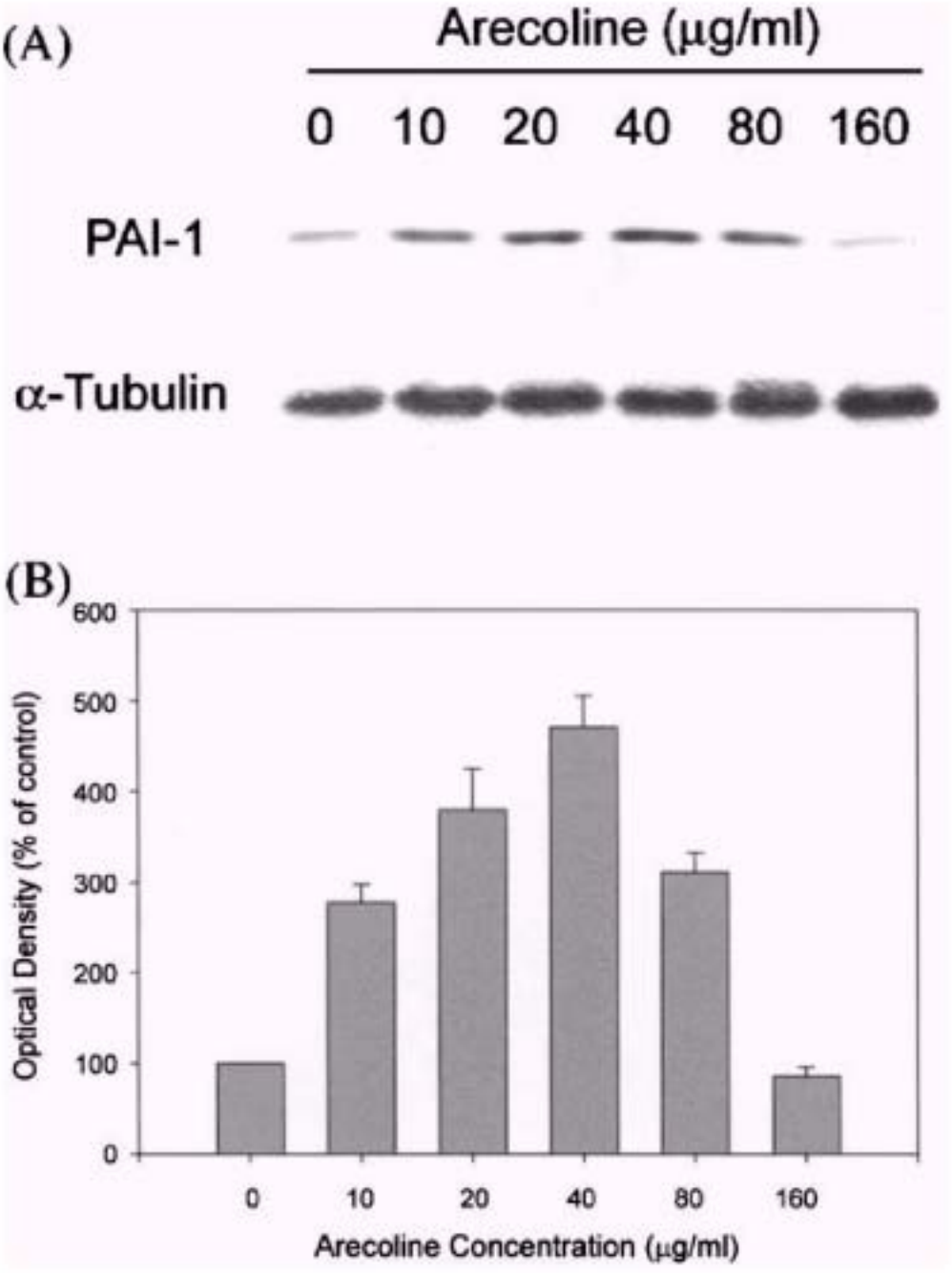

Fig. 5. (A) Expression of PAI-1 in arecoline-treated human BMFs by Western blot. (B) Densitometric analysis of the PAI-1 bands was calculated from their protein activity. Optical density values represent the means of three different BMF ± standard deviations.