TAT-Hsp27이 MDPC-23 상아모세포주의 부착과 이주에 미치는 효과

Effect of TAT-Hsp27 on Adhesion and Migration of Odontoblastic MDPC-23 Cells

2010년 2월 일

조선대학교 대학원

치 의 학 과

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지도교수 황 호 길

이 논문을 치의학 박사학위 논문으로 제출함.

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조선대학교 대학원

치 의 학 과

임 영 신

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위원장 서울대학교 교수 박 주 철 인

위 원 조선대학교 교수 윤 정 훈 인

위 원 조선대학교 교수 김 흥 중 인

위 원 조선대학교 교수 민 정 범 인

위 원 조선대학교 교수 황 호 길 인

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조선대학교 대학원

Contents

ABSTRACT

Effect of TAT-Hsp27 on Adhesion and Migration of Odontoblastic MDPC-23 Cells

Lim, Young-Shin, D.D.S., M.S.D.
Advisor : Prof. Hwang, Ho-Keel, D.D.S., M.S.D., Ph.D.
Department of Dentistry,
Graduate School, Chosun University

Odontoblasts are involved in tooth repair and regeneration as well as dentine formation. Cell adhesion and migration is a critical process for tissue regeneration. In this study, we examined whether delivery of Hsp27 into the cells using a TAT-fusion protein system enhances adhesion and migration of murine odontoblast-like cells, MDPC-23 cells. TAT-fusion protein system delivered successfully Hsp27 into the cells. Transduction of TAT-Hsp27 induced adhesion and migration of MDPC-23 cells in a dose-dependent manner. In addition, transduction of TAT-Hsp27 increased the protein expression of β1 integrin and focal adhesion formation, and induced the phosphorylation of FAK and ERK. TAT-Hsp27-induced migration of MDPC-23 cells was restored by treatment of anti β1 integrin antibody. These findings suggest that TAT-Hsp27 may promote adhesion and migration of odontoblast-like

cells via β1 integrin-mediated signaling and can be considered as a candidate of therapeutic application for tooth regeneration.

Key words: Odontoblast, adhesion, migration, TAT-Hsp27, β1 integrin

Ⅰ. Introduction

Heat shock proteins (Hsps) are a class of conserved and stress-inducible proteins[1]. Hsps play critical roles in signal transduction, cell cycle regulation, and cell proliferation through their activities involved with protein folding, trafficking, degradation, and the fostering of signaling responses[1,2]. According to their molecular size, Hsps have been divided into several families; Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and low molecular mass Hsps[3].

Hsp27 is a member of heat shock protein family and induced in response to a variety stresses including oxidative stress[4,5]. Hsp27 also takes part in cellular protection by preventing apoptosis via direct binding to either cytochrome c or procaspase-3[6,7]. In addition, Hsp27 promotes adhesion, invasion, and migration in a variety of mammalian cell types[8-11].

Regeneration of a functional and living tooth is considered as the most promising therapeutic strategy for the replacement of damaged tooth[12-14]. Odontobalsts that are a part of outer surface of dental pulp are involved in tooth repair and regeneration as well as dentine formation (dentinogenesis) . Cell adhesion and migration are critical for regeneration process in a variety of physiological and pathological conditions as well as tumor metastasis. It has been known that Hsp27 is involved in the cell death of dental pulp cells during stress because

of modification and activation[15]. Although Hsp27 regulates cell adhesion and migration in wound healing or cancer cell migration[9,16], the precise function of Hsp27 in odontoblasts remains to be fully elucidated. Recently, the TAT protein of the human immunodeficiency virus 1 (HIV-1) has been shown to be transduced efficiently into mammalian cells[17]. Many fusion proteins utilizing this TAT leading sequence have been generated that result in effective transduction of functional proteins. Previous study demonstrated that TAT-Hsp40 trasnduced cells were more resistant to cellular cytotoxicity and cell death[18].

In this study, we examined the effect of Hsp27 using a TAT-fusion protein system on cell adhesion and migration of murine odontoblast-like MDPC-23 cells. Possible underlying mechanisms were also investigated.

Ⅱ. Materials and Methods

1. Purification of TAT-Hsp27 fusion protein

The TAT-Hsp27 expression vector was constructed as previously described[19]. Briefly, TAT-fused Hsp27 protein was expressed in E. coli BL21 (DE3) pLysS cells (Invitrogen) and purified using the urea-denaturing protein purification method[19]. The cells were lysed via sonication in lysis buffer (1 mM imidazole, 100 mM NaCl, 20 mM HEPES, pH 8.0) containing 8 M urea. The cell lysates were centrifuged at 12,000 g for 30 minutes at 4 ℃ and 1 mM of Ni2+-NTA agarose was added to the cleared supernatant. After 2 hours of gentle mixing at 4 ℃, the resins were transferred into a column and subsequently washed three times with 10 ml of washing buffer (20 mM imidazole, 300 mM NaCl, 50 mM phosphate buffer, pH 8.0). The proteins were eluted four times with 1 ml of elution buffer (500 mM imidazole, 300 mM NaCl, 50 mM phosphate buffer, pH 8.0). The urea denaturant was removed with a Mono-Q ionic exchange column and desalinated with a PD10 Sephadex size exclusion column. The protein concentration was quantified via the Bradford assay and confirmed by SDS-polyacrylamide gel electrophoresis (PAGE).

2. Transduction and detection of TAT-Hsp27 fusion protein

MDPC-23 cells[20,21], odontoblast-like line, were cultured in Dulbecco's

modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO), 100 μg/ ml streptomycin and 100 units/ml pemicillin, 100 μg/ml non essential amino acid at 37℃ in a 5% CO_2 humidified incubator. One day prior to the addition of the fusion protein, the cells were seeded on 12-well plates at a density of 310^5 cells/ml. The culture media was then removed and replaced with 1 ml of fresh DMEM containing various doses of TAT-Hsp27. 24 hours after transduction, the cells were lysed and 25 μg of total protein was loaded onto SDS-PAGE. The proteins were separated by electrophoresis and Western blotting was accomplished using an anti-Hsp27 antibody (diluted 1:1000; Santa Cruz Biotechnology, USA).

For immunocytochemistry, the transduced cells were washed twice in PBS and fixed with 4% paraformaldehyde in PBS for 10 minutes. After being washed with PBS twice, the cells were incubated with methanol for 2 minutes. For immunostaining, the cells were pre-treated for 30 minutes with 5% BSA in PBS. The cells were then incubated with 2.5% BSA in PBS containing anti-Hsp27 antibody (diluted 1:1000; Santa Cruz Biotechnology, USA) for 1 hour and washed with PBS for 10 minutes. The cells were incubated for 30 minutes with Cy3-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch) in 2.5% BSA containing PBS. Finally, the cells were washed twice for 10 minutes with PBS and mounted with ProLong Antifade mounting medium (Molecular Probes). Fluorescence analysis was conducted by conventional fluorescence microscopy.

3. Cell viability assay

Cell viability was determined using the MTT assay. The cells were seeded into the 12 well plates at a density of 1×10^5 cells/ml. The cells were cultured overnight and treated with TAT-Hsp27 for additional 24 hours. Cells were washed twice with PBS, and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenylte-trazolium bromide reagent diluted in culture media were added. After 3 hours incubation at 37℃, the media was removed and 250 μL of acid-isopropanol (0.04 mol/L HCl in isopropanol) were added to dissolve formazan crystal. The optical density (OD) value of the dissolved solute was then measured by Microplate Autoreader ELISA (Bio-Tek Instruments Inc., Winooski, VT) at 570 nm wavelength.

4. Cell adhesion assay

50 μl of type I collagen diluted in PBS was added to each well of 96-well plates and placed at 4℃ overnight. Subsequently, each well was rinsed with PBS and nonspecific binding sites were blocked with 1% BSA at 37℃ for 1 hour. 5×10^4cells were placed in each well and allowed to adhere at 37℃ for 30 to 90 minutes. TAT-Hsp27 was added to each well for 30 minutes before addition of the cells. Non-adherent cells were rinsed off with PBS, and the remaining cells were fixed with 4% paraformaldehyde for 5 minutes. The cells were

stained with 0.5% toluidine blue in 4% paraformaldehyde for 5 minutes and finally rinsed in water. The cells were solubilized with the addition of 100 μl of 1% SDS and optical density was measured by a Microplate Autoreader ELISA (Bio-Tek Instruments Inc., Winooski, VT) at 595nm. In addition, another experiment to visualize adherent cells was designed. For this, the cells and TAT-Hsp27 were applied to type I collagen-coated well equally with above. After removing non-adherent cells by washing with PBS, the samples were fixed with 200 L 10% formalin for 24 hours. The samples were processed and stained with hematoxylin and eosin for microscopic observation. Adherent cells were counted from five randomly selected areas at 200 magnifications.

5. Cell migration assay

Cell migration assay was performed using a Chemotaxis Cell Migration Assay kit (CHEMICON) according to the manufacturer's instructions. The cells were collected by trypsinization and resuspended in serum-free medium at a density of 2.5×10^4 /ml. The cells were added onto the insert and TAT-Hsp27 was treated in the media. In an experiment to examine the effect of β1 integrin on cell migration, the cells were pre-incubated with 25 μg/ml of anti-β1 Integrin antibody (R&D Systems, USA) for 20 minutes. The cells were then allowed to migrate for 24 hours at 37℃. The cells that migrated to the lower surface of the membrane were fixed with methanol and stained with

hematoxylin for 5 minutes. The number of migrated cells on the lower side of the membrane was counted from five randomly selected high power fields.

6. Western blot analysis

TAT-Hsp27-transduced cells were lysed with 2 SDS sample buffer. The proteins were resolved by SDS-PAGE and transferred onto PVDF membrane. After blocking with 5% skim milk in TBS at room temperature for 1 hour, the membranes were incubated with anti-HSP27, β1 Integrin, FAK, pFAK, pERK, and actin antibodies (Santa Cruz Biotechnology, USA) at 4 ℃ overnight. The membranes were then washed three times with TBS supplemented with 0.05% tween 20 (T-PBS), followed by incubation with secondary antibody at room temperature for 1 hour. Finally, the membranes were visualized with West-ZOL® (plus) (iNtRON Biotechnology Inc., Korea) detection reagent using chemiluminescence system of LAS-1000 Image Reader of Luminescence Image Analyzer (FUJIFILM Life Science, Tokyo, Japan).

7. Counting of focal adhesions

The cells (1×10^4) were seeded in a 4-well chamber slide, and treated with TAT-Hsp27 for 24 hours. The cells were washed with cold PBS,

fixed with 4% formaldehyde for 10 minutes at room temperature. After washing, the cells were blocked with 5% BSA in TBS for 30 minutes, and then incubated with pFAK antibody in 2.5% BSA in TBS for 2 hours. After washing, FITC-conjugated secondary antibody was added with DAPI solution (1 mg/ml). The number of focal adhesions per unit area was determined microscopically.

8. Statistical analysis

Statistical significance between groups was determined by two tailed Student's t-test (Excel, Microsoft). Differences were considered significant when p values were <0.05.

Ⅲ. Results

1. Transduction of TAT-Hsp27 fusion protein and its effect on the cell viability

We examined whether the TAT-Hsp27 fusion protein is capable of traversing the cytoplasmic membranes of cells. The TAT-Hsp27 protein was added to the MDPC-23 cells for 24 hours and then the level of transduced Hsp27 was determined by Western blot analysis. TAT-Hsp27 was delivered successfully into the cells in a dose-dependent manner (Fig. 1A). Immunocytochemistry also verified the significant accumulation of TAT-Hsp27 in both the cytoplasm and nucleus (Fig. 1B). In addition, to examine the effect of TAT-Hsp27 on cell viability, MDPC-23 cells were treated with various concentrations (0.1, 0.5, and 1μg) of TAT-Hsp27. Compared with control, TAT-Hsp27 at the tested concentrations did not affect the viability, showing more than 95% cell viability (Fig. 1C). Thus, we determined this range of concentration as the doses of TAT-Hsp27 to be used for subsequent experiments.

2. Effect of TAT-Hsp27 on the cell adhesion and migration

Previous studies showed that Hsp27 regulates adhesion, invasion, and migration in several cell types[8-11]. Therefore, we examined

whether TAT-Hsp27 influences adhesion ability of MDPC-23 cells to type I collagen. Adherent cells to type I collagen was counted by microscopic observation and additionally, adhesion was quantified by measuring toluidine blue absorbance. The number of adherent cells was significantly increased by TAT-Hsp27 in a dose-dependent manner (Fig. 2A). Moreover, toluidine blue absorbance also gradually increased in TAT-Hsp27-transduced cells in a dose-dependent manner (Fig. 2B). Next, we examined the effect of TAT-Hsp27 on migration ability of MDPC-23 cells using a commercial cell migration assay kit. TAT-Hsp27 treatment significantly enhanced migration of MDPC-23 cells, which was also dose-dependent (Fig. 3A and B).

3. Effect of TAT-Hsp27 on expression of 1 Integrin and phosphorylation of FAK and ERK

Because β1 integrin and its downstream signaling are involved in cell adhesion and migration, we examined whether TAT-Hsp27 induces the expression of β1 integrin and the activation of FAK and ERK. TAT-Hsp27 transduction apparently induced the expression of β 1 integrin in MDPC-23 cells, which was dose-dependent (Fig. 4A and B). And it also slightly increased phosphorylation of FAK and ERK (Fig. 4A and B). These findings mean that TAT-Hsp27 may modulate β1 integrin-mediated signaling in MDPC-23 cells.

4. The role of β1 integrin on TAT-Hsp27-mediated migration of MDPC-23 cells

Next, we examined whether β1 integrin regulates TAT-Hsp27-mediated migration of MDPC-23 cells. Similarly in Fig. 3, TAT-Hsp27 transduction led to increase of cell migration, which was restored by treatment of anti- β1 integrin antibody (Fig. 5). This finding suggests that TAT-Hsp27 may promote the migration ability of MDPC-23 cells via β1 integrin-mediated signaling.

5. Effect of TAT-Hsp27 on focal adhesion formation

As FAK plays crucial roles in cell adhesion, we examined focal adhesion formation in TAT-Hsp27-transduced cells by immunofluorescent staining with anti pFAK antibody. TAT-Hsp27 transduction significantly increased the number of focal adhesions of MDPC-23 cells in a dose dependent manner (Fig. 6).

Ⅳ. Discussion

Cell adhesion, invasion, and migration play a critical role not only in normal homeostasis such as wound healing and regeneration but also in pathological conditions including tumor progression via angiogenesis and metastasis[22]. Because odontoblasts are responsible for tissue regeneration and dentine formation, and cell adhesion and migration are critical processes in tissue regeneration, it is valuable to clarify the precise mechanism for the adhesion and migration of odontoblasts.

In this study, we showed that the TAT-fused Hsp27 was effectively delivered into the mouse odontoblast-like MDPC-23 cells. Furthermore, our results revealed that transduction of TAT-Hsp27 promoted the ability of adhesion and migration of MDPC-23 cells. The treatment of TAT-Hsp27 (1 μg) in MDPC-23 cells increased the migratory and adhesive capacity compared with control cells. However, TAT-Hsp27 at the same concentration had no effect on proliferation or cytotoxicity. This means that enhanced cell migration and adhesion was not simply as a result of an increase in the number of cells.

Human odontoblasts displayed several integrins and these integrins were thought to play a physiological role in maintaining the cohesion of the odontoblast layer necessary to the integrity of the peripheral dental pulp[23]. Integrin activation has been shown to be an essential requirement for cell migration. β1 integrin is a predominant β subunit that can pair with multiple subunits to form heterodimeric integrin receptors. Association of β1 integrin in dental pulp cell adhesion is

anticipated as it has been reported to modulate adhesion and migration in several cell types. In this study, inhibitory action of an antibody against β1 integrin on cell migration supports that such integrin is required for migration of MDPC-23 cells.

It has been shown that overexpression of Hsp27 activates integrin-mediated signaling pathways and enhances focal adhesion formation via FAK activation[9,24]. And phosphorylation of the MAP kinase ERK 1/2 has been linked to alterations in focal adhesion formation via stimulation of receptor tyrosine kinases[25]. In the present study, we revealed that TAT-Hsp27 transduction induced slightly phosphorylation of FAK and ERK and focal adhesion formation. It is likely that TAT-Hsp27 enhances focal adhesion formation via activation of FAK and ERK.

In conclusion, we showed that biologically active, exogenous Hsp27 can be delivered into cells using the HIV-1 TAT protein and promotes adhesion and migration of MDPC-23 cells. Additionally, we have demonstrated its ability to promote the phosphorylation of FAK, ERK as well as expression of β1 integrin. Our results open the possibility of the effective use of TAT-Hsp27 to coat pulp capping materials to promote cell adhesion and migration for biodental applications of dentine repair or regeneration purposes.

Ⅳ. Conclusion

Our results showed that TAT-Hsp27 enhanced cell adhesion and migration through the phosphorylation of focal adhesion kinase (FAK), extracellular signal-regulated kinase (ERK), and its upstream cell surface molecule β1 integrin pathway.

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Figures

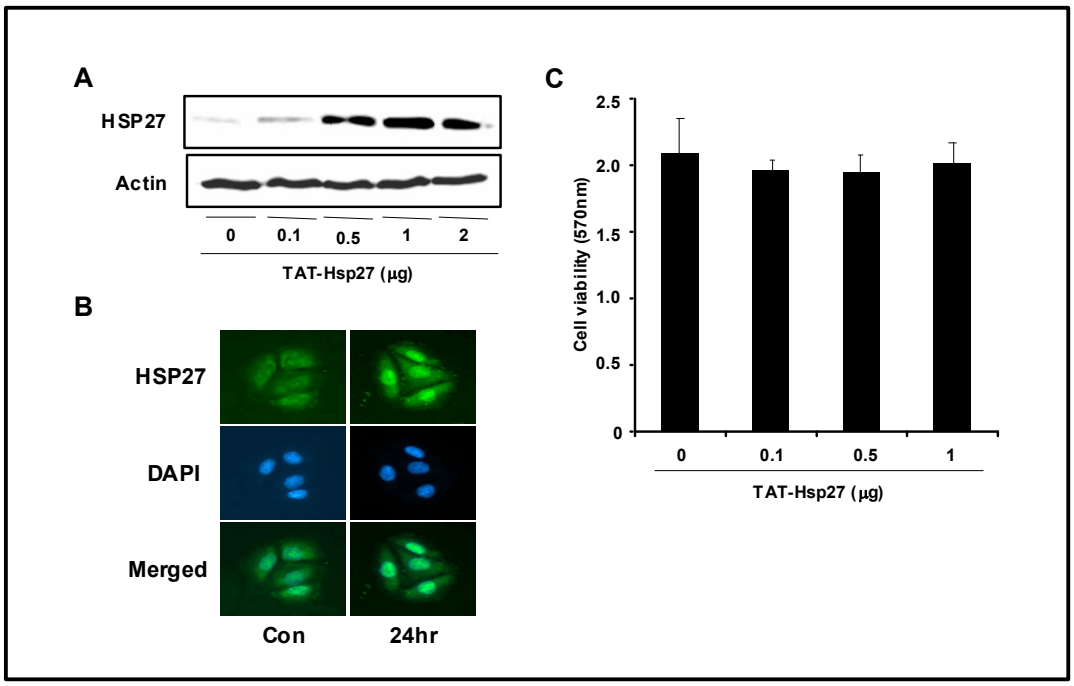

Fig. 1. Transduction of TAT-Hsp27 and cell viability in MDPC-23 cells. (A) MDPC-23 cells were incubated with the indicated concentrations of TAT-Hsp27 for 24 hours. The cell lysates were prepared and the level of transduced TAT-Hsp27 was assessed by Western blot analysis using anti-Hsp27 antibody. (B) MDPC-23 cells were transduced with 1 μg of TAT-Hsp27 for 24 hours. Transduced TAT-Hsp27 was visualized by immunofluorescence microscopy. The nuclei were stained with DAPI. (C) Cell viability was measured by MTT assay. The data are expressed as the means SD.

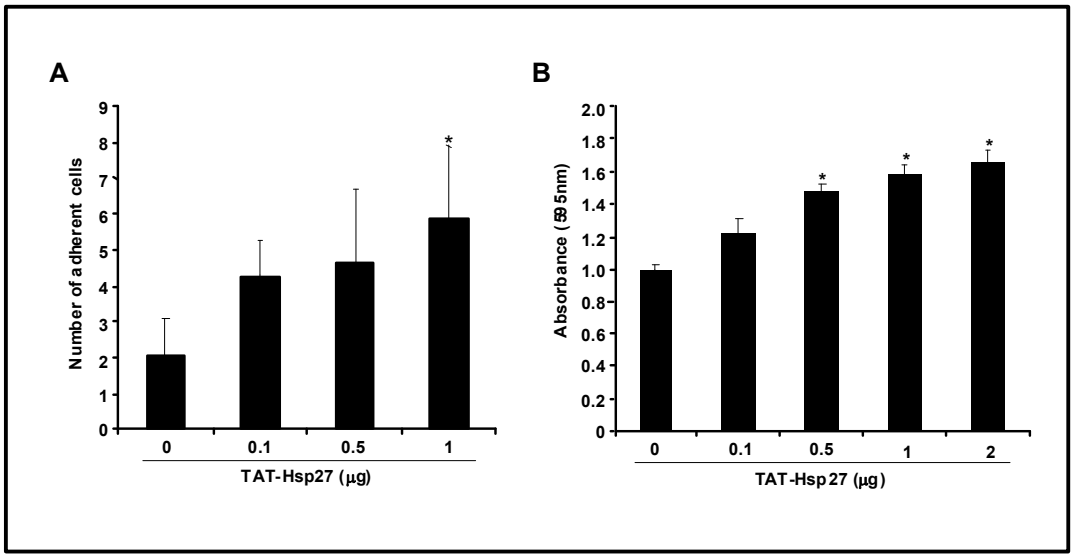

Fig. 2. TAT-Hsp27 promotes the adhesion of MDPC-23 cells to type I collagen. The cells were incubated in type I collagen-coated plate at the absence or presence of TAT-Hsp27. (A) The cells adherent to type I collagen were stained with H&E and counted from five randomly selected areas (200 magnifications). (B) The cells adherent to type I collagen were stained with toluidine blue and solubilized with the addition of 100 μl of 1% SDS. Finally, optical density was measured at 595 nm. Results are expressed as the means SD. *P < 0.05

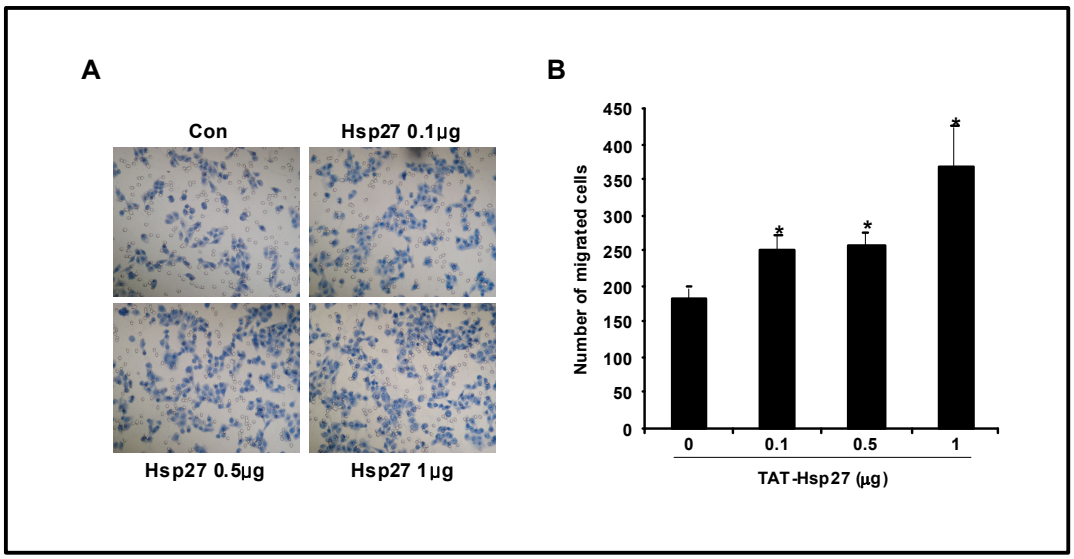

Fig. 3. TAT-Hsp27 promotes the migration of MDPC-23 cells. Migration assay was performed as described in Materials and Methods. (A) Migrating cells were fixed with methanol and stained with hematoxylin. (B) The number of migrating cells was counted from five randomly selected areas (200 magnifications). Results are expressed as the means SD. *P < 0.05

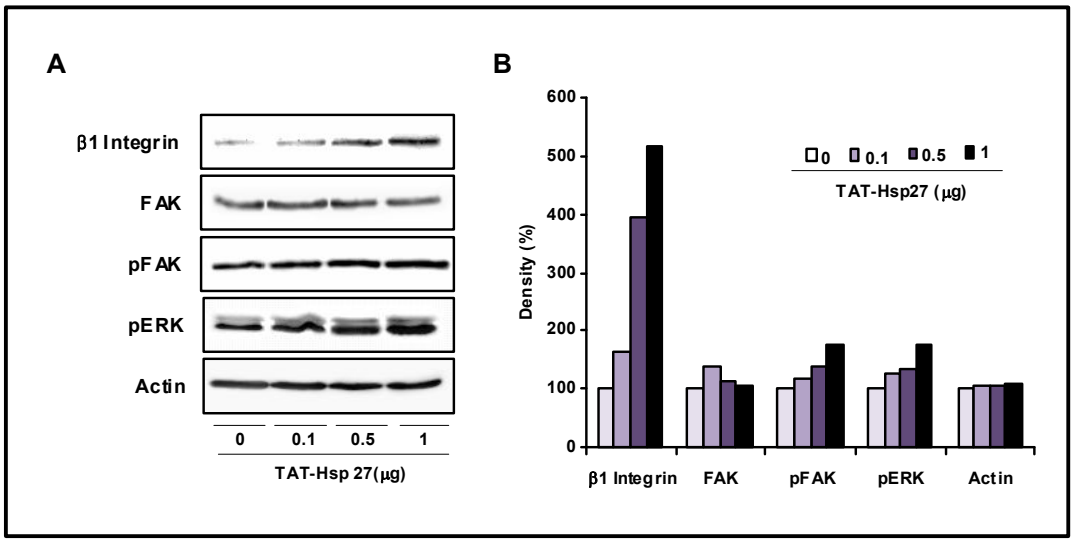

Fig. 4. TAT-Hsp27 up-regulates β1 integrin and phosphorylation of its downstream FAK and ERK. (A) The cells were incubated with the indicated concentrations of TAT-Hsp27 for 24 hours. The cell lysates were prepared and the level of β1 integrin, FAK, p-FAK, ERK, p-ERK, and actin was assessed by Western blot analysis. (B) The band densities were measured and expressed as the percentage to control.

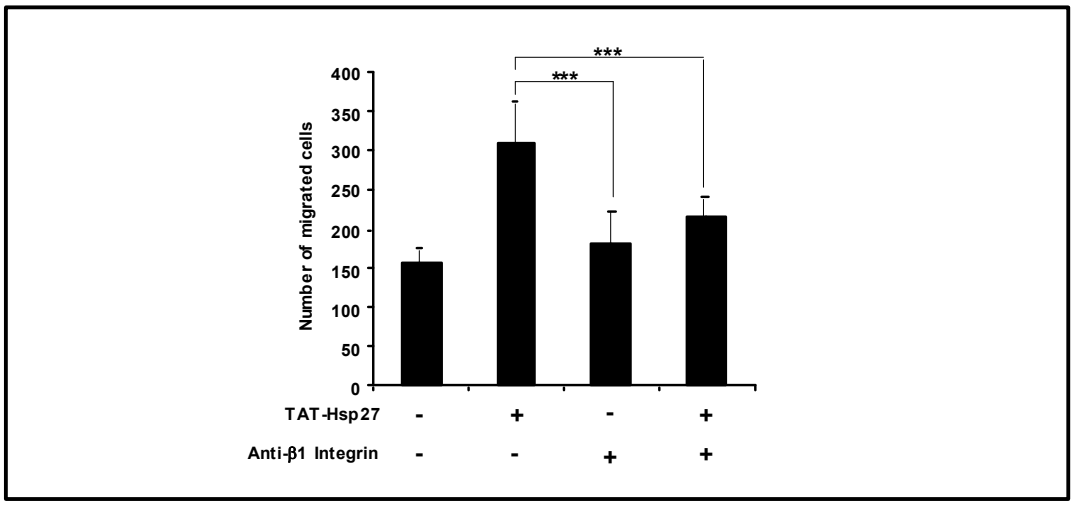

Fig. 5. TAT-Hsp27-mediated migration of MDPC-23 cells was restored by anti-β1 integrin antibody. TAT-Hsp27 was applied to MDPC-23 cells pretreated with and without anti-β1 integrin antibody. The number of migrating cells was obtained as described in Fig 3B. Results are expressed as the means SD. **P < 0.01

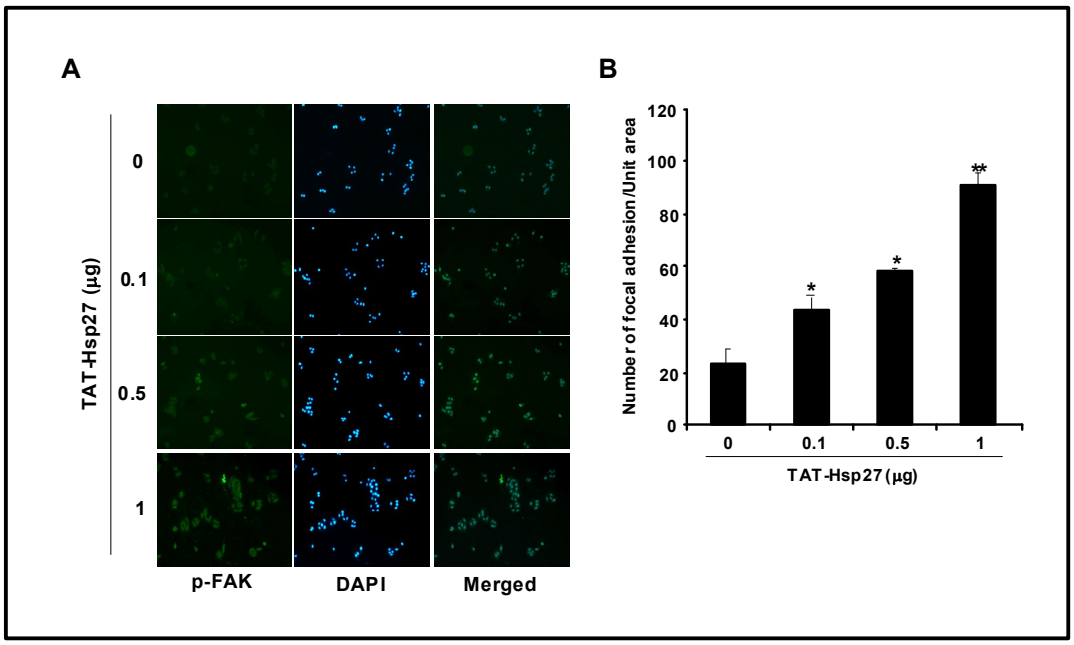

Fig. 6. TAT-Hsp27 increased the formation of focal adhesion. (A) The cells were treated with different doses of TAT-Hsp27 and stained with anti pFAK antibody. The cells were then reacted with FITC-conjugated secondary antibody with DAPI solution. (B) The number of focal adhesions per unit area was determined microscopically. Results are expressed as the means SD. *P < 0.05, **P < 0.01

국문초록

TAT-Hsp27이 MDPC-23 상아모세포주의 부착과 이주에 미치는 효과

조선대학교 대학원 치의학과
임 영 신

상아모세포는 상아질 형성 뿐 아니라 치아의 치유와 재생에도 관여를 한다. 세포 부착과 이주는 조직 재생에 있어서 중요한 과정이다. 본 연구에서는 TAT-fusion protein system을 이용해 세포 내부로 Hsp27을 전달하는 것이 쥐의 MDPC-23 상아모세포주의 부착과 이주를 향상시키는 지를 알아보고자 하였다. 실험방법으로는 TAT-fusion 단백질을 분리하였으며, TAT-Hsp27 융합 단백질을 형질도입하고 탐지하였다. 또한 MTT 분석을 이용해 세포 생존력을 평가하였으며 Microplate Autoreader ELISA와 현미경을 이용해 세포 부착을 관찰하였고 CHEMICON을 이용해 세포 이주 능력을 평가하였다. 마지막으로 Western blot 분석과 국소적 부착도 평가하였다. 실험결과 TAT-fusion protein system은 성공적으로 세포내부에 Hsp27을 전달하였으며 TAT-Hsp27의 형질도입은 용량에 의존하여 MDPC-23의 부착과 이주를 유도하였다. 게다가 TAT-Hsp27의 형질도입은 β1 integrin의 단백질 발현과 국소적 부착 형성을 증가시켰으며 FAK와 ERK의 인산화를 유도하였다. MDPC-23 상아모세포주의 TAT-Hsp27-induced migration은 항β1 integrin 항체 처치에 의해 회복되었다. 결론적으로 TAT-Hsp27이 β1 integrin-mediated signaling을 통해 상아모세포양 세포의 부착과 이주를 촉진함을 알 수 있었으며 치아 재

생을 위한 치료에 적용해 보는 것을 고려해볼 수 있었다.

Key words: 상아모세포, 부착, 이주, TAT-Hsp27, β1 integrin

저작물 이용 허락서

학 과	치의학과	학 번	20087442	과 정	박 사
성 명	한글 : 임영신 한문 : 任榮臣 영문 : Lim, Young Shin				
주 소	경기도 부천시 원미구 상동 646				
연 락 처	010)2200-4824				
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본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

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저작자 : 임 영 신 (서명 또는 인)

조선대학교 총장 귀하