TAT-Hsp27 promotes adhesion and migration of murine dental papilla-derived MDPC-23 cells through ß1 integrin-mediated signaling

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Abstract. Odontoblasts are involved in tooth repair and regeneration as well as dentin formation. The aim of this study was to examine whether delivery of heat shock protein 27 (Hsp27) into cells using a TAT fusion protein system (TAT-Hsp27) enhances adhesion and migration of murine dental papilla-derived MDPC-23 cells. Hsp27 was delivered into cells by the TAT-fusion protein system. To examine whether TAT-Hsp27 affects the viability of MDPC-23 cells, MTT assay was performed. The effect of TAT-Hsp27 on adhesion and migration of MDPC-23 cells was determined using type I collagen-coated plates and a commercial kit, respectively. In addition, a precise molecular mechanism was examined by Western blot analysis and focal adhesion activity. TAT-fusion protein system delivered Hsp27 into cells successfully. Transduction of TAT-Hsp27 induced adhesion and migration of MDPC-23 cells in a dose-dependent manner. Moreover, transduction of TAT-Hsp27 increased the protein expression of ß1 integrin and focal adhesion formation, and induced phosphorylation of FAK and ERK. TAT-Hsp27-induced migration of MDPC-23 cells was restored by treatment of anti-ß1 integrin antibody. These findings suggest that TAT-Hsp27 promotes adhesion and migration of MDPC-23 cells via ß1 integrin-mediated signaling and is a promising candidate for therapeutic application of dental pulp regeneration.

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 the heat shock protein family and is induced in response to a variety of 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 the most promising therapeutic strategy for the replacement of damaged teeth (12-14). Dental pulp cells and/or odontoblasts are involved in tooth repair and regeneration as well as dentin formation. Cell adhesion and migration are critical for the regeneration process in a variety of physiological and pathological conditions as well as tumor metastasis. It has been shown that Hsp27 is involved in the cell death of dental pulp cells during stress because of modification and activation (15). Although Hsp27 regulates cell migration and adhesion in wound healing or cancer cell migration (9,16), the precise function of Hsp27 in dental pulp cells remains to be fully elucidated.

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 and result in effective transduction of functional proteins. This system has many advantages such as the ability to deliver proteins or nucleic acid into all types of cells and into all organs *in vivo*, even into the brain across the blood-brain barrier (18,19). In this study, we examined the effect of Hsp27 using a TAT fusion protein system on cell migration and adhesion of murine dental papilla-derived MDPC-23 cells. Possible underlying mechanisms were also investigated. Our results showed that TAT-Hsp27 enhanced cell migration and adhesion through the phosphorylation of focal adhesion kinase (FAK),

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extracellular signal-regulated kinase (ERK), and its upstream cell surface molecule the ß1 integrin pathway.

Materials and methods

Purification of TAT-Hsp27 fusion protein. The TAT-Hsp27 expression vector was constructed as previously described (20). Briefly, TAT-fused Hsp27 protein was expressed in *E. coli* BL21 (DE3) pLysS cells (Invitrogen) and purified using the urea-denaturing protein purification method (20). Cells were lysed via sonication in lysis buffer (1 mM imidazole, 100 mM NaCl, 20 mM HEPES, pH 8.0) containing 8 M urea. Cell lysates were centrifuged at 12,000 x g for 30 min at 4˚C and 1 ml of Ni^{2+}-NTA agarose was added to the cleared supernatant. After 2 h of gentle mixing at 4˚C, 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). Proteins were eluted four times with 1 ml of elution buffer (500 mM imidazole, 300 mM NaCl, 50 mM phosphate buffer, pH 8.0). Urea denaturant was removed with a Mono-Q ionic exchange column and desalinated with a PD10 Sephadex size exclusion column. Protein concentration was quantified via the Bradford assay and confirmed by SDS-polyacrylamide gel electrophoresis (PAGE).

Transduction and detection of TAT-Hsp27 fusion protein. MDPC-23 cells (21,22) 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 penicillin, 100 μg/ml non essential amino acid at 37˚C in a 5% CO_2 humidified incubator. One day prior to the addition of the fusion protein, cells were seeded on 12-well plates at a density of $3x10^5$ cells/ml. Culture media were then removed and replaced with 1 ml of fresh DMEM containing various doses (0.1, 0.5, 1, and 2 μg/ml) of TAT-Hsp27. After transduction (24 h), cells were lysed and 25 μg of total protein was loaded onto SDS-PAGE. Proteins were separated by electrophoresis and Western blotting was accomplished using an anti-Hsp27 antibody (diluted 1:1,000; Santa Cruz Biotechnology, USA).

For immunocytochemistry, transduced cells were washed twice in PBS and fixed with 4% paraformaldehyde in PBS for 10 min. After being washed with PBS twice, cells were incubated with methanol for 2 min. For immunostaining, cells were pre-treated for 30 min with 5% BSA in PBS. Cells were then incubated with 2.5% BSA in PBS containing anti-Hsp27 antibody (diluted 1:250; Santa Cruz Biotechnology, USA) for 1 h and washed with PBS for 10 min. Cells were incubated for 30 min with FITC-conjugated anti-rabbit secondary antibody (diluted 1:250; Jackson ImmunoResearch) in 2.5% BSA containing PBS. Finally, cells were washed twice for 10 min with PBS and mounted with ProLong Antifade mounting medium (Molecular Probes). Fluorescence analysis was conducted by conventional fluorescence microscopy.

Cell viability assay. To examine whether TAT-Hsp27 induces cytotoxicity in MDPC-23 cells, cell viability was determined using the MTT assay. Cells were seeded into 12-well plates at a density of $1x10^5$ cells/ml. Cells were cultured overnight and treated with different doses (0.1, 0.5, and 1 μg/ml) of TAT-Hsp27 for an additional 24 h. Cells were washed twice with PBS, and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide reagent diluted in culture media was added. After 3 h incubation at 37˚C, the media were removed and 250 μl of acid-isopropanol (0.04 mol/l HCl in isopropanol) was added to dissolve formazan crystals. 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.

Cell adhesion assay. Type I collagen (50 μl) (50 μg/ml; diluted in PBS) was added to each well of 96-well plates and placed at 4˚C overnight. Subsequently, each well was rinsed with PBS and nonspecific binding sites were blocked with 1% BSA at 37˚C for 1 h. Cells ($5x10^4$) were placed in each well and allowed to adhere at 37˚C for 30-90 min. Various doses (0.1, 0.5, 1, and 2 μg/ml) of TAT-Hsp27 were added to each well for 30 min before addition of cells. Non-adherent cells were rinsed off with PBS, and the remaining cells were fixed with 4% paraformaldehyde for 5 min. Cells were stained with 0.5% toluidine blue in 4% paraformaldehyde for 5 min and finally rinsed in water. Cells were solubilized with the addition of 100 μl of 1% SDS and OD was measured by a Microplate Autoreader ELISA (Bio-Tek Instruments Inc., Winooski, VT) at 595 nm. In addition, another experiment for visualizing adherent cells was designed. For this, cells and TAT-Hsp27 were equally applied to type I collagen-coated wells with the above concentrations. After removing non-adherent cells by washing with PBS, samples were fixed with 200 μl 10% formalin for 24 h. Samples were processed and stained with hematoxylin and eosin for microscopic observation. Adherent cells were counted from five randomly selected areas at x200 magnification.

Cell migration assay. Cell migration assay was performed using a Chemotaxis Cell Migration Assay kit (Chemicon) according to the manufacturer's instructions. Cells were collected by trypsinization and resuspended in a serum-free medium at a density of $2.5x10^4$ cells/ml. Cells were placed onto the insert and TAT-Hsp27 was added to the media. In an experiment to examine the effect of ß1 integrin on cell migration, cells were pre-incubated with 25 μg/ml of anti-ß1 integrin antibody (R&D Systems, USA) for 20 min. Cells were then allowed to migrate for 24 h at 37˚C. Cells that migrated to the lower surface of the membrane were fixed with methanol and stained with hematoxylin for 5 min. The number of migrated cells on the lower side of the membrane was counted from five randomly selected high power fields (x200).

Western blot analysis. TAT-Hsp27-transduced cells were lysed with 2x SDS sample buffer. Proteins were resolved by SDS-PAGE and transferred onto PVDF membrane. After blocking with 5% skim milk in TBS at room temperature for 1 h, membranes were incubated with anti-HSP27, ß1 integrin, FAK, pFAK, pERK, and actin antibodies (1:1,000 dilution; Santa Cruz Biotechnology, USA) at 4˚C overnight. Membranes were then washed three times with TBS supplemented with 0.05% Tween-20 (T-PBS), followed by incubation with secondary antibody (1:5,000 dilution) at room temperature

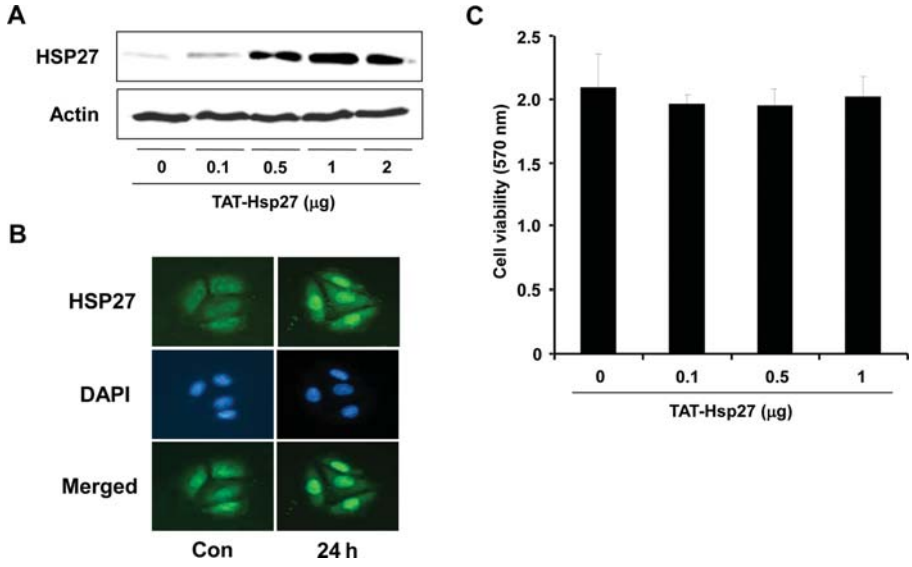

Figure 1. Transduction of TAT-Hsp27 and cell viability in MDPC-23 cells. (A) MDPC-23 cells were incubated with the indicated concentrations of TAT-Hsp27 for 24 h. Cell lysates were prepared and the level of transduced TAT-Hsp27 was assessed by Western blot analysis using anti-Hsp27 antibody. (B) MDPC-23 cells were transduced with 1 μg of TAT-Hsp27 for 24 h. 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.

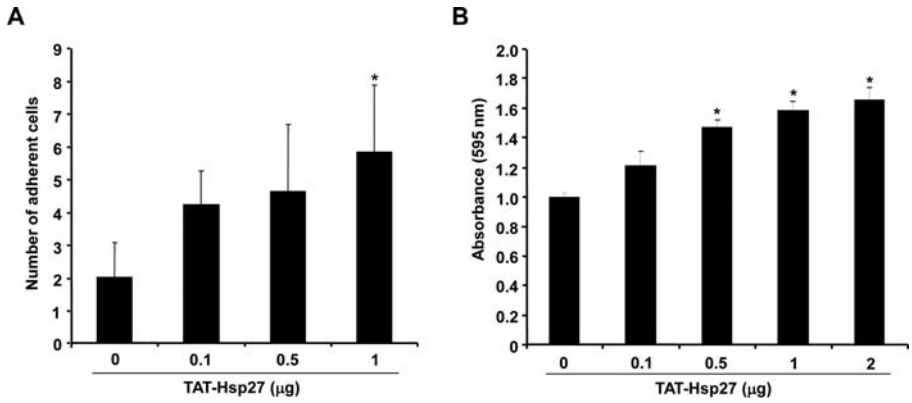

Figure 2. TAT-Hsp27 promotes the adhesion of MDPC-23 cells to type I collagen. Cells were incubated in type I collagen-coated plates in the absence or presence of TAT-Hsp27. (A) Cells adherent to type I collagen were stained with H&E and counted from five randomly selected areas (x200 magnifications). (B) 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.

for 1 h. Finally, 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).

Counting of focal adhesions. Cells ($1x10^4$) were seeded in a 4-well chamber slide, and treated with TAT-Hsp27 (0.1, 0.5, and 1 μg/ml) for 24 h. Cells were washed with cold PBS, fixed with 4% formaldehyde for 10 min at room temperature. After washing, cells were blocked with 5% BSA in TBS for 30 min, and then incubated with pFAK antibody (1:200; Santa Cruz Biotechnology) in 2.5% BSA in TBS for 2 h. After washing, FITC-conjugated secondary antibody (diluted 1:250; Jackson ImmunoResearch) was added with DAPI solution (1 mg/ml). The number of focal adhesions per unit area was determined microscopically.

Statistical analysis. Statistical significance between groups was determined by two-way ANOVA test. Differences were considered significant at $p<0.05$.

Results

Transduction of TAT-Hsp27 fusion protein and its effect on 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 h and then the level of transduced Hsp27 was determined by Western blot analysis. TAT-Hsp27 was delivered successfully into 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

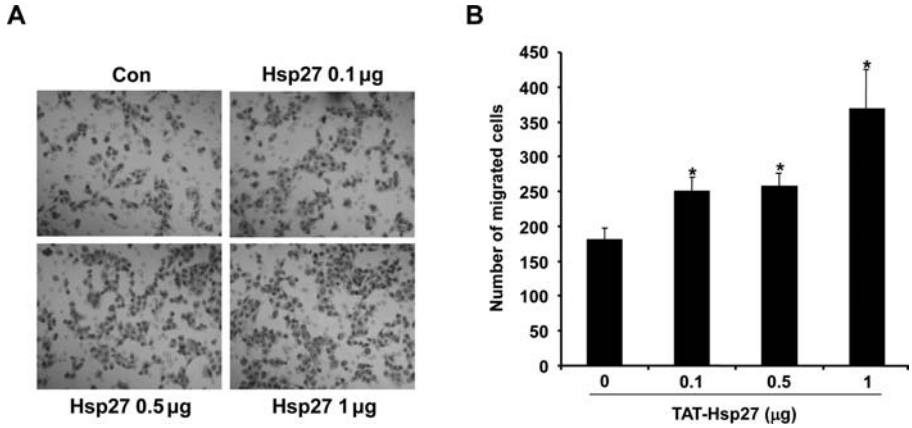

Figure 3. TAT-Hsp27 promotes migration of MDPC-23 cells. Migration assay was performed as described in Materials and methods. (A) Migrating cells were fixed with methanol and stained with hematoxylin. (B) The number of migrating cells was counted from five randomly selected areas (x200 magnification). Results are expressed as the means ± SD. $^{*}P<0.05$.

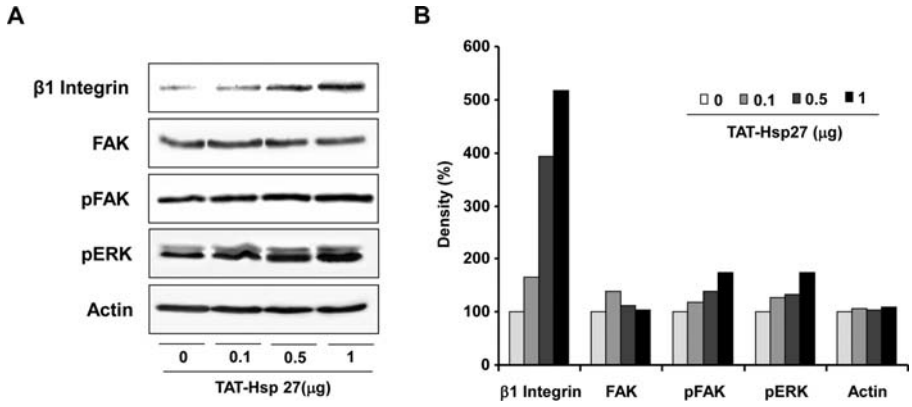

Figure 4. TAT-Hsp27 up-regulates ß1 integrin and phosphorylation of its downstream FAK and ERK. (A) Cells were incubated with the indicated concentrations of TAT-Hsp27 for 24 h. Cell lysates were prepared and the level of ß1 integrin, FAK, p-FAK, ERK, p-ERK, and actin was assessed by Western blot analysis. (B) The band densities were measured and expressed as the percentage to control.

various concentrations (0.1, 0.5, and 1 μg/ml) of TAT-Hsp27. TAT-Hsp27 transduction at the tested concentrations did not affect the viability of MDPC-23 cells, showing >95% cell viability (Fig. 1C). Thus, we determined this range of concentrations of TAT-Hsp27 as the doses to be used for subsequent experiments.

Effect of TAT-Hsp27 on 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. Cells adherent to type I collagen were counted by microscopic observation. 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).

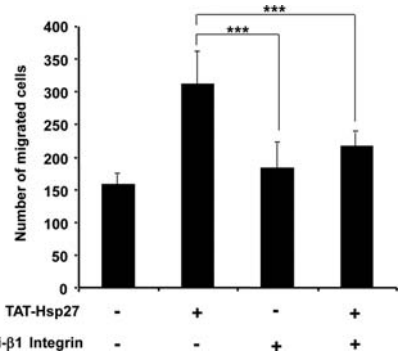

Figure 5. TAT-Hsp27-mediated migration of MDPC-23 cells is restored by anti-ß1 integrin antibody. TAT-Hsp27 was applied to MDPC-23 cells pretreated with and without anti-ß1 integrin antibody. The number of migrating cells was obtained as described in Fig. 3B. Results are expressed as the means ± SD. $^{***}P<0.01$.

Effect of TAT-Hsp27 on expression of ß1 integrin and phosphorylation of FAK and ERK. Because ß1 integrin and its downstream signaling are involved in cell adhesion and migration, we examined whether TAT-Hsp27 induces the

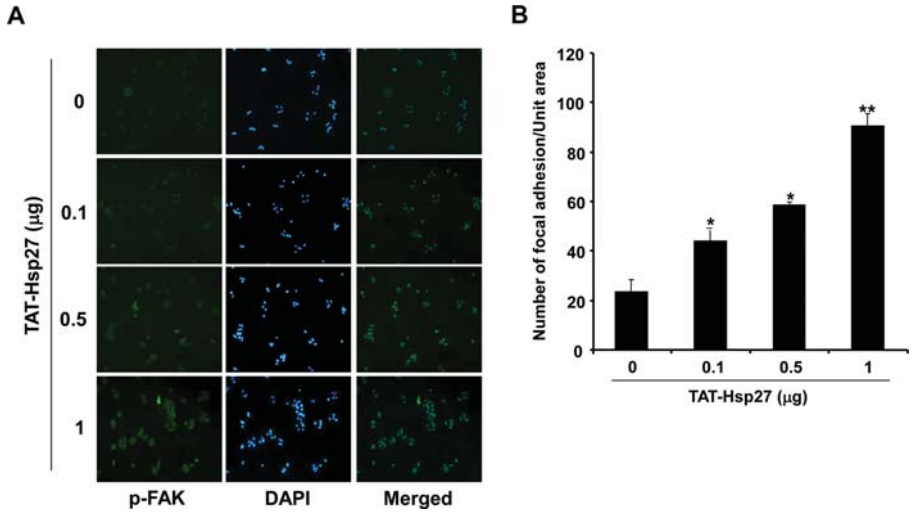

Figure 6. TAT-Hsp27 increases the formation of focal adhesion. (A) Cells were treated with different doses of TAT-Hsp27 and stained with anti-pFAK antibody. Cells were then reacted with FITC-conjugated secondary antibody with DAPI solution. (B) The number of focal adhesions per unit area was determined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

expression of ß1 integrin and the activation of FAK and ERK. TAT-Hsp27 transduction apparently induced the expression of ß1 integrin in MDPC-23 cells, which was dose-dependent (Fig. 4A and B). It also slightly increased phosphorylation of FAK and ERK (Fig. 4A and B). These findings mean that TAT-Hsp27 may modulate ß1 integrin-mediated signaling in MDPC-23 cells.

The role of ß1 integrin on TAT-Hsp27-mediated migration of MDPC-23 cells. Next, we examined whether ß1 integrin regulates TAT-Hsp27-mediated migration of MDPC-23 cells. Similarly to that shown in Fig. 3, TAT-Hsp27 transduction led to an increase of cell migration, which was restored by treatment of anti-ß1 integrin antibody (Fig. 5). This finding suggests that TAT-Hsp27 promotes the migration ability of MDPC-23 cells via ß1 integrin-mediated signaling.

Effect of TAT-Hsp27 on focal adhesion formation. Since FAK plays a crucial role 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).

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 (23). Because dental pulp cells and/or odontoblasts are responsible for tissue regeneration and dentin 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 dental pulp cells.

In this study, we showed that the TAT-fused Hsp27 was effectively delivered into the 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/ml) 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 a result of an increase in the number of cells.

Human dental pulp cells and 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 (24). Integrin activation has been shown to be an essential requirement for cell migration. ß1 integrin is a predominant ß subunit that can pair with multiple subunits to form heterodimeric integrin receptors. Association of ß1 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 ß1 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 (9,25). Phosphorylation of the MAP kinase ERK 1/2 has been linked to alterations in focal adhesion formation via stimulation of receptor tyrosine kinases (26). In the present study, we revealed that TAT-Hsp27 transduction slightly induced 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.

Collectively, our findings have shown here 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 ß1 integrin. Our results demonstrate the possibility of the effective use of TAT-Hsp27 to coat pulp capping materials to promote cell adhesion and migration for biodental applications of dentin repair or regeneration purposes.

Acknowledgements

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