Effect of hBD2 Genetically Modified Dermal Multipotent Stem Cells on Repair of Infected Irradiated Wounds

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Deficiencies in repair cells and infection are two of the main factors that can hinder the process of wound healing. In the present study, we investigated the ability of human beta-defensin-2 (hBD2) genetically modified dermal multipotent stem cells (dMSCs) to accelerate the healing irradiated wounds complicated by infections. An hBD2 adenovirus expression vector (Adv-hBD2) was firstly constructed and used to infect dMSCs. The antibacterial activity of the supernatant was determined by Kirby-Bauer method and macrodilution broth assay. Time to complete wound healing, residual percentage of wound area, and the number of bacteria under the scar were measured to assess the effects of Adv-hBD2-infected dMSC transplantation on the healing of irradiated wounds complicated by Pseudomonas aeruginosa infection. Results showed that the supernatant from Adv-hBD2-infected dMSCs had obvious antibacterial effects. Transplantation of Adv-hBD2-infected dMSCs killed bacteria in the wound. The complete wound healing time was 19.8 ± 0.45 days, which was significantly shorter than in the control groups (P < 0.05). From 14 days after transplantation, the residual wound area was smaller in the experimental group than in the control groups (P < 0.05). In conclusion, we found that transplantation of hBD2 genetically modified dMSCs accelerated the healing of wounds complicated by P. aeruginosa infection in whole body irradiated rats.

INTRODUCTION

The healing of wounds resulting from radiation injuries, severe burns and trauma remains a great challenge to clinicians, especially when they are complicated by infections. Deficiencies in repair cells and infection are important factors that can hinder the healing of such complicated wounds. In our previous study, dermal multipotent stem cells (dMSCs) isolated from rat skin showed promising effects on the repair of non-infected wounds in rats suffering from combined radiation exposure and wounds, but they were less effective in Pseudomonas aeruginosa-infected wounds.1,2 Strategies to control infections are needed in such wounds.

Human beta-defensin-2 (hBD2) is an antimicrobial peptide that was initially isolated and purified in 1997.3 Subsequent studies showed it to have strong, broad-spectrum antimicrobial activity, enabling it to effectively kill Gram-negative bacteria, Gram-positive bacteria, and fungi.4,5 hBD2 thus has the potential to control infection and promote the healing of infected wounds. In order to combine the repair effects of dMSCs and hBD2, we constructed an hBD2 adenovirus vector (Adv-hBD2). dMSCs were then infected with Adv-hBD2 and transplanted into irradiated wounds complicated by P. aeruginosa infection, in order to observe their effects on infection control and the rate of wound healing.

MATERIALS AND METHODS

The experimental protocol was reviewed and approved by the Ethical Committee of the Third Military Medical University, P. R. China.

Materials

Wistar rats were purchased from the Center of Laboratory Animals of the Third Military Medical University (qualified certification number: CQA 0101078#, 0102289# and
0110951#, Chongqing, China). Standard strains of *P. aeruginosa* (ATCC 27853) were provided by the clinical laboratory of Southwest Hospital, Third Military Medical University. All materials used for cell culture were bought from Hyclone (UT, USA). Reagents for polymerase chain reaction (PCR) and reverse transcription-PCR (RT-PCR) were obtained from TaKaRa Bio Inc. (Kyoto, Japan). Primers used were synthesized by Sangon Biological Engineering & Technology Services Co., Ltd (ShangHai, China). Tripure used to isolate RNA was purchased from Promega Corp. (Madison, WI, USA). Anti-hBD2 rabbit polyclonal antibody was from Santa Cruz Biotech (CA, USA). All restriction endonucleases used in the experiment and the DNA ligation kit were purchased from New England Biolabs (Beberly, CA, USA). Lipofectamine 2000 was bought from Invitrogen (Carlsbad, CA, USA). The enzyme-linked immunosorbent assay (ELISA) kit for hBD2 was bought from Phoenix Company (CA, USA), protein extraction T-PER™ reagent was from Pierce Company (IL, USA), and the low-molecular-weight protein marker was from Kerun Biopharmaceutical R&D Co. Ltd (Chongqing, China).

**Construction of Adv-hBD2**

A commercial AdEasy adenoviral vector system was used to construct Adv-hBD2 and it was a kind gift from Dr Bert Vogelstein, Howard Hughes Medical Institute, Johns Hopkins Oncology Center, Baltimore, USA, and the methods used to construct Adv-hBD2 were similar to those previously reported. A macrobroth dilution assay was used to determine the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the supernatant of *P. aeruginosa*. After incubation at 37°C for 24 h, MIC was defined as the lowest concentration that inhibited the visible growth of micro-organisms and MBC was defined as the lowest concentration of supernatant that prevented any visible growth on the plate (99.9% killed). Each concentration of extracts was tested in triplicate. Super-

**Acquisition of hBD2 target gene.** Damaged skin tissue was collected from patients with psoriasis and patients signed informed consent forms. Total RNA was extracted by using Tripure, and hBD2 cDNA was then amplified from 1.0 μg RNA using hBD2-specific primers. The forward primer was 5’-AAGCTT AGG GTG TGG TAT CTC CTC-3’, and the reverse primer was 5’-GCGGCCGC TCA TGG CTT TTT GCA GCAT T- 3’. The underlined bases are *Hind* III and *Not* I restriction sites. The PCR product was 209 bp long.

**Generation of recombinant pAd-hBD2.** Restriction enzyme reactions using *Not* I and *Hind* III were performed to cut the pAdTrack-CMV shuttle vector and the full-length hBD2 cDNA, respectively, and the digested products were then ligated overnight. The region between multiple cloning sites of the pAdTrack-CMV was removed and replaced by the full-length hBD2 cDNA. The resultant vector was verified by restriction endonuclease digestion with *Not* I and *Hind* III, and by PCR.

Then, 120 μl of the newly constructed vector was linearized with *Pme* I and mixed with competent *E. coli* strain BJ5183 cells to allow recombination. The recombinant vector was amplified and verified by restriction endonuclease digestion using *Pac* I, and by PCR. The vector was named pAd-hBD2.

**Assembly of recombinant adenovirus.** Human embryonic kidney 293 cells (HEK 293 cells) were cultured to a confluency of approximately 60–70%. A transfection mixture was then prepared by adding linearized pAd-hBD2 and Lipofectamine 2000 to HEK 293 cells, followed by incubation of the mixture at 37°C. Green fluorescent protein (GFP) expression by the transfected cells was monitored under a fluorescence microscope, suggesting the generation of adenovirus of hBD2 (Adv-hBD2). Seven days after transfection, the cells were collected and lysed using three consecutive freeze-thaw cycles, and Adv-hBD2 was collected from the supernatant. The supernatant was used to infect HEK 293 cells again, followed by three consecutive freeze-thaw cycles. After eight rounds of infection, the supernatant was collected and purified by CsCL density gradient centrifugation. The titer of the purified Adv-hBD2 was determined by plaque assays and it was used in the following experiments.

**In vitro antibacterial assay**

dMSCs were isolated as described previously. Twenty microliters of Adv-hBD2 was used to infect dMSCs at a titer of 2.5 × 10^10 plaque-forming units (PFU)/ml. The cell culture supernatant was collected 5 days after dMSCs were infected by Adv-hBD2. The concentration of hBD2 in the supernatant was determined by ELISA. The supernatant was then collected, de-salted, lyophilized and re-dissolved in appropriate concentration as needed in the following tests.

The antibacterial effect of the supernatant was firstly assessed by Kirby-Bauer method. Briefly, the supernatant was concentrated 10 times by lyophilizing and re-dissolving. Sterile filter papers were soaked in the concentrated supernatant and put onto Luria-Bertoni plates containing *P. aeruginosa*. After incubation at 37°C for 12 hours, photographs were taken to assess the antibacterial effect, based on the width of the vacant zones surrounding the filter papers.

A macrobroth dilution assay was used to determine the minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the supernatant for *P. aeruginosa*. Briefly, Serial two-fold dilutions of lyophilized supernatant (from 1:2 to 1:128 starting from a concentration of 40 μg/ml) were added to an equal volume of Mueller-Hinton broth containing 100 μl *P. aeruginosa* at an inoculum of approximately 5 × 10^8 CFU/ml, followed by incubation at 37°C for 24 h. MIC was defined as the lowest concentration that inhibited the visible growth of micro-organisms after overnight incubation. For MBC determination, an aliquot (100 μl) from tubes containing no visible growth was mixed with sufficient proteinase K to neutralize the residual hBD2, and was then transferred on to a Mueller-Hinton agar plate and subcultured for 24 h at 37°C. The MBC was defined as the lowest concentration of supernatant that prevented any visible growth on the plate (99.9% killed). Each concentration of extracts was tested in triplicate.
natants from non-infected dMSCs and dMSCs infected with a GFP adenovirus vector (Adv-GFP, a vector constructed by Zong Zhao-wen) served as controls.

**Effect of dMSCs infected with Adv-hBD2 on healing of irradiated wounds complicated by infection**

**Animal groups and transplantation procedure.** One-hundred and eighty 6-week-old Wistar rats, weighing about 150 g, were randomly divided into six groups: group A (rats transplanted with Adv-hBD2-infected dMSCs, n = 30), group B (rats transplanted with Adv-GFP-infected dMSCs, n = 30), group C (rats transplanted with non-infected dMSCs, n = 30), Group D (rats treated by Adv-hBD2 injection into the wound, n = 30), Group E (rats treated by Adv-GFP injection into the wound, n = 30) and Group F (rats treated by saline injection into the wound, n = 30).

The radiation injury model was produced as described previously, with some modifications. Briefly, rats in all groups were irradiated over the whole body with 5 Gy of gamma rays from a Co source and the absorbate rate was 31.02–31.98 cGy/min. Thirty minutes after irradiation, rats were anesthetized and a circular wound with a diameter of 2 cm was made on the back. One milliliter of bacterial solution (1 × 10^6 dMSCs of the appropriate type, diluted in 1 ml culture medium. Rats in group D, group E and group F received 1 ml Adv-hBD2, Adv-GFP and saline injection into the wound bed respectively.

Three, 7, 14, and 21 days after injury, six rats from each group were sacrificed and samples were taken for bacteria colony determination and Western blot. Wound healing was assessed in a further six rats from each group.

**Examination of the expression of hBD2 by Western blotting.** One gram of wound tissue was collected at each time point, and was homogenized in 1 ml of phosphate-buffered saline (PBS). Ten microliters of homogenate and ground down in a homogenizer containing 1 ml of phosphate-buffered saline (PBS). Plates were incubated overnight and the numbers of bacterial colonies were then counted. The plate with 30–300 colonies was selected to calculate the number of bacterial colonies in the corresponding sample. The number of bacterial colonies formed from 1 g of wound tissue was calculated by multiplying the number of bacterial colonies on the selected plate by the dilution number and by 10^3. Three different samples were acquired for each rat and the bacterial colony assays were performed in triplicate for each sample.

**Assessment of wound healing.** Time to complete wound healing and residual percentage of wound area were used to assess the speed of wound healing. Complete healing time was indicated by the shedding of scars and complete re-epithelialization of the wound bed.

**Statistical analysis**

All data were expressed as means ± standard deviations and were analyzed using SPSS 11.0 statistical software. Statistical significance was evaluated using unpaired Student’s t-tests for comparisons between two groups, or ANOVA for multiple comparisons. A value of P < 0.05 was considered significant.

**RESULTS**

**Construction of Adv-hBD2**

Full length hBD2 cDNA was amplified (Fig. 1A, Lane 2) and was then ligated into pAdTrack-CMV shuttle vector. PCR analysis (Fig. 1A, Lane 3) and restriction endonuclease digestion (Fig. 1A, Lane 4) confirmed that the hBD2 cDNA was correctly cloned into the pAdTrack-CMV shuttle vector.

The newly constructed shuttle vector was then linearized with Pme I and used to transfect BJ5183 cells to allow recombination to occur. PCR examination showed that the expected product of hBD2 (209 bp) was amplified (Fig. 1B, Lane 2), and restriction endonuclease digestion with Pac I produced the two expected segments (3 kb and 30 kb) (Fig. 1B, Lane 3). These results indicated successful recombination. The recombinant vector was named pAd-hBD2.
Sequence assay showed that the sequences constructed into pAd-hBD2 was correct and no mutation occurred (data not shown).

pAd-hBD2 was then linearized and used to transfect HEK 293 cells to construct the primary adenovirus stock. About 30 hours after transfection, GFP expression was detected under a fluorescence microscope (Fig. 1C), indicating the generation of Adv-hBD2. After eight amplification rounds, about 30 ml purified Adv-hBD2 was obtained. The titer was about $8.0 \times 10^{10} - 3.3 \times 10^{11}$ PFU/ml, as determined by plaque-forming assays.

**In vitro antibacterial assay**

The supernatant from dMSCs was collected on day 5 after infection with Adv-hBD2. The concentration of hBD2 in the supernatant was 743.6 ng/ml, as determined by ELISA. Using the Kirby-Bauer method, the vacant zones surrounding the filter papers soaked in supernatants from Adv-hBD2-infected dMSCs were $21 \pm 2.1$ mm, which was significantly larger than the areas around Adv-GFP-infected dMSCs or non-infected dMSCs, which formed no obvious vacant zones. The MIC and MBC of recombinant hBD2 were 5 $\mu$g/ml and 20 $\mu$g/ml, respectively. These results indicate that Adv-hBD2-infected dMSCs secreted functional hBD2 with both bactericidal and bacteriostatic activity.

**Effect of transplanted Adv-hBD2-infected dMSCs on infected irradiated wounds**

The expression of hBD2 in the wound was examined by Western blotting. As shown in Fig. 2, transplantation of Adv-hBD2-infected dMSCs or injection of Adv-hBD2 into the wound increased the expression of hBD2 3 days after
injury significantly more than in the control groups ($P < 0.05$). The expression of hBD2 in the wound at other time points was similar (data not shown).

The numbers of bacteria under the scars are shown in Fig. 3. There were fewer bacteria under the scars on day 3 in groups A and D than in the other groups ($P < 0.05$). By days 7, 14 and 21 after injury, the numbers of bacteria under the scars in groups A and D were even more dramatically reduced compared with the other groups ($P < 0.01$). These results suggest that hBD2 secreted by Adv-hBD2-infected dMSCs or Adv-hBD2 injection was able to kill bacteria in the wounds. There were no significant differences between groups A and D.

The time to complete wound healing in group A was 19.8 ± 0.45 days, which was significantly less than in group E and group F (27.3 ± 0.96 days and 27.1 ± 0.65 days respectively) ($P < 0.01$). The times to complete healing in groups B, C and D were 24.2 ± 0.85 days, 24.5 ± 0.59 days and 23.7 ± 0.27 days respectively, which were longer than that in group A ($P < 0.05$). The time to complete wound healing in group D was less than in groups B and C, but the differences were not significant.

![Fig. 2. The expression of hBD2 in the wound 3 days after injury.](image)

Fig. 2. The expression of hBD2 in the wound 3 days after injury. (A) Representative Western blot images. (B) hBD2 protein levels are presented as the relative abundance compared with β-actin. **$P < 0.01$, compared with group A and group D at the same time point.

![Fig. 3. The numbers of bacteria under the scars in each group.](image)

Fig. 3. The numbers of bacteria under the scars in each group. Animal group was the same as described in the part of “Materials and Methods”. *$P < 0.05$, **$P < 0.01$, compared with group A and group D at the same time point.
The residual percentages of wound areas are shown in Fig. 4. The residual wound area in group A was smaller than in the other groups, but the difference was not significant until 14 days after injury. After 14 days, the residual wound area in group A was significantly smaller than in the other groups (\(P < 0.01\)). There were no significant differences among groups B, C and D.

**DISCUSSION**

Wound healing is a common process, but some kinds of complicated wounds still present a challenge to clinicians. The widespread use of radioactive materials in medicine, industry and agriculture means that humans more frequently experience the combination of radiation and injuries, resulting in skin wounds hard to heal. The mechanisms underlying this failure to heal include a reduced number of repair cells in the wound, and reduced growth factors and collagen synthesis.\(^1,^2\) Also these wounds are easily colonized by microorganisms. Under such circumstance, deficiency of repair cells and infection together hinder the process of wound healing.

The use of conventional antibiotics provides the most powerful means of treating bacterial infections, but the misuse and overuse of antibiotics has led to the colonization and infection of chronic wounds by antibiotic-resistant organisms.\(^3,^4\) This situation suggests that alternative strategies and therapies are required to fight infection. Antimicrobial peptides have been identified as key elements in the innate host defense against infection. The defensins are a major family of antimicrobial peptides that can be categorized into three families, \(\alpha\), \(\beta\) and \(\theta\), based on differences in their tertiary structures. hBD2 is a defensin that was initially identified in skin keratinocytes of psoriatic patients. It demonstrated potent microbicidal activity against many Gram-negative bacteria and less potent bacteriostatic activity against Gram-positive bacteria. No acquired resistance to hBD2 has so far been reported.\(^4\) Furthermore, hBD2 does not require specific posttranslational modification, and does not require cell type-specific processing.\(^5,^6\) Functional hBD2 has been shown to be produced in various cell types including fibroblasts, IH3T3 cells, fibrosarcoma cell line and mouse submandibular gland cells.\(^7\) hBD2 has often been chosen as a target for the promotion of healing of infected wounds. McFarland *et al.* used cytokine stimulation to increase the expression of multiple antimicrobial protein genes, including hBD2, so up-regulating the innate immunity of cultured skin substitutes to help combat wound infections after grafting onto patients.\(^8\) Glycyrrhizin was applied to restore the reduced levels of beta-defensin in infected wounds, which in turn improved the resistance of burned mice to *P. aeruginosa* wound infections.\(^9\) Adv-hBD2 was constructed in the present study to combine the repair effect of stem cells and the anti-bacterial effect of hBD2, and this was then used to infect dMSCs. The results showed that Adv-hBD2 up-regulated hBD2 expression in dMSCs after infection, and the resulting supernatant had obvious antibacterial effects. In vivo experiments showed that transplantation of Adv-hBD2 dMSCs into wounds was able to kill bacteria in infected wounds and accelerate wound healing.

One concern regarding the use of hBD2 is its potential connection with the formation of neoplasias. Zhuravel *et al.* reported that regulated expression of hBD2 led to a higher growth rate and longer survival in cultured human embryonal kidney cells,\(^10\) and transfection of hBD2 increased the growth rate and changed the malignant phenotype of

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*Fig. 4. The residual percentage of wound area in each group. Animal group was the same as described in the part of “Materials and Methods”. *\(P < 0.05\), **\(P < 0.01\), compared with group A at the same time point.*
Lewis lung carcinoma (3LL) cells. However, conflicting data exist. hBD2 has been reported to inhibit the formation of anal intraepithelial neoplasia and papilloma via its antiviral effect. hBD2 also activates both innate and adaptive immunity to generate a potent antileukemia response, and can inhibit the formation of malignant ascites. In our previous study, we found that Adv-hBD2 infection could moderately accelerate the growth rate of dMSCs, and had no influence on the migration and differentiation ability of dMSCs. No tumors formed 3 months after Adv-hBD2 infected dMSCs was transplanted subcutaneously in nude rats. However, the complex mechanism of neoplasia formation means that further studies are needed to clarify the role of hBD2 in tumor formation.

It should also be noted that infection is not the only factor that hinders the healing of refractory wounds; the lack of repair cells is also a contributory factor. In this study, the wound healing time in rats treated by injection of Adv-hBD2 into the wound was 4 days longer than that in rats transplanted with Adv-hBD2-infected dMSCs. Thus both repair cells (dMSCs) and anti-infective measures (Adv-hBD2) are helpful to promote faster wound healing.

In conclusion, the results of the present study show that Adv-hBD2 infection can up-regulate the expression of hBD2 in dMSCs, and that the resulting product has antibacterial activity both in vitro and in vivo. Transplantation of Adv-hBD2-infected dMSCs could accelerate the healing of wounds complicated by P. aeruginosa infection in whole body irradiated rats.

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