Original Article

Infection can promotes NFκB and c-Src expression during differentiation of osteoclasts and leads to infected nonunion

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Abstract: Infected nonunions pose many challenges to the treating surgeon and the patient. Its researches focus on treatment, the basis studies are less. This study aims to investigate the effect of infection on nonunion and its possible mechanism. In our study, infected nonunion animal model were established, and osteoclasts were isolated and cultured successfully. The results show that the number of osteoclast cells, the degree of differentiation and maturation, the bone-resorption activities and the expression of NFκB and c-Src protein in infected nonunion animal model were significantly higher than those in the control model group (P < 0.05). Therefore, we concluded that infection can significantly promote the differentiation and maturation of osteoclasts, and leads to infected nonunion. Its mechanism may be related to promoting the expression of NFκB and c-Src protein. In brief, NFκB and c-Src may become a new early diagnosis mark and therapy target in infected nonunion.

Keywords: Infected nonunion, osteoclast, differentiation, maturation, NFκB, c-Src

Introduction

Fracture healing depends on mechanical stability, osteogenic cells, osteoconductive scaffolds, growth factors, and vascularity whose deficiency can result in delayed or absent bone regeneration. Sufficient vascularity is essential for fracture consolidation, whereas exaggerated tissue perfusion can be caused by an infection, which can lead to a nonunion [1]. Infected nonunions of tibia pose many challenges to the treating surgeon and the patient. Challenges include recalcitrant infection, complex deformities, sclerotic bone ends, large bone gaps, shortening, and joint stiffness [2]. They are easy to diagnose and difficult to treat. Treatment frequently consists of repeated and radical surgical debridement in combination with prolonged antibiotic therapy [3]. But multiple failed surgeries add fibrosis, shortening, and a weakened morale. Many attempts at union exhaust bone graft donor sites. Osteoporosis results from prolonged inability to bear weight. The incidence of bone nonunion in the clinical treatment of fracture is about 10% [4, 5]. Its researches focus on therapeutic approaches, management and therapeutic evaluation, the basis studies are less. Since the mechanism of infected nonunion is unknown at present, it’s a basic job to establish a well infected nonunion animal model for investigation. Derived from hematopoietic stem cells in bone marrow, osteoclasts are the main bone-resorbing cells and formed by the cell fusion of multiple monocytic macrophages from the bone marrow [6]. Increase in quantity and activity of osteoclasts are closely related to a variety of bone-loss clinical diseases. Osteoclasts play an important role in the homeostasis of bone metabolism and also involved in a series of pathophysiologic processes, such as osteoporosis, disorders of minerals and bone metabolism in chronic kidney disease, the resorption effect of bone graft, inflammatory bone loss and fracture healing etc [7]. Osteoclast differentiation is mainly mediated by the RANKL/RANK/NF kappa B classic pathway and the bone absorption effect is mainly induced by cytokines like cathepsin K and c-Src [8].

In our study, the infected nonunion animal model and control model were established, and
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the osteoclasts were isolated and cultured. Meanwhile, the expression levels of osteoclast differentiation activating factor NFκB and osteoclast bone resorption promoting factor c-Src were measured. Therefore, the effect of infection on the differentiation, maturation and function of osteoclasts in nonunion and its correlation with the expression of NFκB and c-Src were discussed in our research.

Materials and methods

Bacteria and experimental animal

Staphylococcus aureus ATCC29210 were provided by department of clinical laboratory of Shenzhen Second People’s Hospital. To dilute bacterial fluid to $2 \times 10^6$ cfu/ul with casein hydrolysate medium. Healthy New Zealand white rabbits 20 (5-6 months), weighing 2.5-3.0 kg, male and female in half, were provided and fed by the experimental center of Shenzhen Second People’s Hospital. The above research activities were confirmed by the hospital ethics committee approval.

Reagents

RPMU1640, fetal bovine serum, α-MEM culture medium and trypsinase all were bought from Sigma Co., Ltd (USA). Tartrate resistant acid phosphatase (TRAP) staining kit and Toluidine blue staining were purchased from Gibco Co., Ltd (USA). The preparation method 1% toluidine blue staining solution: 1 g toluidine blue powder dissolved in 100 ml distilled water, fully mixed, and storage at room temperature. Rabbit anti-human NFκB and c-Src and mouse anti-human β-actin polyclonal antibody were purchased from Abcam Co., Ltd (USA).

Methods

Establishment of infectious nonunion model of radius: Totally 20 New Zealand white rabbits in half genders were randomly divided into model group and control group, each had 10 rabbits. Operation method: the rabbit was fixed on the operating table with right forelimb exposed. Cut the cony hair off, and then the operation area was disinfected with iodine complex and covered with sterile hole sheets. Infiltration anesthesia was done locally with 1% lidocaine. Take the middle part of the radius in the forelimb and choose longitudinal skin incision with a length of about 2.5-3.0 cm. Then cut open layer by layer and expose the middle part of the radius with periosteum reserved intentionally. After the periosteum cut off and subperiosteal bone exposed, mark the maximal curvature point of the middle part, from which to the distal part cut out 20 mm of the bone using a small sharp osteotome. Vernier caliper was used to measure the cut-off length and the length error should be controlled within 0.3 mm. Finally, use a small rongeur to trim the broken end as flat as possible and at the same time try to keep the cut-off site located at the middle and distal part of radius as possible. As the experimental group, the rabbit of group model group was injected with 5 ul drug-resistance staphylococcus aureus, while control group was injected with sterile saline. With the fracture unfixed, use No.1 silk thread suturing the periosteum, subcutaneous tissue and skin in order. The incision skin was disinfected with iodine complex twice a day. Each rabbit was raised in their own cage with free movement. Amoxicillin was taken orally after surgery for one week and stitches were removed at 12 days after surgery.

Isolation and culture of osteoclasts: After 12 weeks, all the rabbits were given euthanasia and transferred into 75% alcohol for 15 minutes of soak. Under aseptic conditions, isolate the skeleton of the long bones in the limbs and remove the surrounding soft tissue. Cut off epiphyses in the two ends of the bone skeleton and clean the bone skeleton in a medium with pure MEM solution. After transferred to another medium, use a injection syringe to flush the bone marrow cavity repeatedly with pure MEM solution no less than 3 times, and the flush fluid will be filtered two times by a strainer of 200 meshes. After that, the suspension was centrifuged in a sterile centrifuge tube (1000 rpm, 10 min). After the supernatant removed, the cells precipitation was diluted by pure MEM solution. Then count under the microscope and adjust repeatedly until the cell number is $1 \times 10^6$/ml. After that, all the cells were cultured in the α-MEM medium which contains 100 U/ml penicillin, 100 g/ml streptomycin and 10% fetal bovine serum at 37°C in a 5% CO₂ atmosphere and the culture medium were changed every two days.

Detection of the differentiation, maturation and function of osteoclast

The morphological changes of osteoclast cells: After being incubated for 48 h, the morphological changes of the cells in two groups were ob-
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TRAP staining: The osteoclasts of model and control group were inoculated on coverslips by 1000/cm², then cell smears were prepared. After cell attachment for 24 h, RANKL and ZOL were added to model and control group respectively for 5 d; then all the cells were fixed by 2.5% paraformaldehyde and were stained according to the kit instructions. 6 visions were randomly selected under optical microscope of 200 times, then count TRAP plus the number of multinucleated cell (the number of nuclei is less than 3). 5 cell climbing films were detected in each group with a total of 30 datas.

Bone absorption toluidine blue staining: The separated end of radial incision was cut into bone slices with 0.5 cm in diameter and 0.4 mm in thickness, and the 10 total films were handled with high temperature and high pressure sterilization. The cell inoculation density was 1000/cm². After being incubated for 5 d, collect all the bone slices. Each slice went through alternating oscillation in 50 Hz for 5 min in 1 mol/L ammonium hydroxide and distilled water. Repeat 3 times, and remove the surface cells of bone slices. The bone slices were taken out and rinsed by PBS at room temperature. All these were fixed by 2.5% glutaraldehyde for 10 min, then PBS rinsed again. Bone slices were rinsed by ultrasonic in deionized water for 3 times and 3 min for each time. After gradient alcohol dehydration, they were placed in 1% toluidine blue staining solution at room temperature for 20 s; PBS rinsed 1 to 2 times after dyeing. Then they were observed under the inverted microscope and taken photos after drying.

Detection of the NF kappa B, and c-Src gene expression

Immunofluorescence: Using the same inoculation method for cell smear. After the experiment all the cells were fixed with 2.5% paraformaldehyde and was incubated by rabbit-anti-mouse NF kappa B and c-Src polyclonal antibody at 4°C overnight. Secondary antibody was fluorescein isothiocyanate (FITC) labeled goat-anti-rabbit IgG. Then hybridization was done at 37°C for 2 h. Propidium iodide (PI) was used for double staining of the nucleus, the mounting. Laser scanning confocal microscopy (LSCM) and image analysis were used to detect and analyze the change of protein content.

Western-blotting: Total cellular protein was extracted and the protein concentration was measured. The test tube was added with 5X sample buffer and boiled for denaturation, and then gel electrophoresis and transfer membrane were performed. Finally, 5% BSA was added for closure at room temperature for 2 h. Primary antibody used were rabbit-anti-mouse NF kappa B and c-Src. Then these were incubated at 4°C overnight, with GAPDH as the internal standard. Secondary antibody was used and incubated at 37°C for 2.5h. BCIP/NBT was added for 1 min for visualization. Image J color analysis software was used to determine the gray value of each band in the film. The experiment was repeated 3 times.

Statistical analysis

SPSS17.0 statistics software was employed to undergo statistical analysis. The measure data
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are represented by x ± s. The difference in expression between two groups were assessed using Student’s t-test (unpaired). The differences in expression between multiple groups were examined by one-way analysis of variance followed by Tukey’s multiple comparison test. P < 0.05 was considered to indicate a statistically significant difference. The p values were designated as *P < 0.05, and **P < 0.01.

Results

Infected nonunion animal model were successfully established

X-ray examination of operative site of experimental rabbits were performed at 1,12 weeks after operation respectively. Compared with the first week, after 12 weeks, in model group, no fracture got united, X-ray showed that the Sclerotic bone was formed at the end of fracture and the epiphysis was closed to form infective nonunion of radius and the infected nonunion of radius was formed (Figure 1). In control group, all the fractures got united, X-ray showed that callus were formed, cortical is continuous, smooth and high echo, the defects were healed completely (Figure 1). It was suggested that infected nonunion animal model and control model were successfully established.

Osteoclast formation and function detection

Cellular morphology: Osteoclast in model group were in a good state, showing oval-shape with close intercellular adhesion, displaying uniform spindle shape and polarity arranged under inverted microscope, and the cell density was significantly higher than that in control group (P < 0.05, Figure 2). Whereas, The osteoclast in control group were spindle shaped, partly circular, arranged in disorder, and it’s number were sig-

Figure 2. The cellular morphology of osteoclast cells in model group and control group under inverted microscope (n=3).

Figure 3. Tartrate resistant acid phosphatase (TRAP) staining of osteoclast cells in model group and control group (n=3).
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Infected nonunion relate with NFκB and c-Src expression significantly lower than model group. (P < 0.05, Figure 2).

TRAP staining: Two groups of osteoclast were stained by TRAP, and showed TRAP positive multinucleated cells, but the number of positive cells in model group was significantly higher than that in control group, and there was statistical difference between two groups (P < 0.05, Figure 3).

Bone-resorption toluidine blue stain: Two groups of osteoclast were occurred bone resorption with varying degrees, we continue with toluidine blue stain, microscopically, deep blue bone resorption distributed on the surface of the bone with irregular insect bite shape, the boundary is clear and the color is relatively deep. The results showed that the number and area of bone resorption and deep blue bone resorption in model group were significantly higher than that of control group; there were statistical differences between two groups (P < 0.05, Figure 4).

The above results suggest that infection can significantly promote the differentiation and maturation of osteoclasts and increase the bone-resorption activities.

NFκB and c-Src protein expression in osteoclasts

The immunofluorescence results showed that the expression of NFκB were strongly positive in model group, cell nucleus is not expressed; the expression of c-Src were also strongly positive in model group, and cell nucleus is also positive (Figure 5). Whereas, the expression of NFκB and c-Src in control group was significantly lower than that in model group, and only expressed in cytoplasm, but not expressed in nucleus (P < 0.05, Figure 5).

The western-blot results showed that the expression levels of NFκB and c-Src protein in model group were significantly higher than that in control group (P < 0.05, Figure 6). The gray values of NFκB and c-Src protein in model group were 895.5 + 30.2 and 958.3 + 25.5, respectively, while those in control group were 356.8 + 15.8 and 268.7 + 21.5.

The above results suggest that infection can significantly promote the expression of NFκB and c-Src protein.

Discussion

Delayed healing after traumatic fracture occurs via several etiologic pathways and in association with well-documented risk factors. In general, cause for nonunion may be distilled down to two main categories: failure of biology or failure of fracture fixation. Septic seeding of the fracture site, either through contamination of an open injury or fracture bed seeding during the peri-operative window creates a significant risk for failure to achieve bony healing. With the incidence of nonunion at approximately 5-10% after all fractures and with the complexity of predisposing risk factors, identification of patients at increased risk for septic nonunion may represent a means for avoiding potential disastrous fallout and, potentially, avoidable returns to the operative suite [6-9]. With less blood flow and soft tissue surrounding, the tibia and radius are the most commonly involved in Infectious nonunion. The pathological process includes infection and nonunion, which has became important clinical problems currently faced in the treatment of fractures [10]. Effective infection control, reasonable nutritional support, lessening the work of respiratory muscles as well as minimizing the occurrence of complications are the keys of successful treatment [11, 12]. Expanding resection of infectious lesions can effectively control the infection, while bone transport can fill the...
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Figure 5. The expression levels of NFκB and c-Src in osteoclast cells of model group and control group were detected by immunofluorescence assay (n=3). Fluorescein isothiocyanate (FITC), Propidium iodide (PI).

Figure 6. The expression levels of NFκB and c-Src in osteoclast cells of model group and control group were detected by western-blot (n=3). A: Protein blotting stripe. B: The relative of NFκB and c-Src protein. *P < 0.05.

Bone defect after resection of the lesions, therefore, more and more scholars have listed these two methods as standard treatment for Infectious bone nonunion [13]. With mechanism unclear, establishing a good model of Infectious nonunion is the base to discuss its mechanism. There are many experimental studies about animal models of defect nonunion by lots of domestic and foreign scholars. Some scholars took the lateral incision of rabbit radius and used wire saw to cut off the bone and periosteum to establish nonunion models. Other scholars took rabbit forelimb “S” incision and gave osteotomy in middle and distal radius, and 3 mm of periosteum was rejected in each ends of two to establish a model. There are also scholars who took rabbit forelimb radi- al incision. After periosteal stripping, 10 mm of bone and periosteum were cut off in a site 3 cm away from the distal radius to set up this model [14]. Establishing a model in different parts of the same animal and different animal models were reported, in which using the rabbit radius as the experimental sites to set up a model for study was also reported, but all these models had their shortcomings. Based on a kind of closer to clinic method that reserves periosteum, we had cut off the bone of the middle and distal radius in rabbit and adopted drug-resistance Staphylococcus aureus infection bone defect, and finally successfully established a more objective animal model of infectious bone nonunion.

As multinucleated giant cells, osteoclast is derived from hematopoietic stem cells of the mononuclear macrophage precursors [15]. Under the effect of macrophage colony-stimu- lating factor (M-CSF), secreted by cells in or around the bone reconstruction unit (BRUs), and receptor activator of nuclear factor kappa B ligand (RANKL) and other cytokines, the pre-
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cursor cells commonly circulating in the peripheral blood aggregated in bone reconstruction unit and formed osteoclasts [16]. The osteoclasts are the main bone-resorbing cells and play an important role in the homeostasis of bone metabolism. Increase of the number and activity of osteoclasts is closely related to a variety of bone-loss clinical diseases [17]. They are involved in a series of pathophysiologic processes, for examples, osteoporosis, disorders of minerals and bone metabolism in chronic kidney disease, the resorption effect of bone graft, inflammatory bone loss and fracture healing and so on [18].

RANKL/RANK/NFκB pathway is the classical pathway regulates osteoclast differentiation, activation and apoptosis. After RANKL bound to the RANK receptor in the osteoclast surface, the Motif (including Motif 1, 2, 3) in the cytoplasmic end of RANK receptor combined with TRAF-6, which can activate NFκB, Akt/PKB and JNK, ERK, and p38 mitogen activated protein kinase (MAPK) signal pathway. Under the stimulation of RANKL, TGF-β-activated kinase 1 (TAK1) can be combined with TRAF-6 and activated with the help of its connexin 2 and 3 (TAB2, TAB3) [18]. The activated TAK1 further kindled the NFκB and JNK signaling pathway. The NFκB is the key transcription regulatory factor and involved in the expression regulation of many specific genes in osteoblasts, and it’s crucial to the differentiation, cell fusion, adhesion, acidification and bone absorption. The NFκB-knockout mice presented as bone sclerosis; its genetically defective embryonic stem cell was unable to differentiate into mature osteoclasts [17, 18].

c-Src is the key promoter for bone absorption in osteoclast. After RANK induced osteoclast differentiation, the expression of c-Src gene was significantly increased [19]. The high level expression of c-Src in osteoclast can mediated cell adhesion and migration, collagen degradation and other physiological processes during bone absorption. Due to lack of collagen degradation, the c-Src-knockout mice may present as osteopetrosis [20].

In our study, infected nonunion animal model. Furthermore, we explore the changes of gene expression after infection. At the beginning, we assume a variety of possible genetic changes. Such as NFκB, c-Src, CSF1R, RANK, RANKL, TRPV5, NFATc1, COX-2, MAPK. But it has been verified by many experiments that only NFκB and c-Src gene statistically changed in model group. To our best knowledge, this is also the first report of infected nonunion relate with NFκB and c-Src expression.

Conclusions

Therefore, we concluded that infection can significantly promote the differentiation and maturation of osteoclasts, and leads to infected nonunion. Its mechanism may be related to promoting the expression of NFκB and c-Src protein. In brief, NFκB and c-Src may become a new early diagnosis mark and therapy target in infected nonunion.

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Disclosure of conflict of interest

None.

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