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Cox-2 inhibition potentiates mouse bone marrow stem cell engraftment and differentiation-mediated wound repair

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Abstract

Background. Engraftment of transplanted stem cells is often limited by cytokine and noncytokine proinflammatory mediators at the injury site. We examined the role of Cyclooxygenase-2 (Cox-2)-induced cytokine-mediated inflammation on engraftment of transplanted bone marrow stem cells (BMSCs) at the wound site. *Methods.* BMSCs isolated from male *C57/BL6J* mice were transplanted onto excisional splinting wounds in syngenic females in presence or absence of celecoxib, Cox-2 specific inhibitor (50 mg/kg, body weight [b wt]), to evaluate engraftment and wound closure. Inflammatory cell infiltration and temporal expression of inflammatory cytokines at the wound bed were determined using immunohistochemical and quantitative-real time polymerase chain reaction (qPCR) analysis, respectively. Mechanistic studies were performed on a murine macrophage cell line (*J774.2*) to evaluate the effect of interleukin (IL)-17A. *Results.* Celecoxib administration led to a significantly high percent of wound closure, cellular proliferation, collagen deposition, BMSCs engraftment and re-epithelialization at the wound site. Interestingly, recruitment of $CD4^+$T cells and $F4/80^+$ macrophages as well as BMSC transplantation induced up-regulation of Cox-2 and IL-17A gene expression levels were reverted by celecoxib administration. Exogenous supplementation of recombinant interleukin (rIL)-17 to *J774.2* cells significantly increased proliferation and gene expression of cytokines -IL-1β, IL-6, IL-8, IL-18 and tumor necrosis factor (TNF)-α via nuclear translocation of nuclear factor kappa B (NFκB)p65/50 subunit. Conditioned media of rIL-17 treated *J774.2* cells when supplemented to BMSCs depicted a dose-dependent increase in the number of apoptotic cells and proapoptotic protein expression that was perturbed by celecoxib or IL-17 neutralizing antibody. Finally, celecoxib led to a dose-dependent increase in BMSC differentiation into keratinocyte-like cells *in vitro*. *Conclusion.* Celecoxib protects transplanted BMSCs from Cox-2/IL-17–induced inflammation and increases their engraftment, differentiation into keratinocytes and re-epithelialization thereby potentiating wound tissue repair.

Key Words: *bone marrow stem cells, Cyclooxygenase-2, engraftment, interleukin-17, inflammation, nuclear factor kappa B, re-epithelialization, transplantation, wound repair*

Introduction

Wound healing is a dynamic and complex process, precisely regulated by sequential phases of inflammation, proliferation and maturation-remodelling that regulates the presence of different types of cells at the wound bed. Inflammatory cells such as neutrophils, macrophages, lymphocytes and leukocytes get recruited at the site of injury and are involved in the evasion of apoptotic cells as well as infectious agents. These inflammatory cells secrete key regulatory molecules such as cytokines or growth factors, which are essential for blood supply, cellular proliferation and migration [1]. Decline in the inflammatory phase initiates the proliferation of skin fibroblasts and keratinocytes, thereby leading to re-epithelialization. Any perturbation in the order of this sequential event leads to chronic wound.

A chronic wound remains as a constant clinical problem, where the healing process is delayed resulting in lack of structural integrity. Patients with diabetes and/or undergoing chemotherapy or radiotherapy often suffer from chronic wounds. Reports suggest 15% of more than 150 million patients with diabetes suffer from chronic wounds [2]. Conventional treatment strategies available for chronic wounds often fail to heal, thereby necessitating development of improved therapies. New therapeutic strategies involving cell transplantations, especially stem cells, have depicted

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promising results. The literature suggests few studies performed using adipose- and/or bone marrow–derived stem cell [3,4] transplantation therapies resulted with low success due to the harsh injury microenvironment that is infiltrated with proinflammatory cytokines released by immune cells and reactive oxygen species. These factors result in activation of signalling pathways that induce proteases belonging to the caspase family, thereby leading to apoptosis of transplanted stem cells [5]. A study by Molcanyi et al. reported that post-trauma induced inflammatory response significantly impaired the survival and integration of implanted embryonic stem cells [6]. Therefore, we hypothesize that use of any anti-inflammatory agents in concurrence with stem cell transplantation may result in improved stem cell engraftment and survival. To target the inflammatory mediator during transplantation, an understanding of the inflammatory milieu at the injury microenvironment will boost the clinical outcome.

In the normal wound healing cascade, hemostatic plug is formed where platelets and polymorphonuclear leukocytes (PMN; neutrophils) get entrapped and secrete chemo-attractants for inflammatory cells [7]. This is followed by recruitment of macrophages that releases the proinflammatory cytokines during the early inflammatory phase of postwounding days 3 and 4 [8] along with an up-regulation of enzyme Cyclooxygenase-2 (Cox-2) at the wound site [9]. Cox-2 is a metabolite of arachidonic acid pathway and involved in synthesis of prostaglandins (PG) through prostaglandin E2 (PGE_2), a critical mediator of the inflammatory response. Cox-2 is involved in various deregulated inflammatory condition-associated diseases like rheumatoid and osteoarthritis, cardiovascular diseases and cancer [10–12]. Anti-inflammatory drug celecoxib, a selective Cox-2 inhibitor, has been reported to accelerate re-epithelialization in fetal wounds with scarless healing [13]. Thus elucidating regulatory mechanisms of the Cox-2 pathway along with other inflammatory mediators in the wound repair process becomes pertinent because they are known to regulate the outcome of repair [14]. However, the role of Cox-2 inhibition on engraftment and the fate of bone marrow stem cells (BMSCs) during transplantation have not yet been explored.

In the present study, we evaluated immune cellular milieu and inflammatory cytokine expression at injury site in a murine excisional splinting wound model. Anti-inflammatory agent celecoxib enhanced wound tissue repair by decreasing inflammation, increasing engraftment of transplanted BMSCs and re-epithelialization. Mechanistic studies revealed interleukin (IL)-17, a downstream mediator of Cox-2, led to activation of macrophages that in turn up-regulates pro-inflammatory cytokines, which induce BMSC apoptosis. Cox-2 inhibition also enhanced BMSC differentiation into keratinocyte-like cells.

Materials and methods

Isolation of BMSCs

Whole bone marrow mononuclear cells/BMSCs were isolated from femurs of 6- to 8-week-old male *C57BL/6J* mice as described earlier [15]. Briefly, bone marrow was flushed from the excised femurs and processed to remove contamination of red blood cells and cultured as described earlier [16]. Animal experimentation protocols were approved by institutional animal ethics committee (approval no. IICT/CB/AD/25/06/2014/13).

Full-thickness excisional splinting wound model generation

Female *C57BL/6J* mice were used to generate excisional splinting wound model for BMSC transplantation (Tx). Two 5-mm thickness wounds were generated using a biopsy punch on the dorsal side of mice followed by tightly adhering a silicon ring around the wound [16]. As described earlier [17], male BMSCs were injected intradermally (id; 0.6×10^6 cells) around the wound and on the wound bed (0.4×10^6 cells) of each mouse. Wound-generated mice were randomly divided into four groups (n = 15/group): wound-control, celecoxib-*per oral* (po), BMSC Tx and BMSC Tx + celecoxib-po. Each group were euthanized at different time-points: postsurgery day 1, 3 and 7 (n = 5/group). Celecoxib was administered po, 50 mg/kg body weight (b wt)/day × 7 days from the day of surgery (institutional animal ethics committee approval no. IICT/37/2015).

Wound-healing analysis

The morphological healing pattern in various groups was analyzed by using digital images of wounds procured on postsurgery day 1, 3 and 7. The wound areas were quantified using NIH Image J software (NIH). The rate of wound healing was represented as percentage of wound closure [(wound area of original wound—area of remaining wound)/wound area of original wound × 100] as described earlier [18].

Histopathologic analysis

Regenerated wound tissues on postsurgery day 7 were excised from various groups and fixed in paraformaldehyde. Then, 10-µm sections were stained with hematoxylin and eosin and Masson's Trichrome (Sigma) to evaluate the cellular proliferation and collagen deposition, respectively, as described earlier [19]. The images of stained sections were taken using a microscope (Olympus) at 10 × magnification.

Image analysis

The image files were opened using ImageJ software followed by conversion in 16-bit by various sequential