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Preliminary report

The role of kinins in the proliferation of fibroblast primed with TNF in scratch wound assay
Kinins and cell proliferation

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ABSTRACT

The aim of this study was to evaluate the involvement of both B_1 and B_2 kinins receptors (B_1R and B_2R) in the fibroblast proliferation induced by the cytokine tumour necrosis factor (TNF) attempting to establish an *in vitro* model of wound healing. Murine fibroblasts L-929 were cultivated in 24 wells plaque until total confluence (DMEM (Vitrocell®); 5% fetal bovine serum, 5% CO_2, 37 °C) and then submitted to the scratch assay. The cells were treated with PBS, TNF (2 ng/mL) and/or mr-TNF antibody (200 µg/mL), or PDTC. The cells received the second set of treatment (3 h later): PBS; 1 µM HOE-140; 1 µM des-Arg^9-Leu^8-BK (DALBK) or 100 µM PDTC. TNF was able to increase the cell proliferation when compared with the group treated with PBS. The co-treatment with the TNF antibody completely reversed the TNF effect. The TNF-proliferative effect was blocked by B_1 (DALBK) and B_2 (HOE-140) kinin receptor antagonists administered separately or along, suggesting the involvement of both receptors in the TNF mechanism of action. Furthermore, the treatment with a NF-κB inhibitor PDTC completely blocked the cell proliferation. The TNF cell proliferation was incremented with BK (1 µM) treatment, and its effect was totally reversed by HOE-140 treatment. No effect was observed for TNF plus DABK. Eventually, TNF treatment was able to increase TNF level in the growing medium; however, this increase was suppressed by BK treatment. These results suggest that TNF induces cell proliferation and the induced signalling cascade has the B_2R participation. All these events seem to be totally dependent on the NF-κB activation. These inflammatory mediators can improve the wound healing in the resolution of inflammation.

1. Introduction

Tissue damage triggers a series of events involving inflammatory cells chemotaxis, cell division, neovascularization and extracellular matrix synthesis. The coordinated action of these events results in the tissue formation and remodelling [1]. Experimental studies point inflammation as essential process for the restoration of cutaneous homeostasis after injury. The beginning of the inflammatory cascade occurs immediately after the tissue damage by the release of tumour necrosis factor (TNF), a pro-inflammatory cytokine produced by macrophages and activated neutrophils in response to pathogens or chemical mediators, or even by damaged endothelium cells such as fibroblasts and keratinocytes [2,3]. TNF has demonstrated to be directly linked to angiogenesis and transforming growth factor-β (TGF-β) synthesis, interfering with the fibroblast proliferation and, consequently, with the tissue healing [4].

It is also known that TNF induces the synthesis and release of numerous chemical mediators including the kinins [5]. Kinins are biologically active peptides involved in several physiological and physio pathological processes. Both peptides bradykinin (BK) and its metabolites des-Arg^9-BK (DABK) are implicated in inflammation by the activation of their respective receptors B_2 and B_1 (B_2R and B_1R) [6]. Cutaneous inflammatory processes induced by different stimulus, including allergenic substances or ultra-violet radiation, triggers tissue inflammatory response by the release of cytokins and kinins, especially BK. Saini et al. [7] and Chu et al. [8] have recently demonstrated that

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both TNF and BK induce the proliferation of colonic myofibroblasts, corroborating for the hypothesis of the inflammation role in tissue reconstitution.

Our study presents for the first time the interaction of TNF and kinins in fibroblast proliferation using *in vitro* model of mechanical tissue damage, called scratch assay, demonstrating the importance of TNF priming for the kinins effects.

2. Material and Methods

2.1. Materials

Recombinant murine Tumour Necrosis Factor (rm-TNF, R&D System), anti-murine recombinant TNF (ab-TNF, R&D System), Bradykinin (BK, Bachem California, Torrance, CA, USA), des-arg^9-BK (DABK, Sigma Chemical Co., St. Louis, MO, USA), [d-Arg^0-Hyp^3-Thi^5-DTic^7-Oic^8-]BK (HOE-140, Hoechst AG, Frankfurt, Germany), [des-Arg^9-Leu^8]BK (DALBK, Sigma Chemical Co., St. Louis, MO, USA), pyrrolidine dithiocarbamate (PDTC, Sigma Aldrich). Dulbecco's modified Eagle medium (DMEM) from Vitrocell, (Campinas, SP, Brazil); Fetal bovine serum (FBS), phosphate buffer saline (PBS) were from Gibco (Carlsbad, CA, USA).

2.2. Cell culture

NCTC clone 929 [L CELL, L-929] murine fibroblast (derived from normal subcutaneous connective tissue and adipose tissue) cells were obtained from the Rio de Janeiro bank of cells (BCRJ-0188). These were grown in DMEM (Dulbecco's Modified Eagle Medium), supplemented with 10% fetal bovine serum (FBS) and placed in an oven at 37 °C with 5% CO_2. After the culture becomes confluent (approximately 48 h) cells were collected and sub-cultured.

2.3. Proliferation assay of L929-fibroblast

The proliferation of fibroblast L929 cells was examined using a method previously described by Balekar et al. [9]. Briefly, L929 cells (1×10^5 cells/mL) in DMEM containing 10% FBS were seeded into each well of a 24 well plate and incubated at 37 °C with 5% CO_2. After the confluent monolayer of L929, cells were formed, and a sterile pipette tip was used to generate a horizontal scratch in each well. Any cellular debris was removed by washing with PBS and replaced with 1 mL of fresh medium. The cells were treated with TNF (2 ng/mL) [10], Anti-TNF (0,1 μg/mL), BK (1 μM), DABK (1 nM), HOE-140 (1 μM), DALBK (1 μM) or PDTC (100 μM) following the scheme demonstrated in the Fig. 1. The used concentrations were chosen based on pilot experiments previously performed.

Photographs were taken of two views on the left and right of each well at a 4× magnification using a microphotograph (Olympus CK2, Japan) on time 0, then the plates were incubated at 37 °C with 5% CO_2, and photographs were taken 30 h after the treatments. To determine the proliferation of L929 cells, the images were analysed using computing software (ImageJ1.42q/Java1.6.0–10). The program was firstly calibrated and then the uncovered area was delimited with specific tool to calculate the area. The % of covered area was obtained considering the uncovered area from images obtained before (time 0) and 30 h after the treatments.

Cytokines levels (TNF and IL-1β) were evaluated in the growing medium 4 h after PBS or BK treatment of cells previously stimulates with TNF and demonstrated in Fig. 1. The medium was evaluated using an enzyme-linked immunosorbent assay kit according to the manufacturer's recommendations (R&D Systems, Minneapolis, MN).

2.4. Statistical analysis

The results from experiments are presented as mean ± S.E.M.

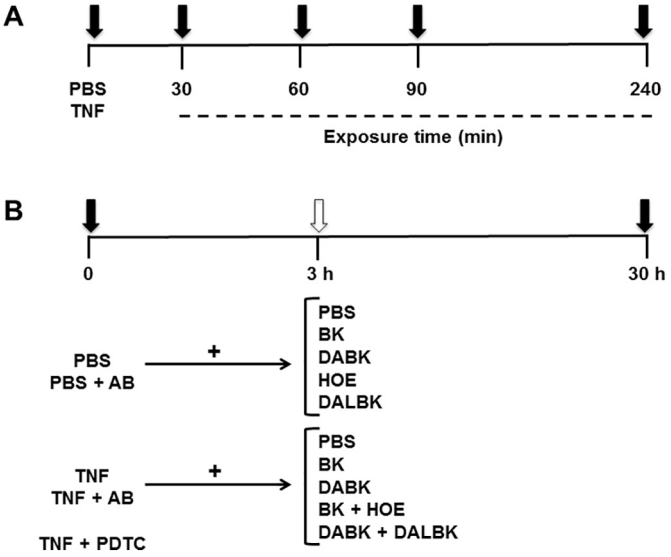

Fig. 1. Scheme of cell treatment. (A) L-929 exposure to TNF in different times. (B) L-929 exposure to TNF and/or BK, followed by different pharmacological tools (ab-TNF, kinins receptors antagonists or PDTC). Black arrows represent the times of photographs, and white arrow represent the time of wells washing with PBS before the second set of drugs treatment.

Statistical comparison of the data were performed using analyses of variance (ANOVA) followed by Tukey's test. p values < 0.05 ($p < 0.05$) were considered significant.

3. Results

Fig. 2 demonstrates that TNF (2 ng/mL) incubation induces fibroblast proliferation in time dependent manner. However, 240-min time of incubation presented an average of 90% of covering already in the first 24 h, not allowing an ideal window of observation for this study. Thus, 90 min of incubation was chosen to perform the following experiments.

TNF effects on fibroblast proliferation seem to be related to its receptor activation, once the co-administration of TNF with anti-TNF antibody blocked the cell proliferation (Fig. 3A). It was also shown that TNF effects depend on the kinin B_2R and B_1R activity, since the antagonists HOE-140 and DALBK prevented the TNF-induced cell

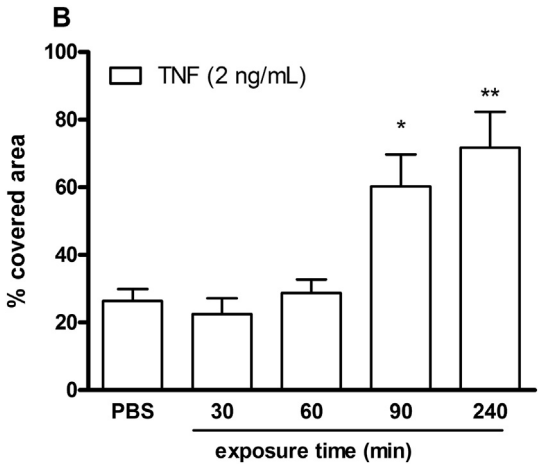

Fig. 2. L-929 migration after exposure to TNF (2 mg/mL). Each group represents the mean of 3 independent experiments and the vertical lines indicate the S.E.M. Significantly different from PBS treated group values * $p < 0.05$ and ** $p < 0.01$ (one-way ANOVA followed by Newman–Keuls post-hoc test).