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Effect of recombinant human endostatin on hypertrophic scar fibroblast apoptosis in a rabbit ear model

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ABSTRACT

Hypertrophic scar (HS) is a dermal fibroproliferative disorder characterized by the excessive proliferation of fibroblasts and is thought to result from a cellular imbalance caused by the increased growth and reduced apoptosis of hypertrophic scar fibroblasts (HSFs). Our recent study demonstrated that recombinant human endostatin (rhEndostatin) plays a key role in the inhibition of HSF proliferation *in vitro*, with a resulting decrease in dermal thickness and scar hypertrophy. However, the effect of this protein on HSF apoptosis is unknown. The present study was undertaken to directly examine the effect of rhEndostatin on HSF apoptosis in the rabbit ear model. Transmission electron microscopy and flow cytometry were used to investigate HSF apoptosis in scar tissues and cultured HSFs *in vitro*, respectively. The expression levels of the *c-jun*, *c-fos*, *NF-κB*, *fas*, *caspase-3*, and *bcl-2* gene products in HSFs were quantified using real-time PCR and Western blotting assays. Our data reveal that rhEndostatin (2.5 or 5 mg/ml) induces HSF apoptotic cell death in scar tissue. Additionally, HSFs treated with rhEndostatin (100 mg/L) *in vitro* accumulated in early and late apoptosis and displayed significantly decreased expression of c-jun, c-fos, NF-κB, fas, caspase-3 and bcl-2. In sum, these results demonstrate that rhEndostatin induces HSF apoptosis, and this phenotypeis partially due to downregulation of NF-κB and bcl-2. These findings suggest that rhEndostatin may have an inhibitory effect on scar hypertrophy *in vivo via* HSF apoptotic induction and therefore has potential therapeutic use for the treatment of HS.

1. Introduction

Hypertrophic scar (HS) is a prevalent pathological outcome of wound healing following dermal injuries. It is characterized by the abnormal activation of fibroblasts, over-secretion and deposition of extracellular matrix, and abnormal neovascularization [1]. The underlying mechanisms of HS formation have not been elucidated. However, it is generally accepted that hypertrophic scar fibroblasts (HSFs) are the predominant effector cells during wound healing and that there may be an imbalance between the growth and death of HSFs, which leads to scar hyperplasia [2]. Therefore, apoptosis induction in HSFs has been proposed as one of the leading strategies for the treatment of HS by way of reducing scar hypertrophy *in situ*.

The rabbit ear model of HS provides reproducible cutaneous scars that parallel human HS conditions in clinical appearance and histopathological structure. Since this model closely simulates HS formation in humans, it is commonly used for anti-scarring modality research [3].

Endostatin, a 20 kDa C-terminal proteolytic fragment of the NC1 domain of collagen XVIII, is a member of a group of endogenous antiangiogenic proteins that was isolated from a conditioned murine hemangioendothelioma (EOMA) cell line without signs of drug toxicity[4]. Endostatin restricts angiogenesis and tumor growth by inhibiting endothelial cell adhesion, migration and proliferation and by inducing apoptosis [5]. Endostatin's anti-tumor effects are mediated by its inhibition of proliferation and migration of partial tumor cells, induction of tumor cell apoptosis, and impedance of pathological angiogenesis [6–8]. Ren et al. have reported that endostatin inhibits HS in a rabbit ear model [9,10]. Recently, we demonstrated that recombinant human endostatin (rhEndostatin) exhibits an inhibitory effect on HSF proliferation *in vitro*, with a resulting decrease in dermal thickness and scar hypertrophy [11]. However, the detailed mechanisms of rhEndostatin action against HS formation are not known. The aim of our current study was to determine whether rhEndostatin could

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induce HSF apoptosis in HS in the rabbit ear. The potential downstream transcriptional targets of rhEndostatin were also investigated.

2. Materials and methods

2.1. Reagents

rhEndostatin was purchased from Simcere-Medgenn Biopharmaceutical Co., Ltd. (Yantai, China). Chloral hydrate and normal saline (NS) were provided by Anhui BBCA Pharmaceuticals Co., Ltd. (Hefei, China). 0.25% trypsinase and Dulbecco's modified Eagle's medium (DMEM) were acquired from Gibco Company (Grand Island, United States). Fetal bovine serum (FBS) was obtained from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). An annexin V fluorescein isothiocyanate (FITC) apoptosis detection kit was purchased from KeygenBiotech.Co., Ltd. (Nanjing, China). Triamcinolone acetonide (TA) was from Zhejiang Xianju Pharmaceutical Co., Ltd. (Hangzhou, China). Propidium iodide (PI), 5-fluorouracil (5-FU), glutaraldehyde, osmium tetroxide, propylene oxide, epoxy resin, diethylpyrocarbonate (DEPC), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody and horseradish peroxidase (HRP)-conjugated secondary antibody were purchased from Sigma Chemical Company (St. Louis, MO, United States). The ShineSybr® quantitative real time PCR (qPCR) Master Mix Kit and all other reagents for qPCR were from ShineGene Molecular Biotechnology Co., Ltd. (Shanghai, China). All primers were synthesized by ShineGene. Polyclonal antibodies to c-jun and c-fos were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, United States), and antibodies to fas, caspase-3, bcl-2, NF-κB were from Biosynthesis Biotechnology Co., Ltd. (Beijing, China). Polyvinylidene fluoride (PVDF) membrane was obtained from Merck Millipore Corp. (Carolina, United States). All chemicals and reagents used here were of high analytical grade.

2.2. HS induction and wound harvesting

Eighteen New Zealand white rabbits weighing between 2.5 and 3.5 kg were purchased from Laboratory Animal Center of Anhui Medical University (Hefei, China). The animals were single-housed under standard conditions at 22 ± 2 °C with a 12-h light/dark cycle and fed *ad libitum*. Rabbits were accommodated to the holding room for at least 1 week before the initiation of experiments. All rabbit experiments were designed to minimize suffering and reduce the number of animals used. Animal treatments were carried out in line with the international ethical clauses concerning the Care and Use of Laboratory Animals of Anhui Medical University, and performed in accordance with the Guidelines for Laboratory Animals of the National Research Council of USA (1996).

The rabbit ear HS model was established according to our previous study [11]. Briefly, the animals were given anesthesia using intraperitoneal injection of chloral hydrate (2.5 ml/kg) under sterile conditions in preparation for wounding, and then, two identical, 7-mm, full-thickness, circular wounds were generated down to, but not including the bare cartilage on the ventral surface of each ear using a biopsy punch (Miltex, Inc. PA, USA). The epidermis, dermis, and perichondrium were carefully removed using a dissecting microscope. Hemostasis was achieved by applying manual pressure with saline-soaked gauze and maintained aseptically. By day 28 postsurgery, the HS had begun to appear, at which point the treatment regimen was initiated.

2.3. Detection of HSF apoptosis in scar tissues using transmission electron microscopy (TEM)

To investigate the effect of rhEndostatin on scar fibroblast apoptosis *in vivo*, TEM examination was carried out using ultrathin sections. Twelve rabbits were randomly divided into the following six treatment groups: untreated, normal saline (NS, 0.9%, w/v), rhEndostatin (1.25, 2.5 or 5 mg/ml), and triamcinolone acetonide (TA, 40 mg/ml). Each group consisted of two rabbits for a total of eight wounds on both the left and right ears. Treatments were administered starting on postwounding day 28. RhEndostatin (1.25, 2.5 or 5 mg/ml) was injected intradermally into each of the wounds on the treatment ears in a radial fashion using a microsyringe (4 injections per wound, 25 μL/injection). The wounds treated with NS and TA received the indicated doses of corresponding material (100 μL intradermally), respectively. Wounds in the untreated group received no injections. The rabbits were treated once every other day with the above mentioned materials until day 40, for a total of 12 days. Any wound displaying evidence of infection, desiccation, or necrosis was excluded from the study.

On postoperative day 47, the rabbits were sedated according to the above mentioned protocol and killed. Scars with a 0.5-cm margin of surrounding unwounded tissue were harvested and cut into with 1-mm^3 volume samples. The samples were then placed in a fixative consisting of 2.5% glutaraldehyde (pH 7.4) in 0.1 M phosphate buffer overnight at 4 °C. After washing with buffer three times, specimens were post-fixed in 1% osmium tetroxide for 1 h and dehydrated using graded solutions of alcohol. Tissues were then cleared in propylene oxide and embedded in epoxy resin. Ultrathin sections were cut at 70-nm using an ultra microtome (Leica, Richmond Hill, Canada) and stained with uranyl acetate and lead citrate prior to visualization with a transmission electron microscope (JEM-1230; JEOL, Tokyo, Japan) equipped with a digital camera.

2.4. Flow cytometry (FCM) analysis of HSF apoptosis

Scar fibroblasts were obtained as described previously [11]. Briefly, on postoperative day 28, the HS specimens were trimmed to remove excessive adipose tissues and epidermis and rinsed with PBS solution three times. The samples were then cut into 0.5–1 mm^3 pieces using a pair of sterile eye scissors. They were placed evenly in 25 cm^2 cell culture flasks and maintained in 5 ml culture medium containing DMEM with 100 U/ml penicillin, 100 μg/ml

Table 1
Primer sequences used in real-time reverse transcriptase polymerase chain reaction.

Genes	Forward	Reverse
c-fos	5′-CCTTCCCTCTGGCTGTGAT-3′	5′-AGGCTCTGCTCTGTGGTCTG-3′
c-jun	5′-TCTACGACGATGCCCTCAA-3′	5′-GTTGCTGGACTGGATGATGA-3′
NF-KB	5′-TCATCTTCCCGGCAGAGCCAG-3′	5′-GTGGGTCTTGGTGGTAGCTGT-3′
Fas	5′-TCATTGAGGAATGCACACAAAC-3′	5′-TGTATCTTCTCAGCCCCAAAAC-3′
caspase-3	5′-AATGCAGCAAACCTCGGG-3′	5′-CCTTCATCACCGTGGCTTAG-3′
bcl-2	5′-CCCACCCCTGGCATCTTC-3′	5′-GCGACGGTAGCGACGAGA-3′
GAPDH	5′-AAGAAGGTGGTGAAGCAGGC-3′	5′-TCCACCACCCTGTTGCTGTA-3′