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Effect of indomethacin and lactoferrin on human tenocyte proliferation and collagen formation *in vitro*

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ABSTRACT

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in patients with injuries and inflammation of tendon and ligament, and as post-surgical analgesics. The aim of this study is to investigate the effect of indomethacin, a classic NSAID and its combinational effect with an anabolic agent of skeletal tissue, lactoferrin, on the proliferation and collagen formation of human tenocytes *in vitro*. A factorial experimental design was employed to study the dose-dependent effect of the combination of indomethacin and lactoferrin. The results showed that indomethacin at high concentration (100 μM) inhibited human tenocyte proliferation in culture medium with 1–10% fetal bovine serum (FBS) *in vitro*. Also, high dose of indomethacin inhibited the collagen formation of human tenocytes in 1% FBS culture medium. Lactoferrin at 50–100 μg/ml promoted human tenocyte survival in serum-free culture medium and enhanced proliferation and collagen synthesis of human tenocytes in 1% FBS culture medium. When 50–100 μg/ml lactoferrin was used in combination with 100–200 μM indomethacin, it partially rescued the inhibitory effects of indomethacin on human tenocyte proliferation, viability and collagen formation. To our knowledge, it is the first evidence that lactoferrin is anabolic to human tenocytes *in vitro* and reverses potential inhibitory effects of NSAIDs on human tenocytes.

1. Introduction

With a considerable morbidity, tendon injuries may cause pain and disability that last for several months [1]. However, the basic cell biology of tendons still has not been fully understood [2], and the management of tendon injury poses a substantial challenge for clinicians [3,4]. Conventionally, non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in patients with musculoskeletal conditions such as sports injuries, inflammation of tendons and ligaments, and also as a post-surgical analgesic [5]. Clinical studies indicate that NSAIDs reduce pain, tenderness and stiffness associated with acute soft tissue injuries [6]. NSAIDs are also used for managing other tendon related conditions such as chronic tendonitis, although histological studies have shown absence of acute inflammatory cells in some of these conditions [7,8]. A number of studies on bone, ligament, and tendon repair have evaluated the effects of NSAIDs in experimental animal tissue. It has been reported that NSAIDs may delay soft tissue healing with unknown mechanisms [9]. Currently, the effect of NSAIDs on tenocytes remains controversial, with both beneficial [10] and harmful [11,12] effects being described.

Indomethacin is the one of the earliest NSAIDs. It inhibits arachidonic acid metabolism via affecting lipoxygenase and cyclooxygenase and therefore is used as an anti-inflammatory drug [13]. It has been reported that indomethacin delays fracture healing [14], and obstructs bone formation [15]. Recently, studies indicate that indomethacin inhibits proliferation of primary tenocytes isolated from rat tendon [16], delays rat tendon healing [17] and impairs rotator cuff tendon-to-bone healing [12]. Controversially, there are also some reports suggesting that indomethacin could improve tendon healing in rats [10]. Therefore, it is of great interest to study the effect of indomethacin on tenocytes, and whether any negative effects can be reversed or prevented.

Lactoferrin is an iron-binding glycoprotein which is present in epithelial secretions, breast milk, and the secondary granules of neutrophils [18]. Bovine and human lactoferrin have been demonstrated to be anabolic to bone and cartilage tissue, stimulating osteoblast and chondrocyte proliferation, as well as increasing bone formation both *in vitro* [19,20] and *in vivo* [21]. However, there is no report of the effect of lactoferrin on human tenocytes.

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The aim of our present study is to investigate the effect of the classic NSAID indomethacin, and its combinational effect with lactoferrin, the anabolic agent of skeletal tissue, on the proliferation and collagen formation of human tenocytes *in vitro*. We found that indomethacin at high concentration (100 μM) inhibited human tenocyte proliferation and collagen formation, whereas lactoferrin at 50–100 μg/ml has a promotion effect. Combinational administration of lactoferrin with indomethacin rescued the harmful effects caused by indomethacin. Hopefully, this study may shed light on the design of therapeutic strategy in clinic.

2. Materials and methods

2.1. Materials

Indomethacin was purchased from Sigma–Aldrich Company Ltd. (Gillingham, UK). Bovine lactoferrin was isolated from fresh skim milk by cation exchange chromatography and gel filtration as described earlier [22]. α-Minimal essential medium (α-MEM) powder were purchased from Invitrogen, Paisley UK; 0.2 μm filter for culture medium preparation was from PALL Lifer Science, PALL Corporation, UK. Filters to remove endotoxin from any solution used were purchased from Sartorius Stedim Biotech, Surrey, UK. Fetal bovine serum (FBS) was purchased from Biosera, East Sussex, UK, and trypsin-EDTA, penicillin and streptomycin from Lonza Wokingham Ltd, Berkshire, U.K. Tissue culture grade polystyrene flasks and plates were obtained from Greiner Bio-One Company in UK. Unless otherwise noted, all other reagents were of analytical grade from Sigma–Aldrich, Poole, U.K., or VWR International Ltd, Lutterworth, U.K.

2.2. Isolation and culture of tendon derived cells

Human tendon biopsies were obtained from consenting, non-smoking, male patients <30 years of age who were undergoing right anterior cruciate ligament (ACL) reconstruction with a hamstring graft at the Nuffield Orthopaedic Centre, Oxford. The protocols were approved by the Oxford Research Ethics Committee C (09/H0606/11). Participants provide their written informed consent to participate in this study. The tendon samples were collected within 1 h following the operation and were kept in 4 °C sterile α-MEM medium before processing.

The tenocyte isolation method was adapted and modified from Bi et al. [23]. In general, 2 cm tendon segments from the middle of the tendon biopsies were diced into 1 mm^3 pieces and were treated in 4 mg/ml dispase (Roche, Hertfordshire, U.K.) and 300 U/ml collagenase type II (Gibco, Invitrogen, Paisley, U.K.) solution in serum free α-MEM (avoiding deactivating of enzymes) at 37 °C incubator for 16 h. After enzymatic digestion, equal volumes of α-MEM medium, supplemented with 10% FBS, were added to quench the collagenase and filtered through cell strainers (70 μm nylon, BD falcon, BD bioscience, California, U.S.A.). The filtered cell suspension was centrifuged at 1500 rpm (380 g) for 5 min, and the supernatant was discarded. Fresh culture medium with 20% FBS was used to boost cell proliferation after enzymatic release and single-cell suspension was cultured in 75 cm^2 tissue culture (TC) flasks (BD falcon, BD bioscience, California, U.S.A.), at 5% CO_2 and 95% air at 37 °C. The cells were sub-passaged after 80% confluence and cultured in the same culture medium supplemented with 10% FBS from passage 1.

2.3. Effect of indomethacin and lactoferrin on human tenocyte proliferation

Passage 3 human tenocytes was seeded in 96 well plates (BD falcon, BD bioscience, Oxford UK) at a density of 5000 cells/well in 1–10% FBS α-MEM medium. Indomethacin at different concentrations (0, 0.01, 0.1, 1, 10 and 100 μM) or lactoferrin at different concentrations (0, 10, 20, 30, 50 and 100 μg/ml) was added to culture medium and cells were cultured for 7–14 days. Culture medium was changed every 2–3 days. Alamar Blue™ assays were performed to determine cell proliferation according to manufacturer's protocol at day 0, day 4, day 7 and day 14. In brief, 200 μl of 5% Alamar Blue™ (Biosource Europe, Nivelles, Belgium) was added in each well after removal of the culture medium. The cells were incubated at 5% CO_2, 37 °C for 2 h. The Alamar Blue™ solution was transferred to another plate with 5 wells of 5% Alamar Blue™ as blanks and the relative fluorescent unit (RFU) was measured with SoftMax Pro software using a SPECTRAmax GEMINI micro plate spectrofluorimeter (Molecular Devices, Berks, U.K.) at the excitation wavelength of 530 nm and the emission wavelength of 590 nm, with a cut off of 570 nm. The estimated cell number was calculated by using a standard curve created by incubating 5% Alamar Blue™ under the same conditions. The cell number was plotted against the RFU. This process was performed repeatedly with each microplate in order to calculate the cell number.

2.4. Effect of lactoferrin on human tenocyte survival in serum free culture

Passage 3 human tenocytes were seeded in 96 well plates at a density of 5000 cells/well in 0% FBS α-MEM medium and were fed with different concentration of lactoferrin (0, 10, 20, 30, 50 and 100 μg/ml) for 7 days. Culture medium was changed every 2–3 days. AlamarBlue™ assay was performed at day 0 and day 7.

2.5. Assessment of the combinational effect of indomethacin and lactoferrin

A full factorial experimental design was employed to study the effect of the combination of the indomethacin and lactoferrin at effective doses selected from the first two experiments. The culture condition was carried out in 1% FBS culture medium. Passage 3 human tenocytes were seeded in 48 well plates at a density of 1×10^4 cells per well in 1% FBS α-MEM medium. The cells were treated with different concentrations of indomethacin and lactoferrin as shown in figures.

A tiered outcome analysis approached assessing (1) human tenocyte number changes (promoting or inhibiting cell proliferation); (2) collagen synthesis; and (3) cell morphology. For morphologic observation, glass cover slips were added into extra wells with cells seeded at the same time and cultured in the same condition. All experiments were repeated three times with consistent results.

2.6. Cell viability assay

Human tenocytes were stained with a fluorescent Live/Dead Viability/Cytotoxicity Kit (Invitrogen, Paisley, UK) at day 7, following the manufacturer's instructions. This fluorescent kit was used for cell labeling, with green for live cells and red for dead cells. Samples were examined by a fluorescent microscope (Carl-Zeiss Axio,) without fixation.

2.7. Evaluation of collagen synthesis

The procedure of Sirius red staining was adapted and modified from Tullberg-Reinert and Jundt [24]. On day 14, the culture medium was removed and the cell layers were washed extensively with PBS before being fixed with 100 μl of Bouin's Solution (71% saturated picric acid, 24% of formalin and 5% 0.5 M acetic acid) for 1 h. After fixation, the cell layers were washed under tap water