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Marine based biomaterial-fish collagen enhances the polarization of human macrophage

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Abstract. Fish collagen have the potential to be used in biomedical fields due to the biocompatible and biodegradable features. However, how hydrolyzed fish collagen regulate human macrophage has not been clearly unraveled. The aim of the current study is to investigate the effects of hydrolyzed fish collagen on the polarization state of human macrophages. The cell viability of human macrophages treated by hydrolyzed fish collagen were determined using CCK-8 assay. The polarization of human macrophages treated by hydrolyzed fish collagen were detected by quantitative real-time polymerase chain reaction (qRT-PCR). it was found that 0.25,0.5 and 1mg/ml hydrolyzed fish collagen did not alter the cell viability of human macrophages. Further, the data showed that hydrolyzed fish collagen inhibited the expression of M1 macrophage marker IL-1β and TNF-α while enhanced the expression of M2 macrophage marker arginase 1(Arg1) and IL-10. Taken together, the results indicated that treatment with hydrolyzed fish collagen result in a significant shift towards an M2 phenotype for human macrophages.

1. Introduction

Collagen has been widely used in cosmetics, pharmaceutical and biomedical fields, the available collagen is primarily obtained from terrestrial animals, such as porcine and bovine[1]. However, mammalian origin collagen present religious constraints and has been linked to a high risk of transmitting certain animal diseases such as foot-and-mouth disease (FMD) and bovine spongiform encephalopathy (BSE) and [2]. Fish collagen has now emerged as a safer alternative to terrestrial collagen due to lack of disease transmission risk [3].

To explore more potential use of fish collagen, recently some groups are focusing on the biological activity of hydrolyzed fish collagen (HFC) [4-6]. It has been found that subcritical water-hydrolyzed fish collagen significantly suppressed LPS-stimulated production of HMGB1(high mobility group box 1) in murine macrophages, along with the reduction of cytosolic translocation of HMGB1[5]. More interestingly, prior studies have indicated that hydrolyzed fish collagen possesses osteoinduction properties [7,8], making it a good candidate as the ingredient of bone implant materials.

Immediately after implantation of a biomaterial, the host acts in a complex cascade of events leading to foreign body reaction mediated mainly by macrophages[9]. Activation of the immune system is an important process for the integration of implantation and wound healing. It is important to elucidate the interaction feature between the biomaterials and the immune related cells. There are two main phenotypes designated proinflammatory M1 macrophage and alternatively activated M2

macrophage [10]. The M1 macrophage is related to the early inflammation phase of wound healing, M1 macrophages produce high levels of pro-inflammatory factors, including interleukin (IL)-6, IL-1β,IL-8 , tumor necrosis factor α (TNF-α) and IL-12, while the M2 macrophage is associated with the late stage of wound healing, M2-type macrophages produce high levels of anti-inflammatory factors such as IL-10 and transforming growth factor-β (TGF-β) in vitro culture conditions [11].

Despite the good biocompatibility of collagen-based biomaterials on fibroblasts[12], these materials are generally associated with the constant and lingering activity of M1 macrophage [13]. Earlier studies have shown that hydrolyzed fish collagen exhibits specific anti-inflammatory effects on mouse macrophages [14,15]. Nevertheless, the effect of hydrolyzed fish collagen on human macrophage are little known. The current study was aimed to evaluate the effect of hydrolyzed fish collagen on the cell behavior of human macrophages. Cell viability and polarization state were investigated in human macrophages in this study.

2. Materials and methods

2.1. Materials

Hydrolyzed fish collagen (HFC) was generously provided by the Shanghai Fisheries Research Institute. HFC has a molecular weight distribution of 700 to 1300 Da, the contact angle of HFC is around 26°, There are many kinds of amino acid, such as glycine (333/1000 residues), proline (115/1000 residues), and hydroxyproline(117/1000 residues) [8].

2.2. Cell culture

The human THP-1 macrophage cell line was purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). Human macrophage was cultured in RPMI 1640 medium (Gibco, Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum (FBS, Hyclone, USA), 100 IU/ml penicillin (Gibco, USA), and 100 μg/ml streptomycin (Gibco, USA). The cells were maintained at 37 °C in a 5% CO_2 atmosphere, and the medium was replaced every 3 days. To induce the differentiation into macrophage, the human THP-1 macrophages were added into 96-well plates at a density of 1.0×10^5/mL, then the cells were treated with 100 ng/ml PMA (phorbol 12-myristate 13-acetate) (EMD Biosciences, USA) for 48 hours at 37℃. To generate the polarized phenotypes, cells were then cultured for 24 hours in M1 polarization inducing media (100 ng/ml LPS) to generate M1 macrophages, or M2 polarization inducing media (20 ng/ml of IL-4, and 20 ng/ml of IL-13) to generate M2 macrophage.

2.3. CCK-8 assay

The cell viability of human macrophages was evaluated using CCK-8 assay (Dojindo, Kumamoto, Japan) according to the manufacturer's protocol. Briefly, cells were seeded onto 96-well plates with a density of 5×10^4 cells/well, and treated with various concentration of HFC (0, 0.25, 0.5, 1, 2 and 4 mg/ml) for 24 h. Then, the medium was removed by aspiration and 100 μl of medium containing 10% CCK8 reagent was added to each well, after incubation at 37 °C incubator for 2 h, the optical density was read at 450 nm. 3 biological replicates were used in CCK-8 assay.

2.4. Real-time PCR analysis

Based on the results of CCK-8 assays, 0.25 mg/ml HFC was used in this part. M0 human macrophage were cultured for 1 day with 0.25 mg/ml HFC to investigate macrophage polarization. Total RNA extraction and purification were performed using TRIzol reagent (ThermoFisher, USA) according to manufacturer's instruction. cDNA was synthesized from 500ng of total RNA using the Prime Script RT reagent kit (TaKaRa, China) according to the manufacturer's protocol. Real-Time PCR was carried out by using SYBR green (PowerUp SYBR Green Master Mix, Life Technologies, USA) according to manufacturer's protocol. The PCR mixture was performed in a 25 μl that consists of the following: 2 μl of cDNA template, 12.5 μl of 2× SYBR Green, 1 μl of forward primer, 1 μl of reverse primer, and

8.5 μl of double distilled water(ddH2O). The ΔΔCt method was used to calculate the gene expression levels after normalization according to the expression of GAPDH. 3 biological replicates were used in Real-time PCR analysis. The primer sequences were shown in Table 1.

Table 1. Primers for real-time PCR.

Target gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
IL-1β	ATGATGGCTTATTACAGTGGCAA	GTCGGAGATTCGTAGCTGGA
TNF-α	ACTTTGGAGTGATCGGCC	GCTTGAGGGTTTGCTACAAC
Arg 1	ACGGAAGAATCAGCCTGGTG	GTCCACGTCTCTCAAGCCAA
IL-10	GCTGGAGGACTTTAAGGGTTAC	GTAGTCTGGGTCTTGGTTCTC
GAPDH	AGCCACATCGCTCAGACA	GCCCAATACGACCAAATCC

2.5. Statistical Analysis

All experiments were performed at least three times, Date were expressed as mean ±SEM. Data analysis was carried out using one-way analysis of variance and Student's t-test. p values less than 0.05 were considered as statistically significant in all experiments.

3. Results

3.1. The effects of different doses of HFC on cell viability of human THP-1 macrophage

CCK-8 assays were used to determine the optimal concentration of HFC on human macrophage. Human macrophage was exposed to various concentrations of HFC for 24 h. Figure 1 demonstrates that HFC at concentrations 0.25 mg/ml, 0.5 mg/ml and 1 mg/ml did not significantly change cell viability in contrast to cells without addition of HFC, suggesting that 0.25, 0.5 and 1 mg/ml HFC were relatively safe for human macrophage (Figure 1). 0.25 mg/ml is the minimum concentration that did not significantly change cell viability in our experiment, based on the principle of minimum dose, 0.25 mg/ml HFC were used for subsequent experiments.

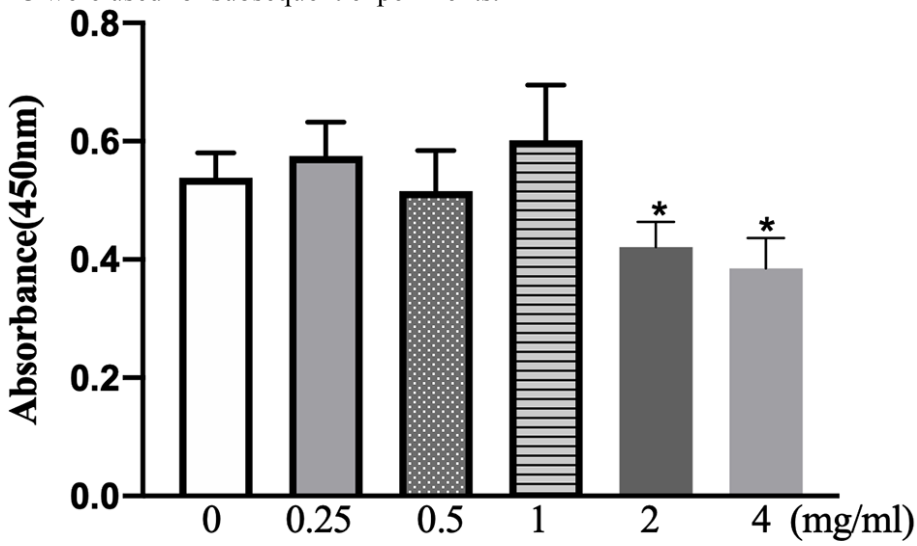

Figure 1. Cell viability of human macrophage treated with different concentrations of HFC. * indicates a significant difference compared with control, $p < 0.05$.

3.2. Macrophage polarization in response to the HFC

The expression of macrophage polarization markers was evaluated by real-time PCR when M0 human macrophage were cultured with 0.25 mg/ml HFC. Both IL-1β and TNF-α, the two important M1 macrophage markers were significantly decreased in HFC group as compared to that in control group (Figure 2A and B), while higher mRNA levels of M2 markers, including Arg1 and IL-10, were

observed in cells treated with HFC when compared with control group (Figure 2C and D). It did show evidence that HFC induced macrophage to become polarized toward the M2 phenotype.

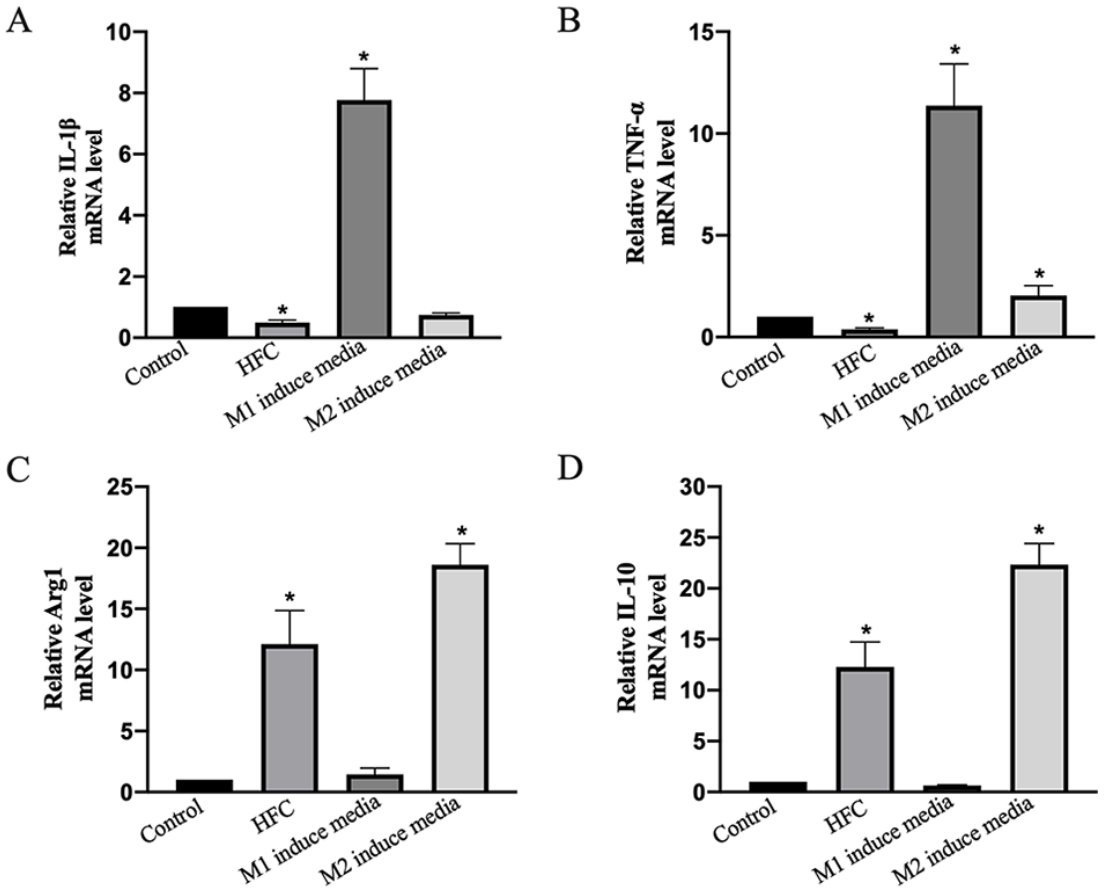

Figure 2. qRT-PCR analyses of IL-1β(A) TNF-α(B), Arg1(C) and IL-10(D) gene expression. *indicates a significant difference between HFC treated groups and control group with p-value < 0.05.

4. Discussion

Amino acids (Proline and Hydroxyproline) are key players in upholding the function of the hydrolyzed collagen. Proline and hydroxyproline are also the most abundant amino acid in mammalian collagen[16], in the present study, since the obtained amino acid composition of HFC was matching to the mammalian, HFC can be definitely used as an alternative to existing commercial collagen, furthermore, it has been shown that HFC possess good bioactivity[17,18].Therefore, HFC is expected to be a good candidate for the biomedical application.

In the present study, 0.25, 0.5 and 1 mg/ml HFC did not alter the cell viability of human macrophage, which suggest the good biocompatibility of HFC at specific concentrations. On the other hand, the recruitment and proliferation of macrophages on the foreign body are triggered by chemokines and cytokines released by the initially adhered macrophages to the implant, which indicates that HFC could potentially serve as a good implant coating material, no interfering the cell viability of macrophages is one of the main characteristics for a good biomaterial[19], but further studies are needed to corroborate this conclusion.

Furthermore, the polarization of human macrophage treated with HFC was evaluated by qRT-PCR. Human macrophage treated with 0.25 mg/ml HFC showed lower expression level of M1 markers (IL-1β and TNF-α), whereas higher expression level of M2 markers (Arg1 and IL-10) compared with that of control. It is commonly believed that the M1 macrophage producing proinflammatory cytokines and

associated with tissue disruption, while the M2 macrophage is anti-inflammatory and linked to tissue repair [20]. M1 macrophages with increased TNF-α, IL-1 expression suggests delayed tissue healing and more severe inflammatory response [21,22]. IL-1β caused an activation of both canonical and noncanonical NF-κB pathway, leading to an increase in intestinal epithelial tight junction permeability [23]. Other group found that IL-1β can cause the destruction of junctions of endothelial cells [24]. Tumor necrosis factor-α (TNF-α) is a cytokine mainly produced by macrophages and monocytes, and plays a central role in inflammatory osteoclastogenesis [25]. Therefore, repression of inflammatory cytokine produced by HFC in our study would be helpful to endothelialization and tissue repair.

In addition, HFC induced M2 macrophage polarization by increasing the level of Arg1 and IL-10. Both Arg1 and IL-10 are anti-inflammatory factors with ability to improve tissue regeneration. Arginase 1 is an enzyme of the urea cycle and join in the regulation of a multitude of cellular processes like cell growth, DNA replication and protein translation [26]. IL-10 can suppress the production of the pro-inflammatory cytokines and inhibit the secretion of nitric oxide [27]. Both Arginase 1 and IL-10 are helpful to implant integration and Integrity. Thus, our results demonstrated that HFC inhibited pro-inflammatory cytokine production by macrophages while promoted the production of anti-inflammatory cytokines, HFC contribute to the development of an anti-inflammatory microenvironment that is favorable to tissue repair and regeneration.

5. Conclusions

In this study, we found that a novel marine biomaterial hydrolyzed fish collagen induced the shift of human macrophage phenotype from M1 to M2, which may lead to the application of hydrolyzed fish in biomedical fields.

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