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Type I collagen or gelatin stimulates mouse peritoneal macrophages to aggregate and produce pro-inflammatory molecules through upregulated ROS levels

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ABSTRACT

Background: Extracellular matrix (ECM) comprising the environments of multicellular society has a dynamic network structure. Collagen is one of the ubiquitous components of ECM. Collagen affects the inflammatory response by regulating the release of pro-inflammatory cytokines from cells. Gelatin, denatured collagen found temporally in tissues, is supposed to be pathophysiologically involved in tissue remodeling, inflammation caused by tissue damage. Previous reports indicate that, phorbol myristate (PMA)-stimulated human U937 (lymphoma cell line) cells that are often used as macrophage-like cells, show cell aggregations when cultured on type I collagen (col I) or gelatin-coated dishes, accompanying the changes of production and release of proinflammatory factors. However, it still remains to be examined whether collagen and gelatin affects normal macrophages as well.

Aim: This study aims to investigate the effect of col. I, the main component of collagenous protein and its denatured product, gelatin, on mouse peritoneal macrophages (MPMs).

Methods: MTT assay, flow cytometric analysis of ROS, biochemical detection of antioxidant levels, ELISA assay, and western blot were used.

Results: MPMs formed multicellular aggregates on col. I - and gelatin-coated dishes with a concentration- and time-dependent manner. Further studies showed that the culture on col. I and gelatin up-regulated the protein expression and secretion of pro-inflammatory molecules such as IL-1β, TNFα and prostaglandin E_2 (PGE_2) in MPMs. The levels were higher in the cells on gelatin than those on col. I. ROS levels are significantly increased in the cells cultured on both col. I- and gelatin-coated dishes, accompanying decreased levels of antioxidant enzyme catalase (CAT) and anti-oxidant glutathione (GSH), and enhanced nuclear translocation of NF-κB.

Conclusion: Col I - or gelatin-coated culture induced the formation of multicellular aggregates and increased production of NF-κB-associated pro-inflammatory molecules in MPMs through up-regulation of ROS levels.

1. Introduction

The extracellular matrix (ECM) plays an important role in regulating development, function and homeostasis of multicellular animals [1,2]. On the maturation process of cell matrix, ECM proteins are crucial in mediating cell adhesion, cell migration, proliferation and differentiation [3].

Collagen is the main component of ECM. Type I collagen (col I) is the most abundant among collagen protein family [4]. Disordered collagen homeostasis leads to the development of many human diseases including fibrosis, which is attributed to excess interstitial deposition of collagen [5]. Excessive and disordered deposition of collagen reflects central and untreatable pathological conditions in many chronic human diseases [6]. Gelatin is the denatured product of collagen with

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essentially identical primary structures [7]. During skin burns, collagen was heat-denatured into gelatin, and at the same time, a large amount of pro-inflammatory cytokines are released [8–10]. Since gelatin is frequently produced along with inflammation, we hypothesize that gelatin might simultaneously be associated with inflammation progression.

Macrophages play an important role in innate cellular immunity with versatile functions [11], participating in both innate and adaptive immunity [12]. Macrophages produce pro-inflammatory cytokines such as TNFα and IL-1β under the inflammatory conditions [13,14].

Macrophages often form aggregates when they encounter with harmful foreign materials or in the presence of cellular byproducts [15]. Vincent et al. reported that host and bacterial determinants lead to the formation of organized macrophage aggregates as part of the host inflammatory response to infected bacteria [16].

Our previous studies demonstrate that both col. I and gelatin induce differentiated U937 cells, human monocyte-like histiocytic lymphoma cell line, to form multicellular aggregates and col. I represses the production of pro-inflammatory cytokines, whereas gelatin increases these cytokines. But the effects on normal macrophages above have not been elucidated [17,18]. We here report that both col. I - and gelatin-coated culture promotes aggregation of MPMs. Col I and gelatin both upregulated the protein expression and secretion of pro-inflammatory molecules such as IL-1β, TNFα and their downstream molecule prostaglandin E_2 (PGE_2) in MPMs. The results confirm that the effect of gelatin on activated U937 is extended to MPM, whereas the effect of col. I on MPM is opposite to that on stimulated U937 cells, suggesting that col. I has different effects on normal macrophages from the cell lines which can be differentiated to macrophage cell types.

2. Materials and methods

2.1. Reagents

FITC-anti-F4/80 antibody was purchased from Biolegend (San Diego, CA, USA). PE-anti-CD11b antibody was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Enzyme-linked immunosorbent assay (ELISA) diagnose kits of mouse IL-1β, TNFα and PGE_2 were purchased from Dakewe Biotech (Shenzhen, Guangdong, China). 2′,7′-Dichlorofluorescein diacetate (DCF-DA), *N*-acetylcysteine (NAC), superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and rabbit monoclonal anti-LC3 antibody were purchased from Sigma Chemical (St. Louis, MO, USA). BAY 11–7082 was obtained from MedChem Express (Monmouth Junction, NJ, USA). SOD, CAT and GSH detection kits were purchased from Nanjing Jiancheng Biotech (Nanjing, Jiangsu, China). Electrochemiluminescence (ECL) reagent was from Thermo Scientific (Rockford, IL, USA). Primary antibodies against IL-1β, TNFα, cyclooxygenase-2 (COX-2), NF-κB, β-actin as well as horseradish peroxidase conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies against Lamin B was obtained from Proteintech (Suite 300 Rosemont, IL, USA).

2.2. Animals

Adult female Kunming mouse weighing 18–22 g purchased from Chang Sheng Biotechnology (Benxi, Liaoning, China) housed under conventional conditions at appropriate temperature (22 ± 0.5 °C) with controlled humidity (50–60%) and 12/12 h light/dark cycle were allowed for free access to food and water. All animal studies were performed in accordance with the China legislation on the use and care of laboratory animals and the guidelines established by the Institute for Experimental Animals at Shenyang Pharmaceutical University. Categorized number: SCXK (Liao) 2015–0001. The experimental protocols were approved by the Animal Care and Protection Committee of Shenyang Pharmaceutical University [19,20].

2.3. Preparation of MPMs

Kunming mice injected intraperitoneally with 4% thioglycolate (Sigma-Aldrich, MO, USA) were left standing for 3 days, when the abdomen became deteriorated. After mice bodies were soaked with 70% alcohol, 10 ml of cold PBS was injected into each mouse peritoneum. Three minutes later, intraperitoneal fluid was aspirated from peritoneum using a syringe. The peritoneal fluid collected was transferred into a 50-ml centrifuge tube on ice. Then the fluid was centrifuged at 400 ×*g* for 10 min at 4 °C. The cell pellets suspended in 10% fetal bovine serum FBS (FBS) (Beijing Yuanheng Shenyang Research Institution of Biotechnology, Beijing, China) [21]-RPMI 1640 medium were placed in 6-well cell culture plates (Corning, NY, USA) with a density of 1×10^6 cells /cm^2 and left standing for 2 h. Thereafter, nonadherent cells including lymphocytes, were removed gently by washing with culture medium. The adherent cells with 98% viability, (as determined by trypan blue staining) (Supplementary Fig. 1D) were recovered for further study.

2.4. Trypan blue staining

Five ml of macrophage suspension was collected and incubated with the same amount of 0.4% trypan blue dye solution. Then, the suspension was placed in an Eppendorf tube. One drop of the stained cell suspension was taken into the counting plate. 200 cells were counted in 3 min, and the percentage of viable cells, which are negatively stained, was calculated.

2.5. Identification of MPMs

MPMs are routinely seeded at 1×10^6 cells/cm^2. Macrophages are typically characterized by large sizes with expression of surface F4/80 glycoprotein and CD11b, one of the chains of the Mac-1 integrin [22,23]. We used F4/80 and CD11b to identify macrophages. Cells were collected and were incubated with 10 μg/ml FITC-F4/80 and 8 μg/ml PE-CD11b (both diluted with PBST) solution in the dark at 37 °C for 30 min, and then analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA). In this study, 1×10^4 cells were analyzed in each case. Data analysis was performed with gating.

2.6. Cell culture on collagen or gelatin-coated plate

Col I and gelatin were prepared in Nippi Research Institute of Biomatrix (Toride, Ibaraki, Japan). Prior to cell culture, col. I or gelatin solution diluted with 0.5 mM acetic acid to the predetermined concentrations were placed in 6-well cell culture plates (Corning, NY, USA) and maintained in an incubator at 37 °C overnight, yielding col. I or gelatin coating on the dishes as a dispersed form. Then the solution was gently removed. The plates washed 3 times with PBS to neutralize acetic acid, were ready for cell culture.

We also used BSA as a negative control. Prior to cell culture, 5% BSA solution in PBS was placed in 6-well cell culture plates and maintained in an incubator at 37 °C overnight. Then BSA solution was gently removed. After the plates were washed 3 times with PBS, the cells were seeded on the 6-well cell culture plates. Cultured cells were observed with an inverted microscope.

2.7. Sirius Red staining of collagen

Collagen and its denatured product gelatin both consist of the polypeptides with (Gly-X-Y) repeating sequences, where G represents glycine and X or Y represents any kind amino acid residues with some post-translational modifications. About one third of X and Y residues are proline and hydroxyproline, respectively. Sirius Red specifically binds to the (Gly-X-Y) structure. Therefore, the Sirius Red Collagen Staining kit (Redmond, WA, USA) was used for determination of

relative amounts of col. I- and gelatin-coated on the plates. Briefly, col. I-or gelatin-coated surface was washed with PBS and incubated at room temperature for 30 min after adding dye solution. The dye solution was carefully removed, and the surface was washed with double-distilled water. After adding the dye extraction reagent (Redmond, WA, USA) and gently mixing, absorbance of extracts at 540 nm was determined and converted to for (Gly-X-Y) index.

2.8. Presentation of the sizes of multicellular aggregates

Macrophages were seeded on the col. I- or gelatin-coated plates at the same density (1×10^6 cells/cm^2) 24 h after seeding, cells on plates were observed with an inverted microscope. The sizes of cell aggregates were measured for each sample in 3 random fields. Cell aggregates with a size of > 30 μm in diameter were employed for statistical analyses. The average sizes of cell aggregates were calculated after analyzing at least six aggregates in each field.

2.9. Determination of cell viability

The MPMs were seeded into 96-well cell culture plate (Corning, NY, USA) at a density of 1×10^6 cells/cm^2 on col. I- or gelatin-coated dishes and cultured for 24 h. Thereafter, the cells were incubated with 100 μl of 0.5 mg/ml MTT (Thiazolyl Blue Tetrazolium Bromide) solution at 37 °C for 3 h. The supernatant was discarded, and the residual cell layer was dissolved with 150 μl DMSO. The optical density (*A* value) was measured at the 490 nm wavelength using a microplate reader (Thermo Scientific Multiskan MK3, Shanghai, China). The cell viability was calculated using the following equation:

$$\text{Cell viability}\ (\%) = (A_{490,sample} - A_{490,blank})/(A_{490,control} - A_{490,blank}) \times 100$$

2.10. Determination of secreted IL-1β, TNFα, and PGE2

The concentrations of IL-1β, TNFα, and PGE_2 in cell conditioned medium were determined by enzyme-linked immunosorbent assay (ELISA) detection kits (Dakewe Biotech, Shenzhen, Guangzhou, China), according to the manufacturer's instruction. Briefly, the cell culture supernatant was carefully collected and placed in the wells provided in the kits with a volume of 100 μl. Then, 50 μl of diluted Biotinylated antibody was added, followed by incubation at 37 °C for 90 min. After washing, 100 μl of diluted streptavidin-HRP was added and further incubated at 37 °C for 30 min. Then the cells were washed and incubated in the wells with 100 μl of TMB (Tetramethylbenzidine) color liquid substrate solution in the dark at 37 °C for 10–30 min. The enzyme-substrate reaction is stopped by quickly pipetting 100 μl of Stop solution into each well and the optical density at 450 nm was measured using an ELISA reader (Thermo Scientific, MA, USA) within 15 min.

2.11. Flow cytometric analysis of intracellular ROS

DCFH-DA is a non-polar fluorescence probe that can penetrate into cells, where it is converted into DCFH. Intracellular ROS oxidize DCFH into high-fluorescence compound DCF. Cells were collected and were incubated with 10 μM DCFH-DA solution in the dark at 37 °C for 30 min, followed by the analysis with a flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). In our study, 1×10^4 cells were analyzed in each case. We set a gate value for DCF intensity. The cells with a DCF fluorescence intensity higher than this gate are taken as DCF-positive cells, reflecting high ROS levels.

2.12. Western blot

Cell samples were collected and lysed on ice with RIPA (radio-immunoprecipitation assay) lysis buffer (Beyotime, Haimen, Jiangsu, China) for 1 h. Then the suspension was centrifuged at 14,000 ×*g* at 4 °C for 10 min. The supernatants were collected, and the protein concentrations were determined by using bicin-choninic acid (BCA) protein assay kit. Eighty μg of total proteins was loaded in each lane for blotting. Samples separated on 10–12% SDS-PAGE gel were transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% nonfat milk in phosphate buffered saline with 0.1% Tween 20 (PBST) at room temperature for 2 h. Then the membranes were incubated with the corresponding primary antibodies (Table 1) at 4 °C overnight. The membranes were washed 10 min with PBST for 3 times, and then incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 2 h. After washing the membranes 3 times with PBST, the protein blots were visualized by the treatment with chemiluminescence (ECL) reagents (Thermo scientific, Rockford, IL, USA).

Table 1
Information about dilution ratios of antibodies.

Antibodies	Full Name	Dilution	Supplier
β-actin [24]	β-actin	1: 500	Santa Cruz Biotechnology
IL-1β [18]	Interleukin-1β	1: 2000	Santa Cruz Biotechnology
COX-2 [17]	Cyclooxygenase-2	1: 2000	Santa Cruz Biotechnology
TNFα [17]	Tumor necrosis factor α	1: 1000	Santa Cruz Biotechnology
Lamin B [25]	Lamin B	1: 1000	Proteintech
NF-κB [26]	Nuclear factor-κB	1: 1000	Santa Cruz Biotechnology

2.13. Statistical analysis

All experiments were repeated at least 3 times. Data are means ± SD. Differences among groups were tested by one-way analysis of variance (ANOVA), followed by Bonferroni's test or Dunnette-*t*-test. All data were analyzed by SPSS 17.0 software (SPSS, Chicago, IL, USA). $P < 0.05$ was considered as significant.

3. Results

3.1. Peritoneal macrophages form multicellular aggregates on col. I- or gelatin-coated surface

In the present study, col. I- or gelatin-coated plates were used for cell culture. To confirm whether col. I or gelatin is indeed coated on plate surface s, we used the staining with Sirius Red, which binds to (Gly-X-Y) structure, to determine relative amount of col. I or gelatin. Results showed that Sirius Red staining increased with the concentration of col. I or gelatin used for coating, suggesting that coated proteins depend proportionally on the protein concentration. (Supplementary Fig. 1A, B).

F4/80 and CD11b, commonly used macrophage surface marker molecules [27,28], were used to identify macrophages. Flow cytometric results showed that, F4/80 and CD11b double positive ratios are up to 92.41% in the cells isolated from mouse peritoneal fluid (Fig. 1A). Considerably highly purified MPMs were used in the following study. On collagen- or gelatin-coated dishes, cells form aggregates with increasing sizes along with the increasing concentrations col. I or gelatin (Fig. 1B). Furthermore, when macrophages were cultured on 500 μg/ml collagen for 6, 9, 12 and 24 h, the sizes of cell aggregates increased time-dependently (Fig. 1C). These results suggest that collagen-coating enhanced the aggregate formation of MPMs in concentration- and time-dependent manners. The similar results were also obtained in the culture on gelatin-coated dishes (Fig. 1D, E). Appearance of multicellular aggregates formed by culturing for 24 h on the dishes coated with a collagen solution at concentration of 500 μg/ml and on the dishes coated with a gelatin solution at concentration of 20 mg/ml is shown in Fig. 1F. To test whether the aggregating effect was specific to col. I and gelatin, but not to “all proteins”, 5% BSA solution in PBS was subjected to coating procedure used for col. I or gelatin. Results showed that BSA

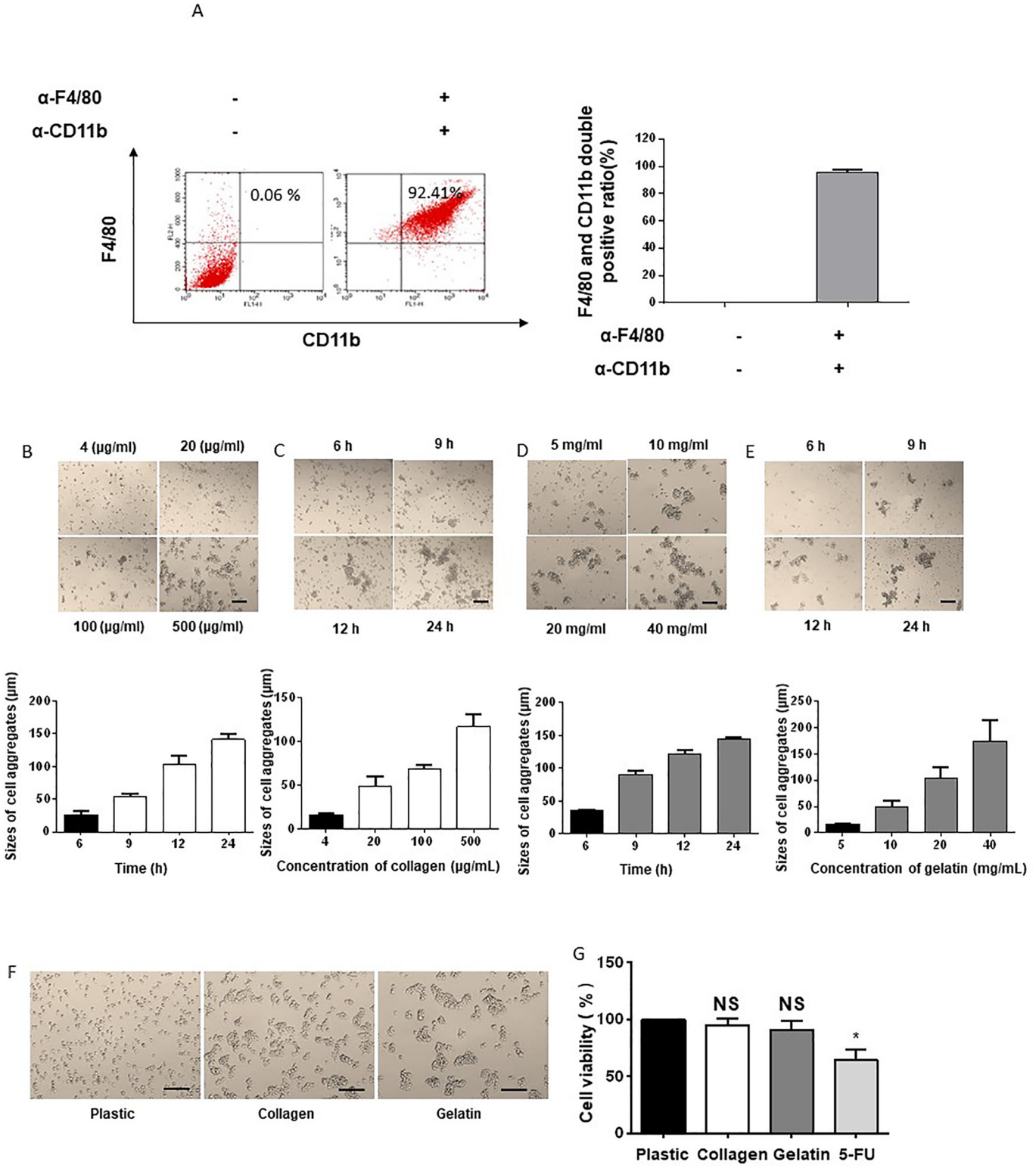

Fig. 1. Collagen I- or gelatin-coated culture promotes cell aggregation in MPMs. (A) Expression of F4/80 and CD11b, macrophage marker and mononuclear cell marker, respectively, on the cells isolated from mouse peritoneal fluid. The cells were examined by flow cytometry. (B-E) Size of cell aggregates in cells cultured with col. I or gelatin. (F) Cell morphology (Scale bar = 50 μm). The concentrations of col. I and gelatin were 500 μg/ml and 20 mg/ml, respectively. (G) Cell viability was examined by MTT assay. 5-Fluorouracil (Fu) at 20 μM serves as a negative control. All groups were compared with non-coated or plastic group. Data in A, B, C, D, E and G are presented as means ± SD of three independent experiments. NS, not significant. *, $P < 0.05$, the P values were determined by one-way ANOVA with Dunnett's post-hoc test.

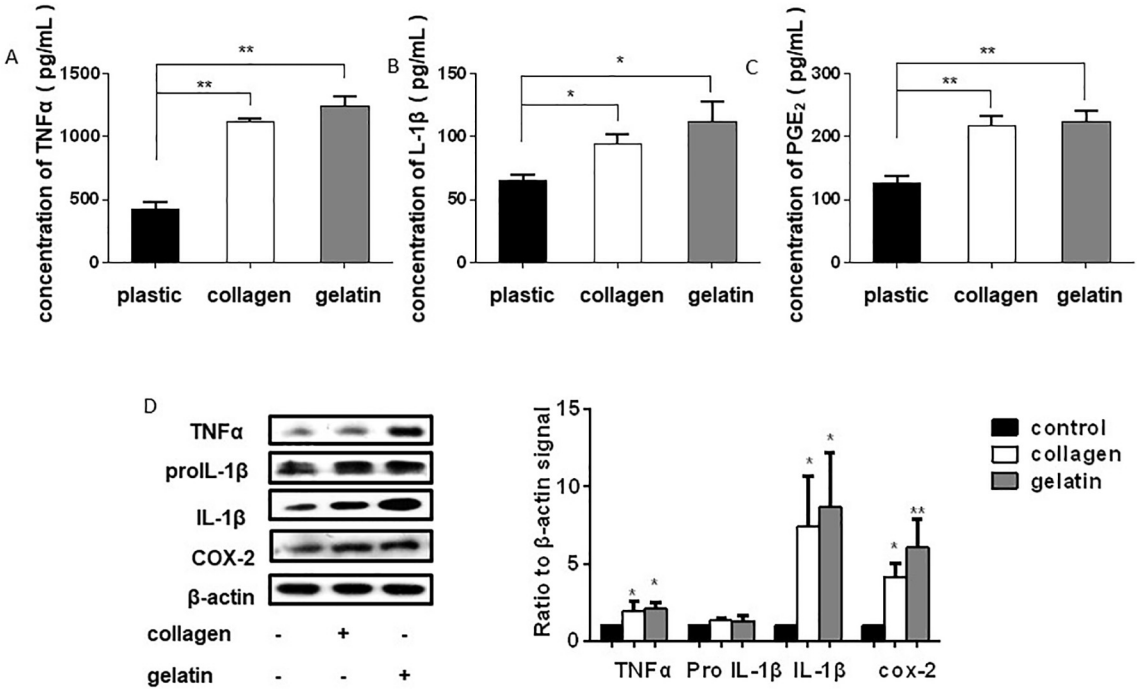

Fig. 2. MPMs cultured on col I- or gelatin-coated plates showed enhanced levels of pro-inflammatory molecules. (A, B, C) The levels of IL-1β, TNFα and PGE_2 in cell culture supernatant were determined by ELISA kits. All groups were compared with the plastic group, and *P* values were determined by one-way ANOVA with Dunnett's post-hoc test. (D) The protein levels of IL-1β, TNFα and COX-2 in the whole cell lysates were determined by western blot analysis. *, $P < 0.05$; **, $P < 0.01$; compared with the protein levels of cells in control group. Data are presented as means ± SD of three independent experiments. All groups were compared with control group. *P* values were determined by one-way ANOVA with Dunnett's post-hoc test.

coating did not induce cell aggregation, indicating the specificity of our study (Supplementary Fig. 1C). To study the cell viability, 5-fluorouracil (Fu) which interferes with the synthesis of cellular DNA was used as a negative control. Results showed that the cell survival ratios are not influenced by the culture on col. I- or gelatin-coated dishes (Fig. 1G).

3.2. Culture on col. I- or gelatin-coated dishes enhances the production of pro-inflammatory factors in MPMs

To investigate the effects of col. I and gelatin on expression of proinflammatory cytokines and immune regulation, the concentrations of IL-1β, TNFα and PGE_2 in the supernatants of cell culture were determined. Results show that compared with non-coated culture, all the levels increased when the macrophages were cultured on the col. I or gelatin-coated dishes (Fig. 2A–C). We further analyzed the protein expressions of IL-1β and TNFα as well as COX-2 which is responsible for the production of PGE_2 by western blotting analysis. The expression levels of these proteins are all up-regulated in the cells cultured in col. I or gelatin-coated plates (Fig. 2D).

3.3. Col I- or gelatin-coated culture increases the production of proinflammatory cytokines through NF-κB signaling pathway

Nuclear factor-kappa B (NF-κB) is a ubiquitous transcription factor that mediates pro-inflammatory responses [29]. The expression level of NF-κB increased in macrophages when cultured on col. I- or gelatin-coated dishes (Fig. 3A). Therefore, we treated the cells with BAY 11–7082 (BAY; an inhibitor of IκBα kinase) to block the NF-κB pathway (Fig. 3B). Treatment with BAY significantly down-regulated the production of IL-1β, TNFα and PGE_2 in the cells cultured on col. I-or gelatin-coated dishes, as shown by ELISA assay (Fig. 3C–E). Then we examined whether the cell aggregation was associated with NF-κB activity. The result was that BAY did not influence the formation of cell aggregates (Fig. 3F). These results suggest that NF-κB signaling pathway mediates the increased production of proinflammatory cytokines in col. I- or gelatin-coated culture, but not the cell aggregation.

3.4. Col I and gelatin both up-regulate ROS levels in MPMs

ROS play a vital role in the development of many inflammatory diseases and tumors [30,31]. Therefore, we examined ROS levels in MPMs. The effect of the culture on col. I- or gelatin-coated on the production of ROS was examined after DCFH-DA staining flowed by flow cytometric analysis. The results show that DCF-positive cell levels significantly increased in the col. I- or gelatin-coated culture (Fig. 4A). Hydrogen peroxide (H_2O_2) and superoxide (O^{2-}) are the main components of ROS. H_2O_2 can be scavenged by catalase (CAT) [32] and glutathione peroxidases (GPX) [33] and O^{2-} by superoxide dismutases (SOD) [34]. The activity of antioxidant enzyme CAT and the level of an anti-oxidant molecule, glutathione GSH [35], decreased when the MPMs were grown on col. I- or gelatin-coated dishes (Fig. 4B, C), while another antioxidant enzyme SOD showed no changes (Fig. 4D). So we think that H_2O_2 but not O^{2-} may stimulate the cell aggregation and the pro-inflammatory cytokine production. To verify this possibility, we added the culture medium with increasing concentration of hydrogen peroxide. Results show that under the stimulation with H_2O_2, cell aggregation (Fig. 4E) and the secretion of IL-1β (Fig. 4F) were increased, suggesting that hydrogen peroxide does affect the activity of macrophages, promoting their aggregation and the release of inflammatory cytokines.

3.5. ROS contribute to the cell aggregation and the production of pro-inflammatory molecules in MPMs on col. I- or gelatin-coated dishes

It is reported that ROS trigger pro-inflammatory processes [36]. We hypothesized that the up-regulation of the ROS levels might be associated with the increased secretion of pro-inflammatory cytokines. Then we treated the cells with NAC, a ROS scavenger (Fig. 5A). The sizes of

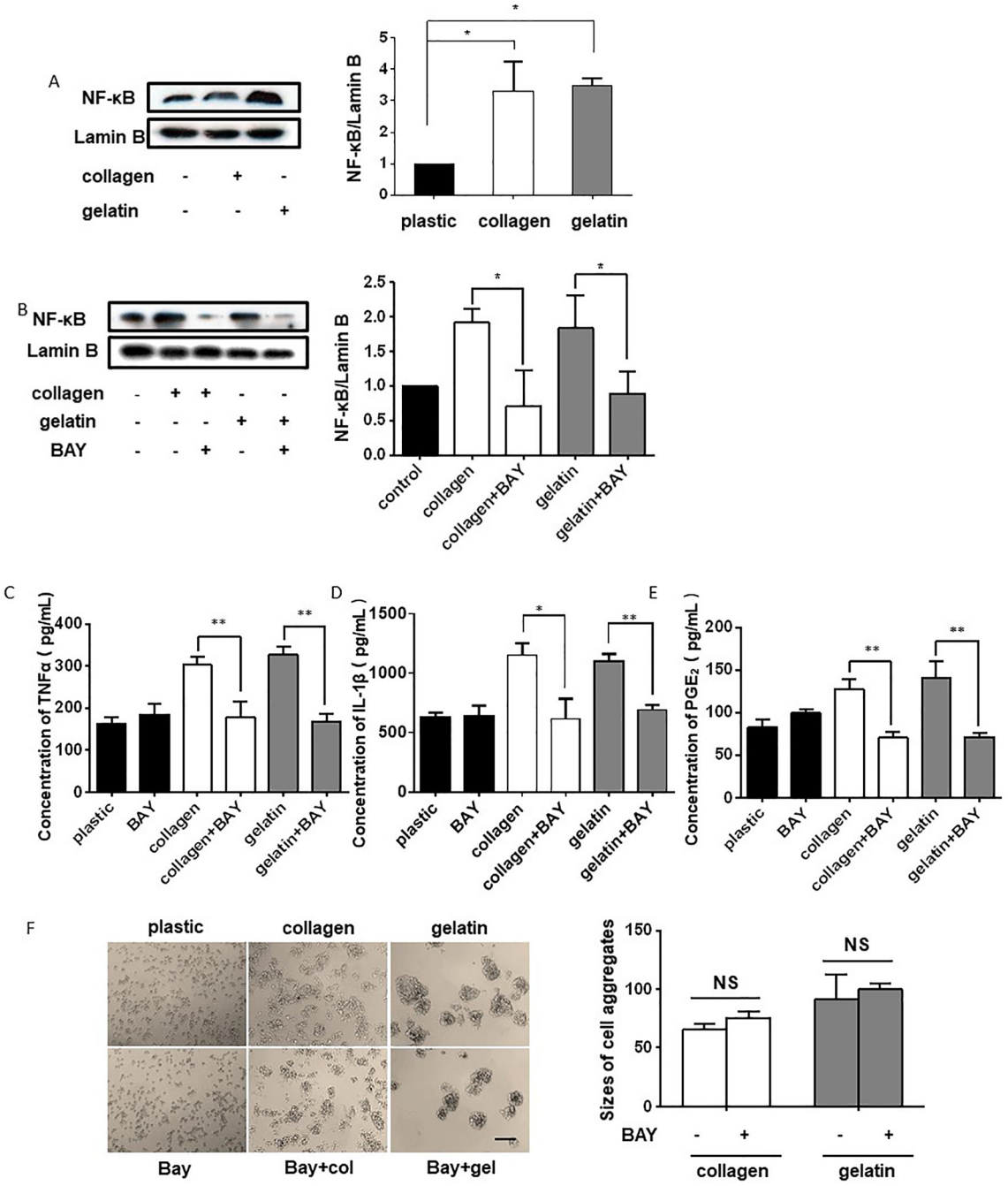

Fig. 3. NF-κB signaling pathway is involved in the increase in the production of pro-inflammatory cytokines by collagen I or gelatin-coated culture. (A, B) The protein levels of NF-κB were determined by western blot analysis. *P* values were determined by one-way ANOVA with Bonferroni's test. (C, D, E) The effects of BAY on the productions of IL-1β, TNFα and PGE2. (F) Sizes of cell aggregates in collagen or gelatin-coated plates with or without BAY. Data are presented as means ± SD of three independent experiments. NS, not significant, *, $P < 0.05$; **, $P < 0.01$. *P* values were determined by one-way ANOVA with Bonferroni's test.

cell aggregates were decreased (Fig. 5B). The increased releases of IL-1β, TNFα and PGE_2 in MPMs cultured on col. I- or gelatin-coated dishes were all reversed by NAC treatment (Fig. 5C–E). Western blot analysis demonstrated that increase in the protein expressions of IL-1β, TNFα and COX-2 on col. I- or gelatin-coated dishes was reversed by NAC treatment (Fig. 5F). The results with increasing concentrations of NAC show increasing repressive activities as shown in the Supplementary Fig. 3. Furthermore, the expression of NF-κB protein was also down-regulated by NAC treatment (Fig. 5G). These results suggest that col. I- or gelatin-coating increases cell aggregation and NF-κB-mediated pro-inflammatory cytokine production through up-regulating ROS levels. However, the sizes of cell aggregates formed on col. I- or gelatin-coated dishes are not associated with NF-κB expression.

4. Discussion

Col I stimulates sclerosing patients to release IL-6 and IL-2 [37]. Collagen fragments (polypeptides) enhance the production of IL-1β by human peripheral blood mononuclear cells [38]. In skin burns, collagen denatures into gelatin, which in turn exacerbates the immune response [39,40]. It was reported that denatured collagen produced in skin burn causes immune responses by the release of pro-inflammatory cytokines [41], showing that under pathological conditions, release of inflammatory factors and change in collagen higher order structure forms

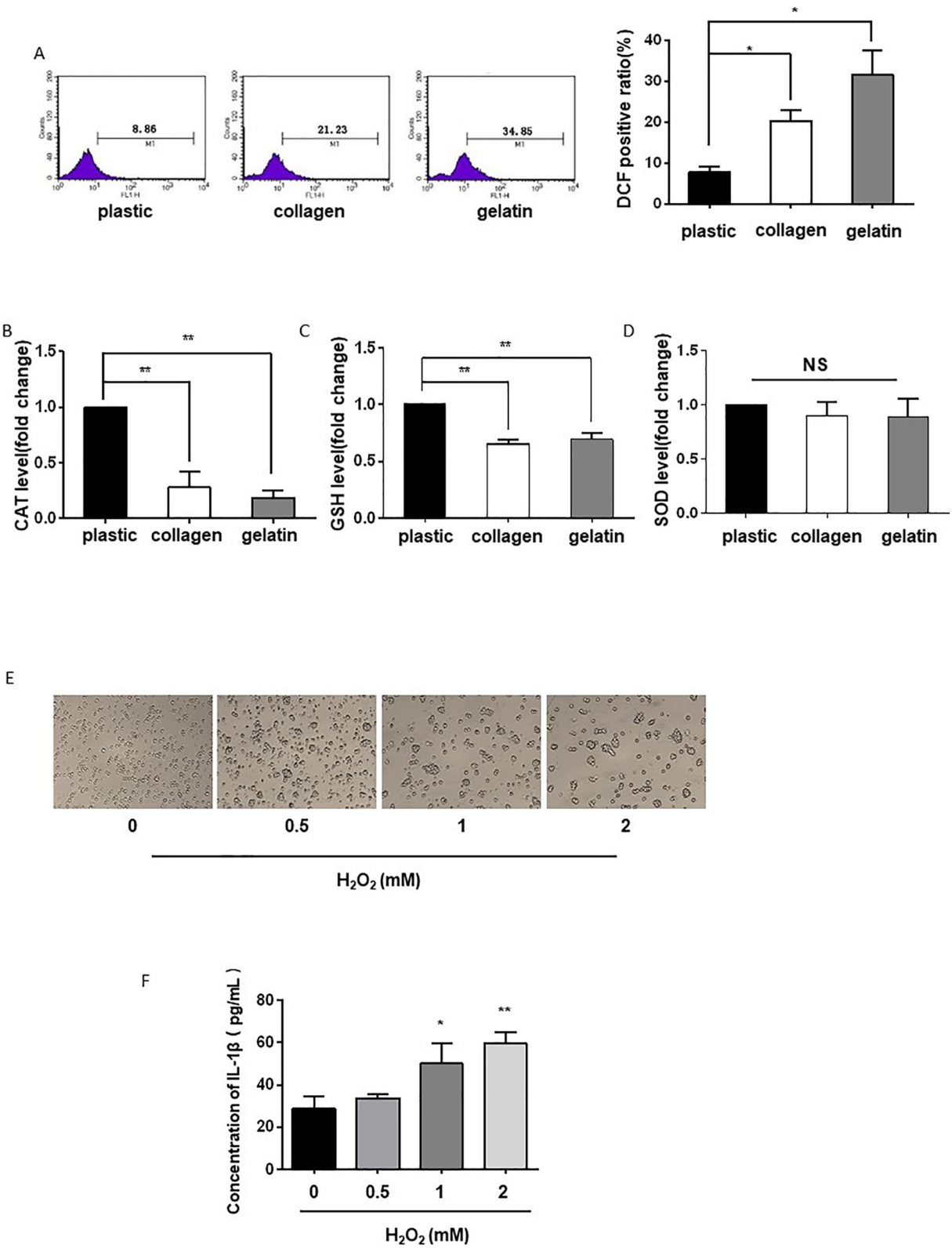

Fig. 4. Col I and gelatin both increase ROS levels of MPMs. (A) ROS levels in the cells were determined by flow cytometry after staining with DCFH-DA. *P* values were determined by one-way ANOVA with Dunnett's post-hoc test. (B-D) The activities of SOD and CAT, and the concentration of GSH were determined by diagnosing kits. **, $P < 0.01$; ***, $P < 0.001$. All groups were compared with the plastic group, and the *P* values were determined by one-way ANOVA with Dunnett's post-hoc test. (E) Effects of different concentrations of hydrogen peroxide on cell aggregation. *, $P < 0.05$; **, $P < 0.01$. (F) The effects of different concentrations of hydrogen peroxide on IL-1β production. All groups were compared with the group without hydrogen peroxide, and the *P* values were determined by one-way ANOVA with Dunnett's post-hoc test. *, $P < 0.05$; **, $P < 0.01$. Data in A, B, C, D and F are presented as means ± SD of three independent experiments.

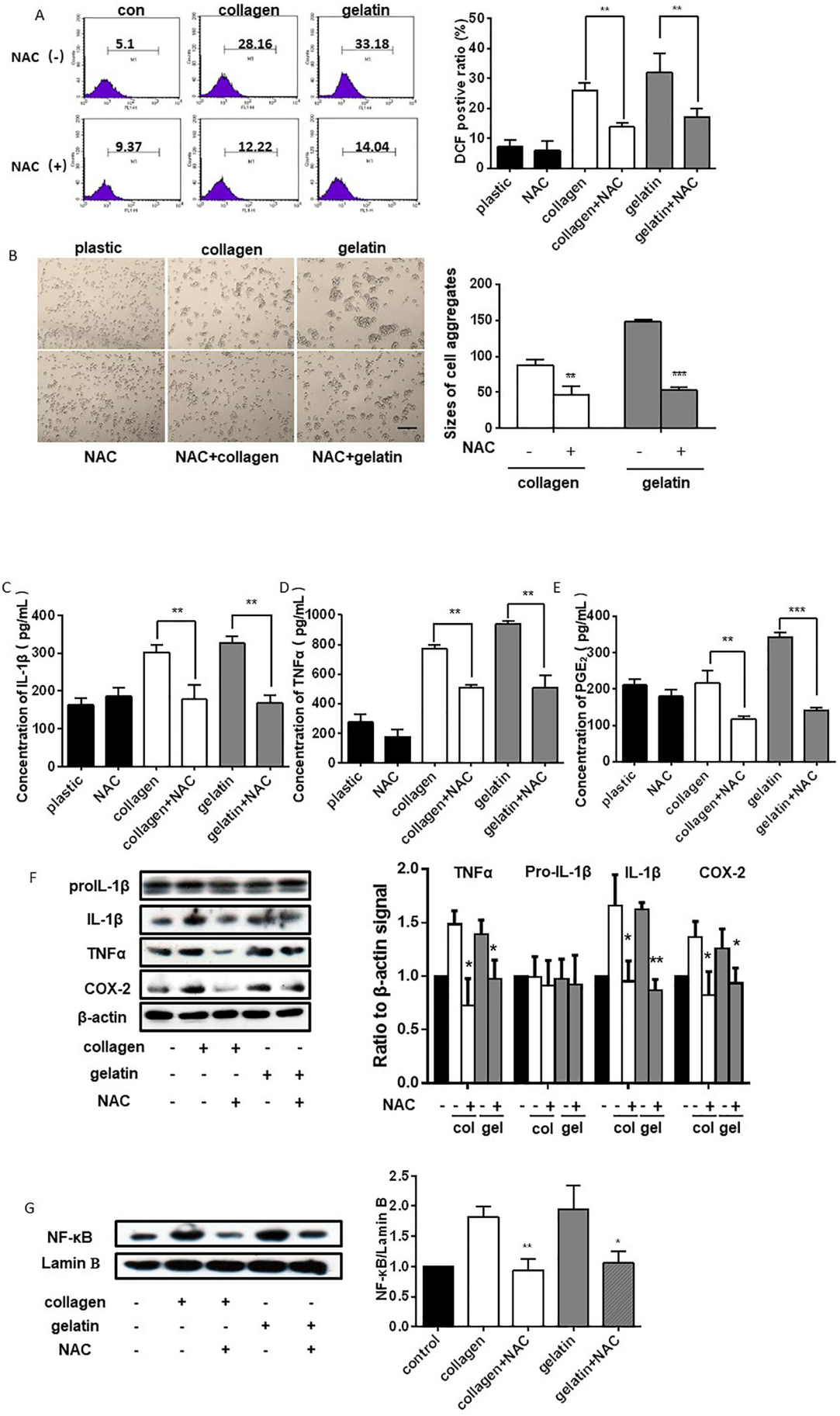

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Fig. 5. ROS are responsible for the production of pro-inflammatory cytokines and cell aggregation in MPMs cultured on collagen I or gelatin-coated culture. (A) The DCF-positive ratios were determined by flow cytometric analysis. (B) Effect of a ROS scavenger, NAC (2 mM), on cell aggregation. The levels of IL-1β (C), TNFα (D) and PGE2 (E) were determined by using ELISA kits. (F, G) The expression levels of IL-1β, TNFα, COX-2 and NF-κB proteins were determined by western blotting. Data are presented as means ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$. P values were determined by one-way ANOVA with Bonferroni's test.

a progressive spiral. The purpose of our experiments was to establish an *in vitro* model to mimic the *in vivo* microenvironment, and to examine the effects of the changes of microenvironment on the immune system. However, there is no uniform standard for the secretion of pro-inflammatory cytokines under physiological or pathological conditions. Under the lipopolysaccharide-stimulation, the amounts of cytokines secreted in cancer survivors and normal humans can reach > 10,000 pg/ml [42]. Pneumolysin synergizes with toll-like receptor agonists stimulate the secretion of proinflammatory cytokines from dendritic cells to several orders of magnitude [43]. In experimental Bacillus endophthalmitis, the amount of IL-1β and TNFα secreted in the eyes of mice can reach several hundred pg/ml [44]. Absolute amount of proinflammatory cytokines secreted under physiological or pathological conditions depends on spacio-temporally. In our experiments, production of proinflammatory cytokines was significantly elevated under the conditions of col. I and gelatin coating, reflecting pathological conditions.

Immune cells play an important role in inflammatory process by producing cytokines and pro-inflammatory mediators such as nitric oxide (NO) and prostaglandins (PGs) [45]. In the injured myocardium, collagen accumulated induces the majority of macrophages to produce pro-inflammatory substances including IL-1β, MMP-9, and NO [46]. Macrophages can be polarized to exhibit different phenotypes by the cytokine production, and accordingly two macrophage subsets (M1 and M2) have been identified. M1 macrophages up-regulate CD86 protein levels, and produce pro-inflammatory cytokines, such as IL-1β and TNFα. M2 macrophages up-regulate the CD206 and generate anti-inflammatory cytokines like IL-10 [47,48]. In the peritoneal macrophage, CD86 and CD206 both exist [49,50]. In addition, macrophages elicited after intra-peritoneal injection of LPS show an inflammatory/M1 phenotype [51], whereas injection of thioglycolate may contain larger amount of M2 macrophages, increased with IL-4 released by other blood cells, that can be directly recovered from the peritoneal cavity [52]. So we think peritoneal macrophages are at transition stages to different polarization types upon different stimulation patterns. In our study, the peritoneal macrophages with the stimulation of thioglycolate secreted a significant levels of pro-inflammatory cytokines without col. I and gelatin coating (data not shown). We thus speculate that MPMs might be a mixture of M1 and M2 types and inclined to the M1 characteristics.

Our previous studies on the differentiated human mononuclear lymphoma cells U937 stimulated with phorbol ester (PMA) show that both col. I and gelatin induce the form of multicellular aggregates, and gelatin promotes the production and release of IL-1β, TNFα and PGE_2, but with col. I coating, production of these pro-inflammatory molecules is down-regulated [17,53]. However, in the present study with MPMs, the production and release of pro-inflammatory molecules in the cells cultured on col. I-coated dishes increase similarly in the cells cultured on gelatin-coated dishes. One explanation for the differences is that U937 is a tumor cell with a certain difference from normal peritoneal macrophages. Another difference is noted, that is, differentiation of human lymphoma cells, U937, is induced by PMA stimulation, which might affect protein kinase C (PKC) of U937 cell [54], while normal peritoneal macrophages were not subjected to the stimulation with PMA. To test the possible role of PMA, we also treated MPMs with PMA. PMA treatment neither affects multicellular aggregation nor the secretion pattern of IL-1β (Supplementary Fig. 2). This shows that these differences between U937 and MPMs are not caused by the treatment with PMA.

NF-κB, a nuclear transcription factor in eukaryotes, serves a critical regulatory function in the expression of a number of genes, particularly inflammatory or immunoreaction-related genes [55]. Wang et al. reported that L929 cells on col. I gel showed higher expression of NF-κB in the nucleus [56], suggesting that NF-κB was activated. Consistent with this, we found that both col. I and gelatin promote the NF-κB translocations into the nucleus of MPMs. When MPMS were treated with Bay, an inhibitor of IκB, NF-κB translocation to the nucleus decreases and pro-inflammatory cytokine production decreases, indicating that the macrophage stimulation with col. I or gelatin in production of pro-inflammatory cytokines involved in inflammatory responses is mediated through nuclear translocation of NF-κB.

ROS activate some signal transduction pathways, such as NF-κB, leading to expression of pro-inflammatory mediators [57,58]. Inflammatory cells are recruited to the injured site to produce a large amount of ROS, and secrete pro-inflammatory cytokines and PGE_2 [59]. Either col. I or gelatin induces both production of proinflammatory cytokines and cell aggregation in MPMs. When treated with NAC, both the increased production of proinflammatory cytokines and cell aggregation are repressed, suggesting that ROS are responsible for col. I- and gelatin-induced aggregation of MPMs and augmented production of pro-inflammatory cytokines by MPMs. The antioxidant enzymes are also important for the balance of oxidative conditions. Reduction of CAT and GSH activities but not SOD activity with col. I or gelatin is detected. H_2O_2 is scavenged by CAT [32] and glutathione peroxidase [33], where as O_2^- is scavenged by superoxide SOD [34]. Therefore, H_2O_2 but not O_2^- may stimulate the cell aggregation and the pro-inflammatory cytokine production. The finding that increasing concentrations of hydrogen peroxide shows similar effect, if not identical, to the effect of col. I or gelatin culture, indicating that H_2O_2 but not O_2^- is involved in further activation of MPMs.

It was reported that the collagen-stimulated generation of ROS regulates signal transduction in platelets [60]. The collagen-induced platelet aggregation has been shown to be closely associated with H_2O_2 production [61]. Few reports are found about gelatin's effect on H_2O_2 production. Our conclusion proves that both col. I and gelatin induce the production of ROS and the increased levels of pro-inflammatory cytokines. These suggest that gelatin as well as col. I aggravates inflammation through upregulation of ROS, H_2O_2 in particular.

Fibrosis is defined by the overgrowth, hardening, and/or scarring of various tissues. It is often attributed to excess deposition of collagen fibrils. If the present finding that col. I- or gelatin-coating stimulates MPMs to form cell aggregates and induce the production proinflammatory cytokines in association with ROS up-regulation *in vitro* is extended to *in vivo*, or fibrotic regions, collagen and/or gelatin spaciotemporarily produced during the metabolic process of fibrosis may well aggravate inflammatory conditions through macrophage aggregation and production of inflammatory mediators, accompanied by activation of myofibroblasts and progression of fibrosis. Our research may be partially answered for the formation and development of fibrosis, providing a theoretical basis for its research and treatment of fibrosis.

In summary, this study demonstrates that col. I and/or gelatin stimulates MPMs to form cell aggregates and induce proinflammatory cytokine production in association with ROS up-regulation.

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