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Type I collagen gel protects murine fibrosarcoma L929 cells from TNFα-induced cell death

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ABSTRACT

Murine fibrosarcoma L929 cells have been used to test efficacy of proinflammatory cytokine TNFα. In the present study, we reported on protective effect of type I collagen gel used as L929 cell culture. L929 cell grew and proliferated well on collagen gel. However, the L929 cells exhibited cobblestone-like morphology which was much different from the spread fusiform shape when cultured on conventional cell dishes as well as the cells tended to aggregate. On conventional cell culture dishes, the cells treated with TNFα became round in shape and eventually died in a necroptotic manner. The cells cultured on collagen gel, however, were completely unaffected. TNFα treatment was reported to induce autophagy in L929 cells on the plastic dish, and therefore we investigated the effect of collagen gel on induction of autophagy. The results indicated that autophagy induced by TNFα treatment was much reduced when the cells were cultured on collagen gel. In conclusion, type I collagen gel protected L929 cell from TNFα-induced cell death.

1. Introduction

Type I collagen is the most abundant component of extracellular matrix (ECM), and it is also known as a good adhesive substrate for many kinds of cells including keratinocyte [1]. As its predominance in the ECM, non-immunogenicity and available methods of isolation from a variety of sources, collagen has been used in a variety of tissue engineering applications. Type I collagen *in vivo* forms fibrils that make up a three-dimensional structure. Isolated collagen molecules reassemble into fibrils under physiological conditions of pH, temperature, ionic strength etc [2,3]. Previous studies reported that fibroblasts showed differences in growth rates, mitogenic responses to growth factors and collagen synthesis by culturing different microenvironments composed of collagen fibrils, collagen molecules or without collagen [4,5]. Proliferation of fibroblasts and responses to growth factors including epidermal growth factor, basic fibroblast growth factor and transforming growth factor-β were dramatically reduced in gel culture, particularly after collagen gel contraction by the cells [6]. Not only fibroblasts but also other types of cells behaved differently by culturing with collagen. Thus, *in vitro* cell culture studies demonstrated that the effects of fibrous collagen on cell growth and differentiation were totally different from those of molecular collagen [3,4]. Madin Darby canine kidney (MDCK) cells performed membrane remodeling and gradually formed lumen in the presence of collagen gel [7] and human intestinal (Caco-2) cells died when cultured on collagen gel [5]. In addition, 3-dimensional collagen type I matrix significantly reduced the anti-migratory effect of doxorubicin on human fibrosarcoma HT1080 cells [8]. All these findings suggest the importance of the matrix supramolecular assembly in the effects on cellular properties and functions.

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Tumor necrosis factor α (TNFα), a pleiotropic cytokine, produces a wide range of biological effects, including production of inflammatory cytokines as well as direct effects on cell proliferation, differentiation and death [9]. Murine fibrosarcoma L929 cells have been used to quantify the cytotoxicity of TNFα, since the cells are reported to be extremely sensitive to TNFα [10–13]. Our previous study demonstrated that TNFα treatment resulted in the necroptosis and autophagy of L929 cells [14]. However, the effects of type I collagen on the cytotoxicity of TNFα in L929 cells are not well elucidated.

In this study we investigated the effect of type I collagen gel on TNFα-induced death and autophagy in L929 cells. We found that type I collagen gel exhibited protective effect on TNFα-induced cell death. As TNFα affected most organs by regulating cell proliferation and cell death, resistance to TNFα-induced cell death may also contribute significantly to tumor formation.

2. Materials and methods

2.1. Cells and cell culture

The murine fibrosarcoma L929 (# CRL-2418) cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in RPMI-1640 medium (Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Beijing Yuanheng Shenyang Research Institution of Biotechnology, Beijing, China), 100 μg/ml streptomycin, 100 U/ml penicillin and 0.03% L-glutamine, and maintained at 37 °C with 5% CO_2 at a humidified atmosphere. Experiments were performed when the cells had reached about 80% of confluence.

2.2. Cell culture on collagen gel

Type I collagen was extracted from calf hide with 50 mM acetic acid, and was purified by salt fractionations under acidic and neutral conditions. Prior to cell culture, 1.0 mg/ml collagen diluted with PBS (−) solution free of Ca^{2+} was added in the 6-well dishes (Corning, NY, USA) and kept in a humidified incubator at 37 °C for 2 h to allow the collagen molecules to assemble into fibrils.

2.3. Reagents

Human recombinated TNFα was prepared from PMAL-C2-TNF/JM109 (*E. coli.*) in our laboratory. Propidium iodide (PI), acridine orange (AO), ethidium bromide (EB), monodansylcadaverine (MDC), necrostatin-1 (Nec-1) and methylthiazolyldiphenyl-tetrazoliumbromide (MTT) were purchased from Sigma Chemical (St. Louis, MO, USA). Rabbit monoclonal anti-LC3 antibody was obtained from Sigma Chemical (St. Louis, MO, USA). Mouse monoclonal anti-β-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). RIPK1 specific polyclonal antibody was purchased from ProteinTech (Wuhan, China). The ATP Assay Kit was from Beyotime (Shanghai, China).

2.4. Cell viability assay

L929 cells (5.0×10^3 cells/well) were cultured in 96-well tissue culture plates with or without collagen fibrils. Cell viability was examined by using the MTT assay. After incubation for 24 h, the cells were treated with increasing doses of TNFα for the indicated time periods. Thereafter, the cells were rinsed twice with PBS and incubated with 100 μl of 0.5 mg/ml MTT solution at 37 °C for 3 h. After removing the supernatant, the formazan crystals formed by MTT were dissolved in 150 μl of DMSO. Absorbance at 492 nm wavelength was measured using a microplate reader (Thermo Scientific Multiskan MK3, Shanghai, China). The cell viability was calculated as follows:

$$\text{Cell viability}\,(\%) = 100-(A_{492,\,\text{control}}-A_{492,\,\text{sample}})/(A_{492,\,\text{control}}-A_{492,\,\text{blank}}) \times 100.$$

2.5. Western blot analysis

L929 cells were plated in 6-well cell-culture plates with or without collagen fibrils for 24 h, and then treated with TNFα for indicated time period. Both adherent and floating L929 cells were collected, washed twice with PBS, and the pellet was lysed in whole cell RIPA lysis buffer (Beyotime, Haimen, Jiangsu, China) supplemented with PMSF (1 mM). After 15,000 × g centrifugation at 4 °C for 15 min, the protein concentration was determined using the Bio-Rad protein assay reagents (Bio-Rad, Hercules, CA, USA). After denaturation with boiling water for 5 min, lysates containing the same account of protein were separated by 12% SDS-PAGE and transferred onto Millipore Immobilon®-P Transfer Membrane (Millipore, Billerica, MA, USA). After blockage with 5% skim milk, the membranes were incubated with primary polyclonal antibodies overnight, followed by corresponding HRP-conjugated secondary antibodies. The blots were visualized using SuperSignal® West Pico Chemiluminescent Substrate (Thermo scientific, Rockford, IL, USA).

2.6. Flow cytometric analysis of membrane integrity

Cell membrane integrity was determined by flow cytometry after being stained with PI. After the treatments as indicated, the cells were collected, then cell pellets were stained with the fluorescent probe solution containing 50 μg/ml PI PBS at 37 °C for 15 min. The PI-stained cells were evaluated with the FACScan flow cytometer.

2.7. Flow cytometric analysis of autophagy

The treated cells were incubated with 0.05 mM monodansylcadaverine (MDC), a marker for autophagic vacuoles, at 37 °C for 1 h [15]. Then, the cells were collected, and the pellets were suspended in 1 ml of PBS. The fluorescent intensity of cells was analyzed by the FACScan flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA) [16].

2.8. AO/EB staining of the nuclear morphology

L929 cells were seeded into 6-well culture plates with collagen-fibrils and incubated with TNFα for indicated periods. The cellular morphology was observed under a phase contrast microscope (Leica, Nussloch, Germany) or a fluorescent microscope (Olympus, Tokyo, Japan) after staining with the fluorescent DNA-binding dye AO/EB. After treatment with indicated reagents, the cells were stained with 20 μg/ml AO/EB (Sigma Chemical, St. Louis, MO, USA) for 15 min. Then, the nuclear morphology was observed under a fluorescent microscope.

2.9. Statistical analysis

All the presented data and results were confirmed in at least three independent experiments. The data were expressed as means ± SD and analyzed by one-way ANOVA using Statistics Package for Social Science (SPSS) software (version 13.0; SPSS, Chicago, IL, USA), followed by LSD post-hoc test. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. L929 cells grown on collagen gel exhibited different morphology but proliferated similarly as those on conventional cell culture dishes

After 24 cell culture on collagen gel, we found that the cells formed a cobblestone-like morphology. In addition, several cells showed aggregation to form colonies, while cells cultured on conventional culture dishes spread to form the fibroblast-like shape and grew separately (Fig. 1A). Then we estimated the cell proliferation rate by determination of relative cell numbers at 24, 48, and 72 h. The cells grown on collagen gel appeared to be smaller in number at all the time points than those grown on conventional culture dishes (Fig. 1B). The cells were delayed in initiating proliferating after seeding on the type I collagen gel in general [17]. Thus, the growth rate counted as the differences in cell numbers after they had started proliferation is the same as found in this study. This was consistent with our findings that the proliferation rate on collagen gel was paralleled to that on conventional dishes.

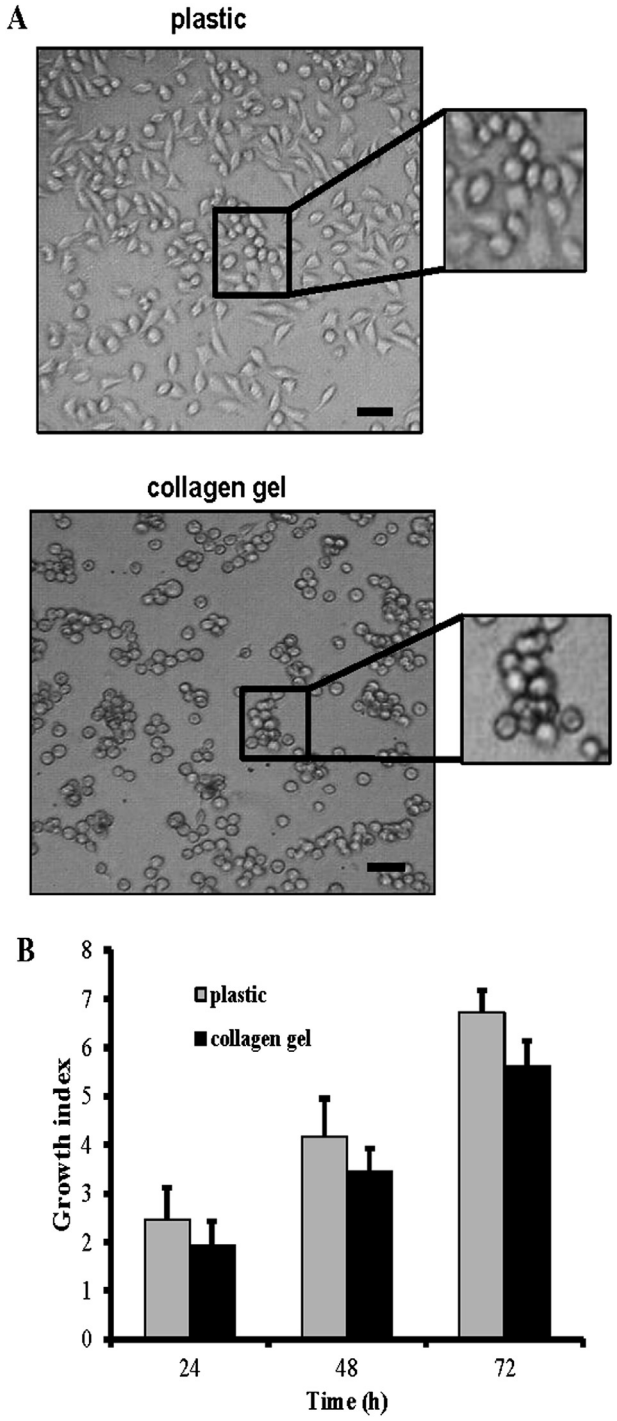

Fig. 1. Morphological changes of L929 cells cultured with or without collagen gel. (A) L929 cells were cultured on collagen gel for 48 h, the morphologic changes were observed by a phase contrast microscopy. Scale bar = 50 μm. (B) Cells were cultured on collagen gel for 24, 48 and 72 h, and the proliferation ratio was determined by MTT assay.

3.2. Cells grown on collagen gel showed resistance towards TNFα-induced bursting morphology and death

L929 cells were sensitive to TNFα treatment and usually used as the model standard cells in TNFα activity assay [13]. Consistently, L929 cells cultured on conventional culture dishes became smaller and bursted, exhibiting markedly injured morphology; however, the cells on collagen gel did not show any impaired morphological changes (Fig. 2A). We further examined the viability of L929 cells by using AO/EB staining. AO is cell membrane permeable and stains cell nucleus green while EB only can permeate only the bursted membrane and stains nucleus red. After treatment with TNFα, the ratio of membrane bursted cells on collagen gel was much lower than that on dishes (Fig. 2B). The amount of formazan crystals generated by MTT is in proportion to the number of living cells. After treatment with TNFα for 24 and 48 h, the viability of the cells cultured on conventional dishes was markedly reduced when compared with the cells cultured on collagen gel (Fig. 2C).

3.3. Collagen gel inhibited TNFα-induced necroptosis in L929 cells

Our previous study showed that L929 cells treated with TNFα underwent the necroptotic cell death that could be totally rescued by necrostatin-1 (nec-1), an inhibitor of necroptosis [14]. In this study, MTT assay showed that nec-1 could reverse growth inhibition induced by TNFα, but cells cultured on collagen gel were not affected (Fig. 3A), indicating that cells grown on collagen gel exhibited resistance towards TNFα-associated necroptosis. Cell membrane integrity was profoundly reduced on conventional dishes when treated with TNFα, but cells cultured on collagen gel were not affected (Fig. 3B). Receptor-interacting serine/threonine-protein kinase 1 (RIPK1) played an important role in necroptotic cell death [18]. For conventional cell culture, RIPK1 expression was increased with TNFα treatment, while it was unchanged if not reduce in the cells cultured on collagen gel (Fig. 3C). Intracellular ATP markedly declined during TNF-induced necroptosis [19]. In our study, intracellular ATP level of cells cultured on collagen gel was elevated compared with these cultured on conventional dishes. TNFα treatment induced loss of ATP in L929 cell cultured on conventional dishes. However, the ATP level of cells cultured on collagen gel was restored to the control level when treated with TNFα (Fig. 3D).

As a semi-solid matrix, its defective effects on TNFα-induced cell death might result only physically from the trap of TNFα by the gel which decreased the concentration of TNFα. To exclude this presumption, we investigated whether the collagen gel could adsorb TNFα and thereby decreasing the concentration of TNFα. TNFα was pre-incubated on collagen gel or plastic surface for 24 h, and then added to cells to measure the cytotoxicity. As shown in Fig. 3E, the cell viability between the plastic group and collagen gel group had no difference. Taken together, collagen gel protected L929 cells from TNFα-induced necroptosis.

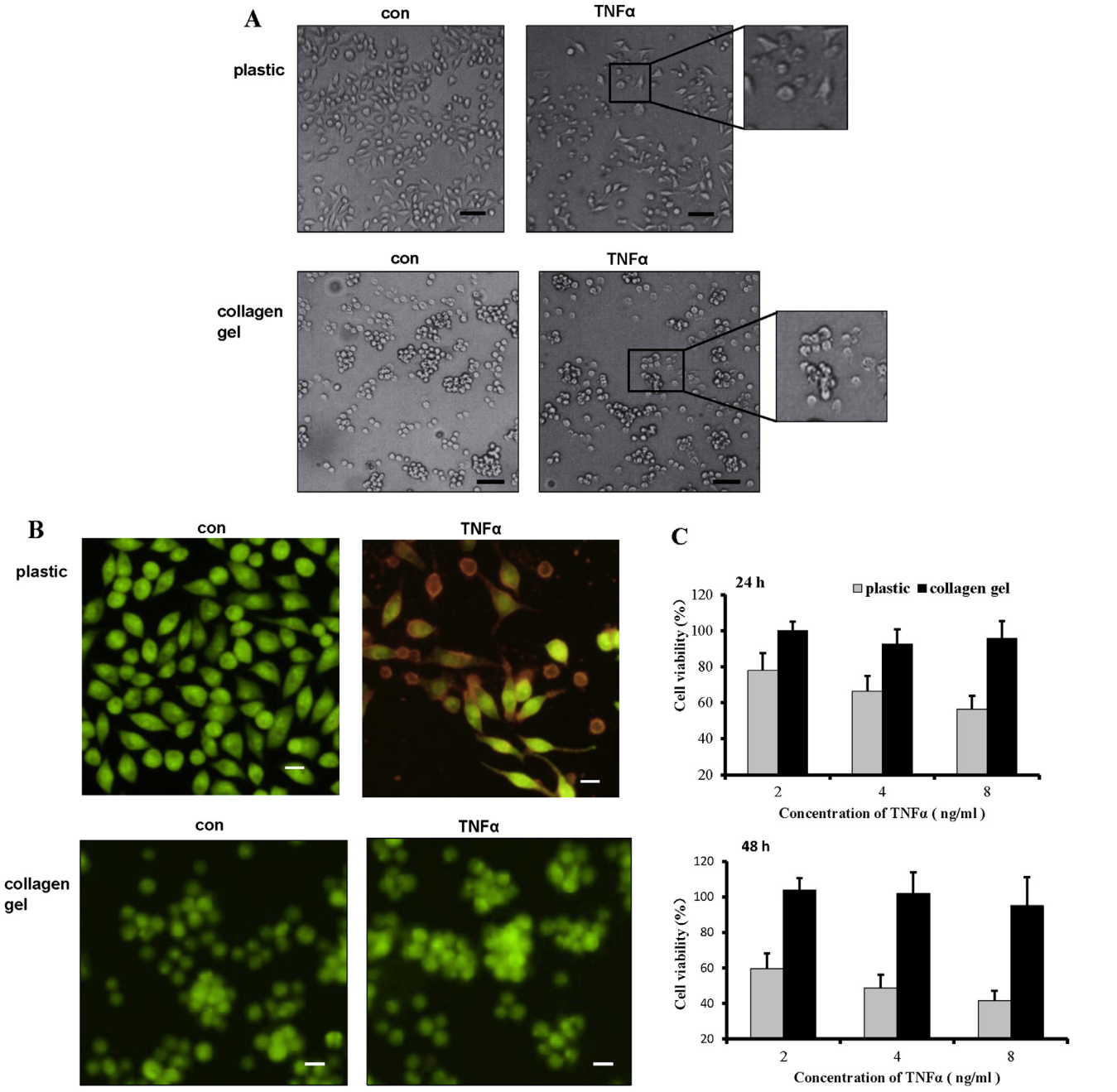

Fig. 2. Morphological changes of cells on collagen gel or conventional cell culture dishes after treatment with TNFα. (A) L929 cells were cultured on collagen gel for 24 h, then treated with 4 ng/ml TNFα for 24 h. The morphologic changes were examined by the phase contrast microscopy. Scale bar = 50 μm (B) AO/EB staining was performed and observed by a fluorescence microcopy. Cell nuclei were stained by AO, and necrotic cellular nuclear were stained by EB. Green: AO staining, red: EB staining. Scale bar = 25 μm (C) L929 cells were cultured on collagen gel for 24 h, then treated with different concentration of TNFα for 24, 48 h, cell viability was measured by MTT assay. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Autophagy induced by TNFα treatment was also inhibited when cells were cultured on collagen gel

In our previous study we proved that TNFα induced autophagy in L929 cells [14]. MDC has been used as the indicator of autophagic flux. In contrast to the marked green punctates found in TNFα-treated L929 cells on conventional cell culture dishes, there were less autophagic punctates in the cells on collagen gel (Fig. 4A). This was confirmed by flow cytometric analysis of the MDC positive cell ratio (Fig. 4B). In consistent with the previous findings [14], the conversion from LC3 I to LC3 II was increased in TNFα-treated L929 cells on conventional plastic cell culture dishes. However, this conversion was less enhanced when the cells were cultured on collagen gel (Fig. 4C).

4. Discussion

The tumor cells cultured on conventional cell culture dishes may not reflect the tumor cell microenvironment *in vivo* where tumor cells interact with their neighboring cells and ECM. The ECM is a major constituent of tumor tissues as well as normal tissues. It is shown that ECM plays a central role in cell development, migration, adhesion, proliferation, survival or other metabolic functions [20]. It was reported that levels of type IV collagen were elevated in most

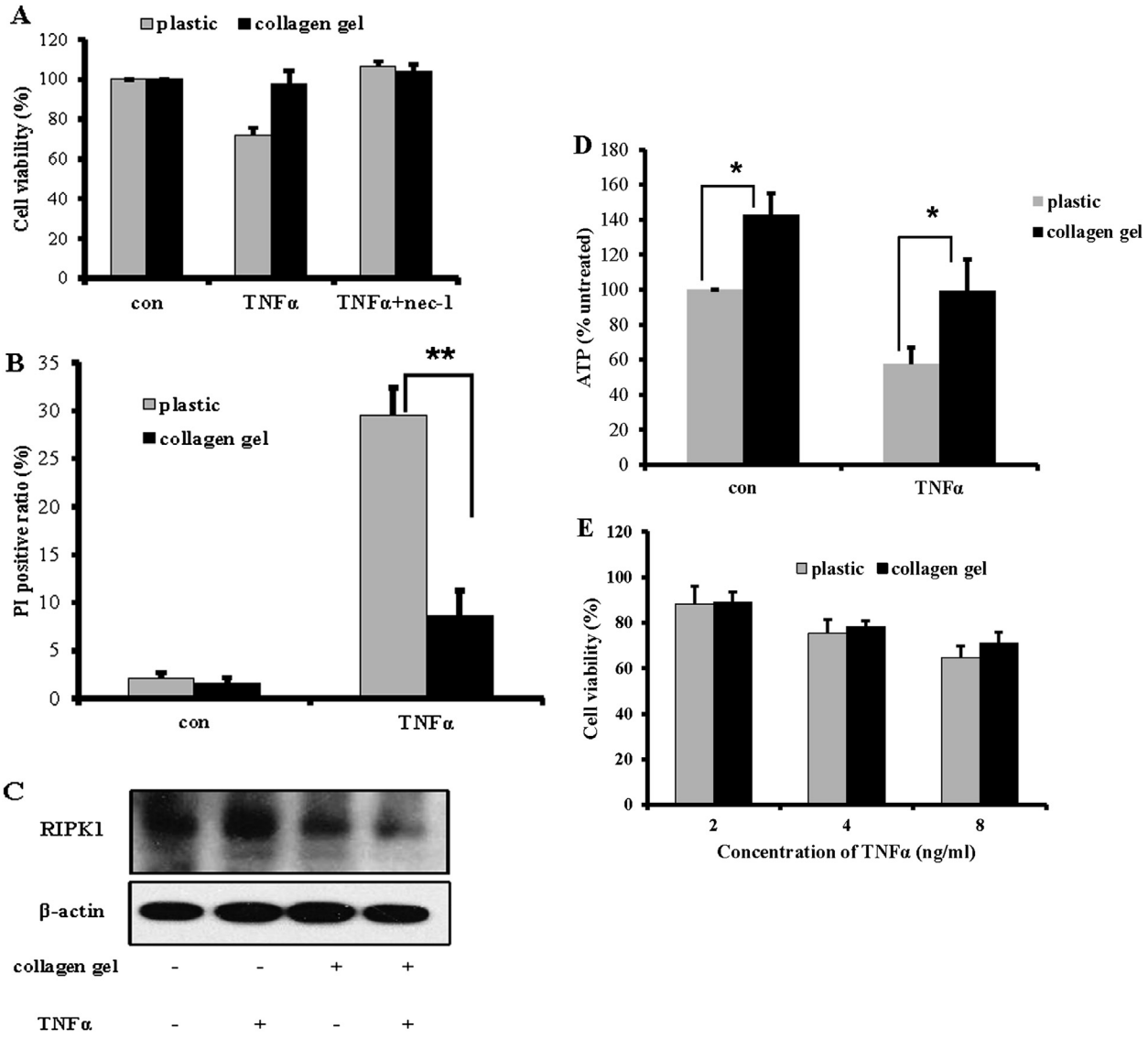

Fig. 3. TNFα did not induce cytotoxicity when L929 cells were cultured on collagen gel. (A) L929 cells were treated with 4 ng/ml TNFα in the presence or absence of 2 μg/ml nec-1for 24 h and cell viability was measured by MTT assay. (B) Cell membrane integrity was measured by PI staining and flow cytometry. (C) Western blot analysis of RIPK1 level. β-actin was used as an equal loading control. (D) Intracellular ATP content was analyzed by ATP luminescent assay. (E) TNFα was pre-incubated on collage gel or conventional dishes without seeding cells for 24 h, then the medium was collected and added it to L929 cells. MTT assay analyzed the cell viability. Data were expressed as mean ± S.D. $^{*}p < 0.05$, $^{**}p < 0.01$.

sections of small cell lung cancer (SCLC). Moreover, it was found to protect SCLC cells from various chemotherapeutic drug-induced apoptosis [21].

TNFα, a proinflammatory cytokine, stimulates the degradation of ECM by enhancing the expression of matrix metalloproteinases [22]. Our previous study indicates that TNFα induces L929 cell necroptosis and autophagy, but not apoptosis, in conventional cell culture [14]. Autophagy, a downstream consequence of necroptosis and had a negative feedback function to necroptosis through activated caspase-6 [23]. The present finding that TNFα did not exert obvious cytotoxic effects on the L929 cells cultured on collagen gel might be related to the morphological change in L929 cells growing on collagen gel with cobblestone-like shape and cell aggregation. By contrast, collagen gel culture did not affect L929 viability and proliferation. On The report that morphological changes of HeLa cells are associated with the reorganization of DNA loop structure domains [24] implies a possibility of that TNFα-stimulation signaling in the L929 cells cultured on collagen gel including the DNA loop structure is altered as well as cell membrane or submembrane arrangement, since the cells cultured on the collagen gel in general take a long time to initiate proliferation even under a condition with rich soluble supplements. The interaction between cell and ECM played an important role in cells' response to TNFα treatment, although detailed mechanism remains to be elucidated. Some intriguing reports include 1) Epithelial and leukemic cell lines cultured on collagen matrices responded differently to taxol treatment [25], and 2) Human fibrosarcoma cells grew on type I collagen gel were also resistant to the anti-migratory effect of doxorubicin [26]. These were consistent with our findings.

Proteins related to cell–matrix interaction, such as integrin [27], focal adhesion kinase (FAK)/phosphatidylinositol 3-kinase (PI3K)/threonine kinase (AKT) pathway [28], ras guanyl nucleotide releasing proteins (RasGRPs) [29] and transforming growth factor type I (TGF) [30], had been reported to function differently in regulating cell proliferation and the responding to drug treatments in cells cultured on different matrix. Whether one or more of these

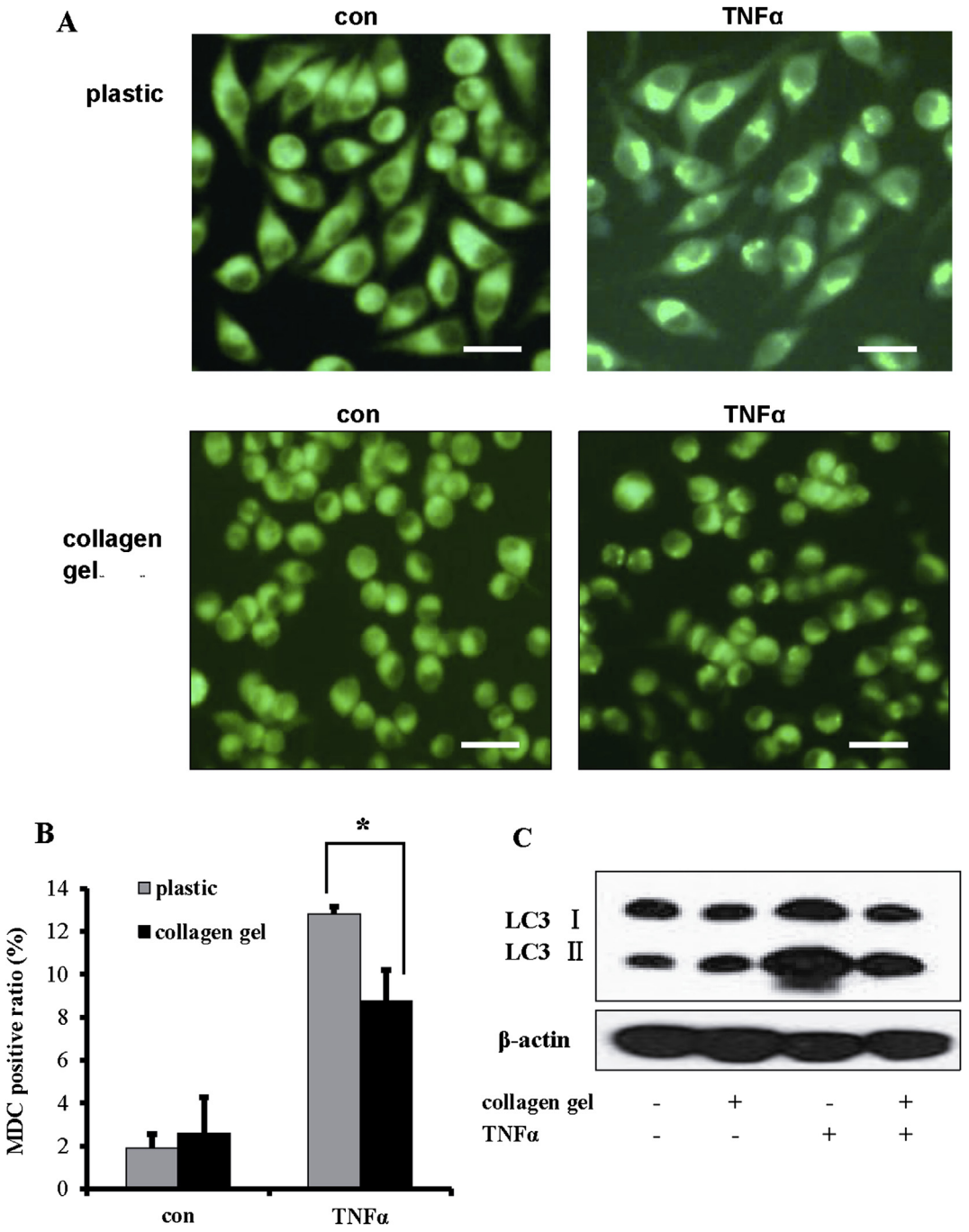

Fig. 4. Autophagy induced by TNFα treatment was markedly inhibited when the cells were cultured on collagen gel. (A) Cells cultured on collagen gel were treated with 4 ng/ml TNFα for 24 h, and then observed under a fluorescent microscope after MDC staining. Scale bar = 25 μm. (B) Flow cytometric analysis for MDC positive ratio was performed. (C) Western blot analysis of LC3 protein. β-actin was used as an equal loading control. Data were expressed as mean ± S.D. $^{*}p < 0.05$.

proteins play a key role remains to be elucidated. Further studies are still needed to fully reveal the mechanism of type I collagen gel protection of L929 cells from TNFα toxicity.

Conflict of interest

None.

Acknowledgments

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