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Regulation on mechanical properties of collagen: Enhanced bioactivities of metallofullerol

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Abstract

Increased mechanical property of extracellular matrix (ECM) around tumor tissue is highly correlated to the progression of cancer, and now its efficient regulation is still a challenge. Here, we report that $Gd@C_{82}(OH)_{22}$-collagen composites greatly suppress the malignant progression of cancer cells *in vitro*, and the metallofullerol can efficiently reduce the mechanical property of collagen matrix. Further study indicates that $Gd@C_{82}(OH)_{22}$ can firmly bind to tropocollagen, facilitate the nuclei and microfibril formation. The interference to interactions among tropocollagens leads to decreased amount and disturbed structure of collagen fibers. $C_{60}(OH)_{24}$, the fullerol counterpart of $Gd@C_{82}(OH)_{22}$, is studied in parallel and their impacts on collagen are strikingly modest. The comparison data reveals that the enhanced bioactivity of $Gd@C_{82}(OH)_{22}$ is highly related with its surface-structure. This study is the first attempt to apply nanomedicines to manipulate the biophysical property of collagen matrix, providing a new sight to target ECM in cancer therapy.

From the Clinical Editor: Increased presence of "harder" collagen in the extracellular matrix (ECM) around the tumor tissue highly correlates with cancer progression. In this paper, a metallofullerol-based approach is reported as an efficient nanotechnology approach in reducing the mechanical properties of the synthesized collagen, paving the way to the development of novel anti-cancer therapies.

Key words: Mechanical property; Collagen; Metallofullerol; Structure-related bioactivity

The tumor microenvironment is a critical determinant for cancer development, amongst which the extracellular matrix (ECM) provides the structural scaffold for neoplastic and non-neoplastic cells.[1] The physical property of ECM has a role in driving the neoplastic evolution. For example, stiffer ECM results in special reciprocity between cells and extracellular environment, leading to a wide range of cellular responses that are critical to tumorigenesis.[2,3] The increased mechanical signals can be transformed to biological signals through force-dependent regulation on expression and activity of integrin and other correlated proteins.[4,5] Accordingly, there is growing interest to probe the tumor treatment approach by decreasing the stiffness of ECM.[6,7] Collagen is the most prevalent proteins in ECM around tumor tissues.[8,9] Several pharmacologic inhibitors of collagen synthesis have been reported to regulate the mechanical property of ECM *in vivo*,[10,11] for collagen density is one of the main factors influencing the rigidity.[12] Meanwhile, the methods to

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directly regulate the mechanical property of ECM *in vitro* were intensively studied, mainly through the regulation of polymerization condition such as temperature and pH.[13–16] Although the method to regulate the stiffness of ECM around tumor *in vivo* by affecting the molecular structure of collagen, especially the fibrils, should be more efficient with less side effects, the studies with this regard are still lacking.

Recent advance in nanotechnology has brought a series of novel approaches to cancer diagnosis and therapy.[17] Due to the enhanced permeability retention (EPR) effect, nanomedicines can passively target the tumor tissue and achieve their antitumor functions. Recently, Gd-metallofullerols are widely studied for their potential application in medicine science.[18,19] The existence of endohedral Gd atom has distinct impact on carbon cage and surface hydroxyl groups. Thus, metallofullerols are different in hydrophility/hydrophobility, surface negative charge and hydroxyl group distribution with their fullerol counterpart,[20] which might result in novel bio-activities.

$Gd@C_{82}(OH)_{22}$ were found to inhibit metastasis in invasive human breast cancer models.[21,22] Previous study showed that $Gd@C_{82}(OH)_{22}$ can induce a thick fibrous cage formation to confine tumor cells in the primary site.[23] Our preliminary work with Masson tri-chrome staining further indicated that collagen layer became thicker with lower density in the $Gd@C_{82}(OH)_{22}$ treated group (Supplementary Figure S1 A and C). The accumulation of $Gd@C_{82}(OH)_{22}$ in the fibrous layer was observed by synchrotron X-ray Fluorescence (μ-XRF) (Figure S1 B and D).[24] Both phenomena hinted that $Gd@C_{82}(OH)_{22}$ might directly interact with collagen instead of inhibiting its synthesis, and the interaction probably affect the assembly process of collagen fibrils *in vivo* and finally lead to changes in their biophysical properties.

Collagen structure is highly hierarchical. Tropocollagen molecules, the basic unit of collagen, can assemble themselves into microfibrils. The microfibrils can be further bundled into collagen fibrils.[25] Recently, some nanoparticles were reported to disturb the assembly process of collagen. For example, Christopher and co-workers found that gold nanorods modified with polyanion can significantly increase the shear modulus of type I collagen gels.[26,27] David et al reported that the presence of silica nanoparticles (12 nm) hindered fibril formation.[28] However, to our knowledge there is still no research work to elaborate the corresponding molecular mechanism.

In this work, we observed the rigidity-reducing effect of $Gd@C_{82}(OH)_{22}$ on collagen hydrogels and investigated the subsequent impacts on the breast cancer cells cultured on/in the gels. We elaborated the corresponding mechanism in the light of their interaction with tropocollagen molecules. To explore the significance of endohedral metal ion, we used $C_{60}(OH)_{24}$ as a comparison group, which was reported to have a similar but more modest tumor-inhibiting effect.[29] A variety of experimental methods including rheology, scanning electron microscopy (SEM) and atomic force microscopy (AFM) imaging, biolayer interferometry (BLI), synchrotron radiation circular dichroism (SRCD), turbidity assay in combination with atomistic molecular dynamics (MD) simulation were used in this work. The results demonstrate the mechanism of conditioning the mechanical property of collagen by affecting its molecular structures with nanoparticles.

Methods

Preparation of collagen gels

Rat tail collagen (4 mg/ml, BD Medical Devices, USA) was mixed on ice with 10X DMEM (Gibco, USA), ddH_2O and fullerol derivatives nanoparticles (f-NPs) in the volumetric ratio of 5:1:3:1, and the pH was neutralized to 7.4 by NaOH. For SEM imaging or cell culture, the collagen mixture was pipetted into a 24-well dish or confocal dish (Corning, USA), and was polymerized for one hour at room temperature.

Rheology

Regidity of type I collagen gels was examined by dynamic torsional shear test on Physica MCR 301 rheometer (Anton Paar, Ashland, USA) with 50-mm parallel plate measurement geometry and a temperature-controlled lower plate. 500 μL of ice-cold collagen solution was placed on the lower plate at 25 °C and polymerized between the two plates for 40 min. All frequency measurements were taken at 3% strain. Then angular frequency sweeps were performed with dynamic strain frequency varying from 1 to 100 s^{-1}. The storage modulus (G′) and the loss modulus (G″) were measured. The complex shear modulus (G*) is calculated from the following equation:

$$G^*(\omega) = G'(\omega) + iG''(\omega) \quad (1)$$

Each sample was repeated three times.

Fluorescent staining and cell counting

Human breast cancer cell line MDA-MB-231 was plated on the collagen gel (about 1×10^6 cells/mL) and cultured in DMEM with 10% fetal bovine serum (FBS, Hyclone, USA) in a humidified 5% CO_2 atmosphere at 37 °C for 24-hour. Then cells were washed twice with pre-warmed phosphate buffer saline (PBS, pH 7.4) and fixed in 3.7% formaldehyde solution for 15 min at room temperature. After three washes in PBS, the cell samples were treated with 0.1% Triton X-100 for 3 min. Cell samples were stained by the working solution including fluorescent phallotoxins and DAPI (Life Technologies Corp., USA), and then were imaged at magnifications of 200× on a fluorescence microscope (Olympus, Japan). Statistical analysis of the attached cells was attained in at least six separated fields in three independent experiments.

Analyzing migration trajectories in collagen gels

MDA-MB-231 cells were incubated with CellTracker Red CMTPX working solution (Life Technologies Corp., USA) for 20 minutes in culture chamber, and then were rinsed with pre-warmed PBS and digested with trypsin. After centrifugation, the cells were resuspended in DMEM with 10% FBS. Neutralized collagen and collagen-$Gd@C_{82}(OH)_{22}$ composites were prepared as described above. After equilibrating the neutralized collagen solution for 10 minutes, we suspended the MDA-MB-231 cells in the solution at a final cells number of 2×10^5. The collagen gel constructions with three-dimension cell culture were first allowed to polymerize for one hour at 37 °C. The dishes were placed on a stage incubated at 37 °C and 5% CO_2, and the