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In vitro toxicity in long-term cell culture of MR contrast agents targeted to cartilage evaluation

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SUMMARY

Objective: Contrast-enhanced magnetic resonance (MR) imaging methods have been proposed for non-invasive evaluation of osteoarthritis (OA). We measured cell toxicities of cartilage-targeted low-generation dendrimer-linked nitroxide MR contrast agents and gadopentetate dimeglumine (Gd-DTPA) on cultured chondrocytes.
Design: A long-term Swarm rat chondrosarcoma chondrocyte-like cell line was exposed for 48-h to different salts (citrate, maleate, tartrate) and concentrations of generation one or two diaminobutyl-linked nitroxides (DAB4-DLN or DAB8-DLN), Gd-DTPA, or staurosporine (positive control). Impact on microscopic cell appearance, MTT spectrophotometric assays of metabolic activity, and quantitative PicoGreen assays of DNA content (cell proliferation) were measured and compared to untreated cultures.
Results: Chondrocyte cultures treated with up to 7.5 mM Gd-DTPA for 48-h had no statistical differences in DNA content or MTT reaction compared to untreated cultures. At all doses, DAB4-DLN citrate treated cultures had results similar to untreated and Gd-DTPA-treated cultures. At doses >1 mM, DAB4-DLN citrate treated cultures showed statistically greater DNA and MTT reaction than maleate and tartrate DAB4-DLN salts. Cultures exposed to 5 mM or 7.5 mM DAB8-DLN citrate exhibited rounded cells, poor cell proliferation, and barely detectable MTT reaction. Treatment with 0.1 μM staurosporine caused chondrocyte death.
Conclusion: Long-term exposure, greater than clinically expected, to either DAB4-DLN citrate or Gd-DTPA had no detectable toxicity with results equivalent to untreated cultures. DAB4-DLN citrate was more biocompatible than either the maleate or tartrate salts. Cells exposed for 48-h to 5 mM or 7.5 mM DAB8-DLN salts demonstrated significant cell toxicity. Further evaluation of DAB8-DLN with clinically appropriate exposure times is required to determine the maximum useful concentration.

Introduction

Osteoarthritis (OA) is the most common and costly joint disease worldwide[1–3] characterized clinically by pain and loss of joint function and structurally by progressive loss of articular cartilage and changes in the underlying bone[2,4–7]. Because articular cartilage has a poor capacity for repair, early cartilage damage diagnosis and intervention, while changes are still minor, is critical to prevent or delay progression to clinical OA. Magnetic resonance imaging (MRI) has proved helpful in assessing articular cartilage with both morphological and compositional techniques[4,5,8–22], however, signal-to-noise and spatial resolution limit the sensitivity to early cartilage degradation and determination of lesion depth, especially with morphological imaging[10,11,18–22].

The extracellular matrix of cartilage is paramount to its biomechanical function and is composed of collagen and glycosaminoglycans (GAG), primarily as glycoconjugates in the form of aggrecan. A hallmark of cartilage degeneration is reflected by alterations in the negatively-charged GAG. Early this may be an up-regulation or down-regulation, however as the disease progresses GAG is lost, resulting in lower fixed charge density (FCD) and loss of

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biomechanical properties. With MRI, the FCD can be calculated directly with sodium (Na^+) imaging or indirectly using positively- or negatively-charged contrast agents that distribute within cartilage relative to the FCD[23–25]. Unfortunately, currently available FCD imaging techniques (Na^+ MRI; delayed gadolinium enhanced MRI of cartilage [dGEMRIC]) are not optimal. Na^+ MRI is inherently difficult with low spatial resolution and requirement of additional hardware and software, including high field strength (>3 T) MR systems[20,24,25]. dGEMRIC exploits the −2 charge on gadopentetate dimeglumine (Gd-DTPA) to measure FCD. However, with dGEMRIC, the cartilage enhancement is not visually obvious with typical MR acquisitions, hence T1 maps must be calculated from multiple MRI acquisitions followed by off-line processing[15–17,20,26,27]. In addition, Gd-DTPA has been associated with nephrogenic systemic fibrosis (NSF) in patients with renal insufficiency presumably because the heavy metal dissociates from its chelate[28–31]. Additionally, an *in vitro* study demonstrated Gd-DTPA toxicity in bovine chondrocytes with induction of apoptosis and an increase in proteoglycan production[32].

Dendrimers to which five-membered ring nitroxides are covalently attached (dendrimer-linked nitroxide, DLN), have been proposed as MRI contrast agents for cartilage imaging[33,34]. DLN MRI contrast agents do not carry the risk of heavy metal ion toxicity and were designed to be positively-charged at physiologic pH with preferential distribution to areas with higher GAG concentrations. Therefore, healthy cartilage has a higher uptake of the contrast agent[33,34] resulting in brighter signal on clinical MR images and potentially enabling early identification of cartilage degeneration without the need of T1 maps.

To date, the toxicity of DLNs on chondrocytes has not been evaluated. Herein, we study the effect of long-term exposure of cultured chondrocytes to diaminobutyl-linked nitroxides, generation one DAB4-DLN [Fig. 1(A)] and generation two DAB8-DLN [Fig. 1(B)], in comparison to Gd-DTPA, staurosporine (positive control) and untreated cell cultures (negative control). The effect of 48-h exposure on cell proliferation, intracellular metabolism, and microscopic cell appearance were compared for different concentrations and for different salts of the DLN contrast agents using a chondrocyte-like cell line derived from the Swarm rat chondrosarcoma (LTC-RCS)[35].

Material and methods

Materials

All reagents used were of highest purity. Cell culture materials included: 10× Trypsin (Gibco-Invitrogen, Grand Island, NY USA), L-Glutamine (Sigma-Aldrich, St. Louis, MO USA), Dulbecco's modified Eagle's medium with low glucose (DMEM; Sigma-Aldrich, St. Louis, MO USA), Penicillin (Sigma-Aldrich, St. Louis, MO USA), and Streptomycin (Gibco-Invitrogen, Grand Island, NY USA); Hank's Balanced Salt Solution without Calcium or Magnesium (Mediatech, Herndon, VA USA); fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA USA); T-75 tissue culture flasks, 96-well and 48-well plates (BD Falcon, Franklin Lakes, NJ USA).

Chondrocyte-cell line

An established long-term culture chondrocyte-like cell line (LTC-RCS) derived from the Swarm rat chondrosarcoma cell line[35,36] was obtained from Dr Veronique Lefebvre (Cleveland Clinic, Cleveland, OH USA) with permission from Dr James Kimura (Henry Ford Hospital, Detroit, MI USA). LTC-RCS chondrocytes were selected because they stably maintain a chondrocytic phenotype under monolayer culture conditions which permits conventional microscopic imaging evaluation[37]. LTC-RCS chondrocytes were cultured in a T75 culture flask using 30 ml of DMEM containing 50 units/ml penicillin and streptomycin and 10% FBS; in a humidified atmosphere of 5% CO_2 at 37°C. The cells were passaged every 4–5 days after reaching approximately 70% confluency (~1.4×10^5 cells/cm^2). For all experiments, LTC-RCS chondrocytes were seeded at 180 cells/mm^2.

Photomicrographs

Phase contrast photomicrographs were taken with an Olympus CKX 41 inverted microscope with an attached DP21 digital camera (Olympus America, Melville, NY USA). Bright field photomicrographs were taken of 96-well cultures following incubation with MTT reagent (details below). For morphological documentation,

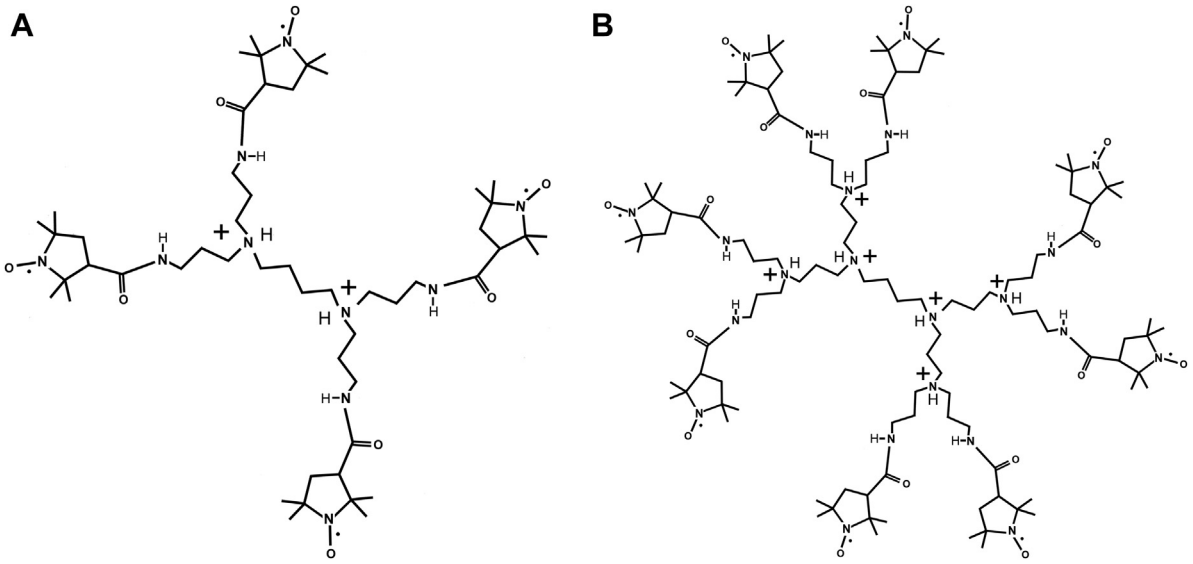

Fig. 1. Chemical structures of DAB-DLN contrast agents. DAB4-DLN (A) has four terminal five-membered nitroxide groups and two tertiary amines that are positively charged (indicated by "+" signs) at physiologic pH while DAB8-DLN (B) has eight terminal nitroxide groups and six tertiary amines.