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Generation and characterization of antibodies against arginine-derived advanced glycation endproducts

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ABSTRACT

Although antibodies reagents have been widely employed for studying advanced glycation end-products (AGEs), these materials have been produced using complex mixtures of immunogens. Consequently, their epitope specificity remains unknown. Here we have generated the first antibodies capable of recognizing each of the three isomers of the methylglyoxal hydroimidazolones (MG-Hs) by using chemical synthesis to create homogenous immunogens. Furthermore, we have thoroughly characterized the epitope specificity of both our antibodies and that of two existing monoclonals by implementing a direct ELISA protocol employing synthetic MG-H antigens. Finally, we employed the reported anti-MG-H antibodies to the detection of MG-Hs in cellular systems using immunofluorescence microscopy. These studies have demonstrated that anti-MG-H1 and anti-MG-H3 staining is concentrated within the nucleus, while anti-MG-H2 affords only minimal signal. These observations are consistent with reported formation preferences for MG-Hs, and may suggest novel nuclear targets for non-enzymatic posttranslational modification. The antibody reagents reported herein, as well as the strategy employed for their creation, are likely to prove useful for the immunochemical study of AGEs in biological systems.

Introduction

Advanced glycation endproducts (AGEs) are posttranslational modifications formed by the non-enzymatic reaction of protein side-chains with sugars and sugar degradation products.[1,2] AGE levels have been shown to be elevated during the aging process,[3] and in various disease states, such as diabetes,[4] cancer,[5,6] and cardiovascular disease.[7] Indeed, several AGEs have been shown to serve as useful diagnostic markers of disease.[8–10] In addition, AGEs are thought to contribute directly to disease pathophysiology by causing protein damage and dysfunction. AGEs have also been shown to induce a number of deleterious effects in cellular systems, such as induction of oxidative stress and pro-inflammatory signaling.[1] Thus, there is considerable interest in the role(s) of AGEs in human health and disease.

The methylglyoxal hydroimidazolones (MG-Hs) comprise the most prevalent arginine-derived AGE.[11] They are formed as a mixture of three isomers by protein glycation with methylglyoxal (MGO), a byproduct of glycolysis (Fig. 1A). MG-Hs are estimated to modify 1–2% of all arginine residues found in lens proteins of elderly human subjects.[12] Levels of this AGE have also been found to be elevated in patients with cardiovascular disease,[13] Alzheimer's disease,[14] and diabetes mellitus (DM).[15,16] Moreover, with respect to DM, increased MG-H levels are strongly correlated with the onset of complications such as retinopathy,[17] highlighting the potential utility of these modifications as biomarkers.

Many techniques are available for the visualization of the MG-Hs and other AGEs, including UV–Vis and fluorescence imaging,[18,19] exhaustive hydrolysis followed by HPLC,[20,21] and mass spectrometry.[22,23] However, these methods suffer from various limitations. For example, UV–Vis and fluorescence detection are usually limited to bulk analysis, and require AGEs that possess suitable chromophores. Chromatography and mass spectrometry are useful in their ability to identify specific AGEs in biological samples, but require specialized instrumentation, and are expensive with respect to both cost and time.

Antibodies have been widely utilized in the study of AGEs, and have enabled histological and immunochemical experiments, providing an attractive alternative to chromatography/mass spectrometry for detecting AGEs in complex biological samples. Despite their widespread usage, however, in general, the epitopic specificity of these reagents is not known. This problem arises for two main reasons. First, immunizing antigens are often produced by reacting a carrier protein (albumin or KLH) with glycating agents, leading to a complex mixture with unknown AGE composition. Therefore, although the resulting MGO-protein mixtures are often assumed to exclusively contain the various MG-Hs,

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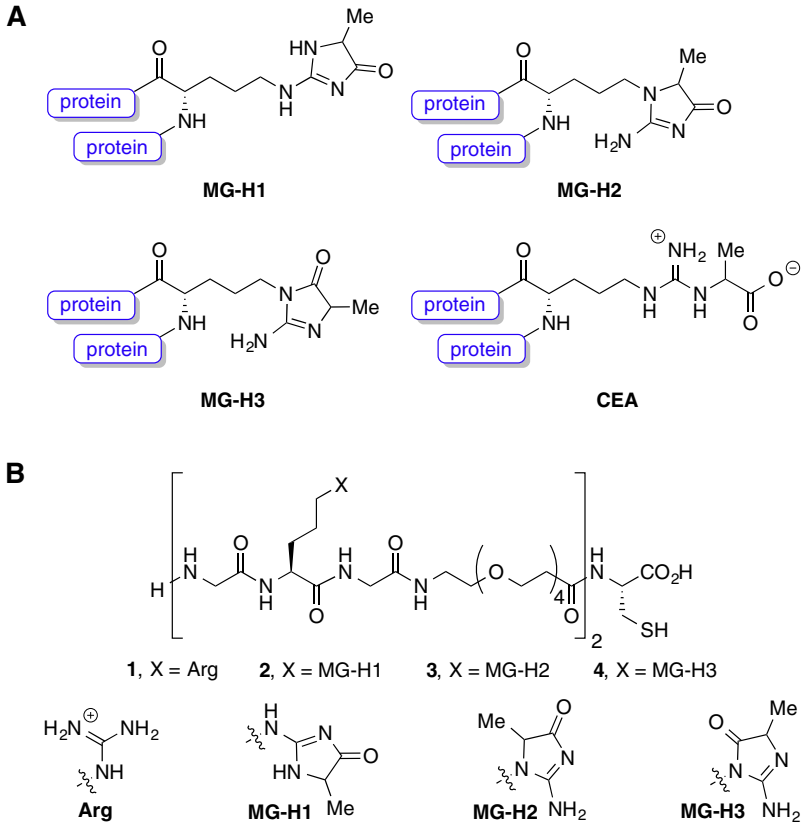

Figure 1. (A) Structures of the methylglyoxal hydroimidazolone (MG-H) isomers and MG-H3 hydrolysis product carboxyethylarginine (CEA). (B) MG-H and arginine-modified peptide constructs used for immunization and antibody purification.

numerous other AGE adducts have also been isolated from these preparations.[11] Second, because chemically-homogeneous synthetic AGE standards have not historically been available for most AGEs, the exact structure of recognition epitopes cannot be rigorously determined. Instead, these antibodies are generally characterized by showing preferential binding to AGE-modified proteins over non-modified counterparts. Such strategies are inherently imprecise because these AGE-proteins contain a heterogeneous array of AGEs. Indeed, while antibodies have been reported to recognize CML,[24–26] CEL,[27,28] pentosidine,[29] and argpyrimidine,[30] for many anti-AGE antibodies, the actual epitope(s) being recognized is not clear.[31]

With respect to the MG-Hs, two mouse monoclonal antibodies, clones 3D11 and 1H7G5 (also referred to as IG7),[32,33] have been reported. Both 3D11 and 1H7G5 were generated using immunogen created by incubation of carrier protein with MGO,[32,34] and these reagents have been used extensively in the literature to study MGO-protein modifications. While 1H7G5 is thought to recognize MG-H1, and to a lesser extent argpyrimidine,[34] the epitopic specificity of 3D11 has not been described in the literature. Well-characterized antibody reagents capable of recognizing the three MG-H isomers independently would therefore be highly useful, both as diagnostics and as research tools.

Herein we report the generation and characterization of the first selective antibodies against each of the three MG-H isomers. These efforts were enabled by our laboratory's recent development of efficient syntheses for each of these isomers as both amino acids and peptide conjugates.[35] Thus, we have constructed chemically homogeneous MG-H preparations, and used these to immunize rabbits for production of selective polyclonal sera. We have also thoroughly characterized the epitopic specificity of both our antibodies, and the two existing anti-MG-H monoclonals, through the use of a direct ELISA employing synthetic antigens. Interestingly, while both anti-MG-H1 and anti-MG-H2 sera proved highly selective for the target antigens, anti-MG-H3 was found to cross-react with carboxyethyl arginine (CEA). Monoclonal antibody 1H7G5 exhibited a similar pattern of cross-reactivity between these two antigens while 3D11 cross-reacted with MG-H1, MG-H3, and CEA. Finally, immunofluorescence microscopy experiments indicated MGO-dependent increases in staining with anti-MG-H1 and anti-MG-H3, but not anti-MG-H2 sera. The staining pattern observed for these antibodies was predominantly nuclear, although faint cytosolic staining was also visualized. This Letter is the first to demonstrate the generation of selective antibodies against individual MG-Hs. These new reagents have the potential to serve as useful tools for both disease diagnosis and fundamental research.

The MG-H class of AGEs is composed of three isomers, as illustrated in Figure 1A. Our goal was to generate antibodies capable of recognizing each MG-H isomer while exhibiting minimal cross-reactivity with the other two. To accomplish this, we designed the immunogens depicted in Figure 1B. Thus, for each isomer, synthetic peptide immunogens were constructed to contain the MG-H modification flanked by glycine and spaced out from the terminal cysteine residue (the site of immunogen protein conjugation) with a flexible polyethylene glycol (PEG) linker (**2**–**4**, Fig. 1B). We employed amino acids with minimal side-chain functionality in order to obtain antibodies capable of recognizing their epitope in a sequence-independent manner. These immunogens were produced using solid phase peptide synthesis (SPPS), purified, and conjugated to keyhole limpet hemocyanin (KLH) for immunization. Antibodies were then isolated through a series of negative and positive affinity purifications. For example, to obtain MG-H3 specific antibodies, the antiserum was first depleted using **1** to remove