

IMARS Highlights



Research Commentaries for Members of The International Maillard Reaction Society

A Non-profit Research and Education Organization in Biomedicine and Food Science:
<http://imars.case.edu>

Volume 3, Number 4

July 15, 2008

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CONTENTS

Editorial comment	i
Special feature	
Protein and nucleotide damage by glyoxal and methylglyoxal in physiological systems - role in ageing and disease <i>Paul J. Thornalley</i>	2
CML content in food stuffs, AGEs or ALEs? <i>Timo Buetler</i>	8
esRAGE, a multipotential guardian of cells <i>Takuo Watanabe and Hiroshi Yamamoto</i>	10
Rapid screening of Maillard reaction inhibitors <i>Rosario Zamora</i>	13
Mental/psychological stress, lipid peroxidation and inflammation <i>Timo Buetler</i>	14
FEATURES	
Highlights of the glycation literature June – July 2008 <i>Naila Rabbani</i>	15
Obituary	17
Corporate Sponsors	18

Editorial comment

Welcome to the July issue of IMARS Highlights. It gives me great pleasure to bring to your attention highlights in glycation research selected by our contributing editors – this month, Toshio Miyata, Timo Buetler, Rosario Zamora and Paul Thornalley. I will encourage the readers of highlights to add their voice and comments related to CML story, debate goes on about the origin of this AGE. Feedback from readers on these and other features in this issue are very welcome. There has been steep rise in publications related to glycation and AGE; references of few selected articles have been included as usual.

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Extract from Drug Metabolism vol 23, 125 – 150, 2008. A special issue on methylglyoxal dedicated to the memory of Professor Albert Szent-Gyorgyi (1893-1986)

Protein and nucleotide damage by glyoxal and methylglyoxal in physiological systems - role in ageing and disease

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Abstract

Glycation of proteins, nucleotides and basic phospholipids by glyoxal and methylglyoxal – physiological substrates of glyoxalase 1 - is potentially damaging to the proteome, genome and lipidome. Glyoxalase 1 suppresses glycation by these α -oxoaldehyde metabolites and thereby represents part of the enzymatic defence against glycation. Albert Szent-Gyorgyi pioneered and struggled to understand the physiological function of methylglyoxal and the glyoxalase system. We now appreciate glyoxalase 1 protects against dicarbonyl modifications of the proteome, genome and lipome. Latest research suggests there are functional modifications of this process – implying a role in cell signalling, ageing and disease.

Letter from Albert Szent-Gyorgyi

“I thank you for your kind lines from May 17th, and the reprints that reached me only now. It was very long ago that I was interested in keto-aldehydes and worked on them. Since then I forgot most of what I knew. So, to my regret I am unable to advise you. Yours truly, Albert Szent-Gyorgyi, MD, PhD, NL.” From a letter to the author dated 7th June 1984.

Above are the words of Albert Szent-Gyorgyi in a letter replying to my request for advice on how to pursue a research career on studies of methylglyoxal and the glyoxalase system. The letter was sent from the National Foundation for Cancer Research, Woods Hole, Massachusetts, USA. It has been framed, along side newspaper obituary tributes to Szent-Gyorgyi when he died in October 1986, hanging in my office for over the last 20 years whilst I grappled with similar problems that once interested him. Our common interest was, for Szent-Gyorgyi, and still is for me the physiological significance of the glyoxalase system and its physiological substrates – primarily the reactive dicarbonyl compounds and physiological metabolites, glyoxal and methylglyoxal^{1,2} – Figure 1.

Methylglyoxal and glyoxal – physiological substrates of the glyoxalase system

The presence and significance of reactive acyclic α -oxoaldehydes, methylglyoxal and glyoxal, in physiological systems was the subject of research before, during and after the research career of Szent-Gyorgyi. Neuberg had discovered the glyoxalase system and metabolism of methylglyoxal in 1913.³ Szent-Gyorgyi advanced his hypothesis of the “retine and promine” theory of control of cell proliferation,¹ and recent research views glyoxalase 1 (Glo1) as part of the enzymatic defence against glycation.² Methylglyoxal has been considered to be an environmental and/or bacterial toxin and metabolite.^{4,5} We now know methylglyoxal is formed spontaneously from triosephosphates in all organisms with anaerobic glycolysis⁶ and from other non-enzymatic and enzymatic pathways of differing significance – depending on the organism.⁷ Glyoxal is formed by lipid peroxidation and the degradation of monosaccharides, saccharide derivatives and glycated proteins.^{8,9}

Modification of proteins by glyoxal and methylglyoxal

Glyoxal and methylglyoxal are potent glycating agents. They react with proteins to form advanced glycation endproduct (AGE) residues directly and relatively rapidly – Figure

2a. Protein glycation adduct residues are formed on extracellular and cellular proteins. The turnover of these proteins by cellular proteolysis releases glycation free glycation adducts (glycated amino acids).¹⁰ Protein damage by glycation is implicated in protein misfolding.

Misfolded proteins are degraded by the proteasome to ensure the high quality of intracellular proteins.¹¹ Protein glycation free adducts are, therefore, the excreted debris of cellular proteolysis of damaged proteins. This appears to be an efficient process since there was little evidence of low molecular mass damaged peptides in peripheral venous plasma. Additional sources of free adducts are direct glycation of lysine and arginine. This probably plays a minor contribution because of the relatively low concentration of free amino acids compared to the concentration of corresponding amino acid residues in proteins.¹⁰

Saccharide-rich and thermally processed foods are a good source of AGE residues.¹² There is a low bioavailability of AGE residues in proteins of ingested foods such that <10% is absorbed¹³ – attributed to resistance to proteolysis of highly glycated proteins in food¹⁴ and some AGEs inhibit intestinal proteases.¹⁵ The highest concentration of absorbed food AGEs is expected in portal venous plasma where we found the hydroimidazolone MG-H1 enriched by 10-fold in peptides.¹⁶ AGEs are therefore probably absorbed from food as both AGE free adducts and AGE-rich peptides; the latter appear to be degraded efficiently after absorption. Hydroimidazolones G-H1 and MG-H1, CML and CEL free adducts had high renal clearances.¹⁰ This suggests that as long as renal function is normal, protein glycation and oxidation adducts absorbed from food may pose little threat – although this remains controversial¹⁷ – Figure 3.

Proteins in mammalian tissues, plasma and extracellular matrix *in vivo* have relatively high MG-H1 residue content (0.1 – 15 mmol/mol amino acid modified), much lower CML and CEL residue content (0.05 – 6 mmol/mol lys) and trace amounts of GOLD and MOLD residues (0.001 – 0.002 mmol/mol lys) - depending on the protein substrate and tissue location, type of AGE. Hydroimidazolone AGEs are found in highest concentrations in lens protein of elderly human subjects; MG-H1 residue content was 1 – 2% of total arginine residues. Stable AGEs such as CML residues accumulate on lens capsule, skin and cartilage collagen with age but reached <6 mmol/mol lys in cartilage in old age.^{18;19}

The physiological importance of protein glycation remains under intensive investigation. Particularly damaging effects are produced by covalent crosslinking of proteins which confers resistance to proteolysis.²⁰ Protein modification is also damaging when amino acid residues are located in sites of protein-protein interaction, enzyme-substrate interaction and protein-DNA interaction (for transcription factors). A bioinformatics analysis of receptor binding domains indicated that arginine residues have the highest probability of being located in such sites (19.6%).²¹ The major modification of proteins by glyoxal and methylglyoxal is on arginine residues. Formation of hydroimidazolones causes structural distortion, loss of side chain charge and functional impairment.²² Two examples we studied recently are methylglyoxal modification of human serum albumin and vascular type IV collagen.

Modification of human serum albumin by methylglyoxal produced hotspot modification of Arg-410 with hydroimidazolone MG-H1 residue formation. Modification of vascular basement membrane type IV collagen by methylglyoxal formed MG-H1 residues at hotspot modification sites in RGD and GFOGER integrin-binding sites of collagen, causing endothelial cell detachment, anoikis and inhibition of angiogenesis. Glycation of proteins by glyoxal and methylglyoxal is not only common in mammalian systems. MG-H1 hydroimidazolone has been isolated from bacteria²³ and we have recently found glyoxal and methylglyoxal-derived AGE residues in proteins of higher plants with variation in light and dark growth cycles.²⁴

Glyoxalase 1 – a critical role in the enzymatic defence against glycation

Accumulation of protein glycation adducts is associated with enzyme inactivation, protein denaturation and a cellular-mediated immune response. Excessive nucleotide glycation is associated with mutagenesis and apoptosis, and excessive lipid glycation with membrane lipid bilayer disruption.²⁵ In 2003, I developed the concept of an enzymatic defence against glycation protecting against glycation-mediated cell damage. It involves the enzymatic activities that suppress the formation of glycation adducts and repair sites of early glycation: Glo1 and certain aldehyde reductase and dehydrogenase isozymes detoxify reactive carbonyl and α -oxoaldehyde glycating agents (glyoxal, methylglyoxal, 3-deoxyglucosone and others)^{26;27}, and amadoriase and fructosamine 3-phosphokinase catalyse the removal of fructosamine glycation adducts formed by glycation of proteins by glucose.²⁸⁻³⁰ The enzymatic defence against glycation suppresses damage to biological macromolecules but it is an imperfect defence; glycation adducts of proteins, nucleotides and basic phospholipids are formed in normal physiological states but at a low level. Steady state levels of glycation adducts are ca. 0.1-1% lysine and arginine residues in proteins, 1 in 10⁵ nucleotides in DNA, and 0.1% basic phospholipids.

Oxidative stress is inextricably linked to glycation because the depletion of GSH and NADPH in oxidative stress also decreases the *in situ* activities of Glo1³¹ and thereby increases the concentrations of glyoxal and methylglyoxal and associated glycation reactions. Glycation of proteins by Glo1 substrates may also increase reactive oxygen species (ROS) – superoxide, hydrogen peroxide and hydroxyl radical, and thereby be a cause or contributory factor to oxidative stress. This is best illustrated by the increased ROS formation associated with mitochondrial dysfunction and methylglyoxal modification of mitochondrial proteins in diabetic nephropathy³² and ageing in *Caenorhabditis elegans*.³³

Concluding remarks: emerging evidence that glyoxalase 1 and glycation by glyoxal and methylglyoxal play a critical role in disease and ageing – the tribute to Szent-Gyorgyi

Glycation by glyoxal and methylglyoxal, and the related influence of Glo1, are now emerging as playing a critical role in ageing³³ and disease processes – vascular complications associated with diabetes^{34;35}, renal failure^{36;37}, Alzheimer's disease^{38;39} and tumourigenesis and multidrug resistance in cancer chemotherapy⁴⁰. They may also have roles in pathologic anxiety^{41;42}, autism⁴³, obesity⁴⁴ and other disorders. The abiding tribute to Albert Szent-Gyorgyi is that to the end of his scientific career he studied biological problems of fundamental importance. It is no disrespect to him that the physiological function of methylglyoxal and glyoxalase was left unresolved at the end of his career since over 20 years later we are still struggling to resolve it.

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Figure 1. a. The glyoxalase system and metabolism of methylglyoxal. B. Advanced glycation endproducts residues formed from glyoxal and methylglyoxal with proteins. AGEs are shown as AGE residues with the peptide backbone $-\text{CO}-\text{CHR}-\text{NH}-$ shown on the left.

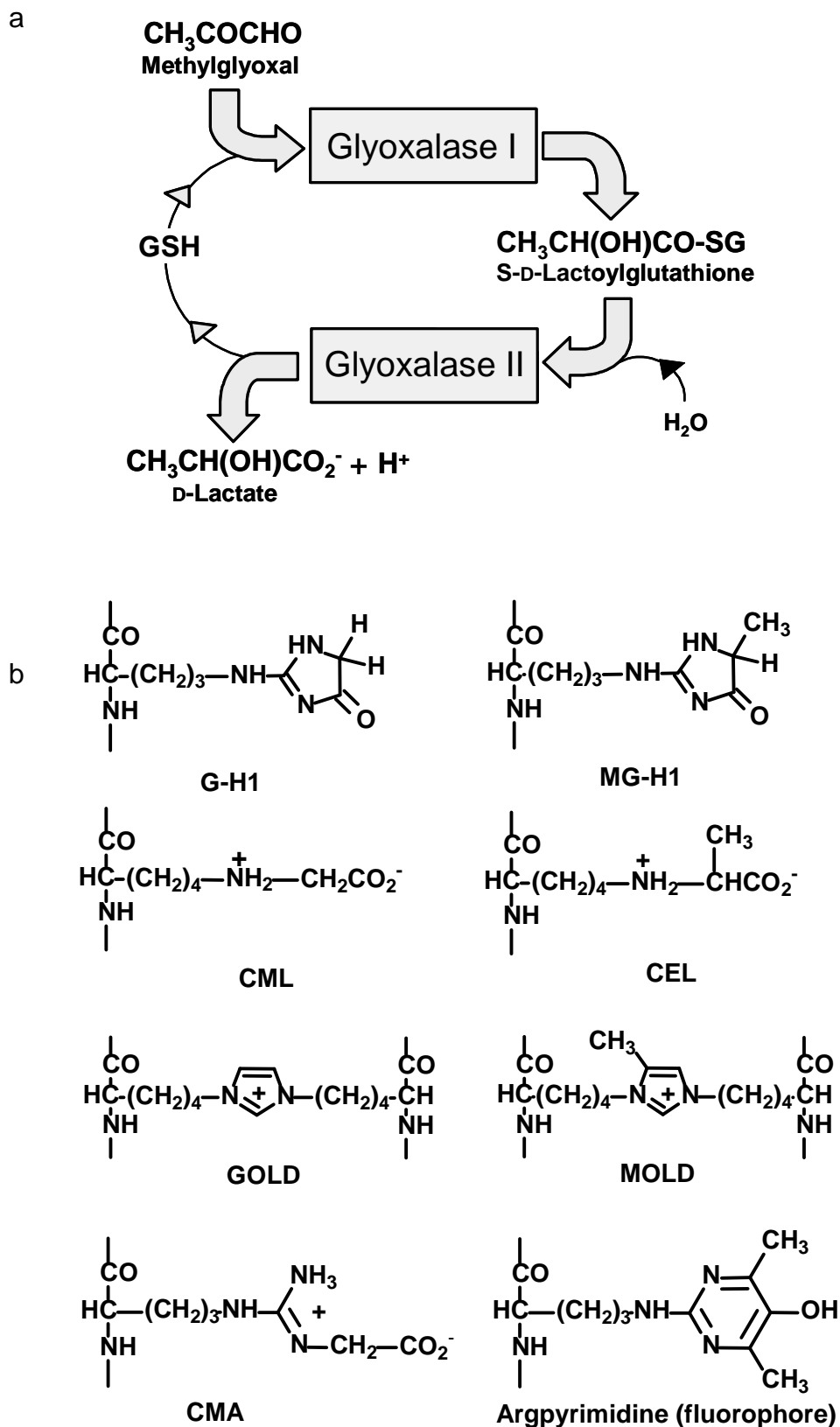
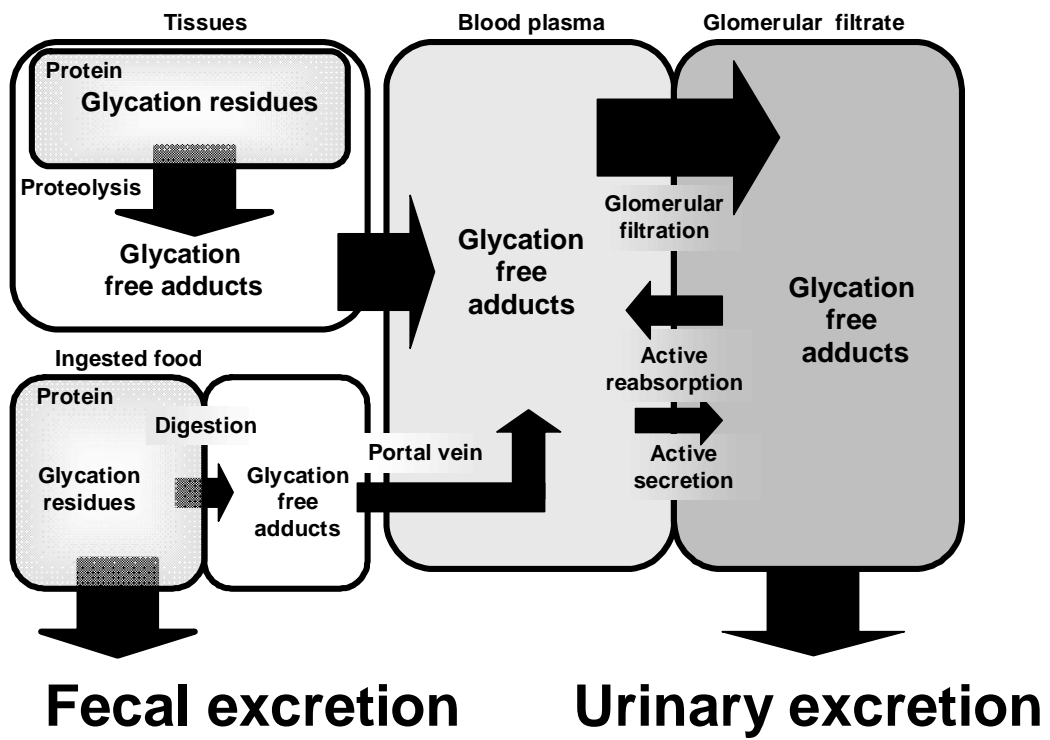


Figure 2. Biodistribution scheme illustrating flows of formation and removal of protein glycation free adducts.



CML content in food stuffs, AGEs or ALEs?

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In the last IMARS Highlights both Baynes and Pischetsrieder discussed the issue of CML content in food. The data published by Goldberg *et al.* (1) reported CML levels in over 250 food items based on a competitive ELISA method. I used this data to compare the reported CML content with the sugar and fat content of some of these food items (2) and draw the graphs shown below on the left side. This analysis suggests that CML is positively associated with the food fat content but not with the food sugar content. This was somewhat surprising because it can be expected that Maillard reaction products should be formed in the presence of sugars. However, CML can also be formed from lipid peroxidation (3) and I argued that in food CML may perhaps be formed primarily from lipid peroxidation products and not via the classical Maillard reaction (2).

Recently, Assar *et al.* (4) have published a similar study that investigated the CML content of various food stuffs with a validated UPLC-MS/MS method. The number of different food items is much smaller than that analyzed by Goldberg *et al.* probably because of the larger effort needed to reliably analyze the CML content in different matrices. From the methods it can be taken that in butter CML was determined in the aqueous phase while in the other foods the total CML was determined (both protein-bound and free).

Plotting a similar comparison of the CML content *versus* the food fat and carbohydrate content reveals a completely different picture (see graphs on the right side). Although only a limited number of food items were analyzed, it is clear that CML levels increase in foods rich in carbohydrate but not in food with high fat content where levels actually are low. This is in outright contradiction with the data published by Goldberg *et al.* (1) but agrees better with the expectations that the Maillard reaction is principally responsible for the formation of CML in food. Interestingly, the fat content does not appear to contribute much to CML formation in this new study. But additional data is needed to confirm this trend.

The question now is, what data can we believe? Most would probably agree that data generated with a validated LC-MS/MS method is generally more trustworthy than data obtained with an un-validated indirect ELISA method (see also Schalkwijk, IMARS Highlights 1 vol 5, 2006, p.8). Several laboratories are currently analyzing different food stuffs for AGE/CML content and this data will soon provide additional support for one or the other data set. In this respect, we also have (unpublished) data on the CML content in butter showing, in agreement with Assar *et al.*, only very low amounts of CML.

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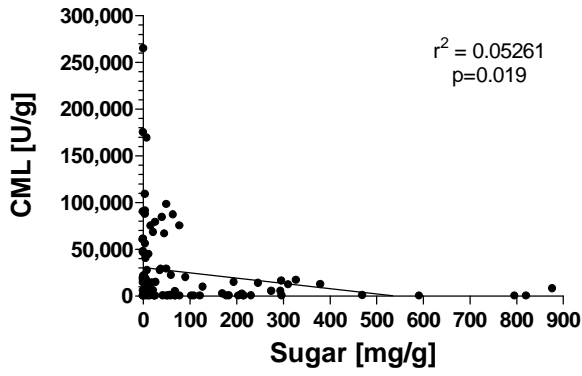
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Data from Goldberg *et al.*

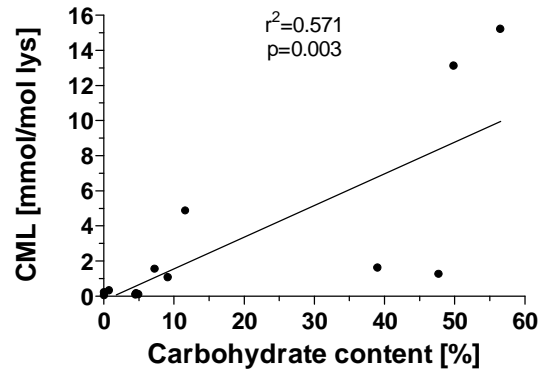
Data from Assar *et al.*

C
A
R
B
S

Correlation CML - sugar content

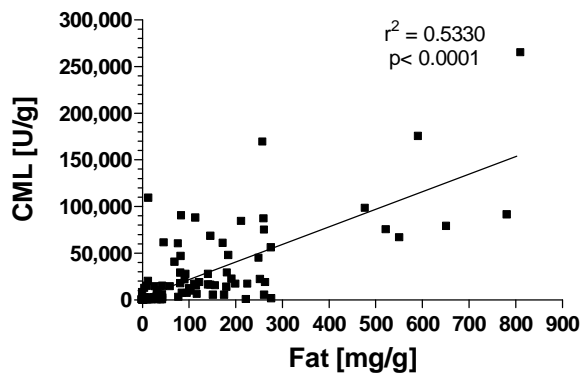


Correlation CML - carbohydrate content

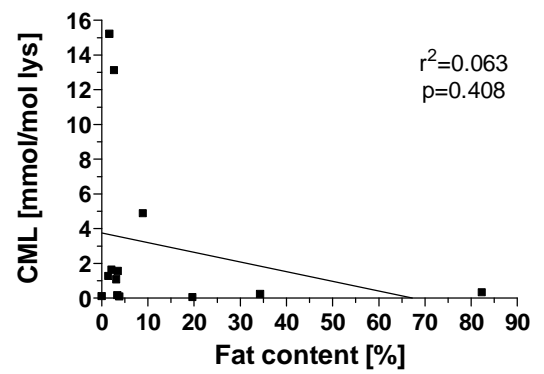


F
A
T

Correlation CML - fat content



Correlation CML - fat content



esRAGE, a multipotential guardian of cells

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Several cellular receptors for advanced glycation endproducts (AGE) have been reported. Most of them, such as class AI/AII (SR-A), cell surface glycoprotein CD36, scavenger receptor class B type I (SRBI), lectin-like oxidized low-density lipoprotein receptor-1, the fasciclin EGF-like, laminin-type EGF-like, and link domain-containing scavenger receptor-1 (FEEL-1) and its paralogous protein FEEL-2 [1], belong to scavenger receptor and the physiological outcomes of their bindings with AGE are mostly unknown. Another AGE receptor, galectin-3, a mammalian lectin is reported to protect tissues from AGE-induced damage by internalization and degradation of AGE [2]. In contrast to galectin-3, receptor for advanced glycation endproducts (RAGE) generates intracellular signals, typically including generation of reactive oxygen species, activation of mitogen activated protein kinase pathways and NF- κ B, which result in tissue damage [3].

RAGE belongs to the immunoglobulin superfamily containing an N-terminal V-type immunoglobulin domain followed by two C-type immunoglobulin domains, a transmembrane domain and a short, 43-amino acid C-terminal cytoplasmic domain. The cytoplasmic domain is reported to be essential for RAGE-mediated signalling [3], although the mechanism that generates intracellular signal is largely unknown. A uniqueness of RAGE is its range of ligands. RAGE interacts with S100/calgranulin, amphoterin (also termed as high mobility group box 1 protein, HMGB1), amyloid fibrils, transthyretin, and a leukocyte integrin, Mac-1. The common characteristics of these ligands are the presence of multiple β -sheets, and RAGE can be regarded as a pattern recognizing receptor [4] like Toll-like receptors. The variety of ligands is reflected in the variety of pathological conditions in which RAGE is involved, such as diabetic angiopathy, Alzheimer's disease, familial amyloid polyneuropathy, cancer, and so on [3].

Another uniqueness of RAGE is the presence of a secretory isoform, endogenous secretory RAGE (esRAGE), that counteracts the membrane-bound form RAGE. esRAGE is generated by alternative splicing of RAGE mRNA that results in unique C-terminal structure lacking transmembrane domain [5]. Because the three immunoglobulin domains are common to membrane-bound form RAGE, esRAGE functions as a decoy receptor that captures the RAGE ligands and inhibits membrane-bound form RAGE-mediated tissue damage [5]. Since the two isoforms, membrane-bound form and secretory form, are generated by mutually exclusive alternative splicing, the balance between membrane-bound RAGE and esRAGE may be critical determinant for susceptibility of cell/tissue to the pathological processes. It should be noted that soluble RAGE (sRAGE) is not the same entity as esRAGE. sRAGE was originally soluble form of RAGE consisting of only the extracellular segment of the membrane-bound RAGE. It was found in tissue and serum, generated by proteolytic cleavage of membrane-bound RAGE and possibly of esRAGE. After identification of esRAGE that has unique C-terminal structure, esRAGE is often categorized as a form of sRAGE. Both sRAGE and esRAGE are considered to function as decoy receptors. An ELISA kit provided by B-Bridge International detects esRAGE specifically, and that provided by R&D Systems detects both sRAGE and esRAGE collectively.

A number of studies suggest levels of esRAGE and/or sRAGE (including esRAGE) in serum is associated with various pathological conditions, *e.g.* decrease of esRAGE in diabetes and metabolic syndrome [6] and decrease of sRAGE Alzheimer's disease [7]. The production and circulation of esRAGE/sRAGE could be altered as a consequence of the pathological condition, or, considering the protective function of esRAGE/sRAGE, the decrease of

esRAGE/sRAGE and reduced protection for cells may trigger or accelerate the pathological conditions. In the case of Alzheimer's disease, we found that the esRAGE expression in hippocampal neurons decreased in early pathological stages in which neuronal loss is not apparent [9]. Taking together, the serum esRAGE/sRAGE might be versatile biomarkers in a wide range of pathological conditions [8].

The esRAGE/sRAGE is also potential therapeutic targets. Induction of esRAGE/sRAGE production should increase resistance of cells against the insults mediated by membrane-bound RAGE. Angiotensin-converting enzyme-1 inhibitor, statin and thiazolidinedione have been reported to increase the serum level of esRAGE/sRAGE [10, 11, 12] although the mechanism is little known. Drugs that induce the esRAGE/sRAGE expression and/or suppress the membrane-bound form expression should be promising candidates of the preventive medicine for any of RAGE-related diseases. Two approaches to find out such drugs are ongoing in our laboratory; elucidation of the regulating mechanism of the alternative splicing RAGE mRNA and development of screening systems for the drugs that alter the RAGE mRNA splicing.

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Rapid screening of Maillard reaction inhibitors

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AGE inhibitors have been studied for a number of years. Now, a simple strategy for the rapid screening of these inhibitors has recently appeared (1). The procedure is carried out by incubating immobilized or soluble RNase with sugar along with the inhibitor at desired conditions. After completion of incubation, the activity of the enzyme is measured. When the immobilized enzyme is employed, the enzyme can be easily isolated and washed, therefore limiting the interfering substances. Although different enzymes have been shown to undergo glycation induced loss of catalytic activity, the use of RNase offers the additional advantage of being remarkably stable. Therefore, reactions can be carried out with moderate heating.

By using this assay, authors have found that DETAPAC was the most effective compound in protecting the enzyme activity, followed by aminoguanidine, EDTA and pencillamine, aspirin and paracetamol. Ascorbic acid and ibuprofen were less inhibitory. For most of these compounds, a good correlation was observed between the extents of protection observed when either soluble or immobilized RNase was used. On the other hand, many plant-derived extracts exhibited different protection when either soluble or immobilized RNase was employed. Among them, those intensively coloured extracts derived from tomato, grapes, and turmeric exhibited a stronger protection when tested with the immobilized enzyme than when soluble RNase was employed.

The work lacks a comparison of the results obtained by using this method with previous procedures, and its usefulness for obtaining potential *in vivo* activities is very limited. On the other hand, the employed strategy is simple and might be useful for the rapid screening of new non-related compounds that can open new insights to the control of AGE formation.

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Mental/psychological stress, lipid peroxidation and inflammation

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In this contribution I would like to discuss the curious, but well documented fact that mental or psychological stress increases lipid peroxidation and circulating markers of inflammation and the acute phase response. Recently, Morimoto *et al.* (1) reported that mental stress induced by the Colour Word Test in postmenopausal women significantly increased blood pressure and lipid peroxidation. This response was not observed in a test of physical stress, i.e. the handgrip test, suggesting that the lipid peroxidation was mainly due to mental stress. There are many examples that mental or psychological stress increases lipid peroxidation and/or inflammatory markers as well as markers of the acute phase response such as CRP (2). An interesting study by Sivonova *et al.* (3) showed that university students undergoing an exam showed significantly elevated levels of oxidative DNA damage while their antioxidant status decreased when compared to the periods in-between exams.

There are no reports that protein glycation was increased during periods of acute or chronic mental or psychological stress but it would not be surprising to find that protein glycation would also be increased under these conditions.

This is to emphasize the importance of environmental factors on markers of oxidative stress and inflammation in humans. Protein glycation is affected by so many factors in humans that it will be very difficult to point out one single factor as cause of adverse health effects. These factors include smoking (4), many kinds of stresses as indicated above, dietary intake of glucose, fat, phytochemicals and antioxidants, general health status, hormonal status, physical activity, etc.

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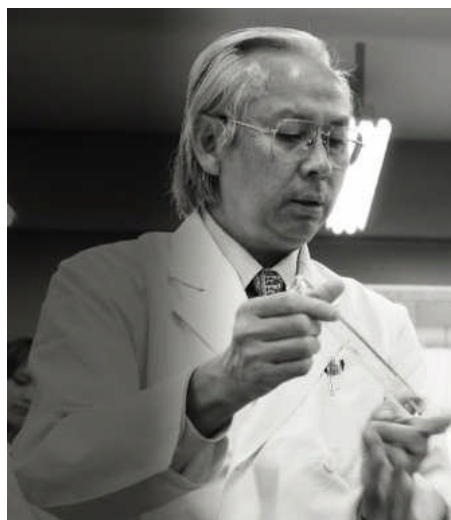
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Obituary

Professor Nguyen van Chuyen (1945 – 2008)



We are sorry to announce the death of Professor Nguyen van Chuyen van Chen. He passed away on June 17, 2008 due to a cerebral haemorrhage at the mid brain area. He became a member of JMARS in 1990 at a very first meeting in Fukui, Japan with his mentor, Professor Kato. He was a distinguished chemist and he had been always interested in the biological significance of the Maillard reaction.

Dr. Teruo Miyazawa, past president of JMARS, and myself respectfully joined a wake the night before his funeral last Thursday held near his home. Many students and faculty members in the Japan Women' University, and many friends participated in his funeral to express their condolences. Many important on-going research projects headed by him remain after his death. We hope his students and colleagues will be able to continue his legacy. We would like to organize his memorial symposium in the JMARS. June 22, 2008.

Naoyuki Taniguchi
(president of JMARS)












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