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Acrylamide induces accelerated endothelial aging in a human cell model

Cyril Sellier [a], Eric Boulanger [a], François Maladry [a], Frédéric J. Tessier [b], Rodrigo Lorenzi [a], Rémi Nevière [a], Pierre Desreumaux [a], Jean-Baptiste Beuscart [d], François Puisieux [c], Nicolas Grossin [a, *]

[a] Inserm U995/Team "Glycation: From Inflammation to Aging", Lille School of Medicine, Lille University, France
[b] Institut Polytechnique de Lasalle Beauvais, EGEAL Unit, France
[c] Geriatric Department, University Hospital of Lille, France
[d] Department of Biostatistics, EA2694, CERIM, Lille School of Medicine, Lille University, Lille, France

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ABSTRACT

Acrylamide (AAM) has been recently discovered in food as a Maillard reaction product. AAM and glycidamide (GA), its metabolite, have been described as probably carcinogenic to humans. It is widely established that senescence and carcinogenicity are closely related. *In vitro*, endothelial aging is characterized by replicative senescence in which primary cells in culture lose their ability to divide.

Our objective was to assess the effects of AAM and GA on human endothelial cell senescence.

Human umbilical vein endothelial cells (HUVECs) cultured *in vitro* were used as model. HUVECs were cultured over 3 months with AAM or GA (1, 10 or 100 μM) until growth arrest. To analyze senescence, β-galactosidase activity and telomere length of HUVECs were measured by cytometry and semi-quantitative PCR, respectively.

At all tested concentrations, AAM or GA reduced cell population doubling compared to the control condition ($p < 0.001$). β-galactosidase activity in endothelial cells was increased when exposed to AAM (≥10 μM) or GA (≥1 μM) ($p < 0.05$). AAM (≥10 μM) or GA (100 μM) accelerated telomere shortening in HUVECs ($p < 0.05$).

In conclusion, *in vitro* chronic exposure to AAM or GA at low concentrations induces accelerated senescence. This result suggests that an exposure to AAM might contribute to endothelial aging.

1. Introduction

Acrylamide (AAM) is an industrial toxic known for its neurotoxic and reprotoxic effects. AAM is classified as probably carcinogenic to humans (Group 2A) by the International Agency for Research on Cancer (IARC) since 1994. In 2002, AAM was discovered in food as a Maillard reaction product (Tareke et al., 2002). This compound is mainly formed in transformed starchy foods (e.g. French fries, crisps, bread and toast) (Törnqvist, 2005) and roasted coffee (Loaëc et al., 2014), during the process at high temperature. The mean dietary AAM intake by an adult was estimated to be 0.4 $\mu g/kg_{BW}$/day (range: 0.3–5 $\mu g/kg_{BW}$/day) (Parzefall, 2008). After ingestion, AAM is extensively absorbed and distributed to all tissues. AAM is partly metabolized into glycidamide (GA) in the liver (Calleman et al., 1990; Sumner et al., 1999). GA is a reactive epoxide metabolite suspected to be responsible for AAM genotoxic effects. AAM and GA have other impacts including pro-oxidative effect (Clement et al., 2007).

Thus, the discovery of this compound in the diet has stimulated research on this subject by extending it to the assessment of health risk associated to dietary exposure. A large number of epidemiological studies have investigated associations between dietary intake of AAM-containing foods and the incidence of cancer. To

Abbreviations: AAM, acrylamide; C_{12}FDG, D-galactopyranoside; CPD, cumulative population doubling; $DiOC_6$-[3], 3,3′-dihexyloxacarbocyanine iodide; GA, glycidamide; HUVEC, human umbilical vein endothelial cell; PI, propidium iodide; SA-β-gal, senescence-associated β-galactosidase; SIPS, stress-induced premature senescence; TL, telomere length.

* Corresponding author. Inserm U995/Team: Glycation: From Inflammation to Aging, Lille2 University, School of Medicine, 1, Place de Verdun, F-59045 Cedex, France.
E-mail address: nicolas.grossin@univ-lille2.fr (N. Grossin).

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date, such association could not be confirmed and remains open to debate (Pelucchi et al., 2011). Even though studies are mostly carried out to elucidate the link between AAM and cancer, other pathological mechanisms could develop in other specific systems including vasculature.

It is widely established that senescence and carcinogenicity are closely related. Many stressors including oxidative stress and DNA damage are common to the pathways of cell senescence and immortality. These latter are involved in endothelial aging by activating senescence (Chen and Goligorsky, 2006). Stress conditions, i.e. oxidative stress, disturbed flow or hypoxia (Coleman et al., 2013), can accelerate the senescence development in endothelial cells, a process also called stress-induced premature senescence (SIPS). Endothelial aging is associated with endothelial senescence and dysfunction and reduction in the regenerative capacity of endothelial cells (Brandes et al., 2005). Endothelial dysfunction, occurring during the aging process, is described as an independent risk factor for the development of atherosclerosis and hypertension (Heitzer et al., 2001). Lifestyle influences age-associated endothelial dysfunction and may have important implications for the prevention of age-related cardiovascular diseases. A common lifestyle factor that interacts with aging to impair arterial function is the food consumption patterns, such as the western diet (Lesniewski et al., 2013) and some of its components. AAM may be one of these.

To date, the effects of AAM on the vascular system have not been studied. The aim of the study was to evaluate the effects of AAM on cell viability and endothelial aging *in vitro*. To explore this question, we have cultured human umbilical vein endothelial cells (HUVECs) exposed to AAM or GA.

2. Experimental procedures

2.1. Cells viability

2.1.1. Cell culture

HUVECs were isolated from human cord vein by collagenase digestion (0.2%) as described (Jaffe and Nachman, 1975). HUVECs were grown on gelatin (0.2%) in M199 culture medium supplemented with 20% of fetal calf serum (FCS; Dutscher, France).

2.1.2. Metabolic activity assay

HUVECs (1.5×10^4 cells/well) were grown in 96-well plates and exposed to increasing concentrations of AAM or GA for 16, 24, 48 and 72 h. The CellTiter 96® AQ_{ueous} assays (Promega) was used to monitor the metabolic activity at 490 nm.

2.1.3. Apoptosis and necrosis

HUVECs ($3 \cdot 10^5$ cells) were grown in 35 mm Petri dishes and exposed to increasing concentrations of AAM or GA for 16 h. Early apoptosis-associated alterations were evaluated by flow cytometry analysis with the potential-sensitive dye 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$-[3]). For necrosis analysis, propidium iodide (PI) incorporation was measured. After incubation with AAM or GA, cells were stained with $DiOC_6$-(3) (40 nM) and PI (0.5 μg/ml) for 30 min at 37 °C in the dark. Fluorescence was measured by FACSCanto II flow cytometer (Becton Dickinson, France). Results were expressed in terms of the percentage of necrotic and apoptotic cells.

2.2. In vitro endothelial senescence

2.2.1. Cell culture

HUVECs cultured *in vitro* have been used in many studies as a model of vascular endothelium aging because they share many characteristics with the other types of vascular endothelial cells *in vivo* including the development of the senescent phenotype (Kurz et al., 2004). HUVECs were grown on gelatin (0.2%) in M199 culture medium supplemented with 20% of fetal calf serum (FCS; Dutscher, France) + endothelial cell growth supplement (ECGS, 50 μg/ml, Sigma) + sodium heparin (50 μg/ml, Sigma) (Terramani et al., 2000). Cells were grown in control condition or in presence of AAM (1, 10 or 100 μM) or GA (1, 10 or 100 μM) up to spontaneous arrest of proliferation or Hayflick's limit. At each passage, cells were harvested by trypsin treatment at subconfluence to avoid contact inhibition of cell growth. Cells were seeded at each passage in 75 cm^2 flask ($4 \cdot 10^5$ cells per flask). Population doubling (PD) was calculated using the following formula:

PD = ln (harvested cells/seeded cells)/ln2, as described (Hastings et al., 2004).

2.2.2. Senescence-associated β-galactosidase activity

Senescence-associated β-galactosidase (SA-β-gal) activity was evaluated by flow cytometry as described (Debacq-Chainiaux et al., 2009). Briefly, $8 \cdot 10^4$ HUVECs/well were seeded in 12-well plate then cultured for 24 h. HUVECs were incubated for 1 h with bafilomycin A1 (100 nM) to raise lysosomal pH to 6. After incubation, D-galactopyranoside (C_{12}FDG, 33 μM; Life Technologies) was added for 1 h at 37 °C. Cells were then harvested by trypsin digestion, washed in cold PBS and centrifugated (200 g, 5 min). Cells were resuspended in cold PBS in the dark until flow cytometry analysis. SA β-gal activity was expressed as median of fluorescence intensity (MdFI).

2.2.3. Telomere length measurement

Telomere shortening was evaluated by semi-quantitative PCR. DNA from 10^6 HUVECs was extracted. Primers for telomere were: forward: 5′-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′; reverse:5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3′ and 36B4:forward 5′-CAGCAAGTGGGAAGGTGTAATCC-3′, reverse: 5′-CCCATTCTATCATCAACGGGTACAA-3′, as described (Cawthon, 2002). PCR reactions using 2 μL of DNA were carried out in a 20 μL volume using the Light-Cycler FastStart DNA Master SYBR Green kit (Roche Diagnostics). Results are expressed as the loss of telomere (%) per population doubling.

2.3. Statistical analysis

Statistical analyses of SA β-gal activities and telomere shortening were analyzed by the Mann–Whitney *U*-test. Population doubling was compared at each passage using the Wilcoxon test for paired samples. All analyses were done using GraphPad Prism 6.0 for Windows®. Differences were considered as statistically significant for p values <0.05.

3. Results

3.1. Time- and dose-dependent toxicity of AAM and GA on HUVEC viability

In order to determine the concentrations of AAM and GA that exhibit cytotoxic effects on HUVECs, metabolic activity and cell death were analyzed after incubation with different concentrations of AAM or GA. For the concentrations above 1 mM of AAM or GA, the metabolic activity was reduced in a time- and dose-dependent manner (Fig. 1A). In our conditions of experiments, it has not been identified cytotoxic effects for concentrations above 1 mM during 72 h of incubation.

Apoptosis was the main pathway of HUVEC death, regardless of the dose of AAM or GA (Fig. 1B). AAM induced apoptosis more significantly at doses higher than 10 mM ($p < 0.05$). In the same