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Original Research

DNA-Damaging Effect of Cadmium and Protective Action of Quercetin

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Abstract

Cadmium is a widespread environmental and occupational pollutant and quercetin is a dietary flavonoid, which is reported to modulate the effects of many mutagens and carcinogens. We investigated the ability of cadmium chloride to induce DNA damage in human lymphocytes in the presence of quercetin using the alkaline comet assay. Cadmium chloride (5-150 μM) evoked dose-dependent DNA damage and quercetin at 50 μM decreased the extent of the damage. The lymphocytes exposed to cadmium chloride were able to remove their DNA damage within a time period shorter than 120 min. The cells treated with quercetin at 50 μM prior to exposure to cadmium required shorter periods of time to recover. Quercetin could chelate cadmium ions, scavenge free radicals produced by cadmium or regenerate cellular DNA-repair enzymes.

Keywords: cadmium, quercetin, DNA damage, DNA repair, comet assay

Introduction

Cadmium is a common contaminant of hazardous waste sites and is released from sources such as fossil fuel combustion and municipal waste incineration and as a component of cigarette smoke [1]. Estimated human tissue concentrations in the low micromolar range for cadmium have been reported, even in individuals without known exposure, thus confirming the high prevalence of environmental background exposure [2]. Cadmium is absorbed by inhalation and ingestion and has a very long biological half-life (> 25 years). It is classified in group 1 of the International Agency for Research on Cancer categories of carcinogens (carcinogenic to humans) [3]. Epidemiological studies identified lung, prostate and, to a lesser extent, kidney and stomach as primary targets for cadmium-induced tumorigenesis [3]. The ability of cadmium to generate oxidative stress has been well documented [4]. This metal can bind sulhydryl groups in proteins and change the levels of cellular thiols such as glutathione, either directly or through the formation of reactive oxygen species [5, 6]. The formation of reactive oxygen species by cadmium suggests that DNA can also be taken into account as a potential target of this metal.

Humans cannot be considered as a subject of exposure to a single environmental chemical such as cadmium. Instead, the human population is continuously exposed to a variety of environmental agents including mutagens, comutagens and antimutagens. Quercetin (Fig. 1) is one of the most abundant dietary flavonoids present in fruits and vegetables and its average human daily intake in various countries is estimated to be approximately 25 mg [7]. A diet rich in quercetin has been reported to inhibit the development of carcinogen-induced rat mammary cancer [8], colonic neoplasia [9] and oral carcinogenesis [10]. Quercetin may account for the beneficial effects of dietary fruits and vegetables on mutagens and carcinogens, including metals.

Recently we could show the induction of the genotoxic effect of cadmium salts using the single cell gel electrophoresis (comet assay) [11], confirming earlier results obtained in other laboratories with the same tech-

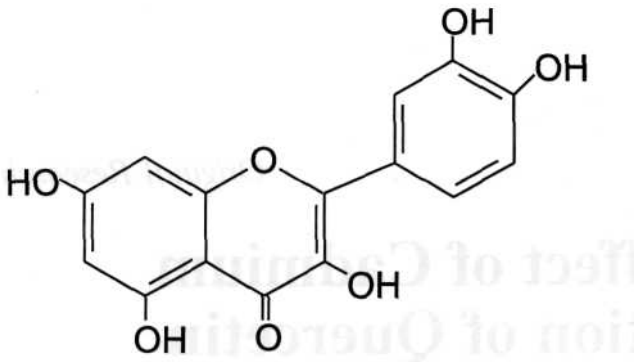

Fig. 1. Chemical structure of quercetin.

nique [12, 13]. The comet assay is a simple and reliable method for identifying agents with genotoxic activity [14] and can also be applied to environmental substances **[15].** Cells with increased DNA breakage show increased migration of the DNA from the nucleus towards the anode and elimination of the lesion as a consequence of DNA repair results in decreased DNA migration. The alkaline version of the assay is capable of detecting DNA single-strand breaks, alkali-labile sites, DNA--DNA/DNA-protein cross-linking and DNA breaks associated with incomplete excision repair sites.

In the present work the ability of cadmium chloride to induce DNA damage in human peripheral blood lymphocytes in the presence of quercetin was investigated using the alkaline comet assay.

Materials and Methods

Chemicals

Cadmium chloride, quercetin (3,3',4',5,7-pentahydroxyflavone) dihydrate, RPMI 1640 medium without L-glutamine, low-melting-point (LMP) and normal-melting (NMP) agarose, phosphate buffered saline (PBS) and DAPI (4;, 6-diamidino-2-phenylindole) were purchased from Sigma Chemicals (St. Louis, MO, USA). Gradisol L was from Polfa (Kutno, Poland). **All** other chemicals at highest purity available were purchased from Sigma Chemicals (St. Louis, MO, USA).

Lymphocyte Isolation

Blood was obtained from young, healthy, non-smoking donors. Peripheral blood lymphocytes were isolated by centrifugation in a density gradient of Gradisol L (15 min, 280g). The viability of the cells was measured by the trypan blue exclusion and was found to be about 99%. Lymphocytes accounted for about 92% of leukocytes in the obtained cell suspension. The final concentration of lymphocytes was adjusted to 1-3 x 10^5 cells/ml by adding RPMI 1640 to the single cell suspension.

Cell Treatment

Cadmium chloride was derived from stock solution and was added to the suspension of lymphocytes to give the desired concentrations. The control cells received only RPMI medium. To examine DNA damage, the lymphocytes were incubated with the drug for 1 h at 37°C. Each experiment included a positive control, which was hydrogen peroxide at 20 μM. H_2O_2 produced pronounced DNA damage, which resulted in comet tail moment of 80-120 μm (results not shown). In experiments with quercetin incubation with cadmium chloride was preceded by the addition of 100 mM quercetin in dimethyl sulfoxide (DMSO) to the suspension of the lymphocytes to give a final concentration of 50 μM. The final concentration of DMSO in the samples was 0.25% and the chemical at this concentration did not influence processes under study (results not presented).

Cell Viability

Cell viability was determined by the trypan blue exclusion analysis. Lymphocytes were incubated with cadmium chloride or quercetin at concentrations from the range 10-200 μM for 1 h at 37°C, washed and resuspended in RPMI 1640. An equal volume of 0.4% trypan blue reagent was added to a cell suspension and the percentage of viable cells was evaluated under a field microscope. Assays were performed in triplicate.

DNA Repair

To examine DNA repair, the lymphocytes after the treatment with cadmium chloride and quercetin, as well as the control samples were washed and resuspended in a fresh RPMI 1640 medium preheated to 37°C. Aliquots of the suspension were taken immediately and at 30, 60 and 120 min later. Placing the samples in an ice bath stopped the repair incubation.

Comet Assay

The comet assay was performed under alkaline conditions essentially according to the procedure of Singh *et al.* [16] with modifications as described previously [17]. A freshly prepared suspension of lymphocytes in 0.75% LMP agarose dissolved in PBS was spread onto microscope slides (Superior, Germany) precoated with 0.5% NMP agarose. The cells were then lysed for 1 h at 4°C in a buffer consisting of 2.5 M NaCl, 100 μM EDTA, 1% Triton X-100, 10 mM Tris, pH 10. After the lysis, the slides were placed in an electrophoresis unit, the DNA was allowed to unwind for 40 min in the electrophoretic solution consisting of 300 mM NaOH, 1 mM EDTA, pH > 13. Electrophoresis was conducted at ambient temperature of 4°C (the temperature of the running buffer did not exceed 12°C) for 30 min at an electric field strength of 0.73 V/cm (30 mA). The slides were then neutralized with 0.4 M Tris, pH 7.5, stained with 2 μg/ml DAPI and

covered with cover slips. To prevent additional DNA damage, all the steps described above were conducted under dimmed light or in the dark.

Comet Analysis

The objects were observed at 200 x magnification in an Eclipse fluorescence microscope (Nikon, Japan) attached to a COHU 4910 video camera (Cohu, San Diego, CA) equipped with a UV-1 filter block (an excitation filter of 359 nm and a barrier filter of 461 nm) and connected to a personal computer-based image analysis system Lucia-Comet v. 4.51 (Laboratory Imaging, Praha, Czech Republic). Fifty images were randomly selected from each sample, and the comet tail moment (a product of fraction of DNA in tail and tail length) was measured. Two parallel tests with aliquots of the same sample of cells were performed for a total of 100 cells, and the mean comet tail moment was calculated. The comet tail moment is positively correlated with the level of DNA breakage and/or alkali labile sites in the cell and is negatively correlated with the level of DNA crosslinks [18]. The mean value of the tail moment in a particular sample was taken as an index of DNA damage in this sample.

Data Analysis

The values in this study were expressed as mean ± SEM from two separate experiments, i.e., data from two experiments were pooled and the statistical parameters were calculated. In the cell viability study all experiments were performed in triplicate. The data were analysed using a Statistica package (StatSoft, Tulsa, OK, USA). If no significant differences between variations were found, as assessed by Snedecor-Fisher test, the differences between means were evaluated by applying the Student *t* test. Otherwise, the Cochran-Cox test was used.

Results

Cell Viability

The results of the viability testing of human lymphocytes after incubation with cadmium chloride or quercetin at various concentrations are displayed in Fig. 2. There was a concentration-dependent decrease in cell viability in the presence of cadmium, but at the highest concentration tested, 150 µM, nearly 90% of the cells remained viable. Quercetin at concentrations up to 200 µM did not change significantly the viability of the lymphocytes.

DNA Damage

Fig. 3 shows the mean comet tail moments for the lymphocytes exposed for 1 h to cadmium chloride in the presence or absence of 50 µM quercetin. It can be seen

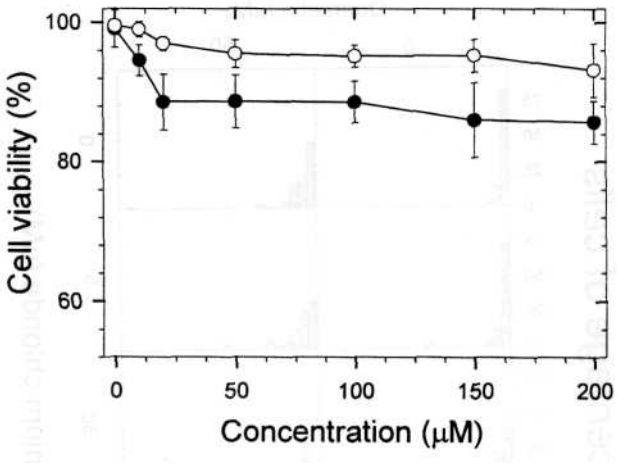

Fig. 2. Effects of cadmium chloride (•) and quercetin (O) on human lymphocytes viability measured by the trypan blue exclusion method. Each point represents the mean of three independent experiments; error bars indicate standard deviation.

from the Figure that cadmium chloride significantly increased the tail moment of the lymphocytes in a dose-dependent manner. At the highest concentration of the chemical, 150 µM, the increase of the tail moment was over four times the initial value (25.18 µm ± 3.89 µm vs. 7.25 µm ± 1.19 µm, $p < 0.001$). Quercetin decreased the tail moment of the lymphocytes exposed to cadmium. The decrease at cadmium chloride concentration 150 µM exceeded 35% ($p < 0.05$). There were not significant differences between values of the comet tail moments of quercetin-treated lymphocytes incubated with cadmium chloride at 5 or 30 µM and control cells.

The most basic way of viewing the data from the comet assay is the distribution of cells according to the percentage of DNA in the tail or to their comet moments [18]. Figure 4 shows the distribution of lymphocytes ac-

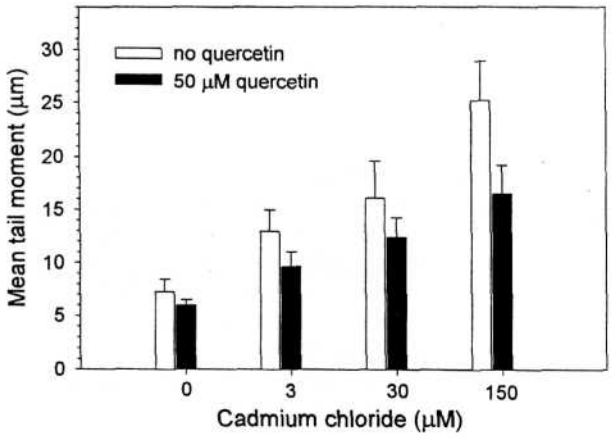

Fig. 3. Mean comet tail moment of human lymphocytes exposed for 1 h at 37°C to cadmium chloride at indicated concentrations in the absence and in the presence of quercetin at 50 µM. The number of cells in each treatment was 100. Error bars denote SEM.

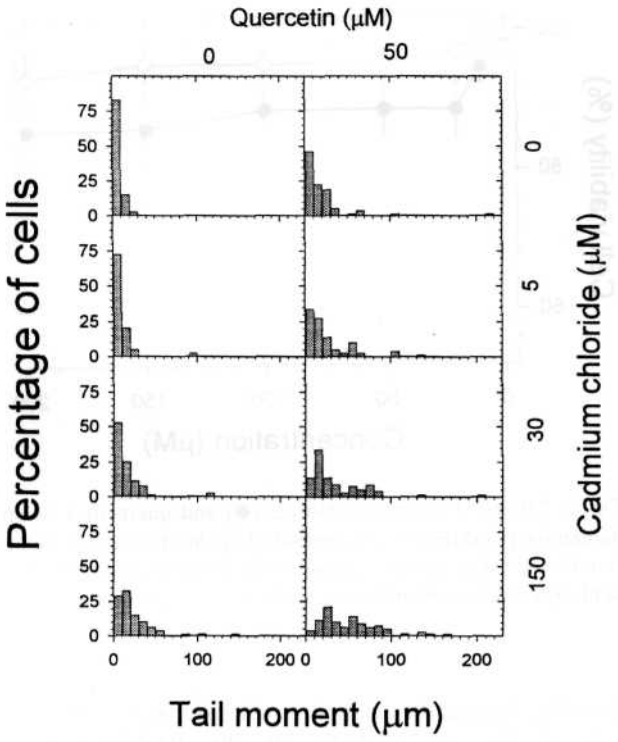

Fig. 4. Histograms of the distribution of comet tail moments in human lymphocytes treated for 1 h at 37° with cadmium chloride at indicated concentrations in the absence or in the presence of 50 μM curcumin. The number of cells scored for each treatment was 100.

cording to their comet tail moments after treatment with cadmium chloride. It can be seen from the Figure that the increasing concentration of $CdCl^2$ caused an increase in the fraction of lymphocytes with greater comet tail moments compared with the unexposed control. These comets contain more DNA in their tails, indicating a greater extent of DNA damage in these cells. The action of quercetin at 50 μM apparently reduced the fraction of lymphocytes with greater tail moment.

DNA Repair

Fig. 5 shows the comet tail moments of the lymphocytes exposed to cadmium chloride immediately after exposure as well as 30, 60 and 120 min thereafter. In all cases the comet tail moment of the control lymphocytes was constant, indicating that preparation and subsequent processing of the lymphocytes did not introduce significant damage to their DNA. The lymphocytes exposed to 20 μM hydrogen peroxide (positive control) were able to recover within the repair incubation time of 60 min (data not shown).

The lymphocytes exposed to cadmium chloride at 30 μM were able to remove their DNA damage within a time period shorter than 60 min (Fig. 5, upper panel). The cells treated with $CdCl_2$ at 150 μM needed about 120 min to recover. The lymphocytes treated with quercetin at 50 μM prior to exposure to cadmium at 30 or 150 μM required shorter periods of time to remove the DNA damage than lymphocytes not treated with quercetin - 30 and 60 min, respectively (Fig. 5, lower panel).

Discussion

The results obtained indicate that cadmium chloride can induce DNA damage in human peripheral blood lymphocytes. This conclusion confirms the results obtained in other laboratories on genotoxicity of cadmium compounds. The main aim of our study was to check whether quercetin, a flavonoid contained in many fruits and vegetables, can modulate the DNA-damaging effect of cadmium. Quercetin decreased the extent of DNA damage evoked by cadmium and increased the effectiveness of the repair of these lesions.

Flavonoids were reported to scavenge free radicals and react with superoxide anion in various experimental systems [19, 20]. Duthie and coworkers, used the comet assay, observed the protective action of quercetin against

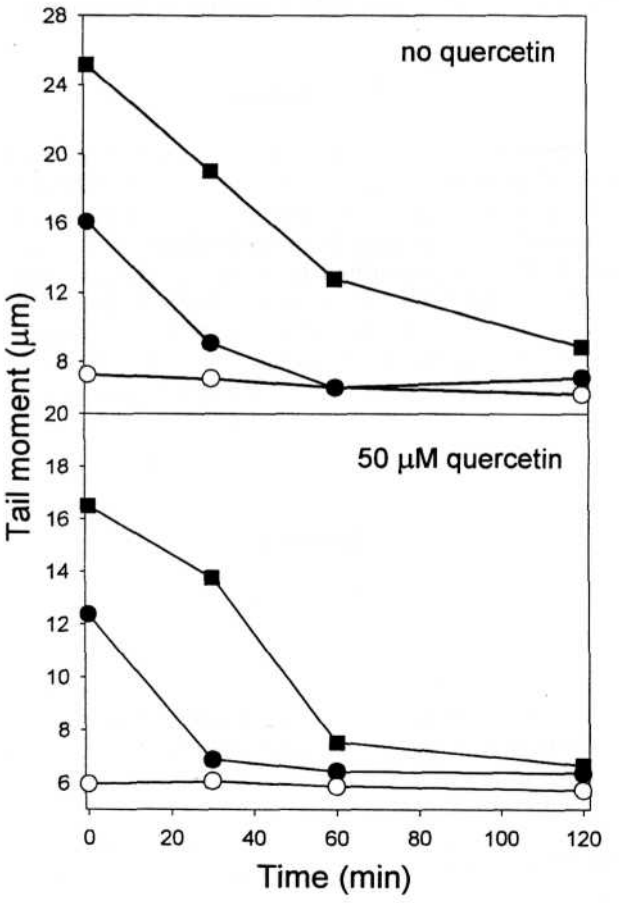

Fig. 5. Time course of the repair of DNA damage in human lymphocytes treated with cadmium chloride at 30 (•) and 150 μM (■) alone (upper panel) and pretreated with quercetin at 50 μM (lower panel) as compared with appropriate controls (O). The number of cells scored for each treatment was 100. Error bars were omitted.

hydrogen peroxide-induced DNA damage in human lymphocytes [21]. The protection in that experiment was probably due to free radical-scavenging efficiency of quercetin, which may be associated with the presence of two hydroxyl groups in the B-ring of its molecule [22]. The presence of polyunsaturated substitution on the B-ring of the molecule of quercetin together with a 2,3 double bond, a free 3-hydroxyl substitution and a 4-keto group confer potent antiperoxidative properties onto the molecule [23].

Quercetin, as other flavonoids, is a metal ion-chelating agent and could have chelated some cadmium ions, preventing them from the interaction with DNA [24]. We showed that the presence of quercetin stimulate DNA repair, so we can speculate that the flavonoid might regenerate DNA repair enzyme in the lymphocytes. However, in a recent study Aherne and O'Brien showed that although quercetin at 50 μM decreased the level of DNA damage induced by hydrogen peroxide, it did not influence the rate of DNA repair in Caco-2, Hep G2 and V79 cells [25]. Furthermore, quercetin was reported to be able to induce DNA damage in various kinds of cells [26, 27]. Therefore, the actual mode of interaction of quercetin with DNA may be complex and depend on many factors, first of all, of the intra- and extracellular environment. The presence of metals, such as cadmium, may additionally complicate the interaction [28].

It was not the purpose of this work to search for the molecular mechanism underlying interaction between quercetin and cadmium. It is well established that fruits and vegetables contain antioxidants such as vitamin E, vitamin C and caretenoids and that these substances may protect against cancer [29]. Quercetin can be the predominant flavonoid in the human diet because it is present in a variety of plant-based foodstuffs and beverages [30]. This should be taken into account when assessment of the true level of the exposure to environmental agents, such as cadmium, is to be made.

Acknowledgement

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