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Hepatoma tissue culture (HTC) cells as a model for investigating the effects of low concentrations of herbicide on cell structure and function

M. Malatesta [a,*], F. Perdoni [b], G. Santin [b], S. Battistelli [c], S. Muller [d], M. Biggiogera [b,e]

[a] Dipartimento di Scienze Morfologico-Biomediche, Sezione di Anatomia e Istologia, University of Verona, Strada Le Grazie, 8, 37134 Verona, Italy
[b] Laboratorio di Biologia Cellulare e Neurobiologia, Dipartimento di Biologia Animale, University of Pavia, Pavia, Italy
[c] Istituto di Istologia e Analisi di Laboratorio, University of Urbino, Urbino (PU), Italy
[d] CNRS, Institut de Biologie Moléculaire et Cellulaire, Immunologie et Chimie Thérapeutiques, Strasbourg, France
[e] Istituto di Genetica Molecolare del CNR (Sezione di Istochimica e Citometria), Pavia, Italy

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ABSTRACT

Previous studies on mice fed genetically modified (GM) soybean demonstrated modifications of the mitochondrial functions and of the transcription/splicing pathways in hepatocytes. The cause(s) of these alterations could not be conclusively established but, since the GM soybean used is tolerant to glyphosate and was treated with the glyphosate-containing herbicide Roundup™, the possibility exists that the effects observed may be due to herbicide residues. In order to verify this hypothesis, we treated HTC cells with 1–10 mM Roundup and analysed cellular features by flow cytometry, fluorescence and electron microscopy.

Under these experimental conditions, the death rate and the general morphology of HTC cells were not affected, as well as most of the cytoplasmic organelles. However, in HTC-treated cells, lysosome density increased and mitochondrial membranes modified indicating a decline in the respiratory activity. Moreover, nuclei underwent morpho-functional modifications suggestive of a decreased transcriptional/splicing activity.

Although we cannot exclude that other factors than the presence of the herbicide residues could be responsible for the cellular modifications described in GM-fed mice, the concordance of the effects induced by low concentrations of Roundup on HTC cells suggests that the presence of Roundup residues could be one of the factors interfering with multiple metabolic pathways.

1. Introduction

Genetically modified (GM) crops, in which new genes have been inserted into the original genome, are nowadays distributed all over the world (Sanvido et al., 2007). Although no direct evidence has been reported so far that GM food may affect human or animal health, the debate is still open (e.g. Paoletti and Pimentel, 2000; Kuiper et al., 2004). In particular, studies on young, adult (2–8 months of age) and old (24 months of age) mice fed GM soybean have demonstrated hepatocyte nuclei modifications involving structural constituents of the transcription/splicing pathways as well as morpho-functional changes in mitochondria (Malatesta et al., 2002a, 2008). The cause(s) of the observed alterations could not be conclusively established but it is worth noting that these modifications disappeared when GM soybean was replaced by a non-GM one in the diet (Malatesta et al., 2005).

The GM soybean used in these studies is tolerant to glyphosate (Padgette et al., 1995) and the plants were consequently treated in the field with the glyphosate-containing herbicide Roundup™; therefore, the possibility exists that the effects observed may be due, at least in part, to herbicide residues. It must be noted that Roundup is almost immediately degraded in the soil, where it gives rise to different metabolites, which not only persist therein (e.g. see Mamy et al., 2008) but that can also be found in the crops (e.g. see Arregui et al., 2004). Unfortunately, the very rapid degradation of Roundup and the heterogeneity of its degradation products make quite problematic their detection even in the crop; consistently, their final amounts in a standard fodder can be under the technical detection limit.

Roundup at high concentrations has been shown to deregulate cell cycle and inhibit transcription (Marc et al., 2002, 2004, 2005), alter cytoskeletal organization (Ovidi et al., 2001), interfere with steroid hormone biosynthesis (Walsh et al., 2000; Richard

Abbreviations: DFC, dense fibrillar component; EDTA, ethylene diamino tetra-acetic acid; FC, fibrillar centre; GC, granular component; GM, genetically modified; HTC, hepatoma tissue culture; NGS, normal goat serum; PBS, phosphate-buffered saline; PG, perichromatin granule.

* Corresponding author. Tel.: +39 045 8027155; fax: +39 045 8027163.
E-mail address: manuela.malatesta@univr.it (M. Malatesta).

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et al., 2005), depress mitochondrial respiratory activity (Peixoto, 2005) and modify liver enzymatic activity (e.g. Hietanen et al., 1983; Jiraungkoorskul et al., 2003).

In an attempt to clarify the potential role of Roundup residues in the occurrence of the cellular modifications found in mice fed glyphosate-tolerant GM soybean, we carried out some experiments on an *in vitro* cell model, which represents a simple, sensitive and reliable system to evaluate pesticide toxicity (El-Demerdash et al., 2001; Bertheussen et al., 1997), provided that its intrinsic limitations in reproducing the complex responsiveness of an organism are taken in the proper account.

Rat hepatoma tissue culture (HTC) cells were incubated with very low concentrations of Roundup for different time periods, with the aim of mimicking the exposure to herbicide traces or residues, and some cellular features were analysed by combining flow cytometry, fluorescence and electron microscopy. The structural and functional modifications induced *in vitro* have been compared with those previously observed *in vivo* on the hepatocytes from mice fed GM soybean.

2. Materials and methods

2.1. Cell culture and treatments

HTC cells (ATCC, Rockville, MD) were grown in Dulbecco's minimal essential medium supplemented with 10% (v/v) fetal calf serum, 1% (w/v) glutamine, 100 U of penicillin and streptomycin (Celbio, Milan, Italy), in a 5% CO_2 humidified atmosphere. Cells were trypsinized when subconfluent and seeded on glass coverslips in multiwell dishes for fluorescence microscopy, or in 25 cm^2 plastic Petri dishes for electron microscopy and flow cytometry.

Twenty-four hours later, cells were treated for 4, 12, 24 or 48 h with Roundup (Monsanto Europe N.V., Antwerp, Belgium), which was dissolved in complete medium at a concentration ranging from 1 to 10 mM. These concentrations were chosen since they are considerably lower than those previously used in the literature (e.g. Peixoto, 2005). The experiments were carried out in triplicate. Whenever not specified, the chemicals used were from Sigma (Buchs, Switzerland).

2.2. Cell viability assay

Viability was assessed by staining both control and Roundup-treated cells with Trypan blue (0.1% in the medium for 2 min). Cells that were permeable to Trypan blue were counted with a hemocytometer and considered as non-viable. Data were expressed as the mean of three independent experiments ± standard deviation.

2.3. Cytometric evaluation of the DNA content

Cells were harvested by mild trypsinization (0.25% (w/v) trypsin containing 0.05% (w/v) ethylene diamino tetraacetic acid (EDTA) in phosphate-buffered saline (PBS)) and stained for 30 min with the DNA-specific dye, propidium iodide (PI; 50 μg/ml in PBS containing 100 U/ml of RNase A, 10 μM EDTA, and 0.015% (v/v) Nonidet P40). To estimate the distribution of cells in the different cycle phases, cytometric measurements were taken with a Partec PAS III cytometer (Partec GmbH, Görlitz, Germany) equipped with an argon ion laser (20 mW output power) at 488 nm, with a 610 nm longpass filter for the PI red fluorescence detection. At least 20,000 cells per sample were measured. The data were handled using FlowMax software from the same company.

2.4. Cell morphometry

Phase contrast micropraphs were taken with a Camedia 5050 digital camera mounted on an Olympys BX51 microscope (Olympus Italia Srl, Milan, Italy). The cellular and nuclear areas of 60 cells per sample were measured using the Image J (NIH) software, and the nucleus to cytoplasm (*N*/*C*) ratio was calculated.

2.5. Organelle immunolabelling

Different immunolabelling procedures for fluorescence microscopy were used to detect microtubules, mitochondria, the Golgi apparatus or lysosomes in cells grown on coverslips and fixed with 4% (v/v) formaldehyde (30 min at room temperature) and 70% (v/v) ethanol in water (30 min at −20 °C). After rehydration with PBS, the samples were incubated with the primary and secondary antibodies reported in Table 1. Microfilaments were also labelled for 60 min at room temperature with an Alexa594-conjugated phalloidin (1:40 in PBS) (Molecular Probes, Invitrogen, Italy). In some experiments, dual-immunolabellings were also performed. All the immunolabelled samples were finally stained for DNA with Hoechst 33258 (0.1 μg/ml in PBS for 10 min), rinsed in PBS, and mounted in Mowiol (Calbiochem, Inalco, Milan, Italy).

To estimate the density of lysosomes in the cytoplasm (number of lysosomes/100 μm^2), micrographs of the immunolabelled cell samples were taken with an Olympus BX51 microscope (see below) and the area of 80 cells from controls or Roundup-treated cells was measured using the software Image J (NIH).

2.6. Conventional and confocal fluorescence microscopy

An Olympus BX51 microscope equipped with a 100W mercury lamp (Olympus Italia Srl, Milan, Italy) was used under the following conditions: 450–480 nm excitation filter (excf), 500 nm dichroic mirror (dm), and 515 nm barrier filter (bf) for Alexa 488 and for JC-1; 540 nm excf, 580 nm dm, and 620 nm bf for Alexa 594; 330–385 nm excf, 400 nm dm, and 420 nm bf, for Hoechst 33258. Images were also recorded with an Olympus Magnifire digital camera system (Olympus Italia Srl, Milan, Italy), and stored on a PC by the Olympus software, for processing and printing.

For confocal laser scanning microscopy, we used a Leica TCS-SP system mounted on a Leica DMIRBE inverted microscope (Leica Microsystems Italia, Milan, Italy); for fluorescence excitation, an Ar/UV laser at 364 nm was used for Hoechst 33258, an Ar/Vis laser at 488 nm for FITC and an He/Ne laser at 543 nm for Alexa 594. Spaced (0.5 μm) optical sections were recorded using a 63× oil immersion objective. Images were collected in the 1024 × 1024 pixel format, stored on a magnetic mass memory and processed by the Leica confocal software.

Table 1
Antibodies used in the immunolabelling experiments

Antibody against	Source	Dilution in PBS	Secondary marker
Alpha-tubulin	Mouse, monoclonal (cell signaling Technology, Danvers, MA, USA)	1:500	Anti-mouse IgG
Mitochondria	Human, autoimmune serum	1:150	Anti-human IgG
Golgi apparatus	Human, autoimmune serum	1:250	Anti-human IgG
Lysosomes	Human, autoimmune serum	1:500	Anti-human IgG

The cells were incubated, at room temperature, for 1–2 h with the primary antibody and for 1 h with the secondary antibodies diluted 1:200 in PBS; the latter were conjugated with either Alexa 488 or Alexa 594.