Ultrastructural Morphometrical and Immunocytochemical Analyses of Hepatocyte Nuclei from Mice Fed on Genetically Modified Soybean

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ABSTRACT. No direct evidence that genetically modified (GM) food may represent a possible danger for health has been reported so far; however, the scientific literature in this field is still quite poor. Therefore, we carried out an ultrastructural morphometrical and immunocytochemical study on hepatocytes from mice fed on GM soybean, in order to investigate eventual modifications of nuclear components of these cells involved in multiple metabolic pathways related to food processing. Our observations demonstrate significant modifications of some nuclear features in GM-fed mice. In particular, GM fed-mice show irregularly shaped nuclei, which generally represents an index of high metabolic rate, and a higher number of nuclear pores, suggestive of intense molecular trafficking. Moreover, the roundish nucleoli of control animals change in more irregular nucleoli with numerous small fibrillar centres and abundant dense fibrillar component in GM-fed mice, modifications typical of increased metabolic rate. Accordingly, nucleoplasmic (snRNP and SC-35) and nucleolar (fibrillarin) splicing factors are more abundant in hepatocyte nuclei of GM-fed than in control mice. In conclusion, our data suggest that GM soybean intake can influence hepatocyte nuclear features in young and adult mice; however, the mechanisms responsible for such alterations remain unknown.

Key words: cell nucleus/liver/genetically modified soybean

Humans have been altering the genome of animals and plants for centuries and selective breeding has been used to produce some desirable characteristics such as yield increase, quality modifications or resistance to diseases. Recently, genetic modification has become the domain of molecular biology and genetic engineering, and genetically modified (GM) organisms have been produced in which new genes have been inserted into the original genome. In particular, genetic engineering has been widely applied in agriculture, thus creating GM crops which are nowadays distributed all over the world.

No direct evidence that GM food may represent a possible danger for health has been reported so far; however, the scientific literature in this field is still quite poor (Schubbert et al., 1994, 1997, 1998; Ewen and Pustzai, 1999; Chiter et al., 2000; Edwards et al., 2000; Halford and Shewry, 2000), especially as to the possible effect of a diet involving a significant amount of GM plants.

The liver is a primary site for biotransformation of the products of digestion and is strategically located between the intestinal tract and the general circulation. Moreover, it degrades and detoxifies toxic compounds received from the intestines or from the general circulation and excretes them in the bile. Finally, it synthesizes many protein components of blood plasma and exercises an important degree of control over the general metabolism. Therefore, hepatocytes
represent a useful model for monitoring one of the targets of the diet.

In the present study, we carried out ultrastructural morphometrical and immunocytochemical analyses on hepatocyte nuclei from mice fed on GM soybean, in order to investigate eventual modifications of nucleoplasmic and nucleolar constituents. Such nuclear components represent the structural counterpart of transcription and processing of messenger and ribosomal RNAs and therefore constitute fine and highly sensitive indicators of cellular activity.

**Materials and Methods**

**Animals and tissue processing**

Pregnant Swiss mice were fed ad libitum on a standard laboratory chow (Mulino & Frantoio del Trasimeno, Castiglione del Lago, PG, Italy) containing wheat, barley, maize, alfa alfa, skimmed milk, minerals and 14% GM soybean obtained by the insertion of the bacterial CP4 EPSPS (5-enolpyruvylshikimate-3-phosphate synthase, an enzyme obtained from Agrobacterium sp. strain CP4) gene conferring a high level of tolerance to glyphosate, the active ingredient of the herbicide Roundup (Padgette et al., 1995). In parallel, other pregnant mice were fed on the same diet with wild soybean. The respective litters were allowed to grow in standard cages (four animals each) under constant environmental conditions (21±1°C, 50±5% moisture, 12L:12D daylight cycle) for different periods on the parental diet. Both animal groups started their respective diets at weaning. Twenty-four female mice from the litter were used: twelve of them were control animals while twelve ate GM food. The mice were weighed and then sacrificed by cervical dislocation when 1, 2, 5 or 8 months old. The liver was quickly removed, weighed and cut in small fragments. For conventional ultrastructural morphology samples of liver from the right lobe were fixed by immersion in a mixture of 2.5% glutaraldehyde and 0.1M Sörensen phosphate buffer, pH 7.4 for 3 h, washed, post-fixed with 1% OsO₄, and 1.5% potassium ferrocyanide at 4°C for 1 h, dehydrated with acetone and embedded in Epon. For morphometrical and immunocytochemical studies on cell nuclei other samples of liver from the same lobe were fixed with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1M Sörensen buffer at 4°C for 2 h. After washing in the same buffer and in phosphate buffered saline (PBS), free aldehydes were blocked in 0.5 M NH₄Cl in PBS for 45 min at 4°C. Following washing in PBS, the specimens were dehydrated through graded concentrations of ethanol and embedded in LRWhite resin polymerised with U.V. light. Semithin sections (2 µm in thickness) were stained with uranyl acetate and lead citrate, while LRWhite-embedded samples were conventionally contrasted with uranyl acetate and lead citrate, while LRWhite-embedded samples were stained with the EDTA method (Bernhard, 1969), which reduces chromatin contrast, thus revealing ribonucleoprotein (RNP) constituents. The specimens were observed in a Philips EM 300 electron microscope operating at 80 kV.

**Morphometry**

Morphometrical analyses were performed both at light and electron microscopic level on LRWhite-embedded samples. Semithin sections of liver were photographed (final print magnification x400) by the Leitz Orthoplan light microscope and 100 hepatocytes per each animal were considered. By using a computerised image analysis system (Image Pro-Plus for Windows 95), nuclear and cellular areas were measured; this allowed us to calculate the nucleus/cytoplasm (N/C) ratio. In order to evaluate quantitatively the fine nuclear features, further morphometrical analyses were performed on a total of 200 randomly selected electron micrographs (final magnification x18,000) of hepatocyte nuclei (10 micrographs from each animal). Areas and perimeters of nuclei were measured to compute an index of nuclear shape irregularity, expressed as the ratio between the perimeter and the circumference of the equivalent circle (P=2πr, where P is the observed perimeter, r is the radius of equivalent circle having the same area A; thus r=A/π). Moreover, areas of nucleoli as well as of each nucleolar component – fibrillar centres (FCs), dense fibrillar component (DFC) and granular component (GC) – were measured and the percentage of FC, DFC and GC area per nucleolus was calculated. Finally, the nuclear pores (NPs) were counted and their density expressed as the ratio between their number and the nuclear membrane length (NP/µm).

For each biological variable a two-way ANOVA test (with age and food factors) was performed. The ANOVA models included an interaction term between the factors. A correction term for multi-level design was introduced to take into account the fact that cellular data from different animals were pooled. When necessary, data were transformed to achieve either normalisation or variance stabilisation, as appropriate. Significance level was fixed at α=0.05.

**Immunocytochemistry**

In order to investigate the fine intranuclear distribution of some splicing factors in GM-fed and control mice, samples of LRWhite-embedded liver were processed for immunocytochemistry. Since our ultrastructural observations have revealed similar nuclear features among control animals as well as among the GM-fed mice regardless of age, we carried out the immunocytochemical study on 5 month-old animals, as a sample. Mouse monoclonal antibodies directed against the (Sm)snRNP (small nuclear ribonucleoprotein) core protein (Lerner et al., 1981), the non-snRNP splicing factor SC-35 (Sigma-Aldrich, Buchs, Switzerland) and the nucleolar protein fibrillarin (Cytoskeleton Inc., Denver, CO) were used. Sections were floated for 3 min on normal goat serum (NGS) diluted 1:100 in PBS and then incubated for 17 h at 4°C with the primary antibodies diluted with PBS containing 0.1% bovine serum albumin (Fluka, Buchs, Switzerland) and 0.05% Tween 20. After rinsing, sections were floated on NGS, and then reacted for 20 min at room temperature with the secondary gold-conjugated antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) diluted 1:10 in PBS. Finally, the sections were rinsed and...
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air-dried. As controls, some grids were treated with the incubation mixture without the primary antibody, and then processed as described above.

In order to assess the presence of the splicing factors quantitatively, the labelling density over some nuclear compartments was evaluated on sections treated in the same immunolabelling experiment. The surface area of each compartment considered – nucleoplasm, nucleolus and, in the case of the anti-fibrillarin antibody, DFC – was measured on fifteen randomly selected electron micrographs (x20,000) from each animal by using a computerised image analysis system (Image Pro-Plus for Windows 95). For background evaluation resin outside the tissue was considered. The gold grains present over the investigated compartments were counted and the labelling density was expressed as the number of gold grains per square micrometer.

The data for each variable were then pooled according to the experimental groups and the mean standard error of the mean (SE) values calculated. Statistical comparisons were performed by the Kruskal-Wallis one-way ANOVA test. Statistical significance was set at P≤0.05.

Biochemistry

At the time of sacrifice some liver samples from the right lobe were quickly removed into ice-cold homogenisation buffer (in mM): 280 mannitol, 10 KCl, 1 MgCl₂, 0.2 Pefabloc SC, 10 Hepes, pH 7.0 adjusted with Tris (Thevenod et al., 1999). The tissue was minced into a fine paste and homogenised manually. The crude homogenate was centrifuged at 10,000 rpm for 10 min in a Microfuge 18 Centrifuge (Beckman Coulter, Inc.), total protein content was determined according to Bradford (1976) and aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactic dehydrogenase (LDH) and gamma-glutamyltranspeptidase (GGT) activities were evaluated enzymatically by means of a Vitros System Chemistry 950 (Ortho-Clinical Diagnostics, Johnson & Johnson Co.).

Statistical comparisons were performed by the non-parametric Mann-Whitney U-test and the significance level was set at P≤0.05.

Results

Body weight of mice at the time of sacrifice ranged from 26 to 38 g without significant differences between control and GM soybean-fed animals; moreover, no macroscopic modifications of liver was observed, its weight ranging from 0.8 g to 1.9 g in all animals.

Electron microscopic examination of Epon-embedded liver samples demonstrated similar structural features of hepatocyte cytoplasmic organelles in control and GM soybean-fed mice (Fig. 1) at all ages considered: the RER was arranged in irregularly oriented cisternae; the Golgi apparata were well developed; mitochondria exhibited ovoid shapes and well-developed transversal cristae;

Fig. 1. Hepatocyte cytoplasm from control and GM soybean fed mice. In both control (a) and GM-fed (b) mice the mitochondria (M) show ovoid shapes with transversal cristae, Golgi apparata (arrows) are well developed and RER cisternae (thick arrows) are irregularly arranged. G: glycogen; L: lipid droplets. Bars: 0.5 µm.
glycogen particles were numerous and mostly gathered in lakes, sometimes in association with lipid droplets.

On the other hand, the ultrastructural observations carried out on LRWhite-embedded EDTA-stained samples revealed modifications of some nuclear features of hepatocytes from mice fed on GM soybean in comparison to control animals. Hepatocyte nuclei from both 1, 2, 5 and 8 month-old control mice (Fig. 2a) generally showed roundish shapes and contained clumps of condensed chromatin distributed both at the nuclear periphery and inside the nucleus. In the nucleoplasm, abundant perichromatin fibrils (PFs) and perichromatin granules (PGs) were distributed along the borders of the condensed chromatin, while interchromatin granule (IG) clusters occurred in the interchromatin space.

Fig. 2. Hepatocyte nuclei from control and GM soybean fed mice. Note the nuclear irregular shape of the nucleus from the GM-fed mouse (b) in comparison to the nuclear roundish shape of the control animal (a). C: condensed chromatin; Nu: nucleolus; IG: interchromatin granules; thick arrows: perichromatin granules; arrows: perichromatin fibrils. Bars: 1 µm.

Fig. 3. Hepatocyte nucleoli from control and GM soybean fed mice. The nucleolus of the control animal (a) shows large fibrillar centres (asterisks) surrounded by dense fibrillar component (thick arrows), while the nucleolus of the GM-fed mouse (b) displays small fibrillar centres (asterisks) and abundant dense fibrillar component (thick arrows). Bars: 0.5 µm.
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The nucleioli generally displayed roundish shapes, with some FCs surrounded by DFC and abundant GC (Fig. 3a). Hepatocyte nuclei from 1 month-old mice fed on GM soybean showed roundish shapes similar to those of control animals (not shown), whereas hepatocyte nuclei from 2, 5 and 8 month-old mice fed on GM soybean (Fig. 2b) frequently showed irregular shapes. This nuclear shape irregularity appeared as a fine waving and was not due to cytoplasmic inclusions distorting the nuclear surface. Moreover, GM-fed mice showed irregular and less compact nucleioli, with many small FCs and abundant DFC (Fig. 3b). On the other hand, the nucleoplasmic components did not show any evident modifications.

Morphometric results are described in Table I. In detail, the nuclear area was generally larger in control than in GM-fed mice; the N/C ratio was lower in 8 month-old animals in comparison to younger ones; the shape index was generally higher in GM-fed than in control mice. The nucleolar area did not change; FC area and GC percentage were generally lower in GM-fed mice than in controls, while the DFC percentage was always higher in GM-fed mice. The FC percentage drastically decreased in older animals with respect to younger ones. Finally, the nuclear pore density was always higher in GM-fed mice than in controls.

Immunocytochemical analysis demonstrated that no difference in snRNP, SC-35 and fibrillarin distribution occurred between GM-fed and control mice. As expected, snRNPs were mainly associated to PFs and, to lesser extent, to IGs, (Fig. 4a), moreover, a few snRNPs were found in nucleioli; SC-35 was specifically associated to PFs and IGs (Fig. 4b); fibrillarin accumulated in nucleolar DFC (Fig. 4c). However, quantitative evaluation of immunolabelling revealed a stronger labelling in GM-fed mice in comparison to controls for all splicing factors investigated (Table II). The background level was 1.02±0.11 gold grains/µm².

Biochemical analyses of AST, ALT, LDH and GGT activities revealed no difference between control and GM-fed mice at all ages considered (Table III).

Discussion

Our observations carried out on hepatocyte nuclei from control and GM soybean-fed mice demonstrate significant modifications of some nuclear features in all GM-fed mice, whereas cytoplasmic organelles do not show any evident alteration.

Modifications of hepatocyte nuclear size are related to both age and food. It is known that cell nuclei become progressively larger as age increases (Schmucker, 1990), but, in our animals, they are also generally larger in control than in GM soybean fed mice. However, the differences in nuclear size between control and GM-fed mice do not imply differences in N/C ratio; conversely, N/C ratio changes are related to the age. In fact, 8 month-old mice show a lower N/C ratio value, probably due to the increased deposition of lipids and glycogen in hepatocyte cytoplasm observed at this age (not shown).

The nuclear shape also is influenced by both age and food. In fact, except for 1 month-old animals, all GM fed-mice showed irregularly shaped nuclei, while control animals generally showed roundish nuclei. An irregular nuclear shape generally represents an index of high metabolic rate (e.g. Aziz and Barathur, 1994; Motohashi et al., 1992; Malatesta et al., 1998); in fact, an increase in the nuclear-cytoplasmic interface may improve the molecular trafficking between the two cellular compartments. Accordingly, an increased nuclear pore frequency has been found in the irregularly shaped nuclei of GM fed-mice in comparison to the roundish nuclei of control animals.

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...tions in GM soybean fed-mice, although their size remains unchanged. In fact, the roundish nucleoli of control animals change in more irregular nucleoli with numerous small FCs and abundant DFC in GM-fed mice. The nucleolus is the site of ribosomal gene transcription and of rRNA processing and assembly with ribosomal proteins (reviews in Smetana and Busch, 1974; Hadjiolov, 1985). The nucleolus is a very dynamic structure that can rapidly adapt its activity, and consequently its architecture, to the cellular metabolic state (for reviews see e.g. Hadjiolov, 1985; Schwarzacher and Wachtler, 1993; Shaw and Jordan, 1995). In particular, it is known that when the metabolic rate increases the number of small FCs as well as the amount of DFC increase (Jordan and McGovern, 1981; Lafarga et al., 1991; Schwarzacher and Wachtler, 1993; Dzidziguri et al., 1994). Interestingly, in our animals the modifications of FC size as well as of DFC and GC amounts are related to food only.

Fig. 4. Hepatocyte nuclei immunolabelled with antibodies against splicing factors. a. The anti-snRNP antibody specifically labels perichromatin fibrils (arrows) and, in a lesser extent, interchromatin granules (IG), while perichromatin granules (thick arrows) are devoid of gold grains. b. The anti-SC-35 antibody specifically labels perichromatin fibrils (arrows) and interchromatin granules (IG), whereas perichromatin granules (thick arrows) appear unlabelled. c. The anti-fibrillarin signal is located in the dense fibrillar component (asterisks) of nucleoli. Bars: 0.2 µm.

Taken together, our morphometrical results suggest that hepatocyte nuclei of mice fed on GM soybean modify their metabolic activities. According to such hypothesis, the nucleoplasmic and nucleolar splicing factors investigated in our study – snRNPs, involved in early pre-mRNA splicing (review in Luhrmann et al., 1990); SC-35, required for spliceosome assembly (Fu and Maniatis, 1990) and fibrillarin, a component of the U3 snRNP complex, involved in several steps of rRNA processing (Kass et al., 1990) – are more abundant in GM-fed mice than in controls. On the other hand, biochemical analyses of major hepatic proteins did
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Table II. QUANTITATIVE EVALUATION OF SPLICING FACTOR IMMUNOLABELING IN CONTROL AND GM-FED MICE

<table>
<thead>
<tr>
<th></th>
<th>Nucleolus (gold grains/µm²)</th>
<th>Nucleolus (gold grains/µm²)</th>
<th>DFC (gold grains/µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Sm)snRNP</td>
<td>2.24±0.25 (C)</td>
<td>1.02±0.23 (C)</td>
<td>-</td>
</tr>
<tr>
<td>SC-35</td>
<td>3.11±1.08 (C)</td>
<td>0.97±0.10* (C)</td>
<td>-</td>
</tr>
<tr>
<td>Fibrillarin</td>
<td>0.86±0.09* (C)</td>
<td>9.94±0.75 (C)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.73±0.08*</td>
<td>19.29±1.10</td>
<td>74.53±4.91</td>
</tr>
</tbody>
</table>

Means±SE values of labelling densities obtained with anti-(Sm)snRNP, anti-SC-35 and anti-fibrillarin antibodies on hepatocyte nuclei from GM-fed and control 5 month-old mice. Values identified with common symbols (*, †) are not significantly different. C: control animals.

Table III. BIOCHEMICAL EVALUATION OF SOME LIVER ENZYMES IN CONTROL AND GM-FED MICE

<table>
<thead>
<tr>
<th>Age</th>
<th>AST (U/g proteins)</th>
<th>ALT (U/g proteins)</th>
<th>LDH (U/g proteins)</th>
<th>GGT (U/g proteins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 month</td>
<td>1952.7±101.3</td>
<td>961.2±174.4</td>
<td>7964.0±683.4</td>
<td>37.3±5.7</td>
</tr>
<tr>
<td>1 month</td>
<td>2019.9±237.3</td>
<td>1027.0±296.2</td>
<td>8650.2±736.7</td>
<td>41.6±4.5</td>
</tr>
<tr>
<td>2 months</td>
<td>2050.2±322.1</td>
<td>811.0±127.3</td>
<td>6975.3±875.2</td>
<td>39.6±6.7</td>
</tr>
<tr>
<td>2 months</td>
<td>1861.7±40.8</td>
<td>791.7±56.7</td>
<td>7727.3±432.8</td>
<td>40.3±2.1</td>
</tr>
<tr>
<td>5 months</td>
<td>2054.6±143.9</td>
<td>815.3±70.8</td>
<td>6749.7±206.7</td>
<td>35.4±3.7</td>
</tr>
<tr>
<td>5 months</td>
<td>1985.7±187.2</td>
<td>727.7±76.6</td>
<td>7185.4±165.5</td>
<td>40.2±3.8</td>
</tr>
<tr>
<td>8 months</td>
<td>2240.3±264.1</td>
<td>832.7±84.1</td>
<td>7338.7±462.1</td>
<td>42.0±5.8</td>
</tr>
<tr>
<td>8 months</td>
<td>2106.7±46.9</td>
<td>819.5±40.6</td>
<td>8086.0±182.5</td>
<td>38.5±7.1</td>
</tr>
</tbody>
</table>

Enzyme levels in liver tissue of the eight groups of animals (mean values±SE). Values in each column are not significantly different from each other. C: control animals.

References

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