Differential response related to genotoxicity in multiple organs of cirrhotic rats

Marcia R. Nagaoka · Luciana Le Sueur-Maluf ·
Odair Aguiar Jr · Glaucia M. Castro ·
Regina C. Spadari-Bratfisch · Daniel A. Ribeiro

Received: 19 October 2009 / Accepted: 16 December 2010 / Published online: 6 January 2011
© Asian Pacific Association for the Study of the Liver 2011

Abstract

Purpose The aim of this study was to use the single cell gel (comet) assay to investigate whether blood, liver, heart, kidney, and brain are particularly sensitive organs for DNA damage in cirrhotic rats to predict genetic instability induced by cirrhosis.

Methods A total of 16 male Wistar rats (negative control, $n = 8$; experimental, $n = 8$) were submitted to bile duct ligation during 28 days.

Results Cirrhosis was able to induce genetic damage in liver and brain cells, as depicted by the mean tail moment. No genetic damage was induced in blood, heart, or kidney cells (i.e., no significant statistically differences were noticed when compared with negative control).

Conclusions In conclusion, our results suggest that cirrhosis could contribute to DNA damage in liver and brain cells.

Keywords Cirrhosis · Rats · DNA damage · Single cell gel (comet) assay

Introduction

The classical picture of acute inflammation and liver damage is hepatitis caused by different agents. Hepatic fibrosis is the common endpoint for most types of chronic liver injury and is considered an irreversible process, especially when the complete picture of cirrhosis is present

[1]. To date, several agents are involved in the etiopathogenesis of liver disease. Among them are viruses, alcohol abuse, insulin resistance, obesity, diabetes mellitus, hypertriglyceridemia, and/or obstruction of the biliary tree for several weeks or months [1].

Current attention has been directed to persistent oxidative and nitrosative stress as well as excess lipid peroxidation, which are induced by inflammatory processes, impaired metal transport, or dietary imbalance, causing accumulation of massive DNA damage together with deregulation of cell homeostasis [2]. As these events appear to play an important role in human chronic disease pathogenesis, DNA damage caused by radical oxygen species, radical nitrosative species, or lipid peroxidation end products provides promising markers for risk prediction and targets for preventive measures [3]. Therefore, patients with chronic degenerative processes, such as cirrhosis, are potential targets for the establishment of some diseases, as in the process of tumorigenesis.

It has been established that DNA damage and subsequent cell proliferation can establish genetic instability through multiple pathways [4]. Accumulation of such abnormalities in the genome is associated with cancer initiation and/or progression [5]. However, little is known about the genotoxicity caused by cirrhosis in rats, especially the relationship between cirrhosis and the systemic host response in different tissues and organs. Particularly, we have no information regarding whether preferential or selective responses occur in different organs during the establishment of cirrhosis.

To date, a variety of assays have been proposed to detect genetic instability, including those that assess metaphase chromosomal aberrations, sister chromatid exchanges, and host cell reactivation. However, these methods are typically laborious and time-consuming or require highly trained

M. R. Nagaoka · L. Le Sueur-Maluf · O. Aguiar Jr ·
G. M. Castro · R. C. Spadari-Bratfisch · D. A. Ribeiro (✉)
Departamento de Biociências, Universidade Federal de São
Paulo, UNIFESP, Santos, SP, Brazil
e-mail: daribeiro@unifesp.br; daribeiro@pesquisador.cnpq.br

technicians to accurately read and interpret slides [6]. A great deal of enthusiasm has been raised by the application of the single cell gel (comet) assay to mammalian cells [7]. In the alkaline version, the assay is a rapid, simple, and reliable biochemical method for evaluating DNA damage in mammalian cells [7]. This technique includes embedding cells in agarose gel on microscope slides and lysing the cells with detergent and high salt concentrations. During electrophoresis under alkaline conditions, cells with damaged DNA display increased DNA migration toward the anode as a result of DNA strand breaks, alkali-labile lesions (including abasic sites), and incomplete repair sites [8]. Broken DNA migrates farther in the electric field and the cell resembles a “comet” with a brightly fluorescent head and a tail region [9]. The extent of the comet is related to the extent of DNA damage. These images can be analyzed and compared on a cell-to-cell basis. Previous studies conducted by our group have demonstrated that the comet assay is an useful tool for detecting DNA breakage in multiple organs induced by some pathological conditions, such as experimental carcinogenesis, parasitic infections, or following exposure to some xenobiotics present in the environment *in vivo* [10–14]. In this study, we used the comet assay to predict genetic instability in multiple organs induced by experimental cirrhosis. Such data will certainly contribute to a better understanding of the pathogenesis of cirrhosis at different cellular levels.

Materials and methods

Animals and experimental design

All the experimental procedures were conducted according to the National Institutes of Health guidelines for the use and care of animals, and the study protocol was approved by the Ethics in Research Committee of the Federal University of Sao Paulo, School of Medicine. Male Wistar rats weighing approximately 250 g were obtained from the Federal University of Sao Paulo, UNIFESP, Brazil, and maintained under controlled conditions of temperature ($23 \pm 2^\circ\text{C}$) and light–dark periods of 12 h with free access to commercial diet (Purina) and water. Biliary cirrhosis in rats was induced by bile duct ligation. Details of the surgery and postsurgical care have been previously reported [15]. The surgical procedure was performed in eight rats. A total of eight age-matched animals served as a negative control.

Sample collection

At the end of the experimental period (28 days), the rats were sacrificed with an overdose of ketamine and xylazine.

Blood samples were collected from the heart into a lightly heparinized syringe. In addition, central fragments from heart, liver, right/left kidneys, and brain were collected and minced in 0.9% NaCl. The supernatant was removed and the cellular suspensions (approximately 10 μl) were used for the comet assay.

Histopathological analysis

To confirm the cirrhotic state, liver fragments from control and experimental rats were collected for histopathological examinations. The samples were fixed in 10% buffered formalin (Merck, Darmstadt, Germany) and embedded in paraplast (Histosec, Merck). Sections of 5 μm thickness were stained with hematoxylin and eosin (H.E., Merck) and observed with a light microscope (CME Leica).

Single cell gel (comet) assay

The protocol used for peripheral blood, liver, heart, kidney, and brain cells followed the guidelines proposed by Sasaki et al. [16] with some modifications. Namely, a volume of 5 μl of peripheral blood was added to 120 μl 0.5% low-melting-point agarose at 37°C , layered onto a pre-coated slide with 1.5% regular agarose, and covered with a coverslip. The supernatant (cellular suspension) (10 μl) of the liver, heart, right/left kidneys, and brain were added to 120 μl 0.5% low-melting-point agarose at 37°C , layered onto a pre-coated slide with 1.5% regular agarose and covered with a coverslip. After brief agarose solidification in the refrigerator, the coverslip was removed and slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl buffer, pH 10, 1% sodium sarcosinate with 1% Triton X-100, and 10% DMSO), for approximately 1 h. Before electrophoresis, slides were left in alkaline buffer (pH > 13) for 20 min and electrophoresed for another 20 min at 0.7 V/cm, 300 mA. After electrophoresis, the slides were neutralized onto 0.4 M Tris–HCl (pH 7.5), fixed in absolute ethanol, and stored until analysis in a fluorescent microscope at 400 $\times$ magnification.

Independent positive controls using cells from peripheral blood, liver, kidneys, brain, and heart were treated *in vitro* with 10 $\mu\text{g}/\text{ml}$ methylmethasulfonate (MMS) for 30 min at 37°C to ensure the reproducibility and sensitivity of the assay.

Genotoxicity data analysis

A total of 50 randomly captured comets per animal (25 cells from each slide) [17] were examined blindly by one expert observer (DAR) at 400 $\times$ magnification using a fluorescence microscope (Olympus) connected through a

black and white camera to an image analysis system (Comet Assay II, Perceptive Instruments, Suffolk, Haverhill, UK) calibrated previously according to the manufacturer's instructions. The computerized image analysis system acquires images, computes the integrated intensity profiles for each cell, estimates the comet cell components, and then evaluates the range of derived parameters. Undamaged cells have an intact nucleus without a tail and damaged cells have the appearance of a comet. To measure DNA damage, two image analysis system parameters were considered: tail intensity (% migrated DNA) and tail moment (the product of the tail length and the fraction of DNA in the comet tail) [17]. Because none of the groups showed significant differences between these parameters, we chose to present the results in terms of tail moment.

Statistical methods

The results obtained in the comet assay were evaluated statistically using the Kruskal–Wallis non-parametric test followed by the post-hoc Dunn's test. A statistical comparison between the negative control and biliary cirrhosis groups was also made with the Mann–Whitney non-parametric test using Sigma Stat for Windows (Jadel Scientific, USA). The level of significance was set at 5%.

Results

In the negative control group, liver had a normal appearance and no remarkable differences were observed among specimens at macroscopic or histopathologic levels (Fig. 1a). However, livers from experimental rats were green in color with a nodular and fibrotic appearance at macroscopic analysis. Under microscopic evaluation, livers displayed intense disorganization of the lobular architecture, characterized by extensive duct proliferation, disarrangement and destruction of hepatocyte cords, and fibrosis. Inflammatory infiltrate and apoptotic cells were also observed (Fig. 1b–d). Taken together, these findings supported the final diagnosis of experimental biliary cirrhosis.

In this study, we were able to evaluate genetic damage induced by cirrhosis in vivo. No statistically significant differences ($P > 0.05$) were found in blood cells of cirrhotic rats when compared with negative control rats (Fig. 2). Statistically significant differences were also absent from other organs evaluated, such as heart and kidney, which displayed no DNA migration (Figs. 3, 4). However, brain cells indicated strong genotoxicity when compared with negative controls (Fig. 5). As expected, liver also showed genotoxicity induced by experimental cirrhosis, although the effect was less pronounced when

Fig. 1 Photomicrographs of rat liver: a control liver with ordinary appearance (a) and a biliary cirrhotic liver showing disorganization of the lobular parenchyma (b). Note the extensive ductular proliferation, especially at the portal area. High magnification of cirrhotic liver reveals proliferating bile ducts (asterisks) and apoptotic cells (arrowheads) (c, d). (H & E, $\times 10$ (a, b), $\times 40$ (c, d))

Fig. 2 DNA damage expressed as mean tail moment in blood cells of cirrhotic rats. Values are expressed as Mean $\pm$ SD. * $P = 0.01$ when compared with negative control

Fig. 3 DNA damage expressed as mean tail moment in heart cells of cirrhotic rats. Values are expressed as Mean $\pm$ SD. * $P = 0.01$ when compared with negative control

compared with brain cells. These results are depicted in Fig. 6.

Rat blood, liver, brain, kidney, and heart cells were further assayed with MMS to ensure the sensitivity of the assay. A clear sensitivity was observed ($P < 0.05$) when compared with the negative control group. No animals died throughout this study. Figure 7 shows an undamaged rat blood cell represented by negative control, a blood cell from a rat with liver disease, and an MMS-induced comet cell (positive control).

Discussion

The aim of this study was to evaluate genetic damage induced in different target organs by experimental

Fig. 4 DNA damage expressed as mean tail moment in kidney cells of cirrhotic rats. Values are expressed as Mean $\pm$ SD. * $P = 0.01$ when compared with negative control

Fig. 5 DNA damage expressed as mean tail moment in brain cells of cirrhotic rats. Values are expressed as Mean $\pm$ SD. * $P = 0.01$ when compared with negative control

Fig. 6 DNA damage expressed as mean tail moment in liver cells of cirrhotic rats. Values are expressed as Mean $\pm$ SD. * $P = 0.05$, ** $P = 0.01$ when compared with negative control

Fig. 7 Representative comet images from negative control (a); a blood cell from a rat with liver disease (b); and an MMS-treated cell (positive control) (c) (DNA stained with ethidium bromide; $\times 40$)

cirrhosis. The investigation was conducted using the single cell gel (comet) assay. To the best of our knowledge, this approach has not been demonstrated so far.

The alkaline version of the comet assay used is sensitive for a wide variety of DNA lesions. Among them are single- and double-strand breaks, oxidative DNA base damage, alkali-labile sites (including abasic and incomplete repair sites), and DNA–DNA/DNA–protein/DNA–drug cross-linking in any eukaryotic cell [7]. Tail moment is a virtual measure, calculated by the computerized image analysis system, which takes into account both the length of DNA migration in the comet tail and the tail intensity. Among the various parameters calculated by this method, tail moment is one of the best indices of induced DNA damage.

On the basis of tail moment data, the results of this study demonstrated that the comet assay in the experimental conditions used failed to detect the presence of DNA damage in blood cells in cirrhotic rats. These data are fully in accord with previous studies showing that experimental cirrhosis is not a genotoxic condition with respect to blood cells [18, 19]. By contrast, others have postulated that increased sister chromatid exchange frequency and low mitotic index may be reflective of a direct genotoxic effect induced by liver damage in human peripheral lymphocytes [20]. Taken together, it seems that cirrhosis is unable to induce genetic damage in blood cells.

The *in vivo* comet assay guidelines [7] recommend the analysis of liver cells because it is a main organ for metabolism. Our results have demonstrated that cirrhosis was able to induce DNA breakage in liver cells. In addition, some studies have indicated that alterations in phenotypic expression of specific P-450 isoenzymes in hepatocytes associated with hepatic inflammation and cirrhosis might increase susceptibility to genotoxicity induced by some xenobiotics [21]. One possible explanation for the DNA damage induced by cirrhosis is based on the hypothesis that cirrhosis and its related reactions may induce oxidative stress, which contributes to alterations in cellular functions, and genotoxic damage. Previous reports on the pathophysiology of biliary cirrhosis have shown that liver lesions are induced by free radicals [22]. Overproduction of nitric oxide in cirrhosis could lead to the generation of peroxynitrite, a source of oxidative DNA damage [23]. In fact, some researchers have postulated that an increased sister chromatid exchange frequency in human

peripheral lymphocytes would contribute to hepatocarcinogenesis as a result of inducing genetic instability [20]. Certainly, DNA damage in liver cells induced by cirrhosis will contribute with liver disease. However, it is not clear how this pathological condition is able to exert these biological actions in the liver, given that the development of tumors in target cells depends not only on the initial levels of induced DNA damage and its repair but also on other contributing factors, including the production of reactive metabolites, their distribution, and their effect on cell proliferation [16]. Furthermore, *in vitro* and *in vivo* genotoxicity tests detect compounds that induce genetic damage directly or indirectly by various mechanisms. Nevertheless, no single test is capable of detecting all genotoxic agents. Therefore, for a more detailed judgment of the genotoxic potential of liver failure, a battery of tests is feasible.

Given that hepatic encephalopathy is a neuropsychiatric manifestation of acute and chronic liver failure, the brain was also evaluated in this setting [24]. Interestingly, we have not found any data in the literature regarding the genotoxic potential of cirrhosis on brain cells using experimental models *in vivo*. Our results clearly showed that genetic damage was induced to a great extent in the brain. Some authors have provided evidence that cirrhotic rats have increased cytochrome oxidase in brain [25]; they also found impaired spatial working memory in animals with cirrhosis [25]. An earlier study conducted by the same research group [26] has revealed that the brains of hyperammonemic rats induced by cirrhosis were able to oxidize RNA in part to perivascular astrocyte processes and to postsynaptic dendritic spines. RNA oxidation may impair postsynaptic protein synthesis, which is critically involved in learning and memory consolidation [24]. Overall, we believe that oxidative DNA damage induced by cirrhosis may contribute to the development of many pathological conditions in the brain, such as encephalopathy.

Accumulating evidence suggests that patients with advanced cirrhosis and rats with short-term bile duct ligation are prone to develop nephrotoxicity from aminoglycosides [27]. In this regard, we were able to evaluate the effect of cirrhosis on kidney cell DNA. Our results demonstrated a lack of genotoxic effect in kidney cells. Some authors [28, 29] have postulated that the experimental model adopted in this study induces progressive renal dysfunction without structural changes in the kidney.

Taken as a whole, our findings support the notion that experimental biliary cirrhosis is unable to induce genotoxicity in kidney cells.

To further elucidate the possible outcomes of liver failure on the cardiovascular system, we were able to evaluate the genotoxicity in heart. Our results revealed that experimental biliary cirrhosis was unable to exert any genetic insult to produce DNA breakage in heart cells. These findings are new and the mechanism is as yet unknown, and therefore difficult to explain. Some published data indicate that the over expression of Galpha (i2), PDE2a, and RGS2 down regulates the beta-adrenergic signaling pathway, thereby contributing to the pathogenesis of cirrhotic cardiomyopathy [30]. Independent of the biological mechanism involved in this phenomenon, it seems that liver disease does not exert noxious activity on heart cells, specifically related to genotoxic damage. However, further studies are necessary to elucidate the issue.

In summary, our results suggest that cirrhosis could contribute to genetic instability as a result of DNA damage in the liver and brain.

Acknowledgements This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) Grant number: 07/01228-4 and 08/55928-0. DAR, MRN, and RCS are recipients of the CNPq fellowship.

References

- Ramadori G, Moriconi F, Malik I, Dudas J. Physiology and pathophysiology of liver inflammation, damage and repair. *J Physiol Pharmacol* 2008;59(Suppl 1):107–117
- Bartsch H, Nair J. Oxidative stress and lipid peroxidation-derived DNA-lesions in inflammation driven carcinogenesis. *Cancer Detect Prev* 2004;28:385–391
- Ohshima H, Tatemichi M, Sawa T. Chemical basis of inflammation-induced carcinogenesis. *Arch Biochem Biophys* 2003;417:3–11
- Limoli CL, Kaplan MI, Phillips JW, Adair GM, Morgan WF. Differential induction of chromosomal instability by DNA strand-breaking agents. *Cancer Res* 1997;57:4048–4056
- Ribeiro DA, Grilli DG, Salvadori DM. Genomic instability in blood cells is able to predict the oral cancer risk: an experimental study in rats. *J Mol Histol* 2008;39:481–486
- Schabath MB, Spitz MR, Grossman HB, Zhang K, Dinney CP, Zheng PJ, et al. Genetic instability in bladder cancer assessed by the comet assay. *J Natl Cancer Inst* 2003;95:540–547
- Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, et al. Single cell gel/comet assay. *Environ Mol Mutagen* 2000;35:206–221
- Singh NP, McCoy MT, Tice RR, Schneider EI. A simple technique for quantification of low levels of DNA damage in individual cells. *Exp Cell Res* 1988;175:184–191
- Olive PL, Banáth JP, Durand RE. Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the “comet” assay. *Radiat Res* 1990;122:86–94
- de Lima Leite A, Santiago JF Jr, Levy FM, Maria AG, Fernandes MS, Salvadori DM, et al. Absence of DNA damage in multiple organs (blood, liver, kidney, thyroid gland and urinary bladder) after acute fluoride exposure in rats. *Hum Exp Toxicol* 2007;26:435–440
- Grassi TF, Camargo EA, Salvadori DM, Marques ME, Ribeiro DA. DNA damage in multiple organs after exposure to chlorhexidine in Wistar rats. *Int J Hyg Environ Health* 2007;210:163–167
- Ribeiro DA, Calvi SA, Picka MM, Persi E, de Carvalho TB, Caetano PK, et al. DNA damage and nitric oxide synthesis in experimentally infected Balb/c mice with *Trypanosoma cruzi*. *Exp Parasitol* 2007;116:296–301
- Ribeiro DA, Fávero Salvadori DM, da Silva RN, Ribeiro Darros B, Alencar Marques ME. Genomic instability in non-neoplastic oral mucosa cells can predict risk during 4-nitroquinoline 1-oxide-induced rat tongue carcinogenesis. *Oral Oncol* 2004;40:910–915
- Ribeiro DA, Pereira PC, Machado JM, Silva SB, Pessoa AW, Salvadori DM. Does toxoplasmosis cause DNA damage? An evaluation in isogenic mice under normal diet or dietary restriction. *Mutat Res* 2004;559:169–176
- Chang SW, Ohara N. Pulmonary circulatory dysfunction in rats with biliary cirrhosis. An animal model of the hepatopulmonary syndrome. *Am Rev Respir Dis* 1992;145:798–805
- Sasaki YF, Kawaguchi S, Kamaya A, Ohshita M, Kabasawa K, Iwama K, et al. The comet assay with 8 mouse organs: results with 39 currently used food additives. *Mutat Res* 2002;519:103–119
- Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, Clay P, et al. Recommendations for conducting the in vivo alkaline comet assay. *Mutagenesis* 2003;18:45–51
- Tieppo J, Vercelino R, Dias AS, Silva Vaz MF, Silveira TR, Marroni CA, Marroni NP, Henriques JA, Picada JN. Evaluation of the protective effects of quercetin in the hepatopulmonary syndrome. *Food Chem Toxicol* 2007;45:1140–1146
- Vercelino R, Tieppo J, Dias AS, Marroni CA, Garcia E, Meurer L, Picada JN, Marroni NP. N-acetylcysteine effects on genotoxic and oxidative stress parameters in cirrhotic rats with hepatopulmonary syndrome. *Basic Clin Pharmacol Toxicol* 2008;102:370–376
- Ucur A, Palanduz S, Cefle K, Ozturk S, Tutkan G, Vatansever S, Erden S, Karan MA, Erten N, Guler K, Tascioglu C. Sister chromatid exchange and mitotic index in patients with cirrhosis related to hepatitis B and C viruses and in chronic carriers. *Hepatogastroenterology* 2003;50:2137–2140
- Kirby GM, Batist G, Alpert L, Lamoureux E, Cameron RG, Alaoui-Jamali MA. Overexpression of cytochrome P-450 isoforms involved in aflatoxin B1 bioactivation in human liver with cirrhosis and hepatitis. *Toxicol Pathol* 1996;24:458–467
- Vallance P, Moncada S. Hyperdynamic circulation in cirrhosis: a role for nitric oxide? *Lancet* 1991;337:776–778
- Ferguson JW, Dover AR, Chia S, Cruden NL, Hayes PC, Newby DE. Inducible nitric oxide synthase activity contributes to the regulation of peripheral vascular tone in patients with cirrhosis and ascites. *Gut* 2006;55:542–546
- Schliess F, Görg B, Häussinger D. RNA oxidation and zinc in hepatic encephalopathy and hyperammonemia. *Metab Brain Dis* 2009;24:119–134
- Méndez M, Méndez-López M, López L, Aller MA, Arias J, Arias JL. Basal and learning task-related brain oxidative metabolism in cirrhotic rats. *Brain Res Bull* 2009;78:195–201
- Méndez M, Méndez-López M, López L, Aller MA, Arias J, Arias JL. Associative learning deficit in two experimental models of hepatic encephalopathy. *Behav Brain Res* 2009;198:346–351
- Ackerman Z, Karmeli F, Pizov G, Ben-Dov I, Pappo O. Renal effects of gentamicin in chronic bile duct ligated rats. *Dig Dis Sci* 2006;51:406–415
- Rivera-Huizar S, Rincón-Sánchez AR, Covarrubias-Pinedo A, Islas-Carbajal MC, Gabriel-Ortiz G, Pedraza-Chaverri J, Alvarez-Rodríguez A, Meza-García E, Armendáriz-Borunda J. Renal

- dysfunction as a consequence of acute liver damage by bile duct ligation in cirrhotic rats. *Exp Toxicol Pathol* 2006;58:185–195
29. Assimakopoulos SF, Vagianos CE. Bile duct ligation in rats: a reliable model of hepatorenal syndrome? *World J Gastroenterol* 2009;15:121–123
30. Ceolotto G, Papparella I, Sticca A, Bova S, Cavalli M, Cargnelli G, Semplicini A, Gatta A, Angeli P. An abnormal gene expression of the beta-adrenergic system contributes to the pathogenesis of cardiomyopathy in cirrhotic rats. *Hepatology* 2008;48:1913–1923