CYCLOOXYGENASE-2 SHORT HAIRPIN RNA LIMITS NONALCOHOLICFATTY LIVER DISEASE BY INDUCING LIVER CELL AGING

Guo-Juan Li[1,2], Meng-Jie Peng[2], Xiao-Bing Wu[2], Hong-Wu Liao[2], Li Zhang[2], Ying Li[2], Xue-Feng Yang[2*], Hong Chen[1*]
[1]Department of Endocrinology and Metabolism, zhujiang Hospital of southern medical university Guangzhou, 510515 (China) - [2]Department of Gastroenterology, Affiliated Nanhua Hospital, University of South China, Hengyang, 421002 (China)

ABSTRACT

Cyclooxygenase-2 (COX-2) has been involved the initiation and development of nonalcoholic fatty liver disease (NAFLD), however the mechanism within has still remained unclear. This aim of this study was observe the effects of COX-2 short hairpin RNA (COX-2 shRNA) on the liver cell aging in rats with NAFLD induced by a high-fat diet. 48 SD rats divided randomly into four group (n=12). The rats was induced NAFLD by high fat diet for 12 weeks together with or without COX-2shRNA. Liver fat degeneration and fibrosis was evaluated by Oil red O, HE and MASSON Staining according to the METAVIR scoring system, and liver tissue cell aging by β -galactose glucoside enzyme staining, COX-2 and a- SMA mRNA expression of liver tissue by real-time fluorescent quantitative PCR. The serum cerealthirdtransaminase (ALT), aspartate transaminase (AST), total cholesterol (TC), trigly-cerides (TG)levels was investigate by enzyme-linked immunosorbent assay (ELISA). The results of this study found liver steatosis degree, liver tissue lipid variable area and liver fibrosis in rats with COX-2shRNA significantly attenuated, and the serum levels of ALT,AST,TC,TG in rats with COX-2shRNA significantly improved. These data suggest COX-2shRNA limits the development of NAFLD through inducing liver cell aging.

***Keywords**: non-alcoholic fatty liver; COX-2; cell aging.*

DOI: 10.19193/0393-6384_2018_2_65

***Received** November 30, 2017; **Accepted** January 20, 2018*

Introduction

It has been confirmed that COX-2 takes part in the initiation and development of NAFLD(1), however the mechanism within has still remained unclear. Recently, some researches show that cell aging is closely related with NAFLD, and liver cell aging is a protection mechanism against alcoholic liver diseases,and COX-2 participate in the regulation of cell aging(2-4). Therefore,we assume that COX-2 promotes the development of NAFLD via regulating liver cell aging. To verify our assumption,we planed to observe the effect of COX-2shRNA to liver cell aging in rats with NAFLD induced by high-fat diet, and make preliminary exploration of the significance of liver cell aging-induced by COX-2shRNA to the initiation and development of NAFLD.

Methods

48 SD rats divided randomly into four group(n=12): Group A (normal control group), group B(NAFLD group), group C(ad-COX-2shRNA group), group D (ad-shRNA group). Apart from control group, the other 3 groups were subjected to adaptive breeding for 1 week and then fed with high-fat diet(80.5% base feed, 2%cholesterol, 7% lard, 10% egg yolk powder and 0.5% bile salt) for 12 weeks for being rat model.C and D group were weekly subjected respectively to tail vein injection of ad-COX-2shRNA and ad-shRNA

(diluted by PBS) ,with dosage of 1×10^{9}pfu per rat; while A and B group were weekly subjected respectively to tail vein injection the same volume of PBS. Liver fat degeneration and fibrosis was evaluated by Oil red O, HE and MASSON Staining according to the METAVIR scoring system, and liver tissue cell aging by β-galactose glucoside enzyme staining ,COX-2 and a- SMA mRNA expression of liver tissue by real-time fluorescent quantitative PCR.Design and synthesis of primer were performed by Changsha YingRun Biotechnologies Co., Ltd, wherein the primer sequence is as Table1. The serum cerealthird-transaminase (ALT), aspartate transaminase (AST), total cholesterol (TC), triglycerides (TG) levels was investigate by enzyme linked immunosorbent assay (ELISA). All experimental data were expressed by (x±s) and subjected to t examination. Intergroup comparison were adopted for the statistics, wherein p<0.05, showing significant difference between groups.

Gene	Gene ID	Gene sequence	Length
Ratβ-Actin	287876	5GACATAAAGGA-GAAGCTGTGC 3	219bp
		5CATGATGGAGTT-GAAGGTGGT 3	
RatCOX-2	117243	5TCAGCCATGCAG-CAAATCCTT3	112bp
		5CCGTAGAATC-CAGTCCGGGT3	
Ratα-SMA	81633	5GACCTT-CAATGTCCCTGCCA3	98bp
		5GATCTCCAGAGTC-CAGCACAATA3	

Table 1: Primer sequence of COX-2 and a- SMA mRNA.

Results

Liver pathologic changes of SD rats in various groups

According to Fig. 1 and Table 2,the structure of normal liver tissue (group A)is complete and clear, and the structures of liver lobule and portal area are in good condition, no fatty degeneration, necrosis or significant inflammatory cell infiltration can be observed within and no fibrous septum is formed within; other groups of rat treated with high fat diet developed severe hepatic steatosis, necroinflammatory activity and fibrosis, as evidenced by the prominent steatosis of hepatocytes, pericellular and periportalbridging fibrosis, and distortion of liver architecture (Table2). However, injection COX-2shRNA per day for 12 weeks significantly improves alterations in liver histology.

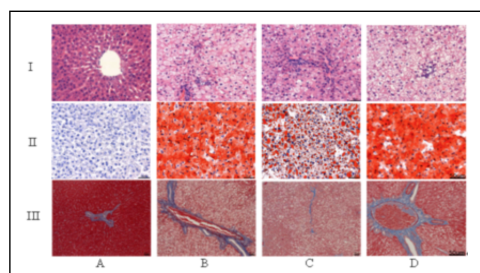

Fig. 1: Liver pathologic changes of rats.
COX-2shRNA group, group D is ad-shRNA group. (I) HE dyeing (original magnification,×200); (II) Oil red O staining(original magnification,×200); (III) Masson dyeing (original magnification, ×200)

Group	n	Fatty degeneration area (%)	Collagen fiber area (%)
Group A	10	0.00±0.00	2.64±0.45
Group B	10	41.32±12.61	25.32±2.07
Group C	10	23.4±11.43	10.39±3.19
Group D	10	37.08±11.89	20.39±4.38

Table 2: Fatty degeneration and Collagen fiber areas of rat liver tissues (%, X±S)
Note: Group A is normal control group, group B is NAFLD group, group C is ad-COX-2shRNA group, group D is ad-shRNA group

Change of serum indexes of different groups

Compared with control group, the ALT,AST, TC, TG levels in rats with NAFLD(group B,C,D) are all increased, there is statistical significance within (p<0.05, p<0.05,p<0.01, p<0.05); while compared with NAFLD model group, the AST, TC, TG levels of COX-2shRNA group are all decreased, indicating the statistical significance (p<0.01,p<0.01,p<0.005, p<0.05) (Table 3).

Group	n	ALT(U/L)	AST(U/L)	TC(mmol/L-1)	TG(mmol/L-1)
Group A	10	38.2±9.92	117.5±43.21	1.05±0.02	0.44±0.12
Group B	10	73.71±43.33	169±100.27	2.37±0.44	0.51±0.19
Group C	10	43.42±23.72	88.92±35.96	2.13±1.06	0.30±0.16
Group D	10	62.51±43.33	149±70.30	2.27±0.54	0.56±0.26

Table 3: Measurement results of ALT, AST, TC, TG of different groups (X±S)
Note: Group A is normal control group, group B is NAFLD group, group C is ad-COX-2shRNA group, group D is ad-shRNA group

Q-PCR Detection of COX-2,α-SMA mRNA expressions in liver tissues among different groups

As shown in Table. 4, compared with control group, the expression COX-2 and α-SMA in liver tissues of NAFLD model group is increased, with statistical significance ($p < 0.05$); while compared with NAFLD model group, the expression level of COX-2 in liver tissues of COX-2shRNA model group is significantly decreased ($p < 0.01$).

Group	n	α-SMA	COX-2
Group A	10	1.046±0.054	1.106±0.048
Group B	10	1.156±0.091	1.396±0.077
Group C	10	0.935±0.094	0.981±0.214
Group D	10	1.172±0.116	1.359±0.113

Table 4: The mRNA expression of COX-2 and α-SMA in liver tissues (X±S)

Note: Group A is normal control group, group B is NAFLD group, group C is ad-COX-2shRNA group, group D is ad-shRNA group

Detection of cell aging conditions based on liver β-galactosidase staining

According to Fig. 2 and Table 5, the positive aging cells are in blue-green. The aging index of Group B is significantly different from that of group A ($p<0.05$), while there is no significant difference in aging index between group C and group B ($p>0.05$), and there is no difference in aging index between group B and group D

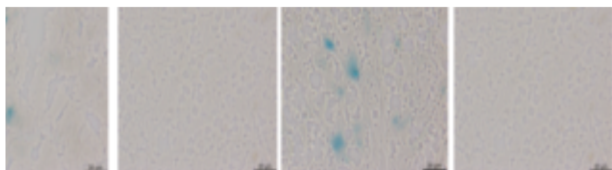

Fig. 2: SA-β-gal staining in rat liver tissues of different groups (×40).

Note: Group A is normal control group, group B is NAFLD group, group C is ad-COX-2shRNA group, group D is ad-shRNA group

Group	Aging index
Group A	14.00±8.41
Group B	0
Group C	18.60±19.55
Group D	0

Table 5: Liver cell againg conditions of different groups.

Note: Group A is normal control group, group B is NAFLD group, group C is ad-COX-2shRNA group, group D is ad-shRNA group

Discussion

As a basic structure unit of an organism, a cell has undergoing aging processes since its born and eventually goes to death. After aging, a cell will show some significant aging characteristics such as the change of metabolic enzymes. β-galactosidase (β-Gal) is widely existed in animals and plants, which can hydrolyze lactose into glucose and galactose. Senescence-Associated β-galactosidase (SA-β-Gal) is a mark of cell aging identification with the strongest specificity that can be found currently. In most aging cells, such mark can be detected using staining method, while in non-aging cells, it cannot be detected[5-6].

Liver, consisting of liver cells, is the biggest parenchymal organ with metabolic functions such as storage, secretion, and detoxification. A liver is one of most vulnerable organs to aging process. Research shows that the individual aging is closely related to organ aging or cell aging. Liver tissue structure will change with the age, resulting in, for instance, the liver cells aging. This research results show that liver cells will show aging phenomenon under normal conditions, which again verfies the objective law that cells aging one of cellular activities[7].

In normal liver, most liver cells are in G0 stage, with very low renewing rate. Almost all liver cells are in resting state, and never enter into proliferation cycle. However, liver cell is the only cell which is highly differentiated while still maintaining certain multiplication capacity. Under destructive stimulations such as the classic partial hepatectomy, the remaining mature liver cells in resting state can quickly enter into cell cycle to regenerate new cells, so as to recover damage or lost liver tissue and recover liver functions. It showed that liver cells can still maintain regenera-tion function even after partial hepatectomies for consecutive 12 times.

The experiment results of serial liver cell transplantation show that primary dissociated live cells can continue to divide and proliferate after being transplanted into liver receptor.

The proliferation capacity remained unchanged even after division for 69 times. Research of repeated hepatectomy and serial liver cell transplantation indicate that during multiple proliferation division processes of liver cells that have entered into cell cycle, there may be certain mechanism that inhibits the occurrence of liver

cells aging[8]. This research shows that the NAFLD liver cell aging speed is significantly decreased as compared to aging speed of normal liver cell, and nearly no SA-β-Gal positive cells can be found in livers of NAFLD model group.

Therefore, can we assume that some liver cells are in fatty degeneration, while remaining mature liver cells in resting state can quick enter into cell cycle, regenerating new cells to recover damage or lost liver tissues and reover liver function, in addition, there may exist certain mechanism causing liver cell proliferation while inhibiting the occurrence of liver cells aging?

Some documents have reported that COX-2shRNA can enhance cell againg[9]. However, domestic research community has never reported of the influence of COX-2shRNA on liver cell aging. This research shows that COX-2shRNA can promote the occurrence of NAFLD liver cells aging.

Why does COX-2shRNA can promote the occurrence of NAFLD liver cell aging? What is the significance of liver cell aging? These questions are remained to be urgently solved. According to former opinion, there may exist a certain mechanism inhibiting the occurrence of aging of liver cells in proliferation state. Based on our experiment data, it can find that the serum levels of AST,TC,TG in COX-2shRNA group are all decreased as compared to those of NAFLD model group, and the difference within in of statistical significance ($p<0.05$, $p<0.001$, $p<0.05$), indicating that the liver cell damage in COX-2shRNA group has been reduced. As liver cell damage reduces, the tendency of recovering damage or lost liver tissue and thus restoring liver function via cell regeneration is weakened, so that proliferation of liver cell is reduced, and thus the inhibiting effect of cell proliferation on cell aging is removed. Hence, we can understand that COX-2shRNA promotes the occurrence of live cell aging, which is basically a knock-on effect of hepatoprotective effect, that is to say, COX-2shRNA inhibits fat-induced liver cell damage, the remaining mature liver cells in resting stage are no longer so hunger to enter into cell cycle for proliferation, and thus the inhibiting effect of cell proliferation on cell aging is removed.

What is the significance of liver cell aging to the initiation and development of NAFLD? To liver cell, it is believed that live cell aging is a protection mechanism. In 2014, Wan et al. found that aging liver cell shows characterisitc anti-apoptosis and anti-fatty degeneration effect. In this experiment, we can find compared with NAFLD model group, the live cell aging degree of rats in COX-2shRNA group is increased, meanwhile the liver cell apoptosis and fatty degeneration are significantly reduced.

Therefore, it can speculate that COX-2shRNA inhibits the NAFLD development via inducing cell aging against cell apoptosis and fatty degeneration. There are two points of view on the influence of aging liver cell on other cells such as hepatic stellate cell (HSCs), wherein one is that the aging liver cell can inhibit the activation of HSCs, while the other is that hepatic fibrotic staging is related to liver cell aging[12-13].

Recently, Aravinthan A et al. from Gastroenterology and Liver Disease Department, School of Medicine, the University of Cambridge, UK, made an research, and the results show that the telomere length of liver cell in NAFLD group is shorter than that of control group ($p < 0.01$); After entering time phase G1／S, the rat liver cells of NAFLD group are subjected to a lack of cell cycle progression; the expression of γ-H2AX increases with fatty degeneration of liver cells ($p=0.01$); the cell cycle inhibitor p21 which is related with cell aging enjoys a high-level expression; the expression of liver cell p21 is related to hepatic fibrotic staging ($p < 0.001$). Some people proposed theory of “infectious aging”[14-15], which means after being stimulated by one or many aging-inducing factors, liver cells can secrete some senescence-messaging secretome (SMS) such as cell growth factor, inflammatory factor, chemotactic factor, and protease. Some of these SMS are maintained in senescence associated secretory phenotype (SASP), while some can cause aging of other cells via “infectious aging” theory[15].

This indicates that once liver cells enter into aging process, the change of secretion function of senescence associated cells will lead to the change of intrahepatic microenvironment, thereby promoting other liver cells to initiate aging processes via feedback regulation mechanism. Nelson et al.[16] found that aging cells can mediate bystander effect and transmit the aging to adjacent cells in normal proliferation condition; aging cell can induce adjacent cell to conduct DNA damage response (DDR) or to be in aging phenotype via gap junction intercellular communication (GJIC).

The research by Krizhanovsky V et al.[17] show that the activation-induced HSCss aging can reduce the secretion of ECM protein while enhancing the degradation of EMC. Kong X[18] et al. made an

attempt to inhibt the development of liver fibrosis through inducing the aging of HSCss. In addition to liver, such similar aging processes also occur in the pancreatic tissue repair process mediated by pancreatic stellate cells aging. Therefore, cell aging may be a certain mechanism which can limit tissue damage, initiate tissue repairing, and help tissue to return to the condition before being damaged. It is worth noting that there is another point of view in which aging liver cell can activate HSCs.

Wiemann SU et al[19] found that telomere length of liver cell in liver fibrosis area is significantly reduced due to various reasons. As entering into aging condition, liver cell can activate other types of cells such as HSCs, leading to the aggravation of liver fibrosis. According to the results of this reseach, there is actually no evidence proving that aing liver cell can activate HSCs, on the contrast, the expression level of -SMAmRNA (a cell proliferation marker for HSCs activation) is reduced when promoting rat liver tissue aging by COX-2shRNA. Therefore liver cell aging is an inhibited factor to activation of HSCs.

References

1) Chen J, Liu D, Bai Q, et al. Celecoxib attenuates liver steatosis and inflammation in non - alcoholic steatohepatitis induced by high - fat diet in rats (J), Mol Med Rep, 2011, 4(5): 811-816.

2) Kim SR1, Park JH, Lee ME,et al.Selective COX-2 inhibitors modulate cellular senescence in human dermal fibroblasts in a catalytic activity-independent manner. Mech Aging Dev. 2008, 129(12): 706-713.

3) Zdanov S, Toussaint O, Debacq-Chainiaux F. p53 and ATF-2 partly mediate the overexpression of COX-2 in H2O2-induced premature senescence of human fibroblasts. Biogerontology. 2009, 10(3): 291-298.

5) Dimri GP, Lee X, Basile G. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci U S A. 1995, 92: 9363-9367.

6) Serra MP; Marongiu F, Sini M, Laconi E. Hepatocyte senescence in vivo following preconditioning for liver repopulation. Hepatology, 2012, 56(2): 760-768.

8) Serra MP, Marongiu F, Sini M, et al. Hepatocyte senescence induced by radiation and partial hepatectomy in rat liver. Int J Radiat Biol. 2014 , 90(10): 876-883.

11) Wan J, Benkdane M, Alons E, et al. M2 kupffer cells promote hepatocyte senescence: an IL-6-dependent protective mechanism against alcoholic liver disease. Am J Pathol. 2014, 184(6): 1763-1772.

12) Aravinthan A, Scarpini C, Tachtatzis P, et al. Hepatocyte senescence pre-dicts progression in non-alcohol-related fatty liver disease. Hepatol. 2013 Mar; 58(3): 549-556.

13) Aravinthan A, Mells G, Allison M, et al. Gene polymorphisms of cellular senescence marker p21 and disease progression in non-alcohol-related fatty liver disease. Cell Cycle. 2014; 13(9): 1489-1494.

14) Serra MP, Marongiu F, Sini M, et al. Hepatocyte senescence induced by radiation and partial hepatectomy in rat liver. Int J Radiat Biol. 2014, 90(10): 876-883

15) Kuilman T, Michaloglou C, Vredeveld LC, et al. Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. Cell. 2008, 133(6): 1019-1031.

16) Nelson G, Wordsworth J, Wang C, et al. A senescent cell bystander effect: senescence-induced senescence. Aging Cell. 2012, 11(2): 345-349.

17) Krizhanovsky V, Yon M, Dickins RA, et al .Senescence of activated stellate cells limits liver fibrosis. Cell. 2008. 134(4): 657-667.

18) Kong X, Feng D, Wang H, et al. Interleukin-22 induces hepatic stellate cell senescence and restricts liver fibrosis in mice. Hepatology. 2012 Sep; 56(3): 1150-1159.

19) Wiemann SU, Satyanarayana A, Tsahuridu M, et al. Hepatocyte telomere shortening and senescence are general markers of human liver cirrhosis. FASEB J. 2002, 16(9): 935-942.

ACKNOWLEDGMENTS
This work was supported by the National Natural Science Foundation of China (No. 81373465).

Corresponding Author:
Hong Chen
Email:chenhong123@smu.edu.cn
Xue-Feng Yang
Email:yxf9988@126.com
(China)