The expression of c-kit and proliferating cell nuclear antigen in oval cells of rats with hepatocellular carcinoma

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OBJECTIVE: To study the relationship between oval cells and primary hepatocarcinoma and the expression of c-kit and proliferating cell nuclear antigen (PCNA) in oval cells of rats with hepatocellular carcinoma.

METHODS: A hundred and twenty clean SD rats were divided into three groups: normal group, cancer-induction group and intervention group. The normal group was fed with standard forage while the rest two groups were fed with 3'-methyl-2-methylamino-azobenzene (DAB) to induce carcinoma for 14 weeks and then fed with standard forage and water. Uscharidin was injected abdominally to the intervention group from the first week to the 14th week. All rats were killed and biopsy specimens were taken from the left and right liver lobes for immunohistochemical staining of c-kit and PCNA on the 2nd, 4th, 6th, 8th, 10th, 12th, 14th, 16th, 18th, 20th, 22nd, and 24th week.

RESULTS: From the 2nd to 14th week after liver infection, c-kit positive cells, mainly oval cells were found in the portal area in the carcinoma-induction group and dotted positive pigmentations in liver lobules. In the 22nd week, a large number of cancerous nodes occurred and nuclei heteromorphism was apparent; the number of positive cell decreased but positive cells could be sparsely observed in cancerous nodes. In the 2nd week of the carcinoma-induction process, PCNA positive cells were oval cells in the portal area. In the 4th week, a lot of hepatic cells were positively stained, especially in the central vein area. In the 6th week, PCNA positive cells could be seen in the lobules of the liver. In the 8th week, the number of PCNA cells decreased comparatively. From the 10th to 14th week, oval cells in the portal area were still over-expressed. From the 16th to 24th week, a large number of cancerous nodes occurred and PCNA was over-expressed in some of them. In necrotic cancerous nodes, the para-cancerous PCNA positive cells were sparsely distributed and their number was less than that of PCNA positive cells of cancerous tissues.

CONCLUSIONS: Hepatic stem cells originating from the terminal biliary plexus of the portal area are involved in the development of hepatocarcinoma because c-kit positive cells expressed in cancerous nodes, accompany the whole process of the development. In the middle inflammatory period of carcinoma-induction, the expression of PCNA in hepatic cells peaked, but the index decreased in the late inflammatory period and in the proliferated fibrosis stage. The expression of PCNA is a tortuous process, going up, down, then up again from normal tissues to cancerous tissues. Combined with pathological findings, PCNA can be considered as a warning index for carcinomatous cells.

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Key words: hepatocellular carcinoma; oval cell; proliferating cell nuclear antigen; c-kit

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Introduction

Hepatocarcinoma (HCC), one of the commonest malignant tumors in China is difficult to treat. Researchers have extensively studied its cellular resource and etiological factors in order to find effective methods for prevention and treatment of the disease.[1] With progress in study of liver stem cells, scientists found that liver oval cell has a potential of proliferation and differentiation, which can not only differentiate into hepatocytes and bile duct epithelial cells but have an immediate relationship with liver cancer cells.[2] At present, two opinions exist:[3] first, HCC is caused by abnormal differentiation and proliferation of oval cells; second, it is resulted from dedifferentiation of matured liver cells. To probe the relations between oval cells and primary hepatocarcinoma, the pathologic characteristics and distribution of oval cells in the liver tissues of rats with hepatocarcinoma and the expression of c-kit and proliferating cell nuclear antigen (PCNA) in oval cells were investigated.

Methods

Animals

One hundred and twenty clean SD rats weighing 100-120 g were used. All the animals were provided by Animal Experiment Center of Zhongshan University and raised at 18-28 ℃ and moisture of 40%-70% in the Animal Laboratory of Zhujiang Hospital, First Military Medical University, Guangzhou, China.

The SD rats were divided into normal group (20), cancer induction group (40), and intervention group (40). Each group was subdivided into 10 groups.

Reagents

C-kit polyclonal antibody was bought from Sigma Corp., USA. Immediately used immunohistochemical reagent kits were purchased from Boster Corp., Wuhan, China, PCNA immunohistochemical reagent kits and DAB developer from Zhongshan Biology Corp., Guangzhou, China, and polylysine binder and neutral resina mountant from Boster Corp., Wuhan, China.

Hepatocarcinoma pattern

With modified cancer induction methods,[4,5] the inducing reagent DAB was evenly mixed with standard forage and the amount was kept at 0.06%. Each mouse was given forage 15-20 g per day equivalent to 0.01 g DAB, and at the same time, it was fed with water regularly and kept at a indoor temperature of 25-28 ℃. From the 14th week, the rats were raised with only standard forage and normal water. The normal group was given standard forage.

Management of rats

The above-mentioned procedures were carried out in the cancer induction group and intervention group to set up hepatocarcinoma.

Uscharidin-injection was given to the intervention group intraabdominally twice a week for 14 weeks and the dosage was in accordance with the weight of each mouse, 2.0×10^4 μl/kg.[6,7]

A subgroup of each group was killed at the 2nd, 4th, 6th, 8th, 10th, 12th, 14th, 16th, 18th, 20th, 22nd, 24th week to get two pieces of tissue (1 cm^3) from the right liver lobe and 2-4 pieces (yellow soybean-size) from the left liver lobe. They were put into 0.5 ml centrifuging tubes separately, fixed by 10% neutral buffer formaldehyde, and imbedded in paraffin. Five μm sections were prepared continuously, baked at 60 ℃ for 3 hours, and deparaffinized and stained with hematoxyin blue. At last, the sections were dried for microscopic observation.

Procedures

Immunohistochemical staining of c-kit and PCNA

Glass slides were treated by polylysine. Five μm paraffin sections were taken and unfolded in 40 ℃ water, sticked, dried with wind, and baked at 60 ℃ for 2 hours. The sections were deparaffinized with dimethylbenzene and gradient alcohol, and washed with 0.01 mol PBS for 2 minutes 3 times. Subsequently, they were treated in mid-

microwave at 100 ℃ to restore antigen for 10 minutes, cooled for 25 minutes, then washed by 0.01 mol PBS 2 minutes each time for 3 times. Unnecessary liquid was removed 10 minutes after instillation of normal second antibody goat blood serum. After incubation under 37 ℃ for 2 hours or 4 ℃ for 12 hours, the sections were washed by 0.01 mol PBS 2 minutes each time for 3 times. After further incubation under 37 ℃ for 20 minutes following instillation of SABC they were washed with 0.01 mol PBS 2 minutes for 3 times.

DAB colorization

A, B, C reagents of the DAB colorant kit were instilled into 1 ml distilled water, and the mixture was instilled to the slice, while controlling the reaction time for about 10-15 minutes. At last, the slice was washed by distilled water to stop staining. Dying by haematoxylin or nuclear-firm-red was performed for 50 seconds, followed by dehydration, transparency, mounting and microscopic observation.

Analysis of image patterns

C-kit protein was stained brown-yellow. C-kit was localized as soon as the liver was injured and its quantity was also reckoned with the development of hepatic injury and HCC. PCNA protein was stained brown yellow. PCNA positive cells in the hepatic lobule and the change of PCNA positive cells were localized when the hepatic injury became more serious and HCC developed. PCNA labelling rate or proliferating index presented by percentage was calculated by counting, i.e., each slice was observed in a 5-high power field randomly. Positive cells among 100 cells were calculated for a mean value.

Statistical analysis

The results were analyzed with software SPSS10.0. Single factor analysis of variance and least significant difference test (LSD) method showed significant difference in the groups ($P < 0.05$).

Results

Staining and localization of c-kit positive cells

C-kit positive staining showed brown-yellow, even, and diffuse location in the cytolymph. Occasionally positive staining was noted in normal liver tissue, mainly in the splitting and proliferating hepatic cells (Fig. 1). In the 2nd week after liver infection, c-kit positive cells mostly oval cells were observed in the portal area and positive pigmentations in liver lobules (Fig. 2). In the 8th week, the oval cells in the portal area showed apparent pigmentation, some of which joined into patch and there were dutted pigmentation around the portal area (Fig. 3). In the 14th week, normal hepatic structure was replaced by a lot of pseudolobules and c-kit positive cells were still expressed in a large amount in the portal area. In the 24th week, a large number of cancerous nodes were formed with apparent nuclei heteromorphism and a decrease in positive cells (Fig. 4). Immunohistochemically c-kit positive pigmentation of the intervened group was identical to that of the carcinoma-induction group.

Distribution and staining of PCNA positive cells

PCNA positive pigmentation in the nucleus was in a granular or diffuse form and sometimes cytolymph pigmentation with big and uneven distribution was noted (Fig. 5).

In the 2nd week of carcinoma-induction process or the early stage of inflammatory lesion, PCNA positive cells were observed first in the oval cells of the portal area, and positive staining of hepatic cells in lobule was rare. In the 4th week of carcinoma-induction process, a lot of hepatic cells were stained positive, highly in the central vein area; in the certain area from the central vein to lobules, the nuclei of positive cells tended to enlarge gradually. The change of PCNA positive cells from the 6th to the 24th week could be observed (Figs. 6-8). In the 8th week, the number of PCNA cells decreased comparatively and in some areas around the portal area, the nuclei of positive cells tended to enlarge. In the 24th week, a large number of cancerous nodes were noted and PCNA was over-expressed in some of them; in cancerous no-

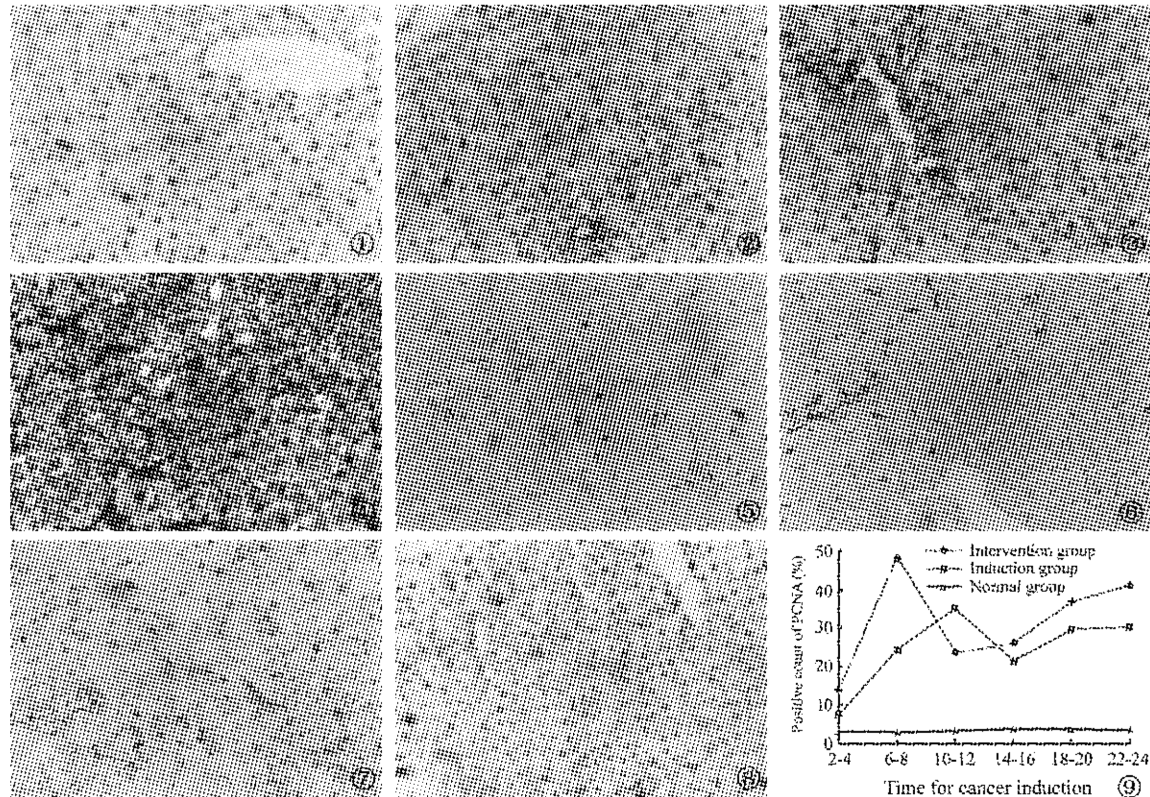

Fig. 1. Expression of c-kit in normal tissue (immunohistochemistry, original magnification ×200).

Fig. 2. C-kit positive staining in oval cells around the portal area in the induction group at the 2nd week (immunohistochemistry, original magnification ×200).

Fig. 3. C-kit positively stained liver tissue showing oval cells around the portal area in the induction group at the 8th week (immunohistochemistry, original magnification ×200).

Fig. 4. C-kit positively stained liver tissue in the induction group at the 24th week showing apparent nuclei heteromorphism, decreased number of positive cells but sparsely located positive cells in cancerous nodes (immunohistochemistry, original magnification ×200).

Fig. 5. PCNA positively stained normal liver tissue showing occasionally positive pigmentation in normal liver tissue, probably in hepatic lobules (immunohistochemistry, original magnification ×100).

Fig. 6. Liver tissue in the later inflammatory period covered by PCNA positive cells in the induction group at the 6th week (immunohistochemistry, original magnification ×100).

Fig. 7. Expression of PCNA in liver tissue in the fibrosis period of liver proliferation at the 12th week of cancer induction and over-expression of oval cells around the portal area (immunohistochemistry, original magnification ×200).

Fig. 8. Expression of PCNA in the tissue of hepatocarcinoma at the 24th week of cancer induction and PCNA over-expressed in some cancerous nodes (immunohistochemistry, original magnification ×200).

Fig. 9. The change of PCNA in the process of cancer induction.

des area with necrosis, a small number of positive cells were seen. The heterogeneity of pericancerous PCNA positive cells was not as clear as cancer cells and the quantity of sparsely distributed pericancerous PCNA positive cells was fewer than that of cancerous tissues.

Immunohistochemical findings in the intervention group showed that PCNA positive cells appeared in the oval cells of the portal area. The increase of cells was dependent on the seriousness of

Table. PCNA count of positive cells in the three groups (%, mean ± SD)

Time (wk)	Normal group (n=4)	Intervention group (n=8)	Induction group (n=8)
2–4	3.0 ± 1.2	7.6 ± 3.3^{a}	14.1 ± 7.5^{b}
6–8	2.9 ± 1.5	24.3 ± 7.4^{b*}	48.2 ± 15.5^{c}
10–12	3.3 ± 0.9	5.1 ± 6.5^{c*}	23.7 ± 9.6^{b}
14–16	3.7 ± 2.1	21.5 ± 4.2$^{b\triangle}$	25.9 ± 5.1^{b}
18–20	3.6 ± 1.3	29.6 ± 7.6$^{b\triangle}$	36.8 ± 6.3^{c}
22–24	3.5 ± 2.2	30.2 ± 8.7$^{b\triangle}$	41.2 ± 8.2^{c}

a: Compared with the normal group, $P > 0.05$; b: compared with the normal group, $P < 0.05$; c: compared with the normal group, $P < 0.01$; *: compared with the cancer induction group, $P < 0.05$; △: compared with the cancer induction group, $P > 0.05$.

hepatic infection and its positive incidence and expression level peaked in the 10th week. The distri bution of PCNA positive cells was the same as that of the induction group when HCC appeared (Table, Fig. 9).

Discussion

C-kit expression and its potential significance

C-kit called proto-oncogene that is able to code stem cell factors. Oval cells show c-kit mRNA in strong positive in FHF study, so c-kit is considered as a specific surface antigen of oval cells. It is no doubt that there are hepatic stem cells in hepatic tissues, but what is their source? Some investigators held that they were from the terminal biliary plexus of the portal area;[8] some believed that they were from the canal of Hering,[9] and some even found evidence that oval cells came from stem cells.[10] To identify the origin of hepatic stem cells and explore their relations to the development of HCC, we conducted immunohistochemical research into the surface marker antigen c-kit. We found that in the early stage of hepatic injury, c-kit positive staining first appeared in the oval cells of the portal area. In the late stage of inflammatory lesion, c-kit of oval cells was over-expressed and positive pigmentation came into patches. In the period of hepatic proliferation and fibrosis, c-kit positive expression of the portal area was less than that in the late stage of inflammatory lesion. During the formation of hepatoma, heteromorphism of cancer cells was obvious and c-kit positive expression was seen in the portal area. With the appearance of inflammatory lesion, c-kit positive cells in small nuclei near the portal area were seen in hepatic lobules. In the period of hepatic proliferation and fibrosis, c-kit positive cells were rarely seen in hepatic lobules. During the formation of hepatoma, c-kit positive cells could be seen both inside and around the cancerous nodes. In the normal hepatic tissues, hepatic cells also presented c-kit positive staining occasionally when they were in the process of splitting and proliferation. According to these results, the following conclusions could be drawn. First, hepatic injury to the whole development period of hepatocarcinoma exhibits the highest expression of c-kit cells in oval cells of the portal area, and it has been proved that the genesis of oval cells is initiated from the cholangioepithlium. The terminal biliary plexus therefore is an important source of hepatic stem cells. Second, in the course of hepatic injury, c-kit positive cells could be found in hepatic lobules near the portal area, which may be related to the phenomenon that oval cells migrate from hepatic fibrosis to hepatic lobules. Third, c-kit is specifically expressed in hepatic stem cells, but splitting and proliferating hepatic cells are expressed c-kit positive. Thus, c-kit might be a specific marker antigen for a type of juvenile cells. Fourth, c-kit positive cells are accompanied with the development of hepatocarcinoma and are expressed in cancerous nodes, so hepatic stem cells are involved in the development of hepatocarcinoma.

Region of expression level of PCNA

PCNA is a type of 36 kDa nucleus proteins only synthesized and expressed in proliferating cells. Cytodynamics studies[11,12] showed that in the cell cycle, PCNA began to increase in the late G_1 period, reached its highest level in S period, began to decrease in G_2-M period, and could regulate the copying of DNA. Therefore, PCNA can effectively reflect the proliferation activity of cells. Our experiment showed that high expression of PC-

NA appeared firstly in oval cells of the portal area in the early stage of inflammation, significantly higher than that in the surroundings. The size of nucleus tended to increase from the interior to exterior in the portal area, which is identical to the result reported by Casse et al.[13] Oval cells are considered as the stem cells of the liver, which have the potential ability of dual differentiation.[14] When the conjugation and proliferation of hepatic cells are hindered, oval cells show the activity of high proliferation firstly, and a large amount of DNA are synthesized in the nucleus. The farther the portal area, the higher level the differentiation maturity of oval cells and the larger the nucleus. Thus the expression of PCNA is distributed scatteredly and regionally around the center of the portal area, and the regional preference is kept for a long time in the development of carcinoma. Since oval cells exist in the whole process of hepatocarcinoma development, we conclude that there might be a close relationship between the development of HCC and the PCNA over-expressed oval cells.

Deflection of expression level of PCNA

Karyokinesia is also an index to study cell's multiplication. Theoretically, PCNA positive indicates that the cell is in the S period or the late G_1, and karyokinesia indicates that the cell is in the M period. In the mid of inflammatory change of hepatocarcinoma, we found PCNA of hepatic cells was at the peak with a highest positive index of 61%, which was not significantly different from the index in the period of hepatocarcinoma formation ($P < 0.05$). PCNA positive index reduced at the late stage of inflammatory change as well as the period of conjugation and proliferation, indicating that a lot of hepatic cells could detain in the S period or in the late stage of the G1 period. The deflection of expression level of PCNA could be explained by the following considerations. DAB can not only inhibit DNA division, but may temporarily promote DNA synthesis in the nucleus of hepatic cells and make DNA skip from the G_2 period directly to the S period. DNA in the nucleus will increase and the nucleus will enlarge if the steps are repeated. Therefore, a large amount of hepatic cells are detained in the period of DNA synthesis in the mid-inflammatory period, which is consistent with the pathologic finding that hepatic cells are in ballooning degeneration and nucleus enlarged in the mid-inflammatory period. Wei et al[15] found that apoptosis of hepatic cells are accompanied with the pattern formation of hepatocarcinoma and peaked in the early stage of hepatic cirrhosis. We consider that apoptosis of hepatic cells might relate to hepatic conjugation and proliferation, which prevent the injury to hepatic cells. This conclusion is supported by the deflection of expression level of PCNA. Pathologically, hepatic cells were in ballooning degeneration and some nuclei were pycnostic, even disappeared in the late inflammatory period in this study, but no infiltration of inflammatory cells was observed around these cystencyte cells. Therefore, it was considered as the apoptosis of hepatic cells. In this stage, the PCNA index decreased significantly compared with that in the mid-inflammatory period ($P < 0.05$).

Time of PCNA expression

We found that in the 2nd week of being infected, i. e. the early inflammatory period, the PCNA index began to increase; in the 6th week, i. e. the middle inflammatory period, the index peaked, and the positive expression of PCNA was related to the inhibition of normal DNA splitting of hepatic cells by DAB. During the period from 16th to 24th week, PCNA expression tended to increase again. All these findings indicate that the expression of PCNA is a tortuous process, going up, down then up again from normal tissues to cancerous tissues. Being combined with pathology, PCNA can be considered as a warning index for the cells changing from normal to carcinomatous.

Origin of hepatocarcinoma and expression of PCNA

It is generally accepted that the origin of hepatocarcinoma is from two ways: one is that cancerous change is due to the abnormal differentiation of hepatic stem cells, and the other is that it is at-

tributable to the dedifferentiation of mature hepatic stem cells. We support the first point of view but not exclude the possibility of the second.

Oval cells treated as the stem cells in the liver have the potential ability of multi-differentiation and they are considered as activating cells.[16] Supportive evidence existed in our experiment. First, PCNA positive cells were dominantly oval cells in the early stage of carcinoma development, indicating that they were an active and proliferating group of cells differentiating toward hepatic cells and cholangioepithleal cells. If a certain part of gene mutated, they might convert to cancerous cells. Second, oval cells appeared in the portal area, migrated and then grew into hepatic lobules via hepatic fibers. Third, precancerous changes like acidophilic body mainly in the portal area were related to the PCNA-positive oval cells. Fourth, the hepatocarcinoma induced in this experiment was mixed cellular carcinoma.

In our experiment, we did not exclude the theory of origin of hepatic cells. HE staining showed that a small part of the precancerous acidophilic bodies was located in the central area of hepatic lobules, which might be related to the inhibition of DNA division in the denatured period. In the late inflammatory period, most of hepatic cells whose DNA had been damaged were apoptosized and a small amount of hepatic cells with gene variation might conjugate and proliferate again, even have cancerous change at last.

Effect of uscharidin on c-kit and PCNA

In this study, the immunohistochemical results of c-kit showed that the intervention of uscharidin on the development of HCC involved by hepatic stem cells had no significant effect. The rules of PCNA expression in the intervened group were similar to those in the carcinoma-induced group, but the time for the peak was delayed as compared with that of the carcinoma-induced group. We consider that uscharidin can prevent inflammatory injury to hepatic cells or delay their proliferation and differentiation. Uscharidin has no siginificant effect on the development of HCC and the recovery of hepatic injury to hepatic stem cells, but it does inhibit the abnormal differentiation resulted from the over-injury of mature hepatic cells. Even though the intervension of uscharidin is unable to prevent the development of HCC, it could delay instead.

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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