Cell apoptosis and regeneration of hepatocellular carcinoma after transarterial chemoembolization

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INTRODUCTION
The efficacy of transarterial chemoembolization (TACE) is greatly determined by the liver function reserve of liver cancer patients. More than 40% patients died of liver failure after TACE, in which most of them were accompanied by poor liver function and damage due to chemotherapy. Therefore, it is important to study the effects of TACE on non-neoplastic liver tissue to decrease the death rate. We have established the animal model of rabbit VX2 hepatic carcinoma. All the rabbits were treated by TACE to detect the effect on cell cycle of non-neoplastic liver tissues after operations.[1]

MATERIALS AND METHODS
Establishment of the animal model
Fifty Japanese alpine hares, weighing 2.5-3.3 kg, provided by the Experiment Animal Center of Tongji Medical College were used in this experiment. The rabbits were implanted hepatocellular carcinoma cell VX2, and observed for 3 wk before experiment[2].

Treatment and radiological examination[3,4]
Fifty rabbits with VX2 hepatic carcinoma were randomly divided into 5 groups: Group A (control group), groups B and C (TACE treatment groups), groups D and E (partial heptectomy groups), 10 rabbits in each group. Three weeks later, at laparotomy of the rabbits, the feeding artery of liver carcinoma was punctured and arteriography was made[3]. A 1 mL of physiological saline was administered to group A. Groups B and C were treated with 0.6-0.8 mL of a mixture of iodine oil (10 mL) and mitomycin (10 mg)[2]. Groups D and E were treated by heptectomy. Groups B and C were performed CT and MRI after TACE[3,4].

Flow cytometry analysis
Rabbits in groups A, B and C were killed on the first day after the procedure, and those in groups D and E were killed on the third day after the procedure. As soon as the rabbits died, a piece of non-neoplastic liver tissue was cut from each rabbit and digested with enzymes. The cells were fixed by 800 mL/L ice ethanol, for 20 min, then flow cytometry was performed. The cells were identified by FAC Sort FCM machine (Beton Dickson Co, USA). The fluorescence of cells was analyzed by Cellquest software and the cell cycle was studied. The other liver tissues were photographed, fixed with 40 g/L formaldehyde, and stained by HE[10].

RESULTS:
Twenty-four hours after operations, compared with control group, the rabbits in TACE group had much higher index of SPF, PI and MI (M: =4.89, P<0.001; SPF: t=5.27, P<0.001; PI: t=4.87, P<0.001). Moreover, the proliferation of liver cells in TACE group was much weaker than that of the cells treated by partial heptectomy, and the differences were significant (M: =7.02, P<0.001; SPF: t=4.06, P<0.001; PI: t=2.70, P<0.05). Seventy-two h after operations, FC showed a small sub-G1 peak in TACE group and PH group, compared with the control group, but there was no difference between them (t=0.41, P>0.05). TACE showed that AI in the treated rabbits was higher than that in control group (t=3.07, P<0.05), and there were no differences between TACE group and PH group, either (t=0.93, P>0.05).

CONCLUSION:
Cell apoptosis and regeneration exist in rabbit liver tissues after TACE to some degree, which may be associated with the selective embolization of iodised oil, chemotherapeutic drug and free radical damage.
The mitosis index (MI) was calculated as the number of mitotic figures per 1,000 liver cells.

**FCM analysis**

S phase cell fraction (SPF) was calculated according to the equation: \( \text{SPF} = \frac{S}{S + G1 + G0 + G2/M} \times 100\% \). Proliferation index (PI) was calculated according to the equation: \( \text{PI} = \frac{S + G2/M}{S + G1 + G0 + G2/M} \times 100\% \). Apoptosis index (AI) was analyzed by TUNEL method\(^{11}\), the number of apoptotic cells was calculated under a microscope\(^{12}\).

**Statistical analysis**

All the data were analyzed by \( t \) test.

**RESULTS**

**General pathological and radiological findings**

Three weeks after tumor implantation, the average size of VX2 liver carcinoma was 3.5±1.6 cm, and it appeared as white nodules near the liver surface. There were no definite margins between carcinomas and normal liver tissues. On DSA, there was increased and irregular angiogenesis of the tumor vessels, with tumor staining mainly at the periphery (Figure 1). On MR, the signal of cancer was slightly lower than that of normal liver tissue on T1WI and slightly higher on T2WI (Figure 2). The iodized oil deposited well after TACE on CT\(^{13}\) (Figure 3).

**Cytological changes**

Red fluorescent light could be seen in the cells under laser confocal microscope (Figure 4). Under light microscope (Olympus), we could find cell mitosis clearly, and mitosis index (MI) could be calculated. Flow cytometry showed that S phase and G2/M phase cells in TACE groups and partial hepatectomy groups increased much more on the first day after operations than in the control group (Figure 5). On the third day after operations, the amount of S-phase and G2/M phase cells in TACE group and partial hepatectomy group was about the same as control group (Figure 6). In the cell apoptosis index (AI) determined by TUNEL method, no significant difference existed between TACE group (Figure 7) and partial hepatectomy group.

The \( t \) test values of MI, SPF and PI of TACE groups and control group are listed in Table 1. The \( t \) test values of MI, SPF and PI of TACE groups and partial hepatectomy groups are listed in Table 2.

**Table 1**

| Index (%) | TACE group | Control group | t value | P value |
|----------|------------|---------------|---------|---------|
| MI (%)   | 1.42±0.55  | 0.51±0.21     | 4.89    | <0.001  |
| SPF (%)  | 12.46±4.18 | 4.90±1.76     | 5.27    | <0.001  |
| PI (%)   | 23.38±8.31 | 9.93±2.68     | 4.87    | <0.001  |

**Table 2**

| Index (%) | TACE group | Partial hepatectomy group | t value | P value |
|----------|------------|---------------------------|---------|---------|
| MI (%)   | 1.42±0.55  | 3.13±0.54                 | 7.02    | <0.001  |
| SPF (%)  | 12.46±4.18 | 21.76±5.92                | 4.06    | <0.001  |
| PI (%)   | 23.38±8.31 | 32.51±6.75                | 2.70    | <0.005  |

**Figure 1** Hepatic arteriography of rabbits. There was increased angiogenesis with thickening and irregularity of the vessels. There was also tumor staining mainly in the periphery of the tumor.

**Figure 2** MRI of rabbit liver. The tumor appeared hypointense on T1WI (A) and hyperintense on T2WI (B).

**Figure 3** CT scan shows massive retention of iodized oil in tumor after TACE.

**Figure 4** Cells giving out red fluorescent light under laser confocal microscope.
Figure 7 Analysis of apoptosis cells by TUNEL method in TACE group. The nuclei of apoptosis cells appeared brown while those of normal cells appeared blue. It was found that part of the cells were apoptotic cells.

The t test values of MI, SPF of TACE groups and control group are listed in Table 3. There was no significant difference between the two groups ($P>0.05$). However, the $P$ value of AI and sub-G1 was less than 0.05. The t test values of MI, SPF, AI and PI of TACE groups and partial hepatectomy groups are listed in Table 4.

Table 3 t test value of MI, SPF, AI, Sub-G1 and PI of TACE groups and control group on the third day after operations

| Index     | TACE group | Control group | t value | P value |
|-----------|------------|---------------|---------|---------|
| MI (%)    | 0.62±0.33  | 0.51±0.21     | 0.88    | $P>0.05$|
| SPF (%)   | 5.70±1.93  | 4.90±1.76     | 0.95    | $P>0.05$|
| PI (%)    | 11.69±1.74 | 9.93±2.68     | 1.68    | $P>0.05$|
| Sub-G1 (%)| 2.31±1.57  | 1.18±0.45     | 2.18    | $P<0.05$|
| AI (%)    | 20.33±2.36 | 13.68±1.97    | 3.07    | $P<0.05$|

Table 4 t test value of MI, SPF, sub-G1 and PI of TACE groups and partial hepatectomy group on the third day after operations

| Index     | TACE group | Partial hepatectomy group | t value | P value |
|-----------|------------|---------------------------|---------|---------|
| MI(%)     | 0.62±0.33  | 0.81±0.30                 | 1.28    | $P>0.05$|
| SPF(%)    | 5.70±1.93  | 6.62±1.56                 | 1.34    | $P>0.05$|
| PI(%)     | 11.69±1.74 | 11.85±2.00                | 0.17    | $P>0.05$|
| Sub-G1 (%)| 2.31±1.57  | 2.05±1.07                 | 0.41    | $P>0.05$|
| AI(%)     | 20.33±2.36 | 22.69±3.79                | 0.93    | $P>0.05$|
DISCUSSION
Transarterial chemoembolization (TACE) has been one of the most important and commonly used methods for treatment of hepatocellular carcinoma. Because most patients died of liver failure after TACE\(^{15,16}\), it is important to study the condition of postoperative normal liver tissues\(^{27}\).

The most precise method to evaluate hepatocyte reproduction and apoptosis has been the study of cell cycle\(^{18}\). In our research, normal hepatocytes were isolated, fixed and stained, flow cytometry (FCM) was performed and DNA was analyzed. Results showed SPF, PI and M1 value of TACE group was much higher than that of control group (\(P<0.001\), Table 1). We could conclude that there existed liver regeneration after TACE. From Table 2 at the same time, we could find that hepatocyte regeneration in PH group was stronger than that in TACE group on the first day (\(P<0.05\)). On the third day after TACE, the difference between TACE group and control group was not significant in all indexes. Furthermore, the difference between TACE group and PH group was not significant either on the third day. From these data, we could conclude that the regeneration process stopped, most of the cells entered the dormancy phase (G\(_1\) phase). However, the value of hypodiploid peak (sub-G\(_1\)) increased on the third day after TACE (\(n=2,18, P<0.05\)). The difference of sub-G\(_1\) between TACE group and PH group was obvious (\(P>0.05\), Tables 3, 4). The apoptosis index (AI) became greater on the third day after TACE.

Cell apoptosis (programmed cell death) means the maintenance of stability of internal environment, i.e. the process of autonomic programmed cell death has been controlled by gene\(^{19}\). After partial hepatectomy, the hepatocytes would regenerate soon. At the peak of regeneration, cell apoptosis begins. Accompanying cell reproduction, cell apoptosis would eliminate the overgrown cells, and rebuilding of the tissue constitution is achieved. Some authors\(^{20}\) pointed out that the process was mitogen→cell reproduction→regeneration→cell apoptosis. It was different from the necrosis caused by liver cytotoxic material, which was in the order of cytotoxic material→necrosis→compensatory hyperplasia→cell regeneration. Liver tissue regeneration after PH has been studied comprehensively. When the liver is resected less than 70%, the regeneration is proportional to the volume of the resected portion. Mitosis reaches the peak in 24 h, and completes in 72 h. Liver tissue regeneration exists also after TACE, this is due to the effect of super-selective embolization with iodized oil. The liver regeneration in TACE group was weaker than that in PH group, the reason was that the extent of “medical hepatectomy” was much smaller\(^{21}\). However, from Tables 3 and 4, we could find the apoptosis levels between TACE and hepatocyte groups. The apoptosis level following liver regeneration was directly proportional to the regeneration level. It is easy to draw the conclusion that there must exist a new mechanism, which promotes cell apoptosis after TACE. The mechanism might be that mitomycin depressed DNA synthesis and promoted cell apoptosis\(^{22}\). Some authors\(^{23}\) pointed out that mitomycin (5 mg/ml) could cause cell apoptosis after 24 h. Almost all chemotherapeutic drugs could induce cell apoptosis by different ways\(^{24}\). For example, vincristine (VCR) and colchicine could induce cell apoptosis by disturbance of microtubule function→loss of hepatocyte function→identification of the injury by hepatocytes→decrease of protein synthesis→increase of [Ca\(^{2+}\)] activation of endonuclease and protein kinases→DNA split→cell apoptosis\(^{25}\). On the other hand, there were ischemia and hypoxia of locally embolized tissue, and release of a large amount of free radicals and cell toxins\(^{26,27}\). They could induce cell apoptosis\(^{28,29}\). For example, free radicals could injure DNA which would lead to activation of polyiodoadenosine phosphate ribotransferase\(^{30}\) and accumulation of p53. A cell calcium ion would cause cell apoptosis. It could activate nucleus transcription factors such as NF-xB which would induce cell apoptosis\(^{31}\). Normal hepatocyte apoptosis was mainly resulted from the effects of chemotherapeutic drugs and free radical injury rather than from normal tissue repair\(^{12,32}\).

The regeneration and apoptosis level of normal hepatocytes usually determine the patients’ prognosis after transarterial chemoembolization. The obvious apoptosis after TACE was due to chemotherapeutic drugs and free radicals, which made the normal process of apoptosis more rapid. Therefore, excessive dosage of chemotherapeutic drugs may promote apoptosis and injure of normal hepatocytes in non-neoplastic area of the liver, and deteriorate the liver function of patients. We can draw the conclusion that it is important to use chemotherapeutic drugs, because they can protect the liver function of patients and improve their survival rate after treatment.

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