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Biology of hepatocellular carcinoma restrained through tumor infiltrating macrophages

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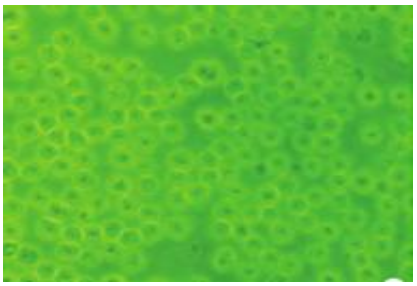
Abstract: **To study the biology of hepatocellular carcinoma restrained through tumor infiltrating macrophages. Mononuclear cells were isolated from healthy adult peripheral blood by density gradient centrifugation and inducedwith IL-4 for selective activation of macrophagesin vitro. Hepatocellular carcinoma cell line HepG2 were cultivate with selective activation of macrophages (M2) for 48h. The Liver cancer cell clone formation were determined by the clone tablet form experiment, and then Using scratches test observed the changes of cells infiltrating migrating ability. We Successful separation cultivate tumor giant cells. In vitro microenvironment of liver cancer, M2 can significantly promote the HepG2 cell proliferation and clone formation ability ($P<0.05$) after co-culture with M2 for 24 hours. The process of cell infiltration and migration were also promoted too ($P < 0.05$). Tumor associated macrophages can promote liver cancer cell invasion and migration. If changing the microenvironment of liver cancer, it can also control the malignant biological behavior of tumors.**



Keywords: Hepatocellular carcinoma; Tumor associated macrophage; Infiltration; Migration

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1. Introduction

The tumor associated macrophage (TAMs) is a source of mononuclear cells which can migrate to tumor micro-environment in the action of tumor cytokines and chemokines, and prove proliferation and differentiation of cancer cells [1-2]. Tumor associated macrophage is an important regulator between inflammation and cancer. It can promote the nature of tumor in the tumor microenvironment by expressing growth factor and matrix protease. TAMs can also promote angiogenesis and suppress the immune response. Therefore, it can be another target for tumor treatment in addition to traditional therapy. Under different micro environmental factors, macrophages had different activated trend and carrying out different functions [3]. Further study the molecular mechanism of tumor associated macrophage in promoting malignant biological behavior of liver cancer, it can improve the effect of the treatment of liver cancer and guide clinical application.

2. Material and Methods

2.1. Material

Hepatocellular carcinoma cell line of Hep G2 and mononuclear cell lines U937 bought from Shanghai cell bank of Chinese academy of sciences. 1640 medium and fetal bovine serum were of Gibco products, trypsin, Buddha wave acetate (P1585, Sigma), human recombinant interleukin 4 (IL-4, PEP-ROTECH) and lipopolysaccharide (LPS, Sigma).

2.2. Cell culture and groups

Cells culture in 1640 completely medium containing 10% fetal bovine serum, 2% glutamine, 100 U/L streptomycin and100 U/m L penicillin at 37 ℃, 5% CO_2 incubator. When the adherent cells reached to 80% ~ 90%, cells were passaged. Experiment is set to 3groups, Hep G2 cells separate training group (Control group), Hep G2 cells with unactivated macrophages (unactivated macrophages, Ua group), Hep G2 cells and selective activation macrophages (alternatively activated macrophages, M2).

2.3. Macrophages induced in vitro

50ng/ml PMA were used to stimulate THP-1 for undifferentiated macrophages M0, and vaccinated in 6 well plates for culture 24h; Collect M0 Cells and detect the cell membrane protein expression using flow cytometry, and then it will be further induced M1 cells using 20ng/mL IFN–γ and 100ng/mL LPS stimulation training for 48h, and M2 type of macrophages were also induced using 100ng/mL IL-4 stimulation training for 48h. TAMs were obtained with EC9706 and M0 co-culture.

2.4. Detect the cell membrane protein expression with Flow cytometry instrument

Collected separately THP 1 source to stimulate 24h after PMA induced adherent cells (M0), and the subsequent induced adherent cells (M1), adjust to 5x 105 M2 and TAMs cells/tube, PBS wash once, 50mu RPMI1640 l + 1% FCS heavy suspension, to add PE-M0 Cy5 CD11b, to join in after induced by

stimulation of M0 FITC CD206 and PE CD16, avoid light incubation for 30min at room temperature, PBS wash 2 times, after reoccupy PBS 400mu l heavy suspension with BD both FACS Calibur flow cytometry instrument detection fixing, Filming pictures and analyzing purposes banding using Bander Leader software.

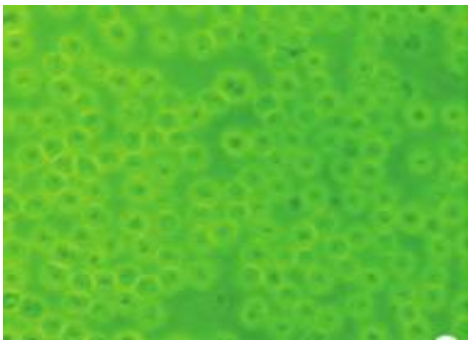

Figure 1. THP 1 cells (not induced).

2.5. Clone tablet form experiment

Each groups cell vaccinated in six holes culture plate for 1, 000 cells/hole, setting up three complex hole, and then gently shaking cells, culturing in 37℃ and 5% CO_2 incubator for 14~15 days. Fixed and dying, and count at low vision for ten vision, calculating more than 50 cell clone number, and take the average value. According to the formula to calculate the clone formation rate (%): cloning formation rate = number/cell number by 100%. The experiment repeated three times.

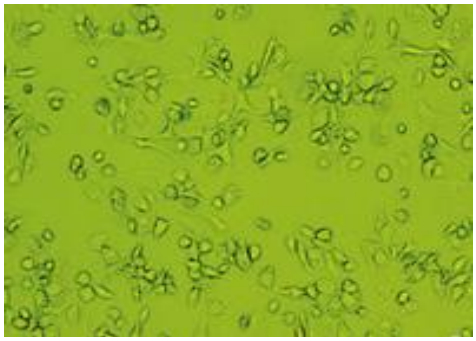

Figure 2. THP-1 cells after PMA induced.

2.6. Statistical methods

Analyzed with SPSS 16.0 statistical software, data were sided to mean±SD. The mean between the two groups were analyzed with independent samples T test, multiple groups comparison using one-way ANOVA, and using least significant difference methods (LSD methods) between groups. The difference had statistically significant when P less than 0.05.

3. Results

3.1. THP 1 macrophages cells induction and identification

THP 1 cells are round and suspended growth in the culture medium (Figure 1), after PMA induced, cells form irregular gradually, the cell body increase, cell intracytoplasmic vacuoles appeared, cell surface extended pseudopodia, begin to stick to wall after 2h, and basic stick wall after 12h. It grows into macrophages (M0, Figure 2) when completely adherent after 24h. Flow cytometry instrument testing shows that macrophage differentiation purity was above 99.57% (Figure 3). M0 to M1 differentiation ratio were more than 96% after IFN-γ+LPS stimulation (Figure 4a). But M0 to M2 cell differentiation rate were also more than 93% after IL-4 stimulus to (Figure 4b). TAMs obtained by M0 induced, and has 90% of the cell phenotype similar to M2 type differentiation (Figure 4c).

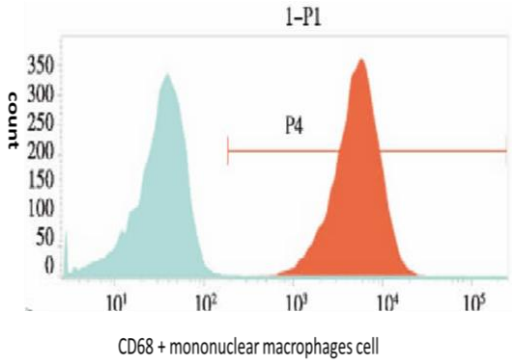

Figure 3. CD68 macrophages identification tags. P4 representative indicators CD68, positive rate was 99.57%.

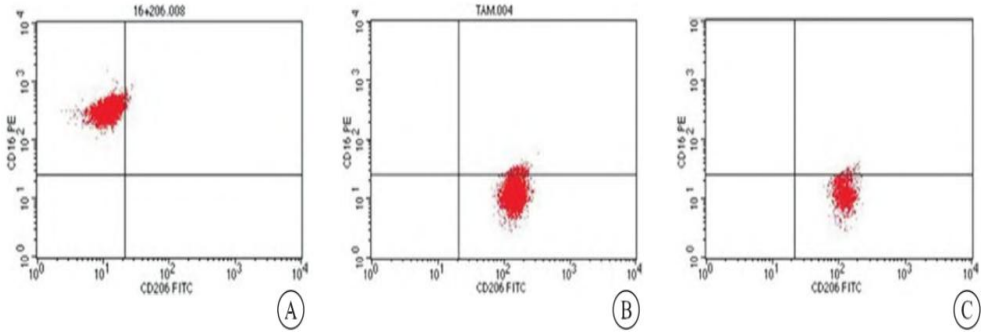

Figure 4. Different types of macrophage.

3.2. Cloning experiments

M2 groups can show obviously clone formation. Compared with control group and Ua group (51 + 0.13) %, the difference was statistically significant (P=0.001) (Figure 5).

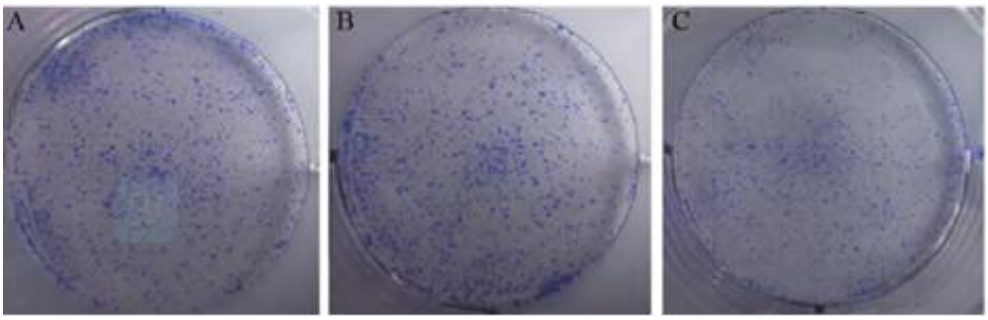

Figure 5. Cloning experiments. note: A: M2 group, B:Ua group, C: control group.

3.3. Scratch test

Compared with the Control group (P=0.004) and Ua group (P=0.005), M2 group cell scratch has been basically covered by the migrated cells. But there was no statistically significant difference between the Control group and Ua group (P=0.175) (Figure 6).

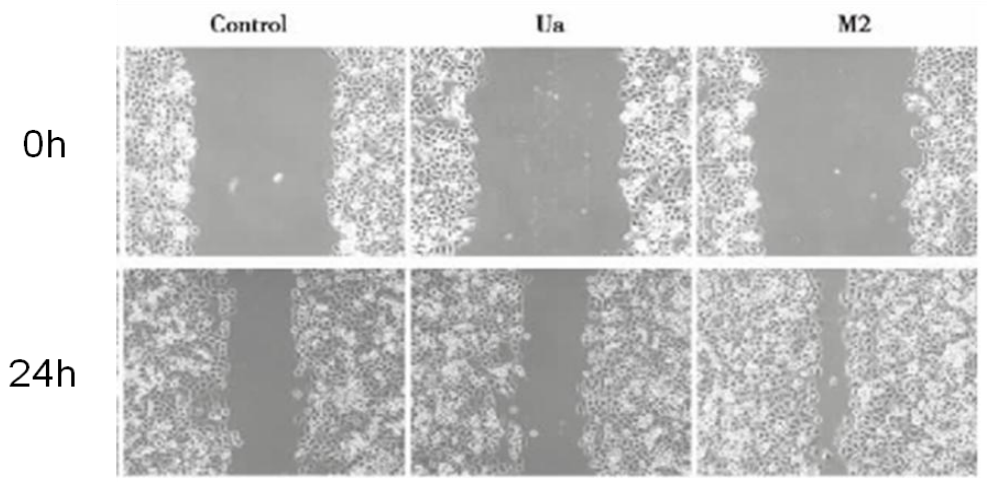

Figure 6. Selective macrophages cells can promote Hep G2 cells' lateral migration.

4. Discussion

As early as in 1889, Stephen Paget based on organ specificity of breast cancer metastasis in clinical observation, put forward the concept of the famous "seed and soil" [4]. The Ioan-nides Anderson cancer research center and the university of Pittsburgh whitesides officially for the first time put forward the concept of "the tumor microenvironment" [5]. Tumor microenvironment is the main influence factors to decided tumor cell behavior in the development of tumor recurrence and transfer, and plays an important role [6]. Tumor associated macrophage is the main immune cells in the tumor microenvironment, a large number of studies have shown that TAMs play an important role in the process of evolution of the occurrence of liver cancer [7].

This experiment adopts the density gradient centrifugation, isolated from healthy adult peripheral blood mononuclear cells, again with IL-4 or LPS in vitro to simulate and differentiated respectively to different activated macrophages for forming a real tumor microenvironment. The experimental results show that the M2 can significantly increase liver Hep G2 cells proliferation after 20h co-culture. M2 can significantly promote the liver cancer cells of lateral migration and longitudinal attack ability. TAMs is the largest number of inflammatory cells in the tumor stroma, account for 30%~50% of the total number of inflammatory cells, famous for its significant phenotypic heterogeneity and functional diversity [8]. M1 mainly secreted pro-inflammatory substances such as IL-1, IL-6, IL-12, IL-23, TNA-a, CXCL-10, ROI [9]. M1 can Present antigen, participate in the positive immune response, immune surveillance function. And M2 is only the weaker antigen presenting ability, which produces a high level of suppression of inflammatory substances such as IL-10, CCL-17, 18,

CCL CCL-22, it can repair tissue, inhibit inflammation and promote tumor [10]. In tumor microenvironment, under the action of stimulating factors such as GM-CSF, M-CSF, IFN-gamma, IL-4, IL-13, lactic acid, macrophages is selectively activated and form of tumor associated macrophages. A large number of studies have shown that M2 is associated with tumor which is similar to the phenotype and function of macrophages. Now vitro experiment was going by M2 simulation entity tumor TAMs [11]. TAMs is a major component of interstitial solid tumors, gathered in the tumor stroma, secretion of chemokines related to tumor cells, including the M-CSF, MCP-1, CCL3, VEGF and uncomplicated 2 [12-13]. Macrophages directly participated in the reconstruction of angiogenesis, tumor cell growth, substrate, energy metabolism evolution process. It plays a two-way adjustment function in tumor. Thus, further clarify the mutual relationship between macrophages and tumor cells, balancing its effect on promoting cancer and suppressing tumor, diagnosis and treatment of the tumor is of great significance [14].

We Successful separation cultivate tumor giant cells. Our study shows that in vitro microenvironment of liver cancer, M2 can significantly promote the HepG2 cell proliferation and clone formation ability after co-culture with M2 for 24 hours. The processes of cell infiltration and migration were also promoted too. However, the specific interaction mechanism in the process of specific transduction and regulation in liver cancer between PI3K/Akt/m signaling pathways and TAMs, and the more the pathway related protein (such as PTEN, EGFR, NF-κB, Notch, PDK-1, 2, Bad, RPTK PDK -, etc.) is needed to further study.

5. Conclusion

To sum up, this study focuses on the influences of TAMs on liver cancer cells of migrating and infiltrating ability. We supplementary interpret the TAMs molecular mechanism on promoting the development of liver cancer, and to provide new guidance and perspective on the targeted of TAMs in inhibiting hepatocellular carcinoma infiltration and migration.

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