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The mitogenic and anti-apoptotic activity of tumor conditioned medium on endothelium

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Abstract. This study was designed to observe the effect of tumor conditioned medium (TCM) on the proliferation and apoptosis of human umbilical vein endothelial cells (HUVECs). HUVECs were exposed to TCM from breast carcinoma cell line MDA-MB-231, then we measured their proliferation, apoptosis and cell cycle distribution by MTT and flow cytometery (FCM). Following the stimulation of TCM, HUVECs showed higher pro-mitogenic and anti-apoptotic ability than did the negative control group (ECGF-free medium with 20% FBS), but a similar ability to the positive control group (medium with ECGF and 20% FBS). From these results, we can conclude that breast carcinoma cell line MDA-MB-231 could secret soluble pro-angiogenic factors that induce HUVEC angiogenic switching, including cell cycle progression, proliferation and growth. The role and character of these factors remain to be further studied.

Keywords: Tumor conditioned medium, endothelium, proliferation, apoptosis, cell cycle

1. Introduction

Angiogenesis, the growth of new capillary blood vessels, was first proposed by Folkman in 1970, and his hypothesis has now been confirmed by molecular studies [1]. This brought renewed hope for cancer patients [2,3]. In 1986, Xiu and her associates also observed the significant angiogenic phenomenon after a 48-hour transplant of Hela and SP2/0 in mice [4,5]. Along with these studies, investigators have found that angiogenesis is a multi-step process and characteristic with heterogeneity [6,7]. Although a "cock-tail" treatment containing various kinds of angiogenesis inhibitor could improve the efficacy of therapy, it costs a lot. These factors force investigators to search for a common target involving angiogenesis for effective therapy.

In previous studies, investigators found that two critical elements play an important role in angio-genesis: pro-angiogenic factors secreted from cancer cells and the surrounding stroma cells; and the angiogenic switch of endothelial cells from a quiescent state to a highly active state. Many studies have been done on the angiogenic factors, and verified in previous work [6], but the latter is still a new field in which few people have set foot.

As is well known, the normal endothelial cells, which are in the inner layer of vessel wall and an play important role in regulating vascular function, are in a high acquiescent state [8]. They may switch to an active proliferative state via stimulation of the angiogenic factor from a tumor, then grow, migrate, and form a new blood vessel that supplies the tumor with oxygen and nutrients [9,10]. Almost all growth factors secreted by tumor cells could induce endothelial cell (EC) proliferation, eventually undergoing

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differentiation to form new blood vessels. As a result, the inhibition of endothelial cell growth can effectively block angiogenesis. So, research into drugs targeting the EC and inhibiting its proliferation have become the focus for blocking angiogenesis. Many angiogenesis inhibitors play a role by inhibiting endothelial proliferation, e.g., Endostatin [11,12], Angiostatin [13], and others. For endothelial hyperproliferation, here we hypothesize that it is also a cell cycle-related disease as well as a tumor cell, where the growth of endothelial cells is beyond control.

To study the rate of proliferation, cell cycle distribution and the ratio of survival and apoptotic cells give other signs of the growth property of cells, especially the former, due to the fact that the three parts of cell proliferation (growth, DNA synthesis and mitosis) end through the cell cycle, so it is important to count the number of cells in different cell cycles and apoptotic cells. The present study was designed to investigate the potency of TCM from breast carcinoma cell line MDA-MB-231 to affect endothelial proliferation, apoptosis, and observe changes in cell cycle.

2. Materials and methods

2.1. Cells and cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated by a mild collagenase treatment as Jaffe has previously described [14]. The cells were cultured and maintained in a complete endothelial growth medium consisting of M199 (Gibco) supplemented with 20% fetal bovine serum (FBS) (Hyclone), 20 μg/ml endothelial cell growth factor (ECGF) (Sigma E9640), 2 mM L-glutamine, 0.2% $NaHCO_3$, 15 mM Hepes, 90 μg/ml heparin, 100 U/ml penicillin and 10 mg/ml streptomycin. Cells were grown at 37°C in a humidified 5% carbon dioxide atmosphere and subcultured by trypsinisation with 0.25% trypsin–0.02% EDTA when confluent monolayers were reached. Endothelial cells were identified by typical phase contrast "cobblestone" morphology and by the presence of von Willebrand factor antigen using the immunohistochemistry technique. HUVECs were synchronized in G_0-G_1 phase by serum starved overnight in M199 added to 1% FBS before stimulation with TCM [15] or by contact inhibition of the confluent monolayers for 24 hours. HUVECs were used between Passage 2 and 3.

The metastatic human breast cell line MDA-MB-231 was purchased from the PUMC Cell Center, China, and resuscitated in medium L-15 supplemented with 15% FBS, 100 U/ml penicillin and 10 mg/ml streptomycin, 2 mM L-glutamine, and then changed gradually to M199 (which is the same as HUVEC without ECGF).

2.2. Preparation of tumor conditioned medium (TCM)

TCM was prepared from the MDA-MB-231 cell culture as follows: MDA-MB-231 cells were grown to subconfluency (roughly 90%). After being washed twice with PBS, cells were incubated in 10 ml serum and an ECGF-free medium (75 cm^2 flask) at 37°C in a humidified 5% carbon dioxide atmosphere for 24 hours. The supernatant was then harvested, centrifuged at 2000 g, 4°C for 10 minutes, filter-sterilized through 0.22 μM pore size filters and stored at -20°C prior to use supplemented with FBS [16].

2.3. Endothelial cell proliferation assay

HUVEC proliferation was assayed by a colorimetric method. Post-confluent endothelial cells were plated into 96 well plates at 5×10^3 cells/well and incubated in M199 complete growth medium overnight

for cell attachment. Then the cells were washed by phosphate-buffered saline (PBS) and incubated in negative control medium (20% BS-M199, 20% FBS-M), trial control medium (TCM-M), positive control medium (M199 with ECGF and 20% FBS, ECGF-M) for 60 hours. Proliferation stimulated by tumor supernatant was measured using the MTT colorimetric assay. After the specified incubation period at 37°C, the cells were washed by PBS and 10 ml of MTT (5 mg/ml PBS, Sigma) was added to each well for 5 hours at 37°C. Supernatants were then carefully aspirated, followed by the addition of 100 ml/well of acidified SDS. The plate was then shaken at room temperature for 1 hour for the complete solution of crystal, and the absorbance at 630 nm (optical density OD) was read on a Bio-Tek microplate reader.

2.4. Flow cytometry for measurement of cell-cycle distribution

HUVEC cells were growth-arrested by contact inhibition for 24 hours, then trypsinized and replanted in a 25 cm^2 flask. Following overnight incubation in complete growth medium for cells to attach, the medium was changed (20% FBS-M, TCM-M, ECGF-M) and the cells were incubated at 37°C for 60 hours. After that, cells were detached by trypsinization, washed by PBS, and centrifuged at 1000 rpm for 10 minutes. The pellet was resuspended in 70% pre-cold ethanol and fixed overnight at 4°C. Next day, the cells was stained in PBS with addition of 50 μg/ml Propidium iodine (PI, Sigma), 25 mg/ml RNase A (Sigma) and 0.1% (v/v) Triton X-100 for 30 minutes at room temperature in the dark. The cells were analyzed using a FACScan flow cytometer (EPICS XL) to observe the effect of TCM on HUVEC proliferation and its distribution in different cell cycles.

2.5. Cell apoptosis analysis

Apoptotic cells were analyzed using flow cytometry according to the recommended procedures described by the Annexin-V Apoptosis Detection Kit (purchased from BaoSai Cot). The HUVECs synchronized by contact inhibition were planted in a 25 cm^2 culture flask at 5×10^5 cells/ml and incubated in a complete growth medium for 24 hours to allow cell attachment. Then the medium was changed to 20% FBS-M, TCM-M, ECGF-M and the cell was treated with 100 mmol H_2O_2 for 24 hours [17]. After treatment, cells were digested, collected, washed by PBS and resuspended in binding buffer with the addition of 10 ml Annexin V-FITC (20 mg/ml) and 5 ml PI (50 μg/ml). The mixture was kept in the dark for 30 minutes at 4°C, then 300 ml of binding buffer was added and analyzed immediately in a flow cytometer.

2.6. Statistic analysis

Data are expressed as mean $\pm$ SE. All statistical assessments were performed with SPSS10.0 software using Student's t-test for unpaired data. $P < 0.05$ was accepted as being statistically significant.

3. Results

3.1. Mitogenic activity and cell cycle progression of TCM

The monolayer confluent HUVEC showed typical cobblestone-forming patterns under a time-lapse microscope and identified via VIII factor immunofluorescene staining (Figs 1 and 2). The growth rate of the HUVECs was measured and it was found that the time of the HUVEC entering a logarithmic growth

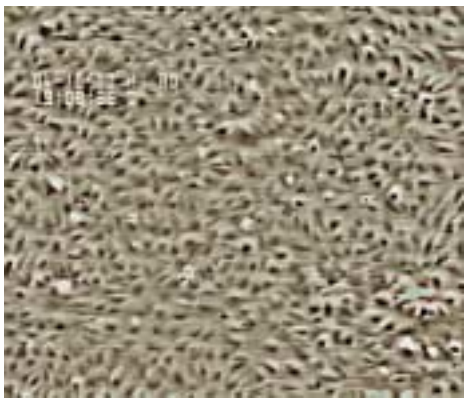

Fig. 1. Confluent HUVECs show the typical cobblestone pattern.

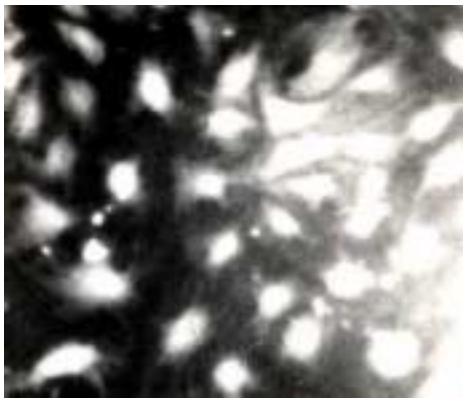

Fig. 2. The confluent monolayer of HUVEC visualized by VIII factor immunofluorescene staining. The positive cells show a high fluorescence label and cobblestone shape; the rate is over 95%.

stage is about 60 hours. So, we treated the cell for that length of time. Following a 60-hour incubation of HUVEC in a different medium, the growth rate was determined using a MTT assay. The results showed that the group cells treated with TCM have a significantly higher OD than control group ($P < 0.05$), but their OD is a little lower than that of ECGF-treated cells with no significance, which means that TCM from the cell line MDA-MB-231 could enhance HUVEC proliferation as well as ECGF (Fig. 3).

3.2. The effect of TCM on cell cycles

In order to observe the effect of TCM on endothelial cell distribution in different cell cycles, HUVECs were exposed to TCM for 60 hours. After that, cell distribution was measured and the same trend was found as for mitogenic activity. There was a higher rate in S and G_2 phases in the trial group than in the negative control group (respectively $13.76 \pm 1.33\%$ and $14.03 \pm 0.39\%$ vs. $6.17 \pm 0.32\%$ and $10.63 \pm 0.43\%$); the difference is significant ($P < 0.05$ in the S phase). The difference between TCM-M and ECGF-M ($15.87 \pm 0.55\%$ and $15.83 \pm 2.00\%$) is not significant (Fig. 4).

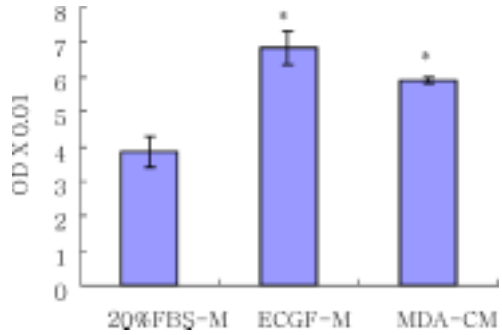

Fig. 3. The optical absorbance after treatment of different medium for 60 h. $^{*}P < 0.05$ vs. 20% FBS-M.

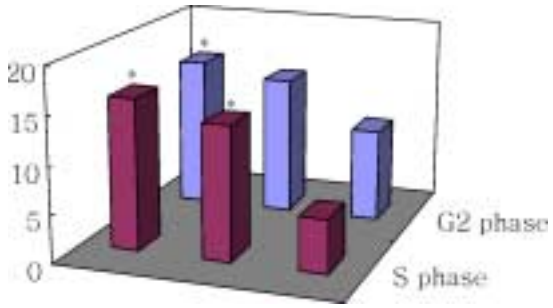

Fig. 4. The percentage of cell cycle distribution in S and G_2 phase of the three group cells. $^{*}P < 0.05$ vs. 20% FBS-M group. The Y-axis represent cell percentage (%), the X-axis order is ECGF-M, MDA-CM, 20% FBS-M (from left to right).

3.3. TCM-inhibiting HUVEC apoptosis induced by H_2O_2

The number of apoptotic HUVECs detected by flow cytometry indicated that MB-231 could prevent HUVEC from apoptosis induced by H_2O_2 as well as ECGF. FCM detection shows a lower rate of apoptosis, and a higher rate of survival appeared in the TCM-treated group than in the negative control group (respectively, 14.9% vs. 40.6% and 85.1% vs. 59.3%), but the rate was similar to that in the positive (ECGF-M) control group (4.6% and 95.2%) (Fig. 5).

4. Discussion

It has been recognized for some time that tumor cells are able to secret soluble products which are mitogenic for endothelium [6,18]. The human tumor cell lines tested in this study were selected on the basic of previous studies that have demonstrated the role of this conditioned medium on angiogenesis and the possible role of surface protein induced by cell proliferation. But no further studies have been undertaken on cell cycles and their relationship with apoptosis. Our results of this *in vitro* study support the role of TCM and further verify the effect of TCM on cell cycle and apoptosis.

Hewett [19] demonstrated using [^{51}Cr]-chromium-relase assays that tumor cells MDA-MB-231 are not significantly affected by 24-hour incubation in serum-free medium and therefore TCM should only contain factors secreted by these cells. Our results show that HUVECs treated with TCM have a higher mitogenic viability than 20% FBS-M ($P < 0.05$), but this is similar to ECGF-medium. The result is consistent with accelerated cell number counting and cell cycle distribution in the S and G_2 phases due

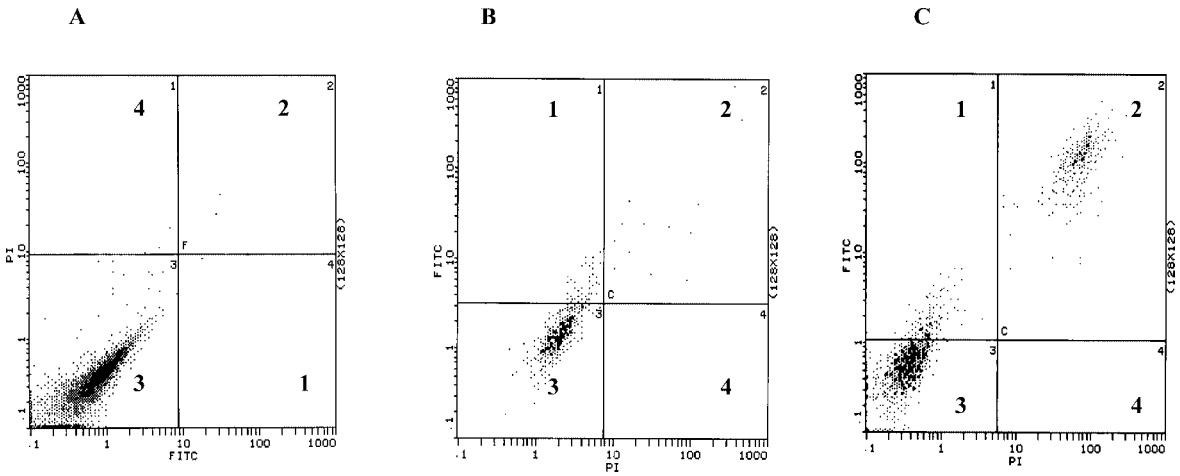

Fig. 5. Flow cytometry detection of apoptotic cells after different conditioned medium treatment A: ECGF-M+H_2O_2 B: MDA-CM+H_2O_2 C: 20% FBS-M+H_2O_2. Region 1: apoptotic cell in early stage. Region 2: apoptotic cell in late stage. Region 3: survival cell. Region 4: dead cell.

to the 60-hour incubation with TCM. All these factors indicate that there are some pro-mitogenic factors secreted from MDA-MB-231 playing a similar role as ECGF, which promotes the cell cycle. Our result is consistent with a previous study [20] which proved that a tumor-conditioned medium prepared from MDA-MB-231 cell cultures contained high concentrations of GM-CSF, VEGF, and IL-8. It is interesting that we found the hetereogenity of TCM from different tumors. The TCM from neuroblastoma has the highest mitogenic ability of three kinds of TCM from MCF-7, SK-NC, MDA-MB-231 (data not shown). The mitogenic effect and cell counting result from TCM from MCF-7 is a little higher than that from MDA-MB-231, despite its lower metastasis and invasion feature (data not shown). This suggests that there are some other factors in TCM from MDA-MB-231 which could promote angiogenesis via other passages than cell proliferation, such as promoting its migration as well as that high concentration of uPA exist in supernatant of MDA-MB-231 but not in MCF-7 [21]. One report [16] also showedthat the supernatant from MCF-7 has a lower mitogenic effect on HUVEC by cell counting. This discrepancy may result from differences in cell type, the collection of TCM and counting time following treatment in the experiment.

Recent evidence suggests that the apoptosis of vascular endothelial cells regulates angiogenesis and promotes vascular regression [22]. In fact, endothelial cells switching to an angiogenic phenotype may involve the inhibition of endothelial cell apoptosis resulting from a growth factor. Tumor cells can produce certain cytokines such as VEGF, a potent endothelial cell-specific mitogen inducer of tumour angiogenesis. It is produced in both humans and animals by many tumor cells [23–25], and promotes endothelial cell survival and angiogenesis. Its role and mechanism involve not only stimulating endothelial cell proliferation but also inhibiting endothelial cell apoptosis. Previous studies have shown that VEGF inhibits the apoptosis of large-vessel endothelial cells induced by tumor necrosis factor-α (TNF-α), Recent studies show the mechanism of this role may via have opposing effects on MAPK/ERK and SAPK/JNK signaling [26,27] and may also support the growth of HUVEC and HDMEC (human dermal microvascular endothelial cells) cultured under serum-free (SF) conditions, while cells in SF conditions without VEGF have shown morphological features characteristic of apoptosis. In our laboratory, Jia [12] found that endostatin gene therapy targeting tumor neovasculature could induce evident endothelial apoptosis with smaller tumor size and lower metastasis rate than the control group.

Previous studies have proved that 100–300 μmol/L H_2O_2 can induce bovine aortic endothelial apoptosis. The apoptotic cells show morphological change rounding, detaching and chromatin condensation, and you can see apoptotic bodies resulting from the fragmentation of dying cells. In order to observe the anti-apoptotic role of MDA-MB-231, we used a type of early apoptosis detection kit using phosphotidylserine (PS) as a marker, which normally locates in the inner cell membrane and comes up to the cell surface when early apoptosis occurs, when it can be detected via flow cytometer, by FITC-labeled Annexin, which binds with PS with high and specific affinity. This kind of cell could be distinguished by the use of PI staining of the dead and late apoptotic cell. From our results, we can see that cells treated with TCM experience apoptosis late and mildly, in contrast to the negative control group, but in a similar way to the positive control group. This further confirmed our conclusion that it is about the existence of growth factors and anti-apoptotic factors in TCM.

5. Conclusion

The result of our investigation proved that breast carcinoma cell line MDA-MB-231 could secrete soluble pro-angiogenic factors that promote HUVEC angiogenic switch including cell cycle progression, proliferation, and anti-apoptosis. Exploration of the mechanism and key regulator in this process will be very interesting. Uncovering their character and function in cell growth would be hard work, but well worth doing.

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