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A proteomic study on cell cycle progression of endothelium exposed to tumor conditioned medium and the possible role of cyclin D_1/E

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Abstract. This study was designed to comprehensively analyze the differential expression of proteins from human umbilical vein endothelial cells (HUVECs) exposed to tumor conditioned medium (TCM) and to identify the key regulator in the cell cycle progression. The HUVECs were exposed to TCM from breast carcinoma cell line MDA-MB-231, then their cell cycle distribution was measured by flow cytometer (FCM). The role of protein in cell cycle progression was detected via two-dimensional polyacrylamide gel electrophoresis (2-DE) and western blotting. Following the stimulation of TCM, HUVECs showed a more cells in the S phase than did the negative control group (ECGF-free medium with 20% FBS), but the HUVECs' level was similar to the positive control group (medium with 25 μg/ml ECGF and 20% FBS). Increased expression of cyclin D_1/E and some changes in other related proteins occurred after incubation with TCM. From our results, we can conclude that breast carcinoma cell line MDA-MB-231 may secrete soluble pro-angiogenic factors that induce the HUVEC angiogenic switch, during which the expression of cell cycle regulator cyclin D_1/E increases and related proteins play an important role in this process.

Keywords: Tumor conditioned medium, endothelium, cell cycle, 2-DE, Western blot

1. Introduction

Angiogenesis was first proposed by Folkman in 1970, and his hypothesis has now been confirmed by molecular studies [1]. This has brought new therapy hope to cancer patients [2,3]. In 1986, Xiu and associates also observed the significant angiogenic phenomenon after 48-hour transplant of Hela and SP2/0 in mice [4,5].

On the mechanism of angiogenesis, much research has focused on the pro- or anti-angiogenic factors secreted in tumor cells, while little work has been done on the endothelial proliferating switch under the action of the growth factor [6]. In contrast to the highly quiescent endothelium in normal vascular walls, tumor-derived endothelium has been shown to be in a rapid proliferating state [7]. Clarification of the key factor regulating the endothelial proliferating switch and its signal pathway will greatly help us in understanding the mechanism of angiogenesis.

Cellular proliferation occurs through cell cycle progression which contains several phases of the cell cycle, regulated by both positive and negative regulators. The former contains cyclins and cyclin-dependent kinase (CDKs). The two factors combine and form a heterogenous complex with protein kinase activity, determine some key events in the G_1 phase and control the pace entering cell cycle. There

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are two kinds of critical cyclins in the cell cycle. One is cyclin D1 which combines with Cdk4 and plays a central role in the regulation of cell cycle transitions in normal cells, thus helping to phosphorylate the product of the retinoblastoma gene (pRb) and allowing G_1 to S transition [8–10]. Cyclin E is another important factor regulating G_1 transition to the S phase. The negative factors contain CDK inhibitors such as p21, p27, p16, p53 and so on. They inhibit the CDKs' activity, and then block the progression of the cell cycle. Recent studies show that cyclin D1 is involved in angiogenesis, for which there are many pro-angiogenic factors such as VEGF. bFGF may promote cyclin D1 synthesis, enhance CDK4 activity, then accelerate angiogenesis [11,12], while many anti-angiogenic drugs such as TNP, ANP, Indomethin, and Kininostatin could inhibit angiogenesis by reducing the expression of cyclin D1 and related protein, e.g., p27 [11–15]. The subcutaneous injection of p21 could significantly inhibit the angiogenesis of breast cancer [16].

The endothelial proliferating switch can result from changes in a series of proteins and the interaction of these proteins. To study this sophisticated process, it would be better to develop a method that could comprehensively analyze the globally differential expression of proteins. Proteomics, which is based on the techniques of two-dimensional electrophoresis (2-DE) and peptide mass fingerprinting (PMF), offers a promising way to resolve hundreds of proteins at the same time.

For endothelial hyper-proliferation, here we hypothesize that it is also a type of cell cycle-related disease as well as s tumor cell, where the growth of endothelial cells are beyond control. We propose that, if we could find the key regulator in the angiogenic switch, it would have inestimable significance for cancer therapy. The present study was designed to investigate the potency of TCM from breast carcinoma cell line MDA-MB-231 to affect endothelial proliferation, apoptosis; to observe changes in the cell cycle, and study the possible role of cyclin D_1/E and other related proteins.

2. Materials and methods

2.1. Cells and cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated by mild collagenase treatment, as previously described by Jaffe [17]. The cells were cultured and maintained in endothelial complete 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 the von Willebrand factor antigen using the immunohistochemistry technique. HUVECs were synchronized in the G_0-G_1 phase by serum starved overnight in M199 added to 1% FBS before stimulation with TCM 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 Centre 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 humidified 5% carbon dioxide atmosphere for 24 hours. The supernatant was then harvested, centrifuged at 2000 g and at 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 [18].

2.3. Flow cytometry for the 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 the overnight incubation in a complete growth medium for cells to attach, the medium was changed to a different medium (20% FBS-M, MDA-CM, ECGF-M) and the cells were incubated at 37°C for 60 hours. After this, 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 were stained in PBS with the 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 FAC Scan flow cytometer (EPICS XL) to observe the effect of TCM on HUVEC proliferation and its distribution in different cell cycles.

2.4. Cell lysate preparation

After treatment with TCM, cells were digested by trypsinisation with 0.25% trypsin-02% EDTA and centrifuged at 150 g at 4°C. The pellet was washed at least 4 times with PBS and after the last wash, the PBS was completely removed, in order not to leave any PBS which would affect the results of the following 2-DE and Western blotting. Then, the cell pellets were solubilized with a lysis buffer solution containing urea (8 M), CHAPS (4%), DTT (70 mM), and resolytes 3–10 (2%), and the mixture was centrifuged at 12000 rpm, at 4°C, for 15 minutes. The supernatant was collected and designated as whole cell lysate and used for Western blotting and 2-DE. The protein concentration was determined using the Bradford method.

2.5. Western blot analysis

Equal amounts of proteins were fractioned by SDS-PAGE, and then one piece of gel was stained by coomassie to observe the electrophoresis state of the cell protein. The other piece of gel was electro-transferred semi-dry onto a polyvinylidene difluoride (PVDF) membrane at 40 mA/cm^2 for 45 minutes. The PVDF membrane was blocked with 5% (w/v) fat-free milk powder in TBS (100 mM Tris-HCl, pH = 7.5, 50 mM NaCl) containing 0.1% (v/v) Tween 20 overnight at 4°C. After washing with TBST, the PVDF membrane was immunoblotted with primary antibodies against cyclin D1 (mouse against human monoclonal antibody 1 : 100) and cyclin E (rabbit against human polyclonal antibody 1 : 200). The bound antibodies were visualized using a secondary horseradish peroxidase-linked anti-mouse or anti-rabbit antibody (Santa Cruz) and a DAB system.

2.6. Two-dimensional cell electrophoresis

2-D PAGE was performed using the Multiphor II horizontal system (Amersham Pharmacia Biotech Int). Immobiline^{TM} gradient isoelectric focusing gels were rehydrated overnight in sample buffer, then the immobiline gels were equilibrated for 25 minutes with 50 mmol/l Tris-HCl, pH $= 8.8$, 6 mol/l urea, 30% glycerol and 2% SDS at room temperature. The immobiline stripes were then washed in distilled water and electrophoresis was carried out at a constant current (60 mA/gel) and temperature (20°C) in 12% SDS-PAGE gradient gels. After electrophoresis, gels were stained with coomassie overnight and decolored 2–30 minutes with 30% thonal and 10% acetic acid for the image analysis and spot identification.

2.7. 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. A level of $p < 0.05$ was accepted as statistically significant.

3. Results

3.1. Effect of TCM on cell cycle

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

3.2. TCM promoting cyclin D_1 and cyclin E protein expression

We also monitored the percentage of cells in the S phase of HUVECs at various times after treatment with TCM, and found that double number cells come into the S phase at the 12–16 hour time points (Fig. 1). To verify whether or not the change of cell cycle of HUVEC was due to cyclin D1/E expression induced by TCM, we next examined the cyclin D1/E protein expression by Western blot of the HUVECs at the 10-hour time point. After SDS-PAGE, we can see that the HUVECs treated with TCM show a higher expression of cyclin D_1/E than the control group (Fig. 2) and there is a different distribution of total protein.

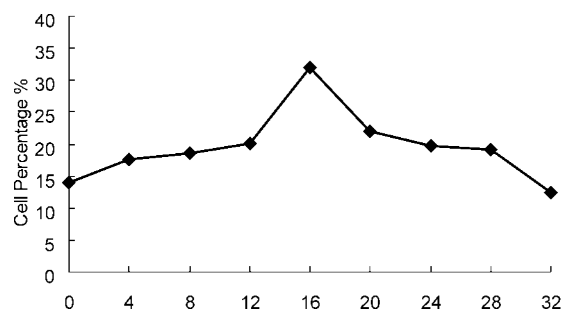

Fig. 1. Time course of the effect of TCM on HUVEC cell cycle $n = 3$.

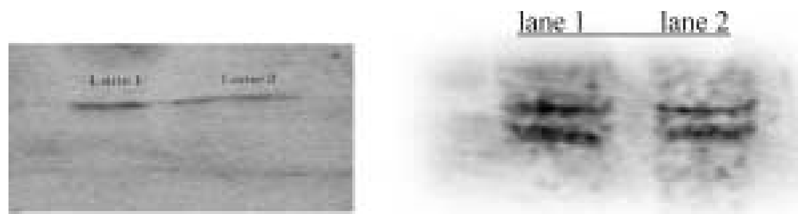

Fig. 2. Expression of cyclin D1/E. Lane 1 MDA-CM group; Lane 2 20%FBS-M group.

Table 1
Expression in treated cells

Spot	SWISS-PROT ACC No	Match rate %	Coverage rate %	Identification of protein
Up-regulated protein				
2	4503571	45	28	Enolase-1
3	P55059	38	15	Phospholipid transfer protein precursor (PLTP precursor)
4	Q00571	53	21	DEAD-box protein
5	P89871	36	12	C1 esterase
8	P14618	62	27	Pyruvate kinase
9	P13010	56	12	ATP-depend DNA helicase II
Down-regulated protein				
6	Q15583	29	18	5′-TG-3′ interacting factor
7	P14207	28	23	FR-beta
10	P27105	25	38	Stomatin

3.3. *Detection of cell cycle related protein induced by TCM*

In order to further verify TCM's role in controlling cell cycle progression, we performed a 2D electrophoresis and image analysis to find the differently expressed proteins. Pairs of samples collected at the same time from normal and treated cells were electrophoresized. Gels were matched in pairs and differences in abundance were calculated for spot. The analysis revealed that 11 spots showed different expressions in treated cells and 9 spots were identified by PepIdent (Fig. 3, Table 1).

4. Discussion

It has been recognized for some time that tumor cells are able to secrete soluble products which are mitogenic for endothelium [6,19]. The human tumor cell lines tested in this study were selected on the basic of previous studies which demonstrated the role of this conditioned medium on angiogenesis and the possible role of surface proteins induced by cell proliferation. But no further studies have been carried out on the mechanism of the angiogenic switch and its regulator, particularly the role of cyclin D1/E. Our results of this *in vitro* study support the importance of the role of TCM and cyclin D1/E in the angiogenic switch. It is a key regulator.

Hewett [20] demonstrated using [^{51}Cr]-chromium-relase assays that tumor cells MDA-MB-231 are not significantly affected by 24-hour incubation in a serum-free medium, and therefore TCM should only contain factors secreted by these cells. Our result shows that HUVECs treated with TCM have a higher

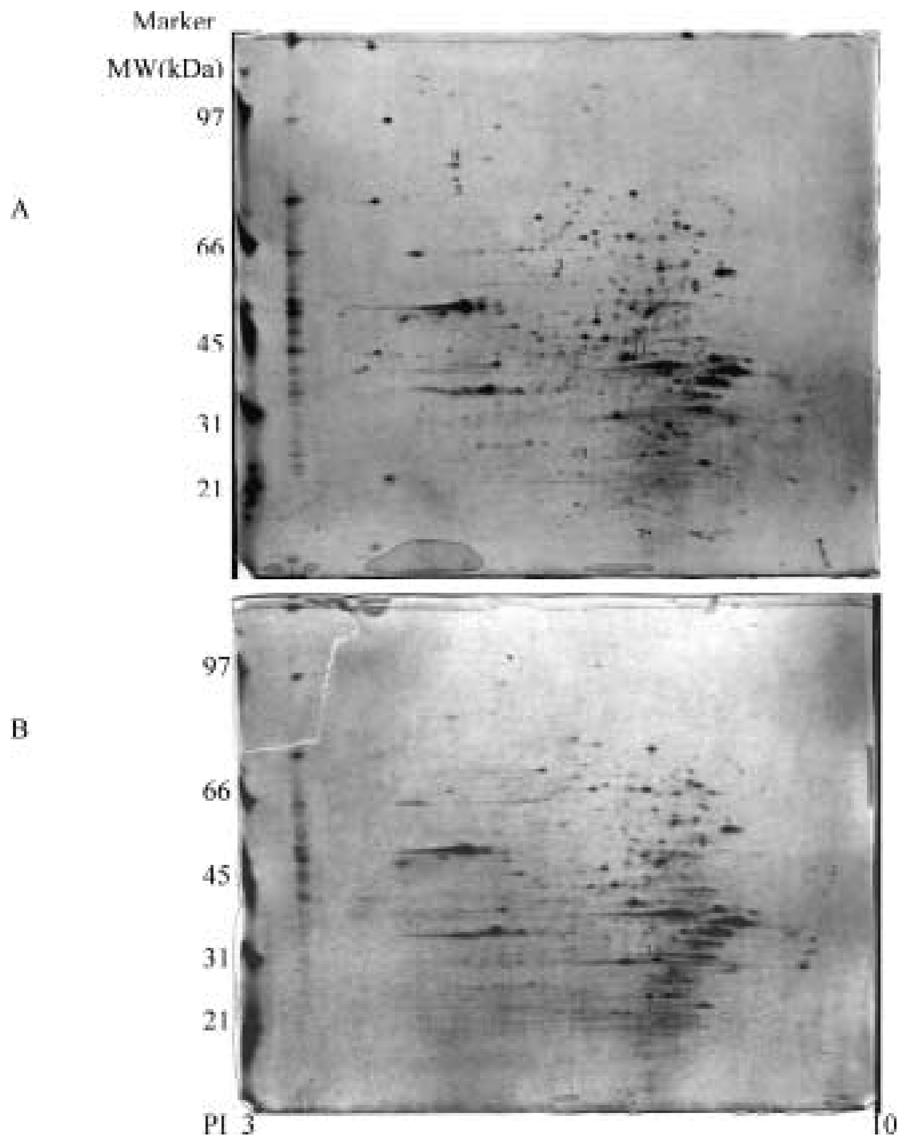

Fig. 3. 2D electrophoresis of total protein extracted from different group cells, load sample 1 mg. The up-regulated spot was labeled in map A (MDA-CM), the down-regulated was showed in map B (20% FBS-M).

mitogenic viability than 20% FBS-M ($p < 0.05$), but this is similar to the ECGF medium. The result is consistent with accelerated cell number counting and cell cycle distribution in the S and G_2 phases owing to the 60-hour incubation with TCM. All these factors indicate 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 the previous study [21], which proved that tumor conditioned medium prepared from MDA-MB-231 cell cultures contained high concentrations of GM-CSF, VEGF, and IL-8.

In order to observe the effects of TCM on cell cycles, we began our study by defining the time course of this effect. At various times after treatment with TCM, we monitored the percentage of cells in the S phase. TCM induced HUVECs into the S phase at the 12–16 hour time points, which is similar to

but a little earlier than the previous study. This discrepancy may result from treatment with different concentrations of FBS.

The importance of cyclin D1 in transitioning cells from the G_1 into the S phase has been amply demonstrated [9]. The cyclin D1 gene product contributes to the regulation of the G_1/S phase transition of the cell cycle and is a candidate oncogene [8]. Cyclin D1 null animals are viable; the abrogation of cyclin D1 expression *in vitro* (e.g., by antisense methods) causes cell cycle arrest or marked inhibition of cell proliferation [22–27]. From our novel observation resulted from western blotting, we suggest that TCM promotes cyclin D1/E expression and then cell cycle progression. Cyclin D1/E is a target of the angiogenic factor secreted in tumor cells. All these factors are consistent with the findings of previous studies that many pro-angiogenic factors such as VEGF and bFGF could promote cyclin D1 synthesis, enhance CDK4 activity, then accelerate angiogenesis [11,12], while many anti-angiogenic drugs such as TNP, ANP, Indomethin, and Kininostatin could inhibit angiogenesis by reducing the expression of cyclin D1 and related protein, e.g., p27 [11–15]. But, for the role of cyclin E in angiogenesis, there are few findings, while our study shows some change and a possible role in the angiogenic switch. The expression of cyclin E in the trial group is higher than the control group. The appearances of two band may be due to the cross-reactivity resulting from polyclonal antibodies or a new change in cyclin E protein. To clarify this, we need further research.

The identification of cyclin D1 has provided an insight into the intracellular signaling events triggered by TCM, but how it connects to cell surface receptors and remains to be elucidated. To further define the other regulators, we performed 2D electrophoresis. The protein expression profile changed significantly after the stimulation of TCM. We identified 8 up-regulated and 3 down-regulated proteins visualized by number, and identified 9 kinds of proteins that are involved in important events in the cell cycle such as DNA synthesis, transcription, cell proliferation and migration, and so on.

5. Conclusion

The result of our investigation has proved that the breast carcinoma cell line MDA-MB-231 may secrete soluble pro-angiogenic factors that promote the HUVEC angiogenic switch, including cell cycle progression, proliferation and growth, during which the increased expression of the cell cycle regulator cyclin D1/E and related proteins play an important role. Our research first tried using proteomic technology to study the protein factors which regulate endothelial active proliferation. Uncovering their character and function in cell growth would be hard work, but well worth doing.

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