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The glycan profile of endothelial cells in the present of tumor-conditioned medium and potential roles of β-1,6-GlcNAc branching on HUVEC conformation

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Abstract Endothelium plays a vital role in the logistics of the immune system, as well as the maintenance of the homeostasis. The major objective of this study is to unravel the relationship between expression changes of carbohydrate structures and the dysfunction of human umbilical vein endothelial cells (HUVEC) stimulated with tumor-conditioned medium (TCM), which is involved in tumor cell extravasation. Using flow cytometry (FCM) assay, the expression profiles of a selected group of 9 carbohydrate structures have been determined in HUVEC under control conditions and TCM-treated conditions, six of which increased significantly in expression after induction. Particularly, the expression level of β-1,6-GlcNAc branching glycan was extremely higher after the stimulation. In parallel, the conformation change of HUVEC monolayer has been detected with inverted phase contrast microscopy and confocal microscopy. Under TCM stimulation, the actin cytoskeleton underwent rearrangement and formed abundant stress fiber within cells; therefore cell contraction was induced, which resulted in paracellular gap formation and barrier dysfunction. We furthered our study to investigate the mechanism underlying the conformation change of HUVEC. The results demonstrated that TCM induced the increase in β-1,6-GlcNAc branching expression of PECAM-1, accompanied by the tyrosine phosphorylation of PECAM-1. The downstream effector RhoA was activated in consequence of the activation of PECAM-1. In conclusion, our results strongly suggested that the carbohydrate composition of endothelial cell surface is very important for the cells to exert their physiological effects correlated with cancer extravasation.

Keywords TCM · Glycosylation · Endothelial cell · β-1,6-GlcNAc branching · PECAM-1 · RhoA

Abbreviations

HUVEC	Human umbilical vein endothelial cells
TCM	Tumor-conditioned medium
FBS	Fetal bovine serum
FITC	Fluorescein isothyocianate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
mAb	Monoclonal antibody
pAb	Polyclonal antibody
HRP	Horseradish peroxidase
ECGS	Endothelial cell growth supplement
PBS	Phosphate-buffered saline
BSA	Bovine serum albumin
TBS	Tris-buffered saline
Fn	Fibronectin
GnT-V	*N*-acetylglucosaminyltransferase V
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazoliun bromide

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Introduction

It is well known that carbohydrates not only act as energy material and structural substance, but also are important bioinformation molecules. Glycosylation is one of the most common post-translational modifications in eukaryotic cell surface. Oligosaccharide units of glycoconjugates fulfill many cellular recognition and modulatory functions. The growing research about glycans on tumor cells demonstrated that alterations in cellular glycosylation may play a key role in metastatic behavior tumor cells [1, 2]. In parallel, increasing evidence shows that the carbohydrates composition of the endothelial cell surface is critical for the cells to exert their physiological functions [3, 4]. However, during the process of tumor cell extravasations, the relationship between HUVEC glycosylation change and HUVEC dysfunction is still unknown.

Tumor metastasis is the most common cause of death in cancer patients,which is thought to consist of a number of distinct steps. In the first step, invasion, neoplastic epithelial cells lose cell–cell adhesion and gain motility, which enables them to invade the adjacent tissue. During the second step, intravasation, tumor cells penetrate through the endothelium of blood/lymphatic vessels and enter the systemic circulation. Only some circulating tumor cells appear to be able to survive the passage through circulation. Some of these survivors manage to complete the third step, extravasation, as they extravasate through the capillary endothelium at distal sites. Finally, in the new host environment, an even smaller subset of such metastasizing cells succeed in proliferating from minute growths (micrometastases) into malignant, secondary tumors [5]. Endothelium is the first defense during the process of tumor cell extravasation. Garcia-Valleji et al. [6] has reported that pro-inflammatory cytokine TNFα secreted during neutrophils transendothelium migration can induce alteration of endothelial cell surface glycans. Tumor cell extravasation is extremely similar to neutrophil transendothelium migration. Both of these two processes require transient disassembly of endothelial junctions [7]. It has been verified that the glycosylation of endothelial cell is a key modulator of the migration of leukocytes [8]. However, it is unknown that whether glycosylation in endothelial cell membrane alters during tumor cell extravasation, and how the glycosylation alteration influences endothelium functions. To reveal the above is our major objective in this research.

In this study, we stimulated HUVEC with TCM to examine whether the glycosylation alteration of endothelial cell can be induced. All nine kinds of examined glycans on the surface of HUVEC increased in different degrees under TCM condition, especially β-1,6-GlcNAc branching, *N*-acetyllactosamine and β-1,4-galactose. By further study of the effects of glycosylated alteration on HUVEC functions, we found that under treatment with TCM, the increase of β-1,6-GlcNAc branching initiated endothelial cell contraction and gap formation, through triggering PECAM-1-RhoA signaling pathway. Thus, we concluded that the increase of β-1,6-GlcNAc branching modification of PECAM-1, and the upregulation of tyrosine phosphorylation of PECAM-1, together with upregulation of RhoA, play the indispensable roles in β-1,6-GlcNAc branching-derived endothelial cell contraction and subsequent biological events.

Materials and methods

Materials

RPMI1640 medium, McCoy's 5a medium, Heparin, Fn, Trypsin, MTT, FITC-conjugated phalloidin and rabbit anti-β-actin mAb were purchased from Sigma, Saint Louis, MO, USA. FBS was obtained from Gibco, Grand Island, USA. The following biotinylated lectins were purchased from Vector Laboratories Inc, Burlingame, USA: Concanavalin A (Con A), Datura Stramonium Lectin (DSL, DSA), Phaseolus vulgaris Leucoagglutinin (PHA-L), Sambucus Nigra Lectin (SNA, EBL), Erythrina Cristagalli Lectin (ECL, ECA), Maackia Amurensis Lectin II (MAL-II), Pisum Sativum Agglutinin (PSA), Wheat Germ Agglutinin (WGA) and Ulex Europaeus Agglutinin I (UEA-I). Streptavidin-R-Phycoerythrin and Streptavidin-HRP were provided by Southern Biotech, Birmingham, Alabama, USA. ECGS, RhoA pull down assay reagent were purchased from Update, Haverhill, USA. Goat anti-human GnT-VpAb, HRP-conjugated goat anti-rabbit IgG, HRP-conjugated goat anti-mouse IgG, rabbit anti-Tyr-p pAb, mouse anti-human RhoA mAb and ProteinA-agrose were purchased from Santa Cruz Biotechnology, USA. HRP-conjugated donkey anti-goat IgG was purchased from Pufei Biotechnology, Shanghai, China. Rabbit anti-human PECAM-1 pAb was provided by Biosen Biotechnology, Beijing, China. Leupeptin and Aprotinin were obtained from Applichen, German. Pepstain was purchased from Amersco, USA.

Cells culture and preparation of TCM

Human colon carcinoma cell line HT29 was a generous gift from Shanghai Institute of Materia Medica. HT29 cells were grown in McCoy's 5a medium supplemented with 10% FBS, penicillin (100 units/ml), streptomycin sulfate (100 μg/ml), 1.5 mM glutamine and sodium bicarbonate (2.2 g/l). HUVEC which purchased from the American Type Culture Collection (ATCC, Manassas, USA.) were

cultured in RPMI1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 0.1 mg/ml heparin and 0.03 mg/ml ECGS. These two kinds of cells were maintained at 37° in a humidified 5% CO_2 atmosphere, and subcultured by trypsinization with 0.25% trypsin–0.02% EDTA when cells became confluent.

TCM from HT29 cells was prepared as follows. HT29 cells were trypsinized when they became confluent, and seeded at a density of 10^5 cells per well in six-well plates. 24 h later, cells were washed three times with PBS and then incubated in McCoy's 5a medium without FBS at 37°C in 5% CO2 conditions for another 24 h. TCM was then harvested, centrifuged at 1,300 rpm for 5 min and supernatantly stored at −20° until used.

Flow cytometry (FCM) assay

To detect the changes of certain carbohydrate determinants, HUVEC were stained with different plant lectins and analyzed by flow cytometry (FACScan; BD Biosciences) following the published method [9]. Briefly, approximately 5×10^5 cells were incubated for 1 h at 4° in 100 μl of assay buffer (10 mM HEPES, 0.15 M NaCl, 0.08% sodium azide, 0.1 mM Ca^{++}, 1% bovine serum albumin) containing different lectins with certain concentrations: ConA (10 μg/ml), PHA-L (20 μg/ml), DSA (4 μg/ml), SNA (20 μg/ml), PSA (15 μg/ml), ECL (10 μg/ml), MAL-II(10 μg/ml), UEA-I(5 μg/ml), and WGA (5 μg/ml). Lectin-stained cells were washed thrice with cold PBS and then incubated with Streptavidin-R-phycoerythrin (2.5 μg/ml) for 0.5 h at 4° in 100 μl PBS. Flow cytometric analysis was carried out immediately after washing cells with PBS. Each lectin was tested in triplicate and the experiments were repeated at least twice in each case.

Toxicity analysis

Evaluation on the cytotoxicity of TCM was carried out using MTT assay. In this experiment, HUVEC were seeded at a density of 2.5×10^5 cells per well in 96-well plates overnight, then the completed medium was removed, after that the cells were washed with PBS. TCM- or FBS-free culture medium was added to different wells and incubated for 24 h at 37°. Then 0.5 g/l MTT solution was added and incubated for additional 4 h. Subsequently, culture supernatant was removed and 150 μl dimethyl sulphoxide (DMSO) was added into each well to dissolve the formazan crystals. Colorimetric determination was made at 570 nm using a microplate reader (Spectra Rainbow, Austria). Six parallel samples were prepared in each group, and each experiment has been replicated thrice.

Immunoblot analysis

2.5×10^6 cells were either stimulated (for specified durations), or kept in control condition. Then they were prepared in NP-40 lysis buffer (50 mM Tris–HCl at pH 7.6, 150 mM NaCl, 10 mM NaF, 1% NP-40, 10% glycerol, 1 mM Na_3VO_4, and 5 mM EDTA, supplemented with 1 μg/ml each of leupeptin, aprotinin, and 1 mM phenyl methylsulphonyl fluoride (PMSF)), and finally cell lysates were formed. After centrifugation at 12,000 rpm for 15 min, the supernatant was harvested as the total cellular protein extract. Protein concentrations were determined with BCA protein assay kit. The total cellular protein extracts were boiled with 2 × electrophoresis sample buffer (3% SDS, 10% β-mercaptoethanol) and separated with SDS-PAGE, before they transferred to nitrocellulose membrane. Membranes were blocked with 5% nonfat dry milk in TBS containing 0.1% Tween-20 (TBS-T) for 2 h at room temperature and incubated with antibodies against GnT-V, PHA-L, PECAM-1, RhoA, p-Tyr and β-actin overnight at 4°. Blots were washed thrice in TBS-T buffer, followed by incubation with the appropriate HRP-linked secondary antibodies for 1 h at room temperature. The specific proteins in the blots were visualized using the enhanced chemiluminescence reagent. The experiments were carried out on three separate occasions.

Immunofluorescence assay

The rearrangement of actin in HUVEC was detected using fluorescence-conjugated antibody and analyzed by confocal microscopy. Cells were grown on fibronectin-coated glass coverslips until they became confluent. Confluent monolayers were washed with PBS and then incubated with TCM at 37° for 18 h. Cells were rinsed in PBS and fixed in 4% paraformaldehyde at room temperature for 20 min. After that, they were permeabilized for 5 min in 0.2% TritonX-100, rinsed thrice in PBS, blocked with 1% BSA. Then, cells were incubated with antibodies and examined under Zeiss Confocal Laser Scanning Microscope (40×, oil).

Immunoprecipitate assay

Cells were grown in six-well plates in culture medium until they became confluent. Then cells were stimulated with TCM or McCoy's 5a medium for different specified durations, and immediately washed with cold PBS, then lysed on ice in lysis buffer (RIPA) containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris (pH 8.0), 20 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin, 2 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate. After 30 min, cells were harvested and

disrupted with Ultrasonic Cell Disruptor. Then the lysate was centrifuged for 10 min at 12,000*g*. The supernatant was collected and quantitated, then incubated with antibody for more than 12 h at 4°. After this, 20-μl ProteinA agarose was added to each sample. 2 h later, precipitated complexes were washed with cold RIPA buffer for three times and boiled in 2 × sample buffer. Precipitates were analyzed on western blotting.

Rho GTPases pull down assay

RhoA activity assays was performed as described elsewhere [10]. In brief, cells were grown in six-well plates in culture medium until they became confluent. Cells were stimulated with TCM or McCoy's 5a medium for different specified durations, and immediately washed with cold PBS, then lysed on ice in lysis buffer (MLB) containing 25 mM HEPES (pH 7.5), 150 mM NaCl, 1% NP-40, 10% glycerol, 25 mM NaF, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM Na_3VO_4, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Cleared lysates were incubated for 45 min at 4° with Rhotekin-coupled agarose beads to precipitate GTP-bound RhoA. Precipitated complexes were washed thrice in lysis buffer and boiled in 2 × sample buffer. Total lysates and precipitates were analyzed on western blotting using antibodies against RhoA.

Statistical analysis

Student's *t* test and analysis of variance (ANOVA) were performed using Statview. $P < 0.05$ was accepted as significant and $P < 0.01$ was regarded as highly significant.

Results

Effects of TCM on carbohydrate expressions of endothelial cell surface

We examined the effects of TCM on the glycosylation of HUVEC by staining cells with different carbohydrate-specific lectins and analyzing by flow cytometry. Lectins are ubiquitous proteins which specifically bind defined monosugars or oligosaccharide structures. Many lectins, mostly of plant origin, were applied as useful tools in studying glycoconjugates. Here nine kinds of lectins, closely related to tumor metastasis, were chosen to identify the membrane glycoconjugates. The specificities of these lectins and corresponding results were detailed in Table 1. Results were expressed by the mean fluorescence index (MFI, i.e., by the ratio between the mean histogram fluorescence intensity generated by incubation with lectins, and mean fluorescence intensity of negative controls) [11]. The

Table 1 Expression levels of cell surface carbohydrates in TCM-stimulated HUVEC (MFI ± SD, $n = 3$)

Lectin	Binding sugar	MFI	
		Control	TCM
PHA-L	β-1,6-GlcNAc branching	31.34 ± 2.03	54.30 ± 1.50***
ConA	High mannose *N*-glycans	25.86 ± 0.37	30.04 ± 1.08*
UEA-I	α-Linked fucose residues	2.18 ± 0.10	2.72 ± 0.04
WGA	*N*-acetylglucosamine	13.68 ± 0.38	15.78 ± 0.53
MAL-II	α-2,3-Sialic acid	1.84 ± 0.02	2.09 ± 0.12
DSA	*N*-acetyllactosamine	9.82 ± 0.11	15.74 ± 0.41***
SNA	α-2,6-Sialic acid	1.57 ± 0.03	2.39 ± 0.10**
PSA	α-Mannose	8.03 ± 0.37	10.72 ± 0.22*
ECL	β-1,4-Galactose	21.01 ± 0.66	44.21 ± 1.68***

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control

results showed that the nine kinds of detected-glycans on the surface of TCM-treated HUVEC increased in different degrees compared with the controls, especially the β-1,6-GlcNAc branching specific lectin PHA-L, *N*-acetyllactosamine specific lectin DSA and β1,4 galactose specific lectin ECL (Fig. 1a, f, i) showed significantly higher staining after TCM treatment. The staining patterns of ConA, SNA and PSA indicated slight increases in the expression of high mannose *N*-glycans, α-2,6 sialic acid, and α-mannose (Fig. 1b, g, h). However, we found there was almost no increase in staining of UEA-I, WGA and MAL-II in TCM-treated cells (Fig. 1c, d, e). That is to say, the expressions of α-linked fucose residues, *N*-acetylglucosamine, and α-2,3-sialic acid, hardly changed after TCM treatment.

Effects of TCM on HUVEC cell viability

To exclude the cell viability on the expression of HUVEC surface oligosaccharides, the effects of TCM on HUVEC viability was assessed by MTT assay. As shown in Fig. 2, there was no significant difference in viability between TCM-treated cells and control cells (0.546 ± 0.010 for TCM-treated vs. 0.577 ± 0.035 for control). Our result indicated that the alteration in glycosylation of HUVEC was not originated from cell damage.

Effects of TCM on β-1,6-GlcNAc branching glycan for different durations

Recently the growing studies of β-1,6-GlcNAc branching glycan focus mainly on the field of cancer. Studies have demonstrated that increased β-1,6-GlcNAc branching glycan expression is associated with enhanced cell motility, invasiveness and in some cases of metastatic potential. However, there are almost no reports about its roles in endothelial cells. Our previous study has shown that β-1,

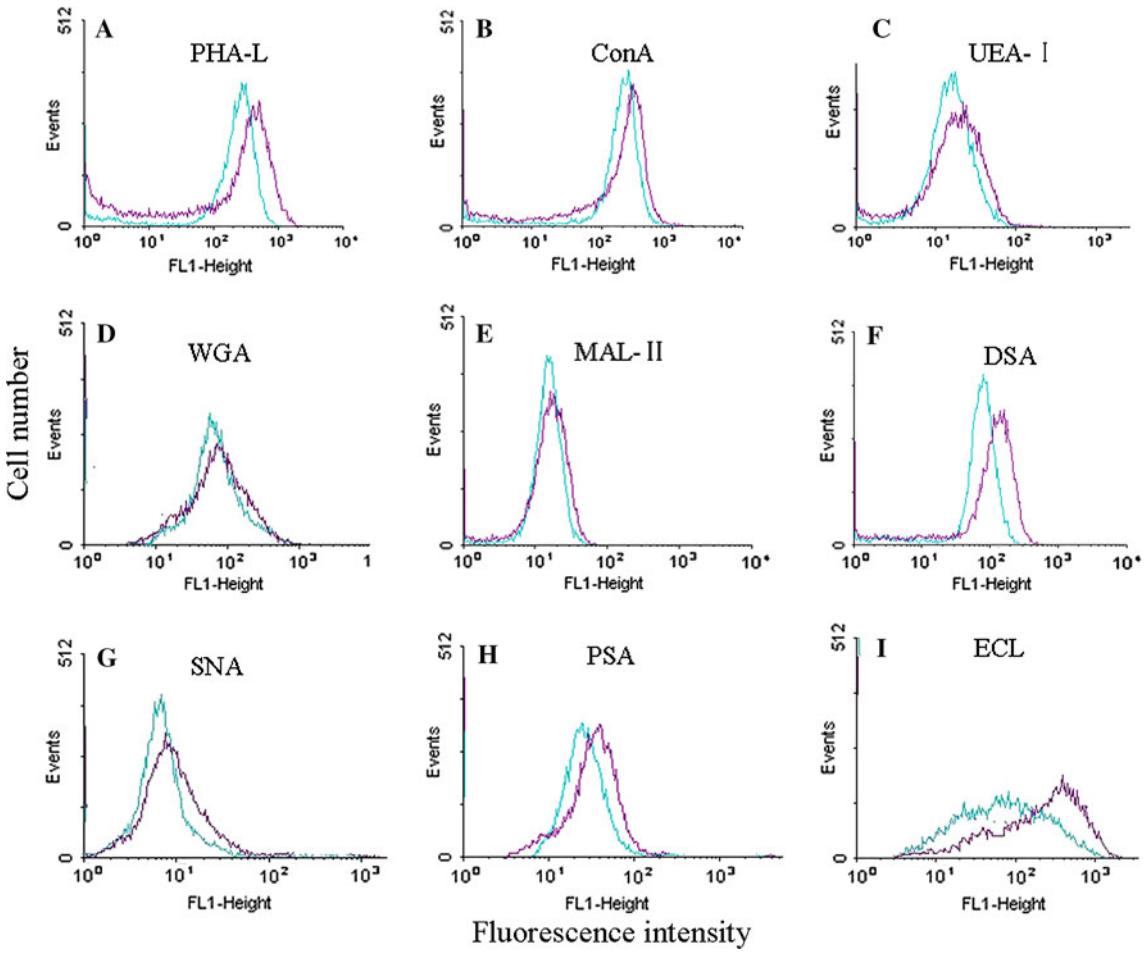

Fig. 1 Expression of cell surface carbohydrates in TCM-stimulated HUVEC. HUVEC were cultured to confluency under standard conditions in six-well plates, and either left untreated or stimulated with TCM for 24 h. After stimulation cells were stained with the indicated lectins, followed by incubation with RPE-conjugated streptavidin. Analysis was performed using FACS Vantage. (control: *blue line*, TCM-treated: *purple line*). The binding specificity of the lectins used was as indicated: PHA-L, β-1,6-GlcNAc branching; ConA, high mannose *N*-glycans; UEA-I, α-linked fucose residues; WGA, *N*-acetylglucosamine; MAL-II, α-2,3-sialic acid; DSA, *N*-acetyl lactosamine; SNA, α-2,6-sialic acid; PSA, α-mannose; ECL, β-1,4-galactose. *x*-axis, fluorescence intensity; *y*-axis, cell number. The results shown are representative of three independent experiments. (Color figure online)

6-GlcNAc branching glycan is one of the most increased carbohydrates under TCM stimulation. So we focus our study on the roles of β-1,6-GlcNAc branching glycan in endothelial cells. First of all, we treated HUVEC with TCM for 12, 18, and 24 h, respectively, and observed the variation of its expression, so that we could select the optimal time points for subsequent experiments. Stimulation with TCM for different durations all induced the increase, but to different extents, in expression of β-1, 6-GlcNAc branching glycan (Fig. 3a). As shown in flow cytometry analysis, the MFI of TCM-treated 18 h group (65.25 ± 2.36) was stronger than that of TCM-treated 12 h group (52.20 ± 2.05, $P < 0.01$) and TCM-treated 24 h group (53.19 ± 3.56, $P < 0.05$), also much stronger than that of control group (38.58 ± 1.70, $P < 0.001$). In other words, the expression of β-1,6-GlcNAc branching reached maximum after treatment with TCM for 18 h.

To determine whether the increased expression of β-1, 6-GlcNAc branching glycan was due to the upregulation of *N*-acetylglucosaminyltransferase V (GnT-V or Mgat5), which is in charge of β-1,6-GlcNAc branching glycan expression, we examined the expression of GnT-V after treating HUVEC with TCM for certain durations. As shown in Fig. 3b, after stimulation with TCM for different durations, the expression level of GnT-V did not vary obviously. Our findings indicated that the increase in β-1, 6-GlcNAc branching glycan expression was not caused by the upregulation of GnT-V.

TCM induced morphological changes in HUVEC monolayer

Endothelial cell monolayer constitutes the main barrier to the passage of macromolecules and circulating cells from

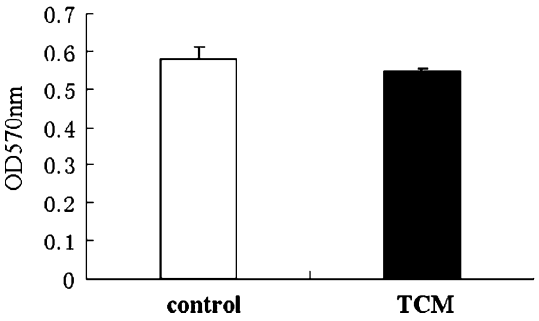

Fig. 2 Effects of TCM on cell viability of HUVEC. HUVEC were seeded at a density of 2.5×10^5 cells per well in 96-well plates overnight, then the complete medium was removed, after that cells were washed with PBS. TCM or FBS free culture medium were added to different well and incubated for 24 h at 37°. Then MTT solution was added at 0.5 g/l and incubated for additional 4 h. Subsequently, culture supernatant was removed and 150 µl dimethyl sulphoxide (DMSO) was added per well to dissolve the formazan crystals. Colorimetric determination was made at 570 nm using a microplate reader. The data, expressed as mean values ± SD of six parallel samples, is a representative of three independent experiments with similar results

blood to tissues. Endothelial cell intercellular junctions play an important role in the regulation of vascular permeability. One of the typical characteristics of endothelial junctions is their dynamic organization. Under physiological condition, endothelial cells need to reversibly change the architecture of their junctions to allow the passage of plasma constituents [12]. Moreover, this mechanism plays a role in regulating the inflammation process or the immune response, by limiting the transendothelial migration of neutrophils [13]. So we observed the morphological changes of endothelial cell monolayer under stimulation with TCM. As shown in Fig. 4a, after treatment with TCM for 24 h, significant morphological changes could be observed. Cells treated with TCM looked round, whereas control cells looked flattened and more spread out. After the 18th posttreatment hour, compared with control cells, cells treated with TCM were significantly contracted; however, they had not become as round as those cells treated with TCM for 24 h. Cells treated with TCM for 12 h did not show visible morphological change. After cells had been treated with TCM for 24 h, TCM was removed. If complete medium had been applied for 20 min, cells spread out again. If complete medium had been applied for 60 min after removal of TCM, there was almost no difference compared with control cells (Fig. 4b). This coincided with the conclusion that TCM did not cause impairment on cell viability. Moreover, our result indicated that barrier disruption of endothelial cell junctions was transient and reversible, which tallied with previous researches [12, 13].

Effects of TCM on intercellular gap formation

The cells contraction may contribute to the increase in endothelial permeability. It is well known that the actin cytoskeleton of endothelial cells consists of at least two morphologically and functionally different systems: the junction-associated actin filament web and the cytoplasmatic system of stress fibers. It has been reported that endothelial intercellular gaps coincided with sites where the junction-associated actin system was disrupted, further conforming to the concept that actin filaments are important for stabilizing intercellular junctions [14]. Subsequently, actin was stained, and analyzed by confocal microscopy using a ×40 oil objective. As shown in Fig. 5, We found that TCM induced a dramatic reduction in intercellular actin filaments with the disappearance of intercellular junctions. With nearly equivalent intercellular space, intercellular actin filaments in control cells were affluent and strong, whereas those in TCM-treated cells were sparse and slender. Besides, TCM induced remarkable increase in stress fibers inside the cells (Fig. 5b).

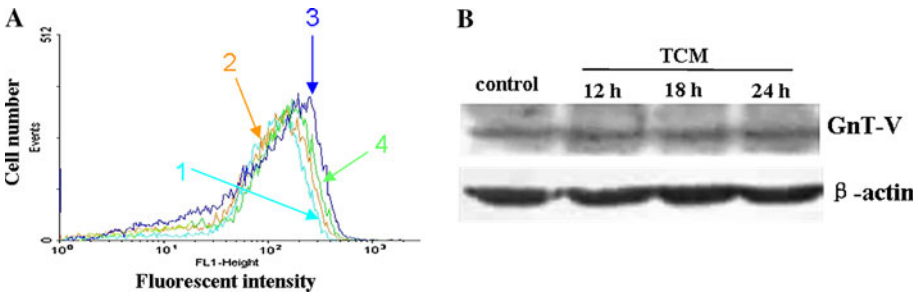

Fig. 3 The changes in expression of β-1,6-GlcNAc branching and GnT-V after TCM treatment for certain durations. **a** HUVEC were incubated with PHA-L which specifically binds with β-1,6-GlcNAc branching, followed by incubation with RPE-conjugated streptavidin. Analysis was performed using FACS Vantage. Three parallel samples were prepared in each group and the data shown is a representative of three independent experiments with similar results. The graphs *1*, *2*, *3*, and *4* represent control cells, TCM-treated 12 h, TCM-treated 18 h and TCM-treated 24 h, respectively. **b** GnT-V expression level under TCM treatment. HUVEC were incubated with TCM for certain durations, then lysed and analyzed by western blotting. The data shown is a representative of three independent experiments with similar results

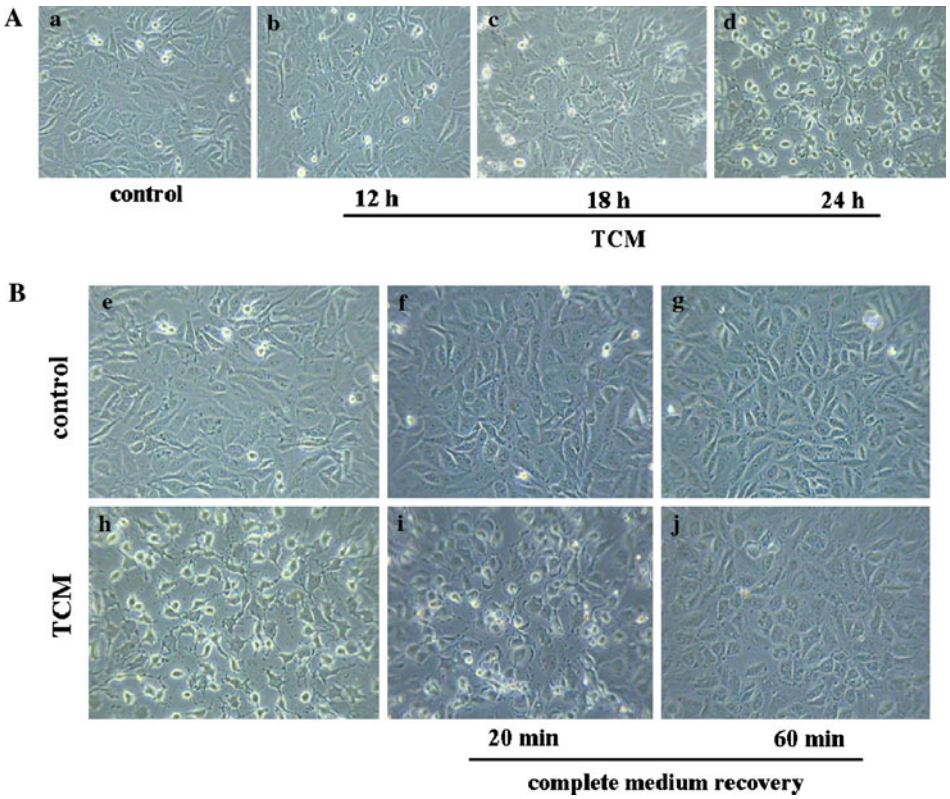

Fig. 4 Effects of TCM on HUVEC conformation. **a** HUVEC treated with TCM for specified durations, and then observed with inverted phase contrast microscope. After treatment with TCM for 24 h, cells looked round, whereas control cells looked flattened and more spread out (**d**). After the 18th posttreatment hour, compared with control cells, cells treated with TCM were significantly contracted; however, they had not become as round as those cells treated with TCM for 24 h (**c**). Cells treated with TCM for 12 h did not show any visible morphological change (**b**). **b** Reverse effect of complete medium on TCM-induced cell contraction. Complete medium recovery caused TCM-induced contracted cell to spread again (**e**). After 60 min, there was almost no difference compared with control (**c**, **f**). The data shown is a representative of three independent experiments with similar results

Effects of TCM on signaling pathways mediated by PECAM-1

PECAM-1 (CD31) is a homophilic adhesion molecule which plays a critical role in regulating endothelium permeability. PECAM-1 is able to bind to and coordinate the assembly of F-actin filaments, especially in association with changes in cell shape [15]. There is growing evidence that PECAM-1 is an important signal transduction molecule regulated by the phosphorylation of tyrosine residues within the cytoplasmic domain in response to numerous forms of cellular stimulation [16]. So we detected the change of PECAM-1 to estimate the effects of TCM on HUVEC. First of all, the change in expression was examined. As shown in flow cytometry analysis (Fig. 6a), the MFI of control (18.93 ± 0.43) was almost equal to those of TCM-treated groups (20.60 ± 0.51, 20.15 ± 0.59, 22.14 ± 0.89 for treat duration of 12, 18, 24 h, respectively). The western blotting analysis also showed that TCM had no influence on PECAM-1 expression (Fig. 6b).

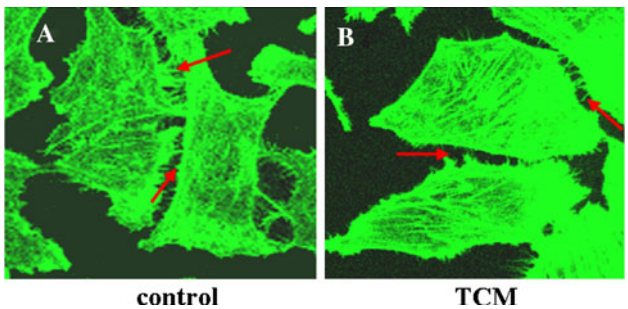

Fig. 5 Effects of TCM on intercellular gap formation. Cells grown on glass coverslips were stimulated with TCM for 18 h and then actin was stained with phalloidine and analyzed by confocal microscopy. TCM induced a dramatic reduction in intercellular actin filaments with the disappearance of intercellular junctions (**b**). As indicated by *red arrows*, with nearly equivalent intercellular space, intercellular actin filaments in control cells were affluent and strong (**a**), whereas those in TCM-treated cells were sparse and slender. Besides, TCM induced considerable stress fiber formation inside cells (**b**). In control group (**a**), actin is diffusive and without stress fiber. The data shown is a representative of three independent experiments with similar results. (Color figure online)

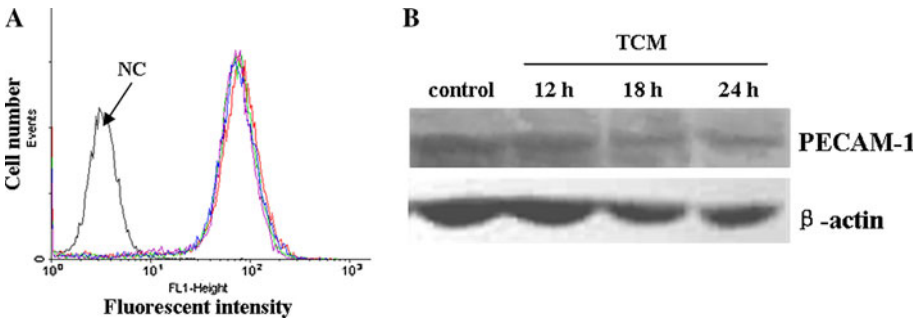

Fig. 6 Effects of TCM on PECAM-1 expression level. **a** HUVEC were stimulated with TCM for specified durations, incubated with rabbit anti-human PECAM-1 pAb and FITC-conjugated goat anti-rabbit IgG, then analyzed by FACS Vantage. Three parallel samples were prepared in each group. The *black graph* on the left is for negative control (NC) stained with second antibody only. The other four graphs in *different colors* on the right represent control cells, TCM-treated 12 h, TCM-treated 18 h and TCM-treated 24 h, respectively. These four curved lines are so nearly coincided that they cannot be differentiated distinctly. *x*-axis, fluorescence intensity; *y*-axis, cell number. **b** After being incubated with TCM for certain durations, HUVEC were lysed and incubated with rabbit anti-human PECAM-1 pAb, then incubated with HRP-conjugated goat anti-rabbit IgG, and finally analyzed by western blotting. The data shown is a representative of three independent experiments with similar results. (Color figure online)

Second, we assumed that there is an increase in β-1, 6-GlcNAc branching glycan of PECAM-1 with TCM treatment. Our study verified that TCM could induce an increase in β-1,6-GlcNAc branching glycan expression. Also, PECAM-1 has abundant glycosylated modification of β-1,6-GlcNAc branching glycan [17]. To verify this assumption, immunoprecipitate assay was performed. As shown in Fig. 7a, β-1,6-GlcNAc branching glycan of PECAM-1 had a significant increase after treated with TCM for 12 and 18 h, especially the latter. But after TCM treatment for 24 h, the expression level of β-1,6-GlcNAc branching glycan became almost equal to that of control cells.

Then, we furthered our study to investigate the effects of TCM on PECAM-1 tyrosine phosphorylation. Immunoprecipitate analysis showed that TCM induced a remarkable increase in tyrosine phosphorylation of PECAM-1, particularly in the case of TCM treatment for 18 h (Fig. 7b).

It is well known that Rho family of small GTPases is involved in signal transduction, which links extracellular stimuli to dynamic actin cytoskeletal rearrangement. Also, activation of Rho leads to stress fiber formation in cultured cells [18]. Moreover, RhoA is the downstream effector of PECAM-1. The PECAM-1 cytoplasmic domain is critical in mediating RhoA activation [19]. So we evaluated the activation of RhoA induced by TCM. Rho GTPases pull down assay revealed that TCM facilitated the activation of RhoA with time dependence (Fig. 7c).

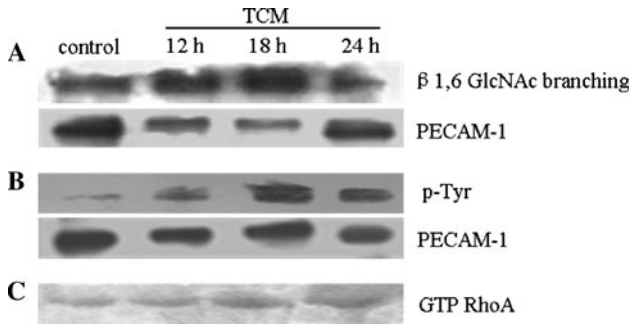

Fig. 7 Effects of TCM on signaling pathways mediated by PECAM-1. **a** Effects of TCM on β-1,6-GlcNAc branching modification of PECAM-1. Endothelial cells were treated with TCM for specified durations, and then analyzed by immunoprecipitation. **b** Effect of TCM on tyrosine phosphorylation level of PECAM-1 by immunoprecipitate assay. **c** Effects of TCM on the activation of RhoA. After treatment with TCM, samples were analyzed following the Rho GTPases pull down assay procedures. Results shown are representative of three independent experiments with similar results

Discussion

It is increasingly verified that the glycosylation of endothelial cells is a key modulator of their physiological functions [8, 10]. In parallel, it has been reported that the inflammatory factors can induce the alteration in expression of HUVEC surface oligosaccharides during inflammatory process. And this change in cellular glycosylation correlates with endothelium barrier dysfunction [6]. In our research, we investigated the change in glycosylation of HUVEC and their functions alteration during tumor cell extravasation by stimulating the cells with TCM. Our results clearly indicated that TCM induced profound changes in glycosylation and morphology of HUVEC. Many kinds of oligosaccharides significantly increased after treatment with TCM. We furthered our study to illuminate the mechanisms and found that TCM induced the increase in β-1,6-GlcNAc branching expression of PECAM-1, thus might facilitate the tyrosine phosphorylation of PECAM-1. The downstream effector RhoA was

activated in consequence of the activation of PECAM-1. Subsequently, the activation of RhoA initiated actin cytoskeleton rearrangement and abundant stress fiber formation within cells. Therefore, cell contraction was induced and resulted in paracellular gap formation and barrier dysfunction.

Most cancer-related deaths are caused by metastasis formation. A potentially rate-limiting step in metastasis formation would be the extravasation process that involves adhesion of tumor cells to endothelial cells and their transmigration through the endothelial cell monolayer and basement membrane. Generally speaking, to study the interaction between the tumor cells and endothelium, the conventional method is to make use of conditioned medium prepared from the tumor cells. Numerous studies have demonstrated that malignant cells produce a host of factors that induce vascular permeability, therefore facilitate tumor spreading [20, 21]. So tumor-conditioned medium is used to mimic the local micro-environmental conditions for cancer extravasation in vitro model. Previously, a great deal of researchers made use of conditioned medium to study the effects of tumor on endothelial cells. Utoguchi et al. [22] reported that tumor-conditioned medium prepared from mouse melanoma B16 can increase macromolecular permeability of endothelial cell monolayer. Castilla et al. also found that cell junctions are widened and cellular areas appear when expose confluent endothelial cells to tumor cell (MG63) conditioned medium [23].

In this experiment, we used TCM to stimulate endothelial cells and obtained the consistent result as above-mentioned reports. After endothelial cells, monolayer had been exposed to TCM for 24 h, endothelial cells retracted to be round, and intercellular junction became disrupted. The above led to intercellular gap formation coinciding with sites where the junction-associated actin web was fragmented. At the same time, we confirmed the reversibility of endothelial cells junctions. After TCM was removed and complete medium was applied instead, contracted endothelial cells would spread out and intercellular junction would form again. This phenomenon is consistent with previous researches: during the process of extravasation, endothelial cells need to reversibly change the architecture of their junctions, to limit the transendothelial migration of tumor cells.

Circulating tumor cells are thought to be capable to promote their own metastasis by interaction with endothelial cells. Tumor cells adherent to endothelial cells secrete inflammatory factors, resulting in the activation of endothelial cells. Thus, endothelial beds undergo changes in permeability, with minimal and transient barrier disruption, in order that tumor cells can fulfill their transmigration [15, 22]. However, the precise mechanisms, by which tumor cells induce vascular endothelium barrier dysfunction, remain unclear. A well-studied phenomenon of cell migration is the entry of leukocytes from the vascular system into sites of injury or infection in the process of inflammation. Moreover, tumor cells extravasation is very similar to leukocytes emigration. Previous studies showed that pro-inflammatory cytokine TNFα, secreted during neutrophils transendothelium migration, can induce alteration of the endothelial cell surface glycans. Also, changes in cellular glycosylation correlate with endothelial cells dysfunction. In this study, we found for the first time that TCM could also induce the alteration of HUVEC surface carbohydrates. Furthermore, we found that DSA, SNA, and ConA ligands increased most conspicuously, but WGA ligand had almost no change after treatment with TCM. However, we did not find remarkable increase in UEA-I ligand which has been reported to be upregulated by TNFα. These results indicated that there were differences in glycan profiles of the endothelial cell surface between transendothelium migration of circulating cells and cancer cells. Meanwhile, with PHA-L, we examined the expression alteration of β-1,6-GlcNAc branching glycan of HUVEC treated with TCM. The experiment results showed that the expression level of β-1,6-GlcNAc branching glycan reached maximum when treated HUVEC with TCM for 18 h, and then decreased. This might be related to the recovery of endothelial cells. Further study indicated that the increase in β-1,6-GlcNAc branching glycan expression was not caused by the upregulation of GnT-V expression. However, the increase in β-1,6-GlcNAc branching glycan expression might be related to the upregulation of enzymic activity of GnT-V.

During the process of transmigration, endothelial cell adhesion molecules provide a scaffold on which leukocytes can migrate as well as stimulate "outside-in" signal transduction in endothelial cells. Signals are activated by the endothelial cell adhesion molecules including PECAM-1, CD99, ICAM-1 (CD54), and VCAM-1 (CD106). Adhesion molecule signals result in alterations in the function of cell junction proteins and/or contractile forces in the endothelial cell, thereby opening an endothelial cell junction and permitting leukocyte migration into the tissue [24]. PECAM-1 which belongs to the Ca^{2+}-independent immunoglobulin superfamily, promotes both homotypic via another identical PECAM-1 molecule and heterotypic adhesion [25]. PECAM-1 is heavily glycosylated including N-glycosylation, about 40% of the molecular mass. PECAM-1 cytoplasmic domain phosphorylation regulates assembly of signaling complexes and, in some cases, interactions with various elements of the cytoskeleton. Garnacho et al. reported that PECAM-1 cytoplasmic domain, more precisely, PECAM-1 tyrosine 686, is critical in mediating RhoA activation and subsequent actin polymerization. Thus endothelial cells contraction was induced as a result

of the increasing of stress fiber [19]. In our study, we found for the first time that the increase in β-1,6-GlcNAc branching modification of PECAM-1 was induced by TCM, which was accompanied by the activation of downstream signaling molecules. That is to say, the alteration in glycosylation of PECAM-1 might influence its biological functions.

In sum, we herein showed for the first time that TCM induces profound changes in glycosylation and morphology of HUVEC. Many kinds of oligosaccharides significantly increased after treatment with TCM. Particularly the increase of β-1,6-GlcNAc branching modification of PECAM-1, and the upregulation of tyrosine phosphorylation of PECAM-1, together with the upregulation of RhoA, play the indispensable roles in β-1,6-GlcNAc branching-derived endothelial cells contraction and subsequent biological events. All these findings substantially release a new insight into the role of β-1,6-GlcNAc branching glycan in PECAM-1 and HUVEC, correlated with cancer extravasation.

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