RESEARCH ARTICLE

Effect of Tea Polyphenols on the Adhesion of Highly Metastatic Human Lung Carcinoma Cell Lines to Endothelial Cells *in Vitro*

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Abstract

Aim: Tea polyphenols are known to play roles in critical steps of human lung carcinoma cell metastasis. For understanding the mechanisms whereby they inhibit tumor metastasis, the present study was conducted to investigate their effects on the adhesion of highly metastatic lung carcinoma cell lines (PG cells) to endothelial cells (EC cells) and adhesion molecule expression *in vitro*. Methods: The expression of CD44 or CD54 in the PG cells was detected by flow cytometry and adhesion of PG cells to EC cells was assessed by confocal microscopy double fluorescence staining. Results: The results showed that tea polyphenols: (1) inhibited the expression of CD44 and CD54, two important adhesion molecules in the PG cells in a dose-dependent manner; (2) significantly blocked the adhesion of PG cells to EC cells not only in a state of rest but also when active; and (3) influenced CD44 and CD54 expression during the adhesion process of PG cells to EC cells. Conclusion: The data indicated that the blocking role of tea polyphenols in the adhesion of PG cells to EC cells is related to CD44 and CD54. The mechanism of tea polyphenol prevention of human lung carcinoma metastasis might be through inhibiting adhesion molecule expression to block cancer cell adhesion.

Keywords: Tea polyphenol - lung carcinoma - metastasis - adhesion molecules

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Introduction

Tumor hematogenous metastasis is a complex multi-step pathological process. One crucial step is the mutual adhesion and interaction between cancer cells and vascular endothelial cells (Orr et al., 2000; Guo et al., 2007). A variety of cell adhesion molecules can mediate the adhesion of cancer cells to vascular endothelial cells (Rice et al., 1988; lower et al., Lowe et al., 1990; Tozawa et al., 1995; limb et al., 1996; Frejns et al., 1997; Guo et al., 2007; Dowlati et al., 2008). CD44 and CD54 are two kinds of important molecules (Fukazawa et al., 1993 Lisignoli et al., 2006; Rokhin et al., 2006). Previous reports have shown that they played an important role in tumor metastasis (Bourrguignon et al., 1995; Grothey et al., 1998).

Tea ranks second only to water as a major component of fluid intake worldwide and has been considered a health-promoting beverage since ancient times. Tea polyphenols, known as catechins, account for 30–42% of the dry weight of the solids in brewed green tea (Mukhtar et al., 1999). They have effects on cancer prevention, inhibition and anti-metastasis (Liang et al., 1999; Lin et al., 1999). The cancer preventive activities have been demonstrated in many different animal models (Yang et al., 1993; Yang et al., 2002; Ju et al., 2007). Results from epidemiological studies as well as laboratory experiments suggest that tea consumption confers protection against the cancer development (Higdon et al., 2003; Crespy et al., 2004;Yang et al., 2009). Out of total 21 studies on the effect of tea on lung tumorigenesis, 19 studies showed inhibitory effects (23-26Mukhtar et al., 1994; Dreosti et al., 1997; Chung et al., 2003; Lambert et al., 2005; Ju et al., 2007). However, few researches have been reported about inhibitory effects of tea polyphenols on hematogenous metastasis of lung cancer cells. The object of the present study was to investigate the mechanism of hematogenous metastasis of lung cancer cells and the anti-metastatic effects of tea polyphenols.

Materials and Methods

Materials

Tea polyphenols were provided by Chinese Tea Institute, Hangzhou, Zhejiang, China. The 0.25% trypsin, RPMI-1640 medium, 5, 6-oxygen fluorescein acetate (5,6–CFDA) and lipopolysaccharides (LPS) were bought from Sigma Co., St. Louis, MO, USA. Mouse

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anti-CD44- or CD54- fluoresceinisothiocyanate (FITC) monoclonal antibody and the isotype goat anti-mouse IgG-FITC for control were purchased from International Immunotech Co. Ltd., France. FACsort flow cytometer was the product of BD Co. USA and the laser scanning confocal microscopy (LSCM) from ZEISS LSM 510 Co. Ltd., German.

Highly metastatic human lung cancer cell lines (PG cells) were provided by Zhejiang Cancer Research Institute, Hangzhou, Zhejiang, China. Human umbilical vein endothelial cells (EC cells, which were digested with 0.25% trypsin), were cultured and generated in RPMI 1640 with 20% fetal bovine serum.

CD44 and CD54 expression measurement

The expression of CD44 or CD54 in the PG cells was detected by the flow cytometer method. The experimental group was added with different doses of tea polyphenols in RPMI -1640 cell culture fluids, which concentrations of tea polyphenols was fixed to 100, 200, 400 or 800μg/ml; the control group was added an equal volume of physiological saline in RPMI -1640 cell culture fluid. After culturing for 72 h and digesting with 0.25% trypsin, the sample was changed into cell suspension fluid. Washed 3 times by PBS (Phosphate Buffered Saline), add with 20 μl of mouse anti-CD44- or CD54-FITC monoclonal antibody for each sample in experimental groups, while the same volume of goat anti-mouse IgG-FITC in the control group, which each reagent was diluted to 1:40. After incubating for 30 min at 4 ˚C, the CD44 or CD54 expression was measured by FACSort flow cytometer.

Test the adhesion of PG cells to EC cells

Groups: The experiment was carried on in 5 groups: Positive control group and 4 tea polyphenol groups. The positive control group was treated with physiological saline only, and the tea polyphenol group was treated with the tea polyphenols which concentration was 100, 200, 400 or 800 μg/ml.

Fluorescently labeled PG cells:PG cells that were in the logarithmic phase were washed 2 times with PBS, and added 0.5 ml of 5, 6-CFDA for incubating 15 min at 37 ˚C. The incubated cells were washed twice with PBS again to clean off the excess extracellular fluorescent dye, and then added a small amount of serum-free medium for the later use. The trypan blue dye exclusion method was used to detect the percentage of the labeled living PG cells.

Adhesion tests: The collected EC cells were washed twice with PBS, and inoculated in the 96-well culture plate. The 50 μl new medium was replaced when the cells were observed to join together under the microscope. Add 5×10^5 PG cells that labeled with 5, 6–CFDA as well as the tea polyphenols and physiological saline into each well, incubate in the 5% CO_2 incubator for 60 min at 37 ˚C, and then discard the inadherent cells. The adherent cells were incubated with 5 μl CD34-phycoerythrin for 30 min at 4 ˚C, and then washed twice with PBS. The adhesion rate of PG cells to EC cells was detected by the laser scanning confocal microscope. The adhesion rate was calculated by the average adherent cell number of accounted 50 cells in 10 random observing fields.

Adhesion molecule expression measurement: After washing with PBS, the cultured PG cells were resuspended in the complete medium to adjust the concentration to 5×10^4/ml. The PG cells were added into the 96-well plates (100 μl/well) that had been covered with EC cells. The tea polyphenols and physiological saline were added into each well to incubate in the 5% CO_2 incubator for 60 min at 37 ˚C, and then discard the inadherent cells. The adherent cells were eluted with 0.25% trypsin, and labeled with CD44- or CD54 -FITC monoclonal antibody separately, later detected by the flow cytometer.

LPS activating EC cell test: EC cells were incubated and activated with LPS (0.5 μg/ml) at room temperature for 6h. The tea polyphenol effects on the cell adhesion tests and adhesion molecule expression were done as above.

Statistical analysis

Each data was the average value of 3 times' repeated experiments. All values were expressed as mean ± standard error of the mean (SEM) and were analyzed between groups by analysis of variance (ANOVA) and t test with SPSS 17.0 software. $P < 0.05$ was considered statistically significant.

Results

Effect of tea polyphenols on CD44 and CD54 expression on the PG cells

Table 1 demonstrated the effect of tea polyphenols on CD44 and CD54 expression on the PG cells. The data illustrated that tea polyphenols could inhibit CD44 and CD54 expression on the PG cells in a dose-dependent manner. Especially in the groups with tea polyphenol concentration of 200, 400 and 800 μg/ml, the CD44 and CD54 expression showed significant difference.

Effect of tea polyphenols on the adhesion of PG cells to resting EC cells

There was a positive correlation between the fluorescence intensity and the living cell number when PG cells were labeled with fluorescent dyes 5, 6-CFDA. Although the survival rate of the labeled cells decreased following the time, it was required to further experiment,

Table 1. Effect of Tea Polyphenols on CD44 and CD54 Expression on PG Cells

Group	n	CD44 expression (counts)	CD54 expression (counts)
Control	12	145.02 ±3.22	45.30 ± 3.03
Tea polyphenols 100 μg/ ml	12	122.01 ±2.11	40.23 ± 2.63
Tea polyphenols 200 μg/ ml	12	108.21 ±2.11*	29.70 ± 1.50*
Tea polyphenols 400 μg/ ml	12	92.35 ±3.05**	20.12 ± 2.42**
Tea polyphenols 800 μg/ ml	12	86.21 ± 4.22***	13.32 ± 2.87***

* P<0.05, **P<0.01 and ***P<0.001 are used for the comparison of the data from control group to the other group

Table 2. Effects of Tea Polyphenols on the Adhesion of PG Cells to EC Cells

Group	n	Adhesion rate (%)	CD44 expression (counts)	CD54 expression (counts)
Control	12	50.51±1.91	417.40±6.12	224.41±0.83
Tea polyphenols 100 μg/ ml	12	47.02±1.53	314.53±4.55	212.55±1.01
Tea polyphenols 200 μg/ ml	12	38.47±2.67*	214.58±5.29*	195.48±1.91*
Tea polyphenols 400 μg/ ml	12	30.47±1.85**	194.93±5.35**	190.47±1.11**
Tea polyphenols 800 μg/ ml	12	29.62±1.63***	189.64±4.59***	180.14±1.47***

*P<0.05, **P<0.01 and ***P<0.001 are used for the comparison of the data from control group to the other group.

Table 3. Adhesion of PG Cells to EC Cells After Activation by LPS

Group	n	Adhesion rate (%)	CD44 expression (counts)	CD54 expression (counts)
Resting PG cells	12	50.51±1.91	417.40±6.12	224.41±0.83
Activating PG cells	12	72.53±0.85***	540.64±40.44***	328.70±1.03***

***P<0.001 are used for the comparison of the data from the resting PG cells to the activating PG cells

Table 4. Effects of Tea Polyphenols on the Adhesion of PG Cells and EC Cells After Activation by LPS

Group	n	Adhesion rate (%)	CD44 expression (counts)	CD54 expression (counts)
Control	12	72.53±0.85	540.64±40.44	328.70±1.03
Tea polyphenols 100 μg/ ml	12	50.51±1.91	480.24±45.14	308.10±1.01
Tea polyphenols 200 μg/ ml	12	48.45±1.32*	435.55±42.50*	216.01±1.35 *
Tea polyphenols 400 μg/ ml	12	41.32±1.34**	345.22±41.03**	165.91±0.78**
Tea polyphenols 800 μg/ ml	12	38.47±2.67***	313.12±37.13***	125.91±1.28 ***

* P<0.05, **P<0.01 and ***P<0.001 are used for the comparison of the data from control group to the other group

because the living cell percentage at 3 h still kept to 95%. Double fluorescent labeling method was used to observe the adhesion rate of PG cells to EC cells in this study, in which PG cells glowing green when labeled with 5, 6-CFDA and EC cells glowing red when labeled with CD34-PE. And the adhesion molecule expressions of PG cells and EC cells were detected by the flow cytometer. Table 2 showed that tea polyphenols exhibited a dose-dependent manner in the inhibitory effect on both the adhesion rate of PG cells to the resting EC cells and the adhesion molecule (CD44 and CD54) expression, especially in the groups with tea polyphenol concentration of 200, 400 and 800 μg/ml, which the data showed a significant difference.

Effect of tea polyphenols on the adhesion of PG cells to activating EC cells

The adhesion rate of PG cells to the activating EC cells and the expression level of the adhesion molecules could be significantly enhanced by LPS (Table 3). Tea polyphenols also had inhibitory effect on both the adhesion rate of PG cells to activating EC cells and the adhesion molecule (CD44 and CD54) expression in a dose-dependent manner, especially in the groups with tea polyphenol concentration of 200, 400 and 800 μg/ml, the data showed significant difference (Table 4).

Discussion

Lung cancer is the most common cancer in the world and represents a major public health problem. As highly metastatic human lung cancer cell lines, PG cells were selected in this experiment to test the anti-metastasis effect of tea polyphenols in vitro. Tea polyphenols are tea extracts that have anti-inflammatory, antioxidant and anti-metastas effects. Researches have proven that tea polyphenols could not only inhibit the invasion and cell adhesion process of the highly metastatic human fibrosarcoma and lung cancer cell lines passing through the single layer of human umbilical vein endothelial cells and its basement membrane, but also inhibit the degradation of the gelatin involved in matrix metalloproteinase MMP-2 and MMP- 9 enzymes. Metastasis remains the principal cause of the deaths of cancer patients despite decades of research aimed at restricting tumor growth (Isemure et al., 1993; Maeda-Yanmamoto et al., 1999; Kim et al., 2005; Khan et al., 2008; Park et al., 2009; Khan et al., 2010; Jayabalan et al., 2011). The present results showed that tea polyphenols could block the adhesion process of PG cells to EC cells in a dose-dependent manner in both resting and activating status. The data also indicated that tea polyphenols might be as candidate of anticancer metastasis drug.

The invasion and metastasis of tumor cells are a complex pathological process, in which the cell adhesion is involved in the entire procedure (Yang et al., 2009; Sandi et al., 2011). The mediating adhesion of cells to other cells is a broad family of cell adhesion molecules, which not only mediate the adhesion of tumor cells to extracellular matrix, but also participate in the interactions between tumor cells and vascular endothelial cells, leucocyte, platelets, solid organ cells or other cells (Albelda 1993; Hamburger et al., 1990; Polley et al., 1991; Gassmann and Haier 2008; Beiras-Fernandez 2009). Many studies have demonstrated that the adhesion molecules, for example CD44 and CD54, play crucial roles in mediating the adhesion of tumor cells to vascular endothelial cells so as to promote hematogenous metastasis of tumor (Lawson and Wolf, 2009; Konstantopoulos et al., 2009). Therefore, the type and quantity of the adhesion molecule ligands on the endothelial cells expressed by tumor cells could reflect the ability of their hematogenous metastasis relatively. The present study discovered that tea polyphenols could inhibit CD44 and CD54 expression on the PG cells in a dose-

dependent manner and the CD44 and CD54 expression level decreased in the adhesion process of PG cells to EC cells. Result suggested that tea polyphenols could inhibit the tumor invasion and metastasis via influencing the adhesion molecule expression.

LPS enhance the adhesion of gastric tumor cells to vascular endothelial cells in a dose- and time-dependent manner (Jiang et al., 2007). Both in vivo and in vitro studies have shown that enhanced adhesion molecule expression in endothelial cells is directly related to activation of leukocytes, increased leukocyte–endothelial adhesion and leukocyte extravasation (Wang et al., 2009). Other studies have also proven that the adhesion molecules expression in endothelial cells was up-regulated by LPS or pro-inflammatory cytokines (Dworakowski et al., 2008). In this study, LPS not only enhanced the adhesion of PG cells to EC cells, but also increased the adhesion molecule expression. The data indicated that that the adhesive ability of tumor cells to vascular endothelial cells was correspond with the ability of hematogenous metastasis. After entering into the blood circulation, the tumor cells might activate the endothelial cells and promote their hematogenous metastasis through triggering the local inflammatory response. In the lung, early metastatic colonies were entirely within the blood vessels, and hematogenous metastases originated from the intravascular proliferation of tumor cells anchored to the endothelia (Khaldoyanidi et al., 2003). Tea polyphenols could act on the adhesion process of PG cells to the activated vascular endothelial cells, whose adhesion ability and the expression level of adhesion molecules were obviously inhibited.

In conclusion, the present study made clear that (1) tea polyphenols inhibited the expression of CD44 and CD54, which the two important adhesion molecules in the PG cells in a dose-dependent manner; (2) tea polyphenols significantly blocked the adhesion of PG cells to EC cells not only in state of rest but also activation; and (3) tea polyphenols could influence CD44 and CD54 expression during the adhesion process of PG cells to EC cells. The data described that the effect of tea polyphenols on anti-adhesion might be related to its effect on anti-metastatic. However, further investigations will be conducted on the exact mechanism of these effects.

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