ELSEVIER

Cancer Letters 173 (2001) 15–20

CANCER Letters

www.elsevier.com/locate/canlet

Inhibitory effect of epigallocatechin gallate on adhesion of murine melanoma cells to laminin

Yasuo Suzuki[1], Mamoru Isemura*

School of Food and Nutritional Sciences, University of Shizuoka, Yada, Shizuoka 422-8526, Japan

Received 14 May 2001; received in revised form 29 June 2001; accepted 5 July 2001

Abstract

We examined the effects of five kinds of green tea catechin on the adhesion of mouse melanoma B16 cells to laminin. (−)-Epigallocatechin gallate (EGCG) and (−)-epicatechin gallate in the culture medium were found to inhibit the cell adhesion. The adhesion to laminin pre-treated with EGCG was also impaired. Affinity chromatography revealed the binding affinity between laminin and EGCG. These data suggest that the inhibitory effect of EGCG on adhesion of melanoma cells to laminin is included in the mechanism(s) of previously reported metastasis inhibition elicited by EGCG and green tea infusion.

Keywords: Epigallocatechin gallate; Laminin; Cell adhesion; Green tea; Melanoma

1. Introduction

Adhesive interactions between tumor cells and extracellular matrix proteins such as fibronectin, laminin and collagens are deeply involved in a tumor growth, invasion and metastasis [1,2]. These extracellular proteins comprise the endothelial basement membrane [3,4], and interruption of cancer cell adhesion may be effective in prevention of blood-borne metastasis. Peroral administration of (−)-epigallocatechin gallate (EGCG), a major constituent of green tea infusion, has been reported to prevent metastasis of mouse Lewis lung carcinoma and melanoma cells [5]. We have reported that gallate-containing catechins, EGCG and (−)-epicatechin gallate, impair adhesion and/or spreading of mouse lung carcinoma 3LL and melanoma B16 F10 cells to fibronectin [6]. Later, we demonstrated the domain-specific interaction of fibronectin with EGCG [7]. However, the effects of EGCG on biological activities of other cell adhesive proteins such as laminin, type I and IV collagens, and vironectin have not yet been characterized. In the present work, we examined whether EGCG inhibits cancer cell adhesion to these proteins.

2. Materials and methods

2.1. Chemicals

(+)-Catechin, (−)-epicatechin, (−)-epicatechin gallate, (−)-epigallocatechin, and EGCG (molecular weight: 458.4) were obtained from Funakoshi Co. Ltd., Tokyo, Japan. Laminin, type I and IV collagens, and Alamar blue (a product of Alamar Biosciences, Sacramento, CA, USA) were from Asahi Techno

* Corresponding author. Tel.: +81-54-264-5531; fax: +81-54-264-5530.

E-mail address: isemura@u-shizuoka-ken.ac.jp (M. Isemura).

[1] Research Fellow of the Japan Society for the Promotion of Science.

0304-3835/01/$ - see front matter
PII: S0304-3835(01)00685-1

Glass Corp., Tokyo, Japan. Vitronectin was prepared from human plasma according to the method described by Yatohgo et al. [8]. Serum-free cell culture medium Cosmedium 001, rabbit anti-laminin antiserum (a product of E.Y. Laboratories, Inc., San Mateo, CA, USA) and a Trypan blue solution were purchased from Cosmo Bio Co. Ltd. Tokyo, Japan. Horseradish peroxidase-conjugated porcine anti-rabbit immunoglobulin G was from DAKO Japan Co. Ltd., Kyoto, Japan, and the peroxidase substrate solution, Colorburst blue, was a product of Aler CHEK, Inc., Maine, USA.

2.2. Cell adhesion to laminin in the presence of catechins

Mouse melanoma B16 cells were obtained from the Health Service Research Resources Bank, Osaka, Japan, and maintained in a culture medium of 10% fetal bovine serum in Dulbecco's modified Eagle's medium (DMEM) with 50 U/ml penicillin, 50 μg/ml streptomycin, 2.5 μg/ml amphotericin B and 50 μg/ml gentamycin at 37°C under 5% CO_2. Adhesion of B16 cells to laminin-coated wells in the presence of (+)-catechin, (−)-epicatechin, (−)-epicatechin gallate, (−)-epigallocatechin or EGCG in solution was examined as follows. Sumilon plastic 48-well multidishes (Sumitomo Bakelite Co. Ltd., Tokyo, Japan) were coated with laminin at 10 μg/ml in DMEM at 37°C for 30 min. After being washed three times with 0.2 ml DMEM, wells were blocked by incubation with 1% bovine serum albumin in DMEM at 37°C for 30 min. After wells were washed three times with serum-free cell culture medium Cosmedium 001, they received 0.1 ml of Cosmedium 001 with or without test catechins at various concentrations. Freshly trypsinized cells were washed three times with Cosmedium 001, $4–6 \times 10^4$ cells in 0.1 ml were plated onto each well, and the mixture was incubated at 37°C in a humidified CO_2 incubator. After 1 h, wells were washed three times with 0.4 ml of Cosmedium 001 and finally received 0.1 ml of Cosmedium 001. Ten microlitres of the Alamar blue solution was added to each well, and the mixture was incubated at 37°C in a CO_2 incubator. After 2 h incubation, fluorescence was measured with excitation at 560 nm and emission at 590 nm as described previously [9].

Cell viability was assessed by the Trypan blue dye exclusion assay as described previously [10].

2.3. Cell adhesion to EGCG-treated laminin

To examine the adhesion to laminin pre-treated with EGCG, the laminin-coated wells were incubated with EGCG at various concentrations. An aliquot of cell suspension was added to each well after it had been washed three times with Cosmedium 001, and cell adhesion was determined as described above.

2.4. Adhesion of EGCG-treated cells to laminin

For pre-treatment, B16 cells were incubated with EGCG at various concentrations in Cosmedium 001 at 25°C for 30 min. The cells were then washed with Cosmedium 001 three times and plated onto laminin-coated wells, and the adhesion was examined as described above. The Trypan blue dye exclusion assay was used to examine the effects of EGCG-treatment on cell viability as described previously [10].

2.5. Affinity chromatography

EGCG immobilized on agarose gel was prepared as described previously [11]. The binding between laminin and EGCG was examined by affinity chromatography as follows. A laminin solution in phosphate-buffered saline (PBS) was loaded onto an EGCG-Sepharose 4B column. After being washed with PBS, the column was eluted with PBS containing 4 M urea and 1 M NaCl. Laminin in the eluates was then monitored by enzyme-linked immunoassay using rabbit anti-laminin antiserum and horseradish peroxidase-conjugated porcine anti-rabbit immunoglobulin G, essentially according to the method described previously [12]. Peroxidase activity was detected using a substrate of Colorburst blue according to the manufacturer's instruction.

2.6. Cell adhesion to type I and IV collagens and vitronectin in the presence of EGCG

Effects of EGCG on adhesion of B16 cells to wells coated with 10 μg/ml of type I collagen, type IV collagen or vitronectin were examined by the method similar to that used for laminin as described above.

3. Results

3.1. Cell adhesion to laminin in the presence of catechins

Mouse melanoma B16 cells were incubated on laminin-coated surfaces in the presence of various kinds of catechin, and cell adhesion was examined. The results showed that EGCG and (−)-epicatechin gallate inhibited cell adhesion to laminin, while other catechins tested had no effect (Fig. 1). The results of Trypan blue dye exclusion assay indicted that these catechins gave no cytotoxic effects on B16 cells under the conditions used here.

3.2. Cell adhesion to EGCG-treated laminin

When the laminin-coated wells were incubated with EGCG at various concentrations, the degree of the adhesion decreased with increasing EGCG concentration; 50% inhibition occurred at 4.8 ± 0.6 μM (an average ± SD from three experiments). Clear morphological changes were observed under a phase-contrast microscope (Fig. 2). Spreading of the cells was markedly inhibited on laminin pre-treated with EGCG at 6.25 μM (Fig. 2). The result suggests the direct binding of EGCG to laminin.

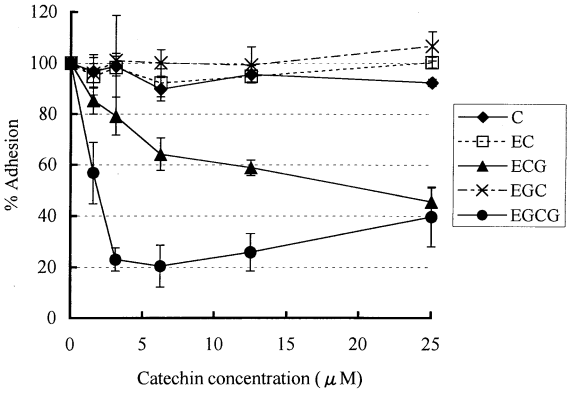

Fig. 1. Adhesion of melanoma B16 cells to laminin-coated surfaces. B16 cells (2×10^5 cells in 0.1 ml Cosmedium 001) were cultured in a 48-well multidish that had been coated with laminin at 10 μg/ml in the presence or absence of catechins at various concentrations. After 1 h incubation, non-attached cells were removed by aspiration, and the number of attached cells was determined by Alamar blue assay. The results are expressed as the relative cell number assessed by the Alamar blue assay, where the value from the cell incubated without catechins is taken as 100%. C, (+)-catechin; EC, (−)-epicatechin; ECG, (−)-epicatechin gallate; EGC, (−)-epigallocatechin.

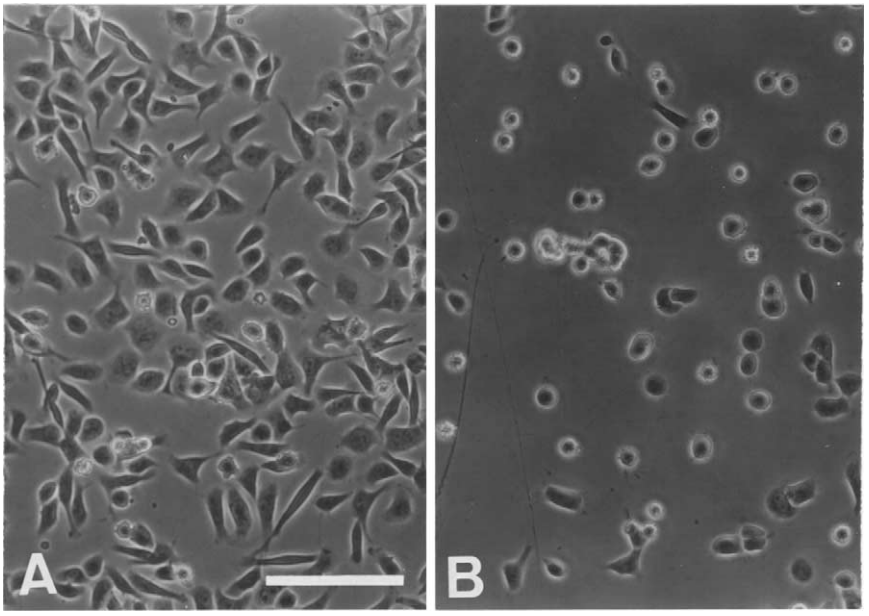

Fig. 2. Adhesion of B16 cells to EGCG-treated laminin. A multidish coated with 10 μg/ml laminin was pre-treated by incubation with EGCG at various concentrations at 37°C for 30 min. A cell suspension was added to each washed well, and attached cells were examined. B16 cells attached to untreated laminin (A) and to laminin pre-treated with EGCG at 6.25 μM (B) are shown. Scale bar, 100 μm.

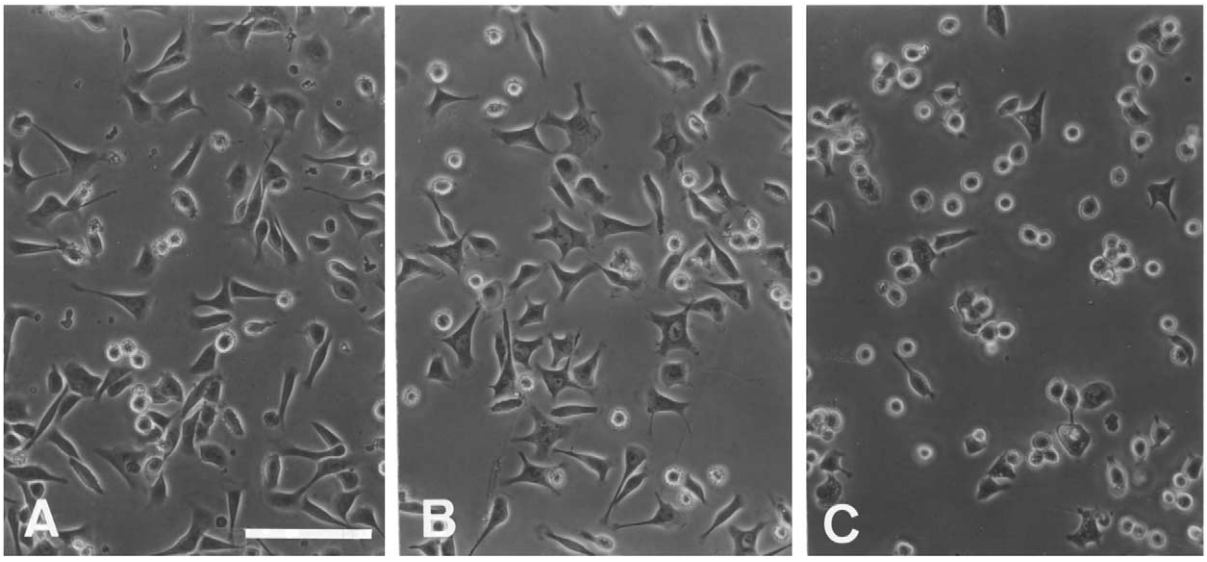

Fig. 3. Adhesion of EGCG-treated cells to laminin. B16 cells were incubated with EGCG at various concentrations at 25°C for 30 min. After being washed three times with Cosmedium 001, EGCG-treated cells were added to wells coated with laminin at 10 μg/ml. Photographs were taken after 30 min incubation of cells treated with EGCG at 50 μM (B) and 200 μM (C). Untreated cells are also shown in (A). Scale bar, 100 μm.

3.3. Adhesion of EGCG-treated cells to laminin

To examine the effects of possible binding of EGCG to the cells, B16 cells were pre-treated with EGCG at various concentrations. For B16 cells pre-treated with EGCG at 1–50 μM, no apparent changes in cell morphology and adhesion to laminin were detected (not shown). The cells pre-treated with EGCG at much higher concentrations, for example, 200 μM, showed deteriorated cell spreading (Fig. 3), suggesting the binding of EGCG to the cells. The Trypan blue dye exclusion assay indicated no significant differences in cell viability between untreated (control) cells (88.1%) and cells treated with 200 μM EGCG (88.5%) under the conditions used.

3.4. Affinity chromatography

Affinity chromatography indicated that laminin was retained by the EGCG-Sepharose 4B column and that it was then eluted with the buffer containing 4 M urea and 1 M NaCl (Fig. 4), demonstrating a binding affinity between them.

3.5. Cell adhesion to type I and IV collagens and vitronectin in the presence of EGCG

When B16 cells were incubated on type I collagen-, type IV collagen- or vitronectin-coated surfaces in the

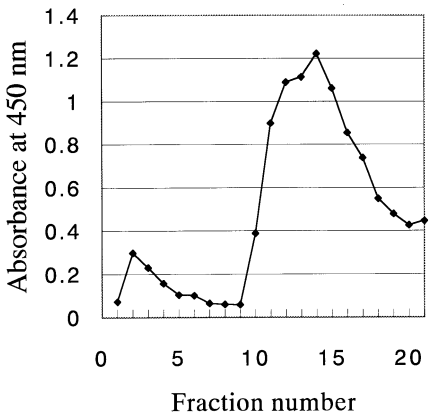

Fig. 4. Binding between laminin and EGCG. A laminin solution (100 μg in 100 μl of PBS) was loaded onto an EGCG-Sepharose 4B column with a bed volume of 1 ml. After washing with 10 ml of PBS, the column was eluted with PBS containing 4 M urea and 1 M NaCl. Fractions of 1 ml were collected and monitored for laminin by the enzyme-linked immunoassay.

presence of EGCG at various concentrations, no effects on cell adhesion was observed in either case at least up to 100 μM of EGCG (not shown). No apparent morphological changes were observed, either (not shown). These results indicate that EGCG has no inhibitory activity for B16 cells to adhere to these proteins.

4. Discussion

The present results showed that gallate-containing catechins inhibit the adhesion of B16 melanoma cells to laminin. Many studies have shown that a gallate group in catechins plays an important role in biological activities including inhibition of matrix metalloproteases [13], apoptosis induction [14,15], and inhibition of telomerase [16].

Cell adhesion to laminin pre-treated with EGCG was markedly reduced, while pre-treatment of the cells with EGCG had much less effect on the adhesion. Affinity chromatography demonstrated binding between EGCG and laminin. We, therefore, concluded that EGCG interfered with adhesion of mouse melanoma cells to laminin in vitro mainly through its binding to this basement membrane component.

The mechanism by which EGCG blocks the adhesion of B16 cells to laminin is not known at present. Our previous results indicated that EGCG inhibited the cell adhesion to fibronectin by its binding not to the cell binding domain but to the heparin binding II domain. Similarly, it may be speculated that EGCG exhibits inhibition by direct binding to a specific domain, though unidentified as yet, in a laminin molecule and that this domain is not necessarily a cell binding domain.

In contrast to the case of laminin, EGCG gave no effects on melanoma cell adhesion to and spreading on type I and IV collagens and vitronectin at least up to 100 μM. This may be explained by possible low affinity of EGCG for these proteins. Alternatively, EGCG may bind to the site, binding to which does not affect the cell adhesion to these proteins.

It has been reported that the peptide Tyr-Ile-Gly-Ser-Arg, which inhibits cell adhesion to laminin, inhibits blood-borne metastasis of melanoma cells [17]. On the other hand, the concentration of EGCG in human plasma is estimated to be 2-4 μM after oral administration [18,19]. Thus, the present findings suggest that the inhibitory effect of EGCG at a few μM level on binding of melanoma cells to laminin may explain, at least in part, previously observed metastasis inhibition elicited by EGCG [5] and green tea infusion [20].

Soluble laminin has been reported to increase the release of type IV collagenase responsible for cancer invasion and metastasis from malignant cells [1]. It remains to be determined if laminin with bound EGCG no more exhibits such activity.

Acknowledgements

This study was supported in part by a Grant-in-aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan (12660119) and by Goto Research Grant from University of Shizuoka. Y.S. is a recipient of a Research Fellowship for Young Scientists.

References

[1] T. Turpeenniemi-Hujanen, U.P. Thorgeirsson, C.N. Rao, L.A. Liotta, Laminin increases the release of type IV collagenase from malignant cells, J. Biol. Chem. 261 (1986) 1883–1889.
[2] L.A. Liotta, C.N. Rao, U.M. Wewer, Biochemical interactions of tumor cells with the basement membrane, Annu. Rev. Biochem. 55 (1986) 1037–1057.
[3] R.O. Hynes, Fibronectin and its relation to cellular structure and behavior, in: E.D. Hay (Ed.), Cell Biology of Extracellular Matrix, Plenum Press, New York, 1981, pp. 295–334.
[4] R. Timpl, S. Fujiwara, M. Dziadek, M. Aumailley, S. Weber, J. Engel, Laminin, proteoglycan, nidogen and collagen IV: structural models and molecular interactions, in: R. Porter, J. Whelan (Eds.), Basement Membranes and Cell Movement, Pitman Press, London, 1984, pp. 25–43.
[5] S. Taniguchi, H. Fujiki, H. Kobayashi, H. Go, K. Miyado, H. Sadano, R. Shimokawa, Effect of (−)-epigallocatechin gallate, the main constituent of green tea, on lung metastasis with mouse B16 melanoma cell lines, Cancer Lett. 65 (1992) 51–54.
[6] K. Ogata, N. Mukae, Y. Suzuki, K. Satoh, K. Narumi, T. Nukiwa, M. Isemura, Effects of catechins on the mouse tumor cell adhesion to fibronectin, Planta Med. 61 (1995) 472–474.
[7] M. Sazuka, M. Isemura, S. Isemura, Interaction between the carboxyl-terminal heparin-binding domain of fibronectin and (−)-epigallocatechin gallate, Biosci. Biotechnol. Biochem. 62 (1998) 1031–1032.

[8] T. Yatohgo, M. Izumi, H. Kashiwagi, M. Hayashi, Novel purification of vitronectin from human plasma by heparin affinity chromatography, Cell Struct. Funct. 13 (1988) 281–292.

[9] Y. Shoji, Y. Aoyagi, T. Kawakami, S. Isemura, M. Isemura, Cell adhesion activity for murine carcinoma cells of a wheat germ 55-kDa protein with binding affinity for animal extracellular matrix proteins, Biochim. Biophys. Acta 1426 (1999) 498–504.

[10] M. Isemura, Y. Suzuki, K. Satoh, K. Narumi, M. Motomiya, Effects of catechins on the mouse lung carcinoma cell adhesion to the endothelial cells, Cell Biol. Int. 17 (1993) 559–564.

[11] M. Sazuka, T. Itoi, Y. Suzuki, S. Odani, T. Koide, M. Isemura, Evidence for the interaction between (−)-epigallocatechin gallate and human plasma proteins fibronectin, fibrinogen, histidine-rich glycoprotein, Biosci. Biotechnol. Biochem. 60 (1996) 1317–1319.

[12] M. Isemura, N. Sato, Y. Yamaguchi, J. Aikawa, H. Munakata, N. Hayashi, Z. Yosizawa, T. Nakamura, A. Kubota, M. Arakawa, Isolation and characterization of fibronectin-binding proteoglycan carrying both heparan sulfate and dermatan sulfate chains from human placenta, J. Biol. Chem. 262 (1987) 8926–8933.

[13] M. Sazuka, H. Imazawa, Y. Shoji, T. Mita, Y. Hara, M. Isemura, Inhibition of collagenases from mouse lung carcinoma cells by green tea catechins and black tea theaflavins, Biosci. Biotechnol. Biochem. 61 (1997) 1504–1506.

[14] H. Hibasami, Y. Achiwa, T. Fujikawa, T. Komiya, Induction of programmed cell death (apoptosis) in human lymphoid leukemia cells by catechin compounds, Anticancer Res. 16 (1996) 1943–1946.

[15] K. Saeki, S. Hayakawa, M. Isemura, T. Miyase, Importance of a pyrogallol-type structure in catechin compounds for apoptosis-inducing activity, Phytochemistry 53 (2000) 391–394.

[16] I. Naasani, H. Seimiya, T. Tsuruo, Telomerase inhibition, telomere shortening, and senescence of cancer cells by tea catechins, Biochem. Biophys. Res. Commun. 249 (1998) 391–396.

[17] Y. Iwamoto, F.A. Robey, J. Graf, M. Sasaki, H.K. Kleinman, Y. Yamada, G.R. Martin, YIGSR, a synthetic laminin pentapeptide, inhibits experimental metastasis formation, Science 238 (1987) 1132–1134.

[18] K. Nakagawa, S. Okuda, T. Miyazawa, Dose-dependent incorporation of tea catechins, (−)-epigallocatechin-3-gallate and (−)-epigallocatechin, into human plasma, Biosci. Biotechnol. Biochem. 61 (1997) 1981–1985.

[19] C.S. Yang, L. Chen, M.J. Lee, D. Balentine, M.C. Kuo, S.P. Schantz, Blood and urine levels of tea catechins after ingestion of different amounts of green tea by human volunteers, Cancer Epidemiol. Biomarkers Prev. 7 (1998) 351–354.

[20] M. Sazuka, S. Murakami, M. Isemura, K. Satoh, T. Nukiwa, Inhibitory effects of green tea infusion on in vitro invasion and in vivo metastasis of mouse lung carcinoma cells, Cancer Lett. 98 (1995) 27–31.