Short communication

Restoration by prostaglandins E₂ and F_{2 α} of resveratrol-induced suppression of hepatoma cell invasion in culture

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Abstract

In our previous study, resveratrol, a polyphenolic compound in grapes with antioxidative property, and resveratrol-loaded rat serum (RS) were found to suppress the invasion of AH109A cells, an ascite hepatoma cell line. The aim of the present study was to investigate whether and which prostaglandins (PGs) would be involved in the invasion of AH109A cells and its suppression by resveratrol and resveratrol-loaded RS, using an *in vitro* invasion assay system. Not only PGE₂ but also PGF_{2 α} stimulated the spontaneous invasion of AH109A cells. They also canceled the resveratrol-induced suppression of hepatoma cell invasion. Results obtained suggest an involvement of PGs, especially PGE₂, in the invasion of hepatoma cells.

Introduction

Invasion is one of the characteristic steps in the progression of malignant cancer cells (Liotta et al. 1988). Since the invasion is important for metastasis and a critical factor for the survival of a cancer patients, blockage of this phenomenon will prolong the life span of an affected host. Resveratrol, 3,5,4'-trihydroxy-*trans*-stilbene, is present in grapes and red wine and has strong antioxidative activity (Fauconneau et al. 1997; Jang et al. 1997; Miura et al. 2003a). In our previous study, reactive oxygen species (ROS) were found to potentiate the invasive activity of the rat ascites hepatoma cell line of AH109A in the co-culture system with rat mesentery-derived mesothelial cells (M-cells), and resveratrol (50 μ M) and resveratrol loaded (5 mg⁻¹ ml⁻¹ 100 g⁻¹ body weight)-rat serum (RS) were shown to inhibit the invasion of AH109A cells due to their antioxidative activity (Kozuki et al. 2001).

Cyclooxygenases (COXs) catalyze the formation of prostaglandins (PGs) from arachidonic acid and PGs are reportedly involved in the invasion and metastasis of certain tumor cells (Liu et al. 1996; Reich and Martin, 1996; Damtew and Spagnuolo, 1997). There are two isoforms of COX, COX-1 and COX-2. COX-1 is expressed constitutively and COX-2 is inducible in response to various mitogenic and inflammatory stimuli (Miyamoto et al. 1976; Seibert and Masferrer, 1994). Resveratrol directly inhibits COX-1 activity (Jang et al. 1997), while it suppresses an increase in COX-2 mRNA expression (MacCarrone et al. 1999). The aim of the present study, was to investigate whether and which PGs would be involved in the invasion of AH109A cells and its suppression by resveratrol and resveratrol-loaded RS, using an *in vitro* invasion assay system. Not only PGE₂ but also PGF_{2 α} stimulated the spontaneous invasion of AH109A cells and canceled the resveratrol-induced suppression of hepatoma cell invasion. Results

obtained suggest an involvement of PGs, especially PGE₂, in the invasion of hepatoma cells.

Materials and methods

Materials

Resveratrol was purchased from Sigma Chemical Co. (St. Louis, MO), and PGE₂ and PGF_{2 α} were from Cayman Biochemicals (Ann Arbor, MI, USA). These compounds were dissolved in dimethyl sulfoxide (DMSO, Sigma) and added to the medium at a final DMSO concentration of 0.5%. Control medium contained 0.5% DMSO alone.

Culture of AH109A hepatoma cells

AH109A cells were provided by the Cell Resource Center for Biomedical Research, Tohoku University, Sendai, Japan. AH109A cells were cultured *in vitro* in 10% calf serum (CS), obtained from JRH, Lenexa, KS, USA (10% CS/MEM) as described previously (Miura et al. 1997).

Ex vivo assay

This experiment was conducted in accordance with guidelines established by the Animal Care and Use Committee of Tokyo Noko University. Male Donryu rats (4 weeks of age) were purchased from NRC Haruna (Gunma, Japan). *Ex vivo* assay was performed with resveratrol-loaded rat sera. Resveratrol was suspended in a 0.3% aqueous solution of carboxymethyl cellulose sodium salt (CMC, Wako Pure Chemical Industries, Osaka, Japan) at a concentration of 5 mg ml⁻¹. Resveratrol suspension or 0.3% CMC (vehicle control) was intubated at a dose of 1 ml 100 g⁻¹ body weight to male Donryu rats (5 weeks old) which had been fasted overnight, blood was collected 1 h after oral administration, and left to clot at room temperature for 2 hrs. RS was prepared by centrifugation, sterilized by filtration, and added instead of CS to the culture medium at a concentration of 10%.

In vitro invasion assay

The invasion assay was based on the method described (Akedo et al. 1986) with slight modifications as also described previously (Miura et al. 1997). Briefly, M-cells were isolated from male Donryu rats. After trypsin digestion, 1.2×10^5 cells were plated in a 60 mm $\varnothing$ culture dish with 2 mm grids (Nalge Nunc International Co., Tokyo), and cultured for 7–10 days to attain a confluent state. Then, 2.4×10^5 cells of AH109A were overlaid on a confluent M-cell monolayer in MEM containing either 10% CS or 10% RS in the absence or presence of various concentrations of PGE₂ or PGF_{2 α} , and cultured for 24 h. After washing non-adherent AH109A cells with Ca²⁺, Mg²⁺-free phosphate buffered saline [PBS(-)], the invaded AH109A cells and colonies were fixed with 0.25% glutaraldehyde in PBS(-) and 10 areas were counted with a phase contrast microscope. The invasive activity of the cells was indicated by the numbers of invaded cells and colonies ⁻¹/cm⁻².

Statistical analysis

Data were represented as means $\pm$ SEM. Multiple comparison was performed by one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons test, and $p < 0.05$ was considered statistically significant.

Results

Effect of PGE₂ on spontaneous invasion of AH109A cells and resveratrol-induced suppression of their invasion

To investigate the involvement of PGs in the invasion of hepatoma cells, we first examined the effect of PGE₂ on the spontaneous invasion of AH109A. As shown in Figure 1a, addition of PGE₂ at concentrations of 0.1–10 nM to the control medium containing 10% CS dose-dependently stimulated the AH109A invasion and the stimulatory effect attained a plateau at 10 nM. We next examined whether or not PGE₂ could restore the suppression of AH109A invasion by resveratrol treatment. As depicted in Figure 1b, addition of resveratrol (50 μ M) to the control medium containing 10% CS significantly suppressed the

Figure 1. Effect of PGE₂ on spontaneous invasion of AH109A cells (a, c) and resveratrol-induced suppression of their invasion (b, d). The invasive activity of AH109A cells was determined by invasion assay. Each value represents the mean $\pm$ SEM of 10 areas. Values not sharing a common letter are significantly different at $p < 0.05$ by Tukey–Kramer multiple comparisons test.

spontaneous invasion of these cells, and addition of 0.1, 1 and 10 nM PGE₂ to this state significantly canceled the resveratrol-induced suppression of the invasion in a dose-dependent manner.

To know whether serum from rats orally given resveratrol has the same inhibitory mechanism as that of resveratrol itself against the AH109A invasion, the invasion assay was carried out using resveratrol-loaded RS. Addition of 0.1, 1 and 10 nM PGE₂ to the control medium containing 10% RS significantly and dose-dependently increased the invasion (Figure 1c). Resveratrol (5 mg 100 g⁻¹ body weight)-loaded RS significantly restrained the invasion of hepatoma cells, and PGE₂ canceled the inhibitory action partially but significantly even at 0.1 nM and completely at 1 and 10 nM. In this study, PGE₂ was found to restore the resveratrol- or resveratrol-treated rat serum-induced suppression of hepatoma cell invasion to the control level.

Effect of PGF_{2α} on spontaneous invasion of AH109A cells and resveratrol-induced suppression of their invasion

The effect of another prostaglandin, PGF_{2α}, on the AH109A invasion was also examined. Figures 2a

and c indicate the effect of PGF_{2α} on the spontaneous invasion of AH109A cells cultured in 10% CS/MEM and 10% RS/MEM, respectively. Addition of PGF_{2α} to the control medium containing CS or RS stimulated the invasion, but a significant effect was observed only at 1 nM PGF_{2α}. Figures 2b and d depict the effect of PGF_{2α} on the suppressed invasion of hepatoma cells caused by resveratrol or resveratrol-loaded RS. PGF_{2α} significantly canceled the suppressive effect at 1 and 10 nM, but did not restore the suppression to the control level.

Discussion

In our previous experiments, resveratrol commenced to significantly inhibit the invasion of AH109A at 25 μM and dose-dependently suppressed it up to 200 μM, whereas this compound exerted no influence on the proliferation of AH109A up to 50 μM and then dose-dependently suppressed it at 100–200 μM (Kozuki et al. 2001). Thus, 50 μM was employed as a proper concentration of resveratrol in the present study, since at this concentration it inhibited the invasion without affecting the proliferation of AH109A cells. Both PGE₂ and PGF_{2α} canceled the resveratrol-medi-

Figure 2. Effect of PGF_{2α} on spontaneous invasion of AH109A cells (a, c) and resveratrol-induced suppression of their invasion (b, d). The invasive activity of AH109A cells was determined by invasion assay. Each value represents the mean $\pm$ SEM of 10 areas. Values not sharing a common letter are significantly different at $p < 0.05$ by Tukey–Kramer multiple comparisons test.

ated suppression of AH109A invasion (Figures 1b and 2b), although the former was more effective. These PGs enhanced the invasive activity when added to the control medium containing 10% CS (Figures 1a and 2a), although, again, PGE₂ was more effective than PGF_{2α}. Likewise, PGE₂ and PGF_{2α} canceled the suppression of AH109A invasion caused by resveratrol-loaded RS (Figures 1d and 2d), and enhanced the invasive activity when added to the control medium containing 10% RS (Figures 1c and 2c). Again, the potency of PGF_{2α} was not as strong as PGE₂. These results suggest that PGs, especially PGE₂, are involved in the invasion of AH109A and that resveratrol and its intracorporal metabolite(s) block the syntheses of PGs by inhibiting either COX-1 activity or COX-2 gene expression. Hepatoma cells do produce and secrete PGs (endogenous PGs) in the absence of resveratrol or resveratrol-loaded RS in the medium, whereas the cells do not produce them in the presence of resveratrol or resveratrol-loaded RS in the medium. In the former case, PGs added to the medium (exogenous PGs) are believed to noticeably stimulate the hepatoma cell invasion in combination with “endogenous PGs.” In the latter case,

“exogenous PGs” have to stimulate the invasion by themselves without the aid of endogenous PGs. This seems to be one reason why higher concentrations (1 or 10 nM) of exogenous PGs failed to restore the resveratrol-induced suppression of the hepatoma invasion over the control (resveratrol-free) level.

Hepatocyte growth factor (HGF) is reported to be identical to the scatter factor and to be deeply involved in tumor cell invasion (Parr and Jiang, 2001). In our previous study, ROS were found to enhance the HGF expression and to stimulate the autocrine action of HGF in invasion of AH109A cells (Miura et al. 2003). Furthermore, we found that the antioxidative property of resveratrol and its metabolite(s) contributed to its anti-invasive activity via the suppression of HGF expression (Miura et al. 2003b). In addition, HGF was reportedly able to trigger activation of the COX-2 gene in gastric mucosa cells or gastric cancer cells through phosphorylation of c-Met receptor and activation of the extracellular signal kinase (ERK2) signaling pathway (Jones et al. 1999; Chen et al. 2001). If this is true of AH109A cells, the antioxidative property of resveratrol or sera from rats orally given this substance is involved in depression

of the COX-2 gene expression and hence the suppression of AH109A invasion. It is well known that PGE₂ is a main product of COX-2 and required for invasive activity (Nagatsuka et al. 2002). However, the possibility that resveratrol and its metabolite(s) inhibit COX-1 is not ruled out. Further studies are required to clarify this aspect.

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