Inhibitory effect of curcumin on the invasion of rat ascites hepatoma cells *in vitro* and *ex vivo*

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Abstract

Curcumin, a yellow pigment in turmeric, is a food factor with antioxidative activity. The effect of curcumin on the proliferation and invasion of the rat ascites hepatoma AH109A cells was studied *in vitro* and *ex vivo* assay systems. Especially, a co-culture system of the hepatoma cells with mesothelial cells derived from rat mesentery was employed to investigate the invasive motility. Curcumin suppressed the hepatoma slipping motility in a dose-dependent manner up to 5 μ M and thereafter maintained the effect up to 20 μ M, whereas this substance exerted little influence on the proliferation of the hepatoma cells at the same concentrations. Sera obtained from rats orally given curcumin also inhibited the AH109A cellular invasive movement when added to the culture medium. Hepatoma cells previously cultured with hypoxanthine and xanthine oxidase showed a highly invasive activity. Curcumin and curcumin-loaded rat sera suppressed this reactive oxygen species-potentiated invasive capacity by simultaneously treating AH109A cells with hypoxanthine, xanthine oxidase and either of curcumin samples. These results suggest that the antioxidative property of curcumin may be involved in its anti-invasive action.

Abbreviations: CMC, carboxymethyl cellulose salt; CS, calf serum; HX, hypoxanthine; M-cells, rat mesentery-derived mesothelial cells; THC, tetrahydrocurcumin; XO, xanthine oxidase

Introduction

The invasion of cancer cells is an important and characteristic step of metastasis (Liotta et al., 1983). The blockage of this biological event by drugs or food factors will prolong the life span of an affected host. Certain *in vitro* assay systems to study tumor cell invasion, e.g., the Boyden chamber assay system with the extracellular matrices-coated membrane (Albini et al., 1987; Welch et al., 1989), have often been used for discovering such factors. There are, however, two reasons for difficulties in finding an effective way to prevent tumor cell invasion. First, tumor invasiveness in such systems and the invasive behavior of tumor cells in their original host were reported to be poorly correlated (Noel et al., 1991; Poste et al., 1980). Akedo et al. (1986) developed a model culture system in which rat ascites hepatoma AH130 cells invaded un-

derneath the monolayer of cultured mesentery-derived mesothelial cells (M-cells), and showed the clear correlation between the *in vitro* invasiveness of AH130 cells in their culture model and the *in vivo* invasive behavior of their cells in host peritoneal cavity. Second, some factors that show the inhibitory potential on tumor cell invasion *in vitro* often exert no effect on tumor cell invasion *in vivo*. The assay method, however, overcomes the discrepancy between the *in vitro* and *in vivo* effects using *in vitro* and *ex vivo* assay systems in combination (Miura et al., 1997a).

AH109A cells, parallel with AH130, grow in the rat peritoneal cavity and invade the mesentery during the course of growth. Thus, the invasion assay system using M-cells and rat ascites hepatoma AH109A cells has been employed in our researches. Our previous studies carried out by this assay system revealed that extracts of teas and coffee, and sera obtained from

Figure 1. Structures of curcumin and tetrahydrocurcumin.

rats given either of these beverage extracts orally suppressed the invasion of the hepatoma cells *in vitro* (Miura et al., 1997a, b; Zhang et al., 1999; Yagasaki et al., 2000). Since both teas and coffee contain polyphenolic components with antioxidative activity, the results of these and other works led us to assume that widespread antioxidants would suppress the invasion of cancer cells. In fact, we have demonstrated that certain carotenoids, food factors with antioxidative activity, inhibit the invasion of AH109A cells (Kozuki et al., 2000).

Curcumin (Figure 1), a yellow pigment, is an antioxidant found in turmeric. This food factor has anti-carcinogenic activity (Huang et al., 1997; Kim et al., 1998; Kawamori et al., 1999). Menon et al. (1995) reported the inhibitory effect of curcumin on the metastasis in mice injected with B16F10 melanoma cells and Lin et al. (1998) reported that the inhibitory effect of curcumin on the invasion of SK-Hep-1 cells using *in vitro* assay with Matrigel matrix. However, it is not known if antioxidative activity of curcumin is involved in the invasion of cancer cells.

In this study, the effect of curcumin on the invasion of AH109A cells of the rat ascites hepatoma cell line was investigated in a co-culture system of the hepatoma cells with M-cells. Using AH109A cells pre-treated with hypoxanthine (HX) and xanthine oxidase (XO), we attempted to show an importance of the free radical-scavenging activity of curcumin to inhibit the tumor cell invasion.

Materials and Methods

Materials

Curcumin (Wako Pure Chemicals Industries, Osaka, Japan) was dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA). The curcumin solution was added to the medium at the final DMSO concentration of 0.5%. Control medium contained 0.5% DMSO alone. All other reagents were of the best grade commercially available.

Culture of AH109A hepatoma cells

A rat ascites hepatoma cell line of AH109A cells was provided by the Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan. AH109A cells were maintained in peritoneal cavities of male Donryu rats (NRC Haruna, Gunma, Japan), isolated from accumulated ascites and then cultured in Eagle's minimum essential medium (MEM, Nissui Pharmaceutical Co., Tokyo, Japan) containing 10% calf serum (CS, obtained from JRH, Lenexa, KS, USA) (10% CS/MEM) for up to 2 months. To eliminate contaminated macrophages and neutrophils, these cells were cultured at least for 1 week after isolation and employed for the assays described below.

In vitro proliferation assay

Effect of curcumin on AH109A proliferation was examined by using WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium-Na) method as described previously (Zhang et al., 1999) or by measuring incorporation of [methyl-³H]thymidine (20 Ci mmol⁻¹, New England Nuclear, Boston, USA) into DNA as described previously (Yagasaki et al., 1992).

In vitro invasion assay

Effect of curcumin on AH109A invasion was examined by the co-culture system as described previously (Akedo et al., 1986) with slight modifications (Miura et al., 1997a). Briefly, M-cells of Donryu rats (4–6 weeks old) were isolated by trypsin digestion, seeded at a density of 1.5×10^5 cells in a 60 mm ϕ culture dish with 2 mm grids (Corning, Cambridge, MA, USA), and cultured for 7–10 days to attain a confluent state in 10% CS/MEM. AH109A cells (2.4×10^5 cells per dish) were applied on the M-cell monolayer in 10% CS/MEM with curcumin and cultured for 24 h.

Under a phase-contrast microscope, penetrating cells and colonies underneath M-cell monolayer were seen as flattened tumor cell islands (Figure 2). Invading cells and colonies were counted in ten random areas, and the invasive activity of AH109A was indicated as the number of invading cells and colonies cm^{-2} .

Ex vivo invasion assay

Curcumin was suspended in 0.3% carboxymethyl cellulose sodium salt (CMC, Wako Pure Chemicals Industries) aqueous solution at a concentration of 100 mg ml^{-1} . Curcumin suspension or 0.3% CMC (vehicle control) was intubated at a dose of $1.0 \text{ ml } 100 \text{ g}^{-1}$ body weight to male Donryu rats (5 weeks old) which had been fasted overnight and blood was collected at the times indicated. The sera were prepared, sterilized by filtration, and added to culture media at a concentration of 10% instead of CS for invasion assay. The invasive activity of AH109A in the presence of these sera was measured as described above.

Treatment of AH109A cells with hypoxanthine and xanthine oxidase

AH109A cells were cultured for 4 h in the absence or presence of $5 \text{ } \mu\text{M}$ of curcumin and/or $4 \text{ } \mu\text{g ml}^{-1}$ of hypoxanthine (HX, Sigma) with $7 \times 10^{-4} \text{ U ml}^{-1}$ of xanthine oxidase (XO, Sigma) (Shinkai et al., 1986; Tanaka et al., 1997). After the treatment, the hepatoma cells were washed once with the medium and applied on the M-cell monolayer in 10% CS/MEM with or without $5 \text{ } \mu\text{M}$ of curcumin, and cultured for another 24 h. To investigate the effect of curcumin-loaded rat sera, the same treatment described above was performed with the medium containing 10% curcumin ($100 \text{ mg } 100 \text{ g}^{-1}$ body weight)-loaded sera or vehicle (0.3% CMC)-loaded sera instead of the medium containing 10% CS in the presence or absence of curcumin ($5 \text{ } \mu\text{M}$). These sera were prepared from blood collected 1 h after oral intubation of curcumin or vehicle.

Statistical analysis

Data were expressed as means $\pm$ standard errors. Differences between group means were compared by Student's *t*-test. Multigroup comparisons were carried out by one-way analysis of variance followed by Tukey or Dunnett test.

Results

Effect of curcumin on the proliferation and invasion of AH109A cells

Curcumin (Figure 1) was tested to evaluate its effect on the proliferation and invasion of AH109A cells. The WST-1 method revealed little influence of curcumin on the hepatoma proliferation at concentrations of 2.5 to $20 \text{ } \mu\text{M}$ (Figure 3A). Viable cells, however, can make WST-1 produce highly yellow colored formazan dyes, and curcumin is a yellow pigment. For this reason, we confirmed the data from the WST-1 method by measuring the incorporation of [methyl- ^3H]thymidine into acid-insoluble fraction of AH109A cells at same concentrations (Figure 3B). Two methods showed little effect of curcumin on the proliferation of the hepatoma cells. On the contrary, curcumin suppressed the invasion of the hepatoma cells in a dose-dependent manner at concentrations of 0 to $5 \text{ } \mu\text{M}$, and the inhibitory activity reached a plateau at $5 \text{ } \mu\text{M}$ or more (Figure 3C).

Ex vivo effect of curcumin on the invasion of AH109A

To investigate whether or not the serum from a rat given curcumin orally has the inhibitory effect on the invasion of AH109A, the invasion assay was carried out with the curcumin-loaded rat serum. Rats were given oral intubation of curcumin-suspension at a dose of $100 \text{ mg ml}^{-1} 100 \text{ g}^{-1}$ body weight and blood was collected at 0, 1, 3, 6 and 12 h thereafter. Serum was prepared and the invasion of AH109A was evaluated by adding the serum to the co-culture medium at a concentration of 10% instead of CS. As shown in Figure 4, the serum commenced to significantly inhibit the hepatoma invasion 1 h after oral intubation as compared with 0-h value, and this effect gradually weakened. All the curcumin-loaded sera significantly suppressed the hepatoma invasion when compared with the corresponding control (vehicle alone) groups.

In vitro and ex vivo effect of curcumin on the invasion of AH109A pre-treated with hypoxanthine and xanthine oxidase

To examine whether or not curcumin inhibits the invasion of tumor cells by its antioxidative activity, the invasion assay was performed with AH109A cells pre-treated by the HX-XO system which generates reactive oxygen species (ROS). As shown in Figure 5, the invasive activity of AH109A cells pre-cultured in

Figure 2. Growth patterns of AH109A cells on and underneath M-cell monolayer. Phase-contrast micrographs were taken at 0 h (left) and 24 h (right) after seeding the tumor cells. Arrow heads, tumor cells penetrating underneath M-cell monolayer; open triangles, tumor cells on M-cell monolayer. Bars, 50 μ m.

Figure 3. Effect of curcumin on the proliferation and invasion of AH109A. Curcumin was dissolved in dimethyl sulfoxide (DMSO). The curcumin solution was added to the culture medium at a final DMSO concentration of 0.5%. The proliferation of AH109A cells was evaluated by WST-1 method (A) or by measuring incorporation of [methyl-³H]thymidine into DNA (B) as described in Materials and Methods. The control value of $100.0 \pm 4.29\%$ is equal to the determined absorbance of 1.313 ± 0.056 (A), whereas the other control value of $100.0 \pm 3.94\%$ equal to the determined radioactivity of 37520 ± 1478 dpm (B). Data are the means $\pm$ SEM of six wells. The invasive activity of AH109A cells (C) was determined by invasion assay as described in Materials and Methods. Data are the means $\pm$ SEM of ten areas in one 60 mm ϕ dish with 2 mm grids. Values not sharing a common letter are significantly different at $p < 0.05$ by Tukey test. Each result is a representative of at least three similar experiments.

Figure 4. *Ex vivo* effect of curcumin on the invasion of AH109A. Curcumin was suspended in 0.3% carboxymethyl cellulose sodium salt (CMC). Curcumin ($100 \text{ mg } 100 \text{ g}^{-1}$ body weight)- or vehicle (0.3% CMC)-loaded rat sera were obtained at the indicated times after oral intubation. With the medium containing 10% each serum, the invasion assay was performed as described in Materials and Methods. Data are the means $\pm$ SEM of ten areas in one 60 mm ϕ dish with 2 mm grids. Asterisks indicate significant differences ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$) compared with corresponding vehicle groups by Student's *t*-test. Sharps show values are significantly different ($\#p < 0.05$, $##p < 0.01$) from 0-h value of the curcumin-loaded rats by Dunnett's test. This result is a representative of three similar experiments.

ROS-generating medium was significantly higher than that of AH109A cells with no treatment. Curcumin (Figure 5A) and curcumin-loaded rat sera (Figure 5B) inhibited the ROS-potentiated invasive activity of the pre-cultured cells in a parallel manner when added to the medium on pre-culturing the hepatoma cells with HX and XO as well as on co-culturing them with M-cells.

Discussion

In this report, we studied the *in vitro* and *ex vivo* effect of curcumin on the proliferation and invasion of AH109A cells. Curcumin suppressed the invasive motility of AH109A at concentrations of 2.5 to $20 \mu\text{M}$ in spite of ineffectiveness on the proliferation of the cells at the same concentrations (Figure 3). Furthermore, curcumin (100 mg ml^{-1} 100 g^{-1} body weight)-loaded rat sera significantly inhibited the invasion of AH109A (Figure 4). These results revealed that curcumin and/or its metabolite(s) in blood inhibited

the AH109A invasion. Holder et al. (1978) have reported that tetrahydrocurcumin (THC; Figure 1) is one of the main metabolites of curcumin *in vivo*. Osawa and collaborators have suggested that THC is one of the derivatives from curcumin modified during absorption through the intestine, and antioxidative activity of THC proved to be stronger than that of curcumin (Osawa et al., 1995; Sugiyama et al., 1996). We previously found that certain carotenoids suppressed the AH109A invasion in the same manner as curcumin did, whereas these substances had little effect on the proliferation of the hepatoma cells (Kozuki et al., 2000). Since curcumin and THC, like carotenoids, have antioxidative activity, we assumed that antioxidative property of THC as well as curcumin itself would be involved in the anti-invasive action of this yellow pigment and its metabolite(s) in our assay system. To verify this assumption, the invasion assay was performed with AH109A cells pre-treated by the HX-XO system. This system generates ROS, especially superoxide anion, in a medium, and potentiates invasiveness of certain tumor cells (Shinkai et al., 1986; Tanaka et al., 1997; Kozuki et al., 2000). Curcumin and curcumin-loaded rat sera suppressed the ROS-potentiated invasive ability by simultaneously treating AH109A cells in the medium containing HX and XO with curcumin or curcumin-loaded rat sera (Figure 5). These results suggest that the free radical scavenging activity, especially superoxide scavenging activity, of curcumin (Reddy and Lokesh, 1994; Sreejayan and Rao, 1996) may be responsible for its counteracting effect on the ROS-potentiated slipping motility of the hepatoma cells beneath the M-cell monolayer.

Recent studies showed the involvement of arachidonic acid metabolites in tumor cell invasion and metastasis (Liu et al., 1996; Reich and Martin, 1996; Damtew and Spagnuolo, 1997). Since curcumin is a lipoxygenase inhibitor (Huang et al., 1991; Began et al., 1998), another possibility that curcumin inhibits the AH109A cell movement by suppressing lipoxygenase activity cannot be ruled out. Further studies are required to clarify this aspect.

In conclusion, we demonstrated that curcumin and curcumin-loaded rat sera inhibited the invasive motility of AH109A cells without influence on the proliferation of the cells, and suppressed the ROS-potentiated invasive capacity of the hepatoma cells treated with HX and XO. Our results suggest that the antioxidative property of curcumin, or curcumin metabolite(s), may be involved in their anti-invasive action.

Figure 5. *In vitro* and *ex vivo* effects of curcumin on the invasion of AH109A cells pre-treated with hypoxanthine (HX) and xanthine oxidase (XO). A; AH109A cells were cultured for 4 h in the absence or presence of 5 μ M of curcumin and/or HX (4 μ g ml⁻¹) with XO (7 $\times$ 10⁻⁴ U ml⁻¹). After the treatment, the cells were washed and applied on the M-cell monolayer in the medium with or without 5 μ M of curcumin. The invasive activity was determined as described in Materials and Methods. B; The same treatment described above was performed with the medium containing 10% curcumin (100 mg 100 g⁻¹ body weight)-loaded or vehicle (0.3% CMC)-loaded rat sera instead of the medium containing 10% calf serum in the absence or presence of curcumin (5 μ M). Data are the means $\pm$ SEM of ten areas in one 60 mm ϕ dish with 2 mm grids. Values not sharing a common letter are significantly different at $p < 0.05$ by Tukey's test. Asterisks indicate statistically significant differences (* $p < 0.01$, ** $p < 0.001$). Each result is a representative of at least three similar experiments.

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