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Inhibition of *in vitro* Tumor Cell Invasion by Transmethylation Inhibitors

Kiyoko Shinkai,[1] Mutsuko Mukai,[1] Takeshi Horai,[1] Hiroaki Ohigashi,[2] Seiji Nishikawa,[3] Hideo Inoue,[3] Yoshiro Takeda[3] and Hitoshi Akedo[1]

[1]Department of Tumor Biochemistry, Research Institute, [2]Department of Surgery, Hospital, The Center for Adult Diseases, Osaka, Osaka 537 and [3]Department of Biochemistry, School of Dentistry, The University of Tokushima, Tokushima 770

Three inhibitors of *S*-adenosylmethionine-mediated transmethylation, 5′-methylthioadenosine (MTA), 2′-deoxyadenosine and sinefungin, inhibited *in vitro* invasion by a highly invasive clone (Cl-30) of rat ascites hepatoma cells, AH 130 (AH cells). Difluoromethylthioadenosine (DFMTA), a non-metabolizable derivative of MTA, also caused strong inhibition of invasion at concentrations that did not suppress the growth of the tumor cells. Cl-30 cells precultured in methionine-depleted medium showed decreased invasiveness. DFMTA was also effective on the invasion by fibrosarcoma, B16 melanoma and human lung carcinoma cell lines.

Key words: Tumor cell invasion — Transmethylation inhibitors

Invasion and metastasis are serious obstacles in the treatment of cancer. We previously established a culture system for *in vitro* assay of tumor cell invasion and are using it to investigate determinants of tumor cell invasion.[1-4] One of these determinants appears to be a directed migration of tumor cells. In our assay system, AH cells penetrated under M-cell monolayers by extending pseudopodia-like processes and by active cellular movement. Colchicine and cytochalasin B blocked this penetration.[5] A factor (IIF) that strongly inhibits AH cell invasion both *in vitro* and *in vivo* has been found in rat liver.[6] IIF was shown to bind to the surface of AH cells and to block their directed migration. Leukocytic chemotaxis is a typical directed migration of cells and is suggested to involve metabolism of cell membrane phospholipids.[7-9] For example, this migration of leukocytes is suppressed by adenosine or 3-diazaadenosine, inhibitors of phosphatidylethanolamine methylation. 5′-Methylthioadenosine (MTA), generated in the process of polyamine biosynthesis, is known to be a natural inhibitor of *S*-adenosylmethionine (SAM)-mediated transmethylation.[10] This paper reports our recent finding that inhibitors of SAM-mediated transmethylation inhibited the *in vitro* invasion by several kinds of tumor cell lines at concentrations that did not affect cell growth.

Rat ascites hepatoma cells, AH 130 (AH cells) were maintained by serial ip transplantation in male Donryu rats. Cells were usually obtained 7 days after transplantation and cultured in Eagle's minimum essential medium with two-fold the normal concentrations of amino acids and vitamins (EM) supplemented with 10% calf serum to eliminate host cells. The highly invasive clone Cl-30 was isolated by plating the cultured AH cells in soft agar. Mesothelial cells were isolated from rat mesentery by trypsin digestion and cultured as monolayers (M-cell monolayers) in the medium described above. The AS-653 spindle cell sarcoma (rat fibrosarcoma) subline clone A with a high lymph-node metastatic potential was established by Dr. K. Koyama[11] and was kindly provided by Dr. N. Saijo (National Cancer Center, Tokyo). The clone (G_6) from B16 melanoma cell line was isolated by Dr. H. Tanaka[12] in our institute. The human small cell carcinoma subline OC-7 was established from a pleural metastatic nodule of a patient with small cell lung carcinoma obtained at autopsy. The cultured cells grew as floating aggregates and had similar cytological and xenograft-histological appearances to small cell carcinoma.

The *in vitro* invasive capacity of the tumor cells was assayed by counting the number of penetrated single tumor cells and colonies formed from the penetrated tumor cells as reported previously,[2,6] except that the coculture was terminated at 24 h. Briefly, 2.4×10^5 AH cells were seeded on an M-cell monolayer (35 mm culture dish) and cultured for 24 h in EM supplemented with 10% calf serum. Although the penetration of tumor cells had been completed in 24 h, not all the penetrated cells started to divide (colony formation) at 24 h. Therefore, the number of penetrated single tumor cells and colonies formed under the monolayer in 60 different visual fields (1.13 mm^2 each) was counted under a phase contrast microscope. The *in vitro* invasive capacity was expressed as the number of penetrated single cells and colonies/cm^2. Since the tumor cells used are in the form of a single cell suspension and each colony developed from each penetrated single cell, the sum of the number of

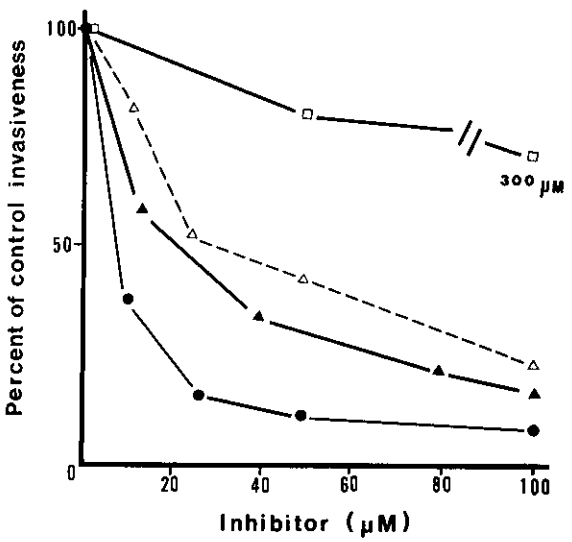

Fig. 1. Effects of transmethylation inhibitors on *in vitro* invasion by Cl-30. Inhibitors were added to the invasion assay medium.

$$\% \text{ Control} = \frac{\text{number of colonies formed with the inhibitor}}{\text{number of colonies formed without the inhibitor}} \times 100.$$

□: sinefungin, △: 2′-deoxyadenosine, ●: MTA, ▲: DFMTA. Number of penetrated single cells and colonies without inhibitors: $554 \pm 27/cm^2$ (mean ± SD).

penetrated single cells and colonies corresponded to the number of tumor cells penetrated. Sinefungin and 2′-deoxyadenosine were purchased from Sigma Co., St. Louis; MTA and difluoromethylthioadenosine (DFMTA)[13] were gifts from Meito Sangyo Co., Nagoya.

MTA, 2′-deoxyadenosine, and sinefungin, which are known to inhibit SAM-mediated transmethylation,[10, 14] inhibited the *in vitro* invasion by Cl-30 cells to different extents, when added to the assay system (Fig. 1). DFMTA, a non-metabolizable derivative of MTA, also caused dose-dependent inhibition of invasion at 15–100 μM. The varying degree of effect of these transmethylation inhibitors, especially the lower effectiveness of sinefungin, appears compatible with the results obtained by Aksamit *et al.*[15] and Pike *et al.*[9] who examined the effect of transmethylation inhibitors on the chemotactic movement of macrophages. They reported that D-homocysteine and 3-deazaadenosine but not sinefungin inhibited the chemotaxis which was associated with the membrane changes in phospholipid methylation. The inhibitor constant (Ki) for sinefungin in phospholipid methylation has been reported to be much larger than those for the other inhibitors tested.[14]

To see whether the inhibitors affected tumor cells or M-cells, Cl-30 cells were preincubated with 300 μM DFMTA for 1 h, washed and inoculated onto M-cell monolayers. Results showed that this treatment caused 52% inhibition of invasion *in vitro*. Moreover, the pretreatment of the M-cell layer with 300 μM DFMTA for 1 h did not significantly modify the invasive behavior of untreated Cl-30 cells. MTA, an inhibitor of spermine synthase, inhibits human lymphocyte blastogenesis[16] and suppresses growth of SV 40-transformed 3T3 cells.[17] Addition of 100 μM MTA or DFMTA, however, did not cause any appreciable suppression of *in vitro* growth of Cl-30 cells after 24 h, though about 10% suppression of cell growth was seen after 72 h. No significant inhibition of growth by sinefungin and 2′-deoxyadenosine was observed at the concentrations tested. A trial to inhibit the *in vivo* invasion of Cl-30 cells by using the transmethylase inhibitors was unsuccessful. Since MTA is supposed to be quickly hydrolyzed by MTA-phosphorylase, DFMTA was injected at the dose of 400 μg/rat/day after the implantation of Cl-30 cells in the peritoneal cavity of rats. No appreciable inhibition of the *in vitro* invasion measured in terms of the formation of invasive nodules in the peritoneum was observed. Additional experiments are under way to develop an appropriate administration protocol.

The methyl groups participating in methylation are mainly supplied from methionine. Therefore, we examined the effect of reducing the reservoir of methyl groups. For this, Cl-30 cells were precultured for 24 h in a medium with 1/10 of the normal methionine concentration and their *in vitro* invasiveness was determined in the same medium. This treatment did not suppress the growth of the cells but caused about 50% inhibition of their invasion. DFMTA caused an additional inhibitory effect (Fig. 2).

The possibility that the reduced invasiveness of Cl-30 cells in the methionine-depleted medium is the result of general disturbance of metabolism in the tumor cells can not be excluded. However, the methionine depletion did not suppress the growth of Cl-30 cells, suggesting that the disturbance may not be so severe as to affect cell growth. The mechanism by which the transmethylation inhibitors suppressed the *in vitro* invasion is not clear at present. MTA is known to inhibit *S*-adenosyl-homocysteine (AdoHcy) hydrolase and to inhibit methylation of proteins, lipids and nucleic acids.[9] DFMTA at concentrations that inhibited invasion was found to have no appreciable effect on the incorporation of the methyl group into RNA, DNA or protein from L-[methyl-^{3}H]-methionine, but did inhibit the methyl group incorporation into phospholipids (Inoue, unpublished results). Recently, Breillout *et al.*[18] showed that 5′-deoxy-5′-*S*-isobutyl-adenosine, another AdoHcy hydrolase inhibitor,

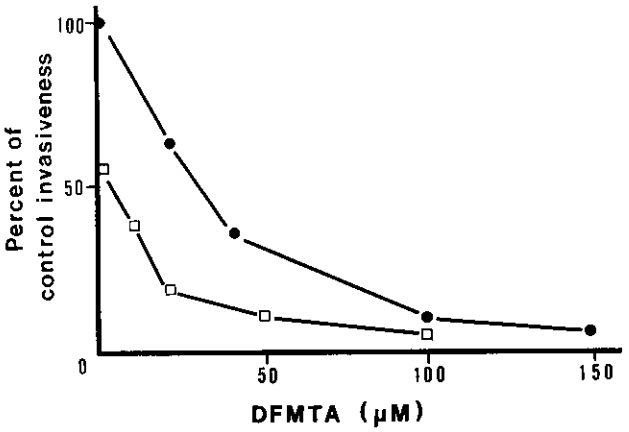

Fig. 2. Effects of DFMTA and methionine depletion on *in vitro* invasion by Cl-30 cells. Cl-30 cells were precultured with methionine-depleted medium for 24 h, and then assayed for their invasive capacity in methionine-depleted assay medium containing various concentrations of DFMTA (□). ●: invasive capacity of Cl-30 cells in the complete medium assayed in the presence of DFMTA. Number of penetrated single cells and colonies without DFMTA in the complete medium: 551±21/cm^2 (mean±SD).

Table I. Effect of DFMTA on *in vitro* Invasion by Various Tumor Cell Lines

Cell line	% inhibition
AH 130 (Cl-30)	93.5
Fibrosarcoma AS-653 (Clone A)	81.0
B16 melanoma (G_6)	66.0
Small cell carcinoma (OC-7)	77.0

Cells were tested for their invasive capacities in the presence and absence of 150 μM DFMTA. Number of penetrated single cells and colonies formed without DFMTA: Cl-30, 570±25/cm^2; fibrosarcoma, 27±6/cm^2; B16 melanoma (G_6), 13098±1532/cm^2; small cell carcinoma (OC-7), 143±7/cm^2 (mean±SD).

suppressed pulmonary metastases of RMS-J and 3LL cells. It has no inhibitory effect on proliferation of the tumor cells at primary foci, so its block of metastases of tumor cells in the lungs must be due to its selective inhibition of tumor cell proliferation at metastatic foci or inhibition of tumor spread from the primary foci.

Liotta *et al.*[19] isolated a factor from highly invasive melanoma cells that enhanced tumor cell motility (an autocrine motility factor: AMF). They reported that AMF could promote cell movement via the methylation of membranous phospholipids and that 3-deazaadenosine blocked AMF-enhanced migration of human melanoma cells (A2058). The mechanism of inhibition of invasion by the block of phospholipid methylation is not known at present, but the reported elevation of membrane fluidity and Ca^{2+} influx[8] followed by phospholipid methylation may participate in the cell motility.

DFMTA inhibited the invasion by not only Cl-30 cells but also rat fibrosarcoma cells, murine melanoma cells (B16) and human lung small cell carcinoma cells (Table I). No appreciable inhibition of growth of these tumor cells was observed at the level of DFMTA used. Transmethylation inhibitors may provide useful information on the mechanism of tumor cell invasion

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