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Prevention of experimental hepatic metastasis with thromboxane synthase inhibitor

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Abstract. To investigate the effectiveness of thromboxane (Tx) synthase inhibitor in the prevention of experimental hepatic metastasis, an in vivo study was designed. Hepatic metastasis was brought about by injection of 1×10^5 cells of colon 38 tumor into the portal vein of C57 B1/65 mice. Seven groups ($n=16$ in each group) received different treatments: with TxA_2 synthase inhibitor (sodium ozagrel), 5, 10 or 15 mg/kg BW before tumor inoculation, and daily for the following 3 days, (groups A, B and C, respectively); with acetyl salicylic acid (aspirin), 1.0, 1.5 or 2.0 mg/kg BW (groups C, D, and E, respectively); a control group, inoculated with vehicle only. Serum TxB_2, a stable metabolite of TxA_2, and prostaglandin $F_{1\alpha}$ were measured. Labeling index for tumor proliferation by bromodeoxy-uridine radio-immuno-assay was also studied. Incidence of metastasis in groups A (60.5%), B (49.5%), C (43.0%), D (80.5%), E (66.0%) and F (58.4%) was less than that in the control group (100%). Tumor size, number or labeling index did not differ among the groups. Serum TxB_2 (pg/ml) levels were significantly lower in all of the groups than in the control. Serum $PGF_{1\alpha}$ levels in the groups with aspirin were lower than those in sodium ozagrel. Tx synthase inhibitor is effective in the prevention of experimental hepatic metastasis when it is given before and immediately after tumor inoculation. As Tx synthase inhibitor leaves metabolic pathway to PGI_2 production intact, it is more effective in the prevention of metastasis than aspirin since aspirin inhibits both thromboxane and PGI_2.

Key words: Hepatic metastasis – Thromboxane synthase inhibitor – Aspirin

Introduction

The most malignant characteristic of cancer, called metastasis [1], is the ability of cells to disseminate and form new foci of growth at noncontiguous sites. Growing

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evidence has revealed that the process of cancer metastasis consists of a series of sequential steps: growth and invasion of the primary tumor into the venules and/or capillaries; transport of the malignant cells in the circulation; arrest in the liver and finally growth and invasion as a metastatic tumor. Surgical manipulation during an operation to remove a tumor can initiate the metastatic process since it may disperse cancer cells in the blood and/or lymph circulation [2–4]. Theoretically, these cells are trapped in the secondary organ such as the liver and the lung to establish metastasis. Tumor cell arrest and growth in the secondary organs require various cellular and non-cellular interactions [5, 6]. Obviously one of the important associated factors is an alternation in the coagulation mechanism that must play an important role in the metastatic process [7].

In the present study, the authors have investigated the interaction of particular arachidonic acid metabolites in cancer metastasis and the value of thromboxane A_2 synthase inhibitor in the prevention of hepatic metastasis using an experimental animal model.

Materials and methods

Mice and tumors

Five- to six-week-old male C57B1/6J mice were obtained from the Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu, Japan. A mouse bearing colon 38 tumors (murine colon adenocarcinoma MCA 38) was kindly supplied from Shionogi Pharmaceutical Co., Osaka, Japan. The tumor had been maintained by subcutaneous implantation in the back of the animals every 4 weeks over 100 passages.

Tumor cell preparation

Colon 38 tumor was minced and filtered through a no. 100 mesh filter. The tumor cells were treated with trypsin and suspended in RPMI-1640 at a density of 1×10^4 cells/ml. Viability of the cells was tested by the trypan blue dye exclusion method. Cells were maintained as a monolayer stock culture at 37 °C in a humidified atmosphere containing 5% CO_2 and were fed twice weekly with RPMI 1640 medium, supplemented with 10% fetal calf serum.

In vivo study of hepatic metastasis

Mice were anesthetized by ether inhalation. Through a small midline skin incision, one branch of the superior mesenteric vein was exposed and 0.1 ml of suspension containing 1×10^5 viable tumor cells was injected into one of the branches of the portal vein. Hemostasis was obtained by gentle compression of the inoculation site with a cotton swab. On day 21 after tumor inoculation, all the animals were killed. The total number of metastases of the liver (which was cut and sliced) and the size of the largest tumor were measured.

Drug administration and grouping of the animals

Thromboxane synthase inhibitor, sodium ozagrel, was supplied by Ono Pharmaceutial Co., Osaka, Japan. Seven different groups of animals were identified ($n=16$ in each group). The scheduled dose was administered immediately before tumor inoculation and daily for the following 3 days in every group. Groups A, B and C received sodium ozagrel 5 mg/kg, 10 mg/kg or 15 mg/kg

BW in distilled water, respectively. Groups D, E and F received acetyl salicylic acid (aspirin) 1.0 mg/kg, 2.0 mg/kg or 3.0 mg/kg BW, respectively at the same intervals. The dosage was based on the knowledge that low-dose aspirin inhibits tumor proliferation more effectively than high dose [8]. In the control group of animals an equivalent volume of vehicle was given with the same dosing schedule. Serum samples ($n=6$ in each group) were taken from some measurement animals for thromboxane B_2 and 2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$ ($PG F_{1\alpha}$) measurement 12 h after the last dose on the 3rd day after tumor inoculation. They were measured by radioimmunoassay with an antibody whose cross reactivity with heterological prostanoids was less than 0.1% [9].

Bromodeoxyurinide (BrdUrd) immunohistochemistry

In other animals of each group, BrdUrd 100 mg/kg in normal saline was injected intraperitoneally on the 10th day after tumor inoculation ($n=3$ in each group). These animals were killed and the livers were excised immediately and fixed in 70% ethanol. Sections were made and stained by the avidin biotin peroxidase complex method, using a Histofine SAB-PO (M) kit (Nichirei, Tokyo, Japan). The labeling index of the tumor was expressed as the ratio of the numbers of the cells positively stained to those of the entire number of cells examined (over 2,000) by light microscopy.

Statistics

Data were expressed as the means ± standard error of the mean. Two-way ANOVA was used for comparison of the mean values between the groups. P values lower than 0.05 were considered significant.

Results

Typical observations of hepatic metastasis on gross inspection showed tumors of varying sizes, maintaining a nearly spherical shape. Metastatic tumors in the sectioned specimens of the liver at termination were distributed preferentially at the peripheral surface in the liver. The distribution pattern of the metastatic foci in the liver did not differ significantly among the groups. Under microscopic observation, the tumor invariably showed a central necrosis when its size was greater, on average, than 2 mm in diameter. There was minimal reaction of inflammatory cells in or around the tumor. The percentage of hepatic metastasis in groups A (60.5%), B (49.5%), C (43.0%), D (80.5%), E (66.0%), and F (58.4%) was significantly lower than in the control group ($P<0.05$). Also, the incidence was significantly lower in group B than in group C ($P<0.05$). The mean numbers of tumors in groups A (2.5 ± 0.3), B (2.3 ± 0.4), C (2.2 ± 0.4), D (2.5 ± 0.3), E (2.4 ± 0.3) and F (2.5 ± 0.3) were significantly ($P<0.05$) lower than that in the control group (3.3 ± 0.4). The greatest diameter of the largest tumor in each animal ranged from 1.0 cm to 5.2 cm. There was no significant difference in the mean between the groups.

Levels of Tx B_2 were significantly lower in groups A (780 ± 265 pg/ml), B (485 ± 175 pg/ml), C (345 ± 160 pg/ml), D (1,150 ± 340 pg/ml), E (570 ± 190 pg/ml) and F (590 ± 200 pg/ml) in the control (1,750 ± 530 pg/ml), ($P<0.05$), (Fig. 1, left). $PFG_{1\alpha}$ levels in groups D (530 ± 60 pg/ml), E (575 ± 50 pg/ml) and F (505 ± 60 pg/ml) were significantly lower than those in A (1,150 ± 395 pg/ml), B (1,200 ± 355 pg/ml), C (1,170 ± 380 pg/ml) and the control group (1,540 ± 655 pg/ml), ($P<0.05$), (Fig. 1, right).

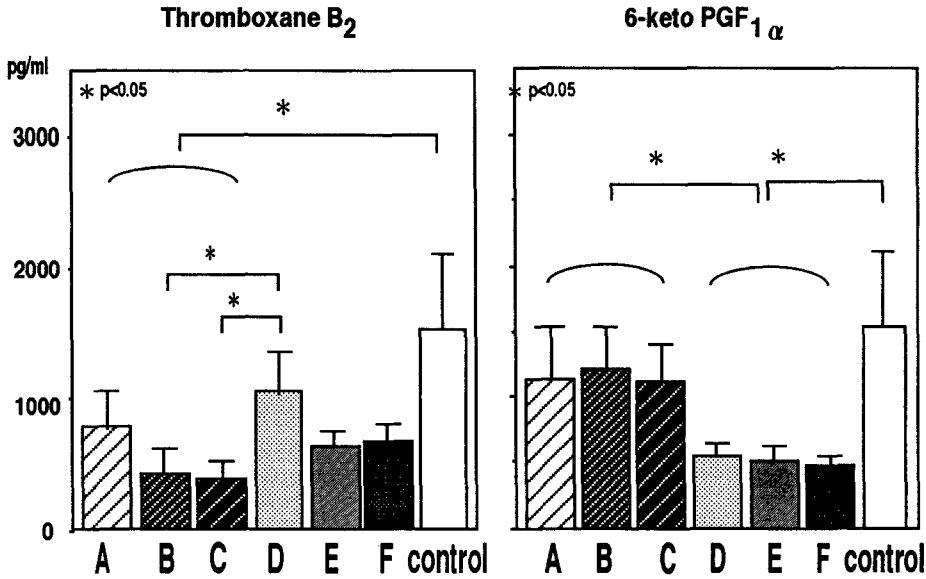

Fig. 1. Serum thromboxane (Tx) B_2 (*left*) and 6-keto $PGF_{1\alpha}$ (*right*) levels 12 h after the last dose of the drug given immediately before tumor inoculation and daily for 3 days in each of groups *A* (Tx synthase inhibitor, sodium ozagrel, 5 mg/kg BW), *B* (sodium ozagrel 10 mg/kg BW), *C* (sodium ozagrel 15 mg/kg BW), *D* (aspirin 1.0 mg/kg BW), *E* (aspirin 2.0 mg/kg BW) *F* (aspirin 3.0 mg/kg BW) and *control* (vehicle). Serum TxB_2 levels in groups *A*, *B*, *C*, *D*, *E* and *F* were lower than those in the control group ($P<0.05$). Serum Tx B_2 levels in groups *B* and *C* were also lower than that in group *D* ($P<0.05$). Serum 6-keto $PGF_{1\alpha}$ levels in groups *D*, *E* and *F* were lower those in groups *A*, *B*, *C* and *control* ($P<0.05$)

Labeling indexes of the tumor cells represented as the ratio of BrdUrd-positive cells at 2 weeks after tumor inoculation were 10.1 ± 6.1%, 11.6 ± 5.2%, 10.5 ± 5.4%, 12.9 ± 5.1%, 11.9 ± 4.3%, 11.5 ± 24.0% and 10.5 ± 4.3% for groups A, B, C, D, E, F and the control group, respectively. No difference was noted among the groups. The labeling index of normal hepatocytes was less than 1% in all specimens examined. There was no significant difference in the labeling index of the normal hepatocytes among the groups.

Discussion

The initial step in hematogenous metastasis to a secondary organ is dispersion of tumor cells into the systemic or portal circulation from the original tumor. In the clinical setting, this situation can be artificially created at or immediately after the time of surgery. Indeed, it has been demonstrated that a significant number of tumor cells are present in the circulation after surgical procedures [2–4]. An interesting observation is that recurrent hepatic tumors tend to recur in the newly grafted liver after liver transplantation for hepatocellular carcinoma [10]. Therefore, the use of an anti-metastatic agent both before surgery and after eradication of the primary tumor can contribute to the prevention of metastasis [11].

Accumulating evidence has shown that cellular interactions between tumor cells, platelets and vascular endothelial cells are of prime importance in the successful completion of the metastatic process [12, 13]. Among these cells, platelets are thought to play a central part in the mechanism of the tumor cell-host interactions [14]. Corresponding to these cellular interactions, various soluble stimulators are generated. Of these, the most important regulatory mediators produced by activated platelets are the arachidonic acid metabolites.

The arachidonic acid cascade generates a family of bioactive lipids. Their oxidized metabolites contribute to specific steps in the process of cell transformation, tumor growth and metastasis [15–17]. Arachidonic acid is oxidized by cyclooxygenase to produce prostaglandin (PG) G_2. PGG_2 is then converted to PGH_2 by peroxidase. Thromboxane (Tx)A_2 is diverted from PGH_2 by TxA_2 synthase. TxA_2 is a potent vasoactive, pro-aggregatory and chemotactic prostanoid [18]. TxA_2 is unstable, so that TxB_2, a more stable hydrolysis product, can be measured in the serum [9]. Platelets are considered to be the main site of Tx production, although it has been shown that there are other possible sources, such as the polymorphonuclear leukocytes, macrophages, Kupffer cells, and endothelial cells [19]. A recent investigation has shown that most of the organs contain Tx synthase in varying degree, with the highest content in the platelets followed by the lung and liver in decreasing order [20].

TxA_2 is involved in the process of cancer metastasis in two ways. One is its procoagulatory effect, inducing platelet and leukocyte interaction that facilitates tumor cells to be trapped and become adherent to the endothelial cells [21]. This interaction also contributes to the production and generation of various cytokines that activate further recruitment of leukocytes. This results in tumor cells penetrating and invading the basement membrane through the gaps between the endothelial cells [31]. The other role of TxA_2 in cancer metastasis is its extremely vasoconstrictive activity [18]. Consequently, distal blood flow diminishes, thereby creating an ideal situation for cancer cells to be embolized in the sinusoids of the liver. It is also conceivable that the ischemic change of the parenchymal and nonparenchymal cells distal to this microembilization leads to a further liberation of Tx, most likely induced by endothelial cells in paracrine and autorcrine fashion [22].

In contrast to TxA_2 production as one aspect of PG metabolism occurring in the platelets, there is another in the vascular endothelial cells, which convert PGH_2 mainly to PGI_2 an inhibitor of platelet aggregation and vasodilatation [23]. Inhibition of PG biosynthesis, e.g. by inhibition of its cyclooxygenase activity by aspirin, is known to cause a decreased incidence of tumor metastasis [24]. However, aspirin also blocks production of PGI_2, which can counteract progression of tumor metastasis, as opposed to the action of TxA_2. Indeed, a number of experiments have confirmed that PGI_2 and its analogues have potent antimetastatic effects on various tumors [25–27]. Honn et al. proposed that the PGI_2-to-TxA_2 production ratio modulates tumor cell adhesiveness [13]. Thus, the importance of PGs, specifically TxA_2 and PGI_2, lies in the regulation of platelet function as the basis of the strategy for interruption of the metastatic cascade.

Sodium ozagrel is a specific inhibitor of TxA_2 synthase, leaving a pathway for PGI_2 production intact. It was clearly shown in the present study that the use of this drug significantly decreases serum TxB_2 while $PGF_{1\alpha}$ level is not affected. The latter is an intermediate metabolite of PGI_2. This indicates that the effectiveness of the inhibition of tumor cell adhesiveness is maximized with the use of so-

dium ozagrel, since a positive action for metastasis by TxA_2 suppressed while a counteractive action for tumor metastasis by PGI_2 is preserved. The use of TxA_2 synthase inhibitor is further supported by the finding in a previous experimental study that TxA_2 exhibits a direct cytoproliferative action on tumor cells in vitro [27, 28]. Also, tumor proliferation is inhibited by a TxA_2 receptor antagonist and administration of the same receptor antagonist prevents growth of the tumor in vivo [29].

Selective inhibition of TxA_2 production leaves a few other metabolites that may affect tumor growth, such as PGD_2 and PGE_2. The former is an antiaggregatory agent [30] that may inhibit tumor seeding, whereas the latter can induce tumor promotion [31].

Another mechanism by which cyclooxygenase inhibitors may reduce tumor metastasis is by modification of the immune response. PGE_2 inhibits blastogenesis of T cell and the cytotoxic activity of natural killer cells [32]. Increased Tx synthesis causes platelet attachment to tumor cells in the bloodstream that may protect them from being attacked by natural killer cells, enhancing their metastatic capability. Thus, understanding the mechanisms of eicosanoid interaction with the tumor cells is complicated by the multistage nature of the metastatic transformation of the tumor.

In conclusion, the result of the present study indicates the importance of PGs, specifically TxA_2 and PGI_2, in the regulation of platelet function, which is the basis for a strategic approach to the interruption of the metastatic process. Of particular importance is the observation that the chance of tumor metastasis can rise dramatically in a specific clinical setting, e.g. during a surgical procedure for tumor eradication, therefore TxA_2 synthase inhibitor may be clinically applicable for the prevention of metastasis on specific occasions when cancer cells are dispersed during tumor surgery.

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