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Short communication

Effects of antitumor agents on inducible prostacyclin production in vascular smooth muscle

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Abstract

The effects of various antitumor agents were examined on prostacyclin production induced by interleukin-1β in rat aortic smooth muscle cells. Stimulation of the cells with interleukin-1β (2.5 ng/ml) resulted in a great increase of prostacyclin production, which was abolished by indomethacin (1 μM) or cycloheximide (2 μM). Daunorubicin at 0.1–1 μM inhibited the inducible prostacyclin production in a concentration-dependent manner. However, other antitumor agents (cyclophosphamide at 1–100 μM, 5-fluorouracil at 1–100 μM and vincristine at 1–100 nM) tested did not significantly affect it. Protein expression of cyclooxygenase-2 induced by interleukin-1β was inhibited by daunorubicin at 0.1–1 μM, but was not affected by other antitumor agents. These results suggest that daunorubicin inhibits induction of cyclooxygenase-2 and subsequent prostacyclin production in rat aortic smooth muscle cells.

Keywords: Prostacyclin; Cyclooxygenase-2; Daunorubicin; Smooth vascular muscle cell

1. Introduction

Therapy with antitumor agents is known to be complicated by vascular diseases such as thrombotic microangiopathic syndrome, Raynaud's phenomenon, and acute ischemic events (e.g., myocardial infarction and cerebrovascular accidents). Putative mechanisms for their toxicity include drug-induced damage to the vascular endothelium, perturbation of the clotting system, platelet activation and autonomic dysfunction (Doll and Yarbro, 1992). Prostacyclin is mainly produced in vascular endothelial cells and plays important roles in the regulation of blood coagulability and vascular tonus (Moncada, 1982). Antitumor chemotherapy sometimes causes thrombotic events following disseminated intravascular coagulation, which is common especially in blood malignancies (DeLoughery and Goodnight, 1996). Prostacyclin has been reported to prevent metastatic tumors from spreading (Schirner and Schneider, 1991). Some antitumor drugs show cardiotoxicity (Shan et al., 1996), which may be aggravated by disturbance of the coronary blood flow. Therefore, it is of interest to investigate the effects of antitumor drugs on prostacyclin production in vascular cells. However, there have been only a few studies investigating the effects of antitumor drugs on it. Mitomycin C inhibits thrombin-stimulated prostacyclin synthesis in human umbilical cord vein endothelial cells without affecting cell viability (Duperray et al., 1988). On the other hand, 5-fluorouracil causes release of prostacyclin in bovine aortic endothelial cells due to endothelial injury, while methotrexate does not affect it (Cwikiel et al., 1996).

Cyclooxygenase, the enzyme which transforms arachidonic acid into endoperoxides, exists in both constitutive (cyclooxygenase-1) and inducible (cyclooxygenase-2) isoforms (Mitchell et al., 1995). Vascular smooth muscle cells contain both isoforms of cyclooxygenase and the inducible one is responsible for the majority of prostacyclin release during inflammatory events in blood vessels (Rimarachin et al., 1994). The induction of cyclooxygenase-2 may thus represent a defense mechanism to compensate for endothelial dysfunction in damaged vessels. However, it is not known whether cyclooxygenase-2-mediated prostacyclin

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synthesis is affected by antitumor drugs. The purpose of the present study was to investigate the effects of various antitumor agents with different cellular mechanisms on prostacyclin production and cyclooxygenase-2 expression induced by stimulation with interleukin-1β, an inflammatory cytokine in rat aortic smooth muscle cells.

2. Materials and methods

2.1. Preparation of rat aortic smooth muscle cells

Rat aortic smooth muscle cells were isolated from the thoracic aorta of male Wistar rats (10–12 weeks old) by outgrowth of explants. Segments of thoracic aorta were dissected and placed on 60-mm plastic dishes containing Ca^{2+}, Mg^{2+}-free phosphate buffer saline (PBS), and then the fat and connective tissues were removed carefully. After longitudinal dissection, the intima was removed with a scraper. Next, 1–2 mm^2 sections of the media were prepared and transferred into 35-mm wells in a 6-well plate containing Dulbecco's Modified Eagle's Medium with 5% fetal calf serum, 4 mM glutamate, 100 U/ml penicillin and 100 μg/ml streptomycin. The dishes were incubated in a humidified atmosphere at 37°C under 5% CO_2–95% air. After 2–3 weeks of incubation, cell layers showing the pattern of hills and valleys, a characteristic of smooth muscle cells grew to confluency. Cells from passages 15–30 were used for the experiments

2.2. Cell culture

Rat aortic smooth muscle cells were cultured in Dulbecco's Modified Eagle's Medium containing 5% fetal calf serum, 4 mM glutamate, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere at 37°C under 5% CO_2–95% air. The cells were spread in 24 well or 6-well culture plates and cultured until they reached confluency. The confluent cells were used for the assays. Cyclooxygenase-2 was induced in rat aortic smooth muscle cells by incubation with interleukin-1β (2.5 ng/ml) for 24 h. In order to check the possibility of endothelial cell contamination, we examined a staining of the cultured cells by 1,1′-dioctadecyl-1-3,3,3′,3′-tetramethylindocarbocyanine percholate (Dil)-acetyl LDL, a dye which stains endothelial cells but not smooth muscle cells. None of the cultured cells observed by fluoromicroscope was detected to be stained by the dye. Thus, we confirmed that vascular endothelial cells hardly contaminated the cultured vascular smooth muscle cells in the present study.

2.3. Measurement of 6-keto prostaglandin F1α

Rat aortic smooth muscle cells were cultured in 24-well plates. Prostacyclin generation was determined by measuring the amount of its stable metabolite, 6-keto prostaglandin F1α, in the supernatant. Aliquots of culture medium were sampled and stored at −20°C until the assay. The 6-keto prostaglandin F1α concentration of the aliquot was measured with enzyme immunoassay using a commercial kit (6-keto prostaglandin F1α EIA system, Amersham Life Science). The amount of prostacyclin production was expressed as the % 6-keto prostaglandin F1α release of the control treated with vehicle or the absolute value of 6-keto prostaglandin F1α (pg) per well.

2.4. Protein separation and Western blotting

Rat aortic smooth muscle cells were cultured in 6-well plates. Following incubation with the requisite treatments, the culture medium was removed, and the cells were washed twice in cold PBS, then solubilized in hot (70°C) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The samples were dispersed by repeated passage through a 25-gauge needle and then transferred to eppendorf tubes. The samples were boiled for 10 min and then stored at −80°C until analysis. Aliquots (20 μg protein) were subjected to SDS-PAGE on 7.5% polyacrylamide slab gels and then blotted onto polyvinylidene difluoride membrane. Polyvinylidene difluoride blots were blocked for 1 h in tris-buffered saline (TBS: 150 mM NaCl, 20 mM Tris, pH 7.5), containing 5% non-fat milk, and then incubated overnight at 4°C with anti-(mouse cyclooxygenase-2) IgG (1:250 dilution) (Transduction Laboratory) in TBS containing 5% non-fat milk. The membrane was then washed in TBS and incubated with goat anti-mouse alkaline phosphatase-conjugated antibody (1:3000 dilution) for 2 h. After further washing with TBS, blots were detected by the enhanced chemiluminescence method using an immuno-blot assay kit (Immuno-Blot Assay Kit, Bio-Rad).

2.5. Measurement of lactate dehydrogenase (LDH) activity

LDH release into the cell culture medium was used to assess the cytotoxic effect of daunorubicin. LDH was measured using a commercially available kit (Liquitech LDH, Boehringer Mannheim). Briefly, 10 μl of the sample was added to 350 μl of a solution containing 0.6 mM pyruvate and 50 mM phosphate buffer (pH 7.5), and incubated at 37°C for 5 min. Next, 70 μl of 0.18 mM NADH solution was added and incubated for further 5 min. Then, absorbance at 340 nm was measured by a spectrophotometer.

2.6. Statistics

The data were expressed as the mean with standard error. Statistical analysis was performed with analysis of variance followed by Dunnett's post-hoc test. *P*-values less than 0.05 were considered significant.

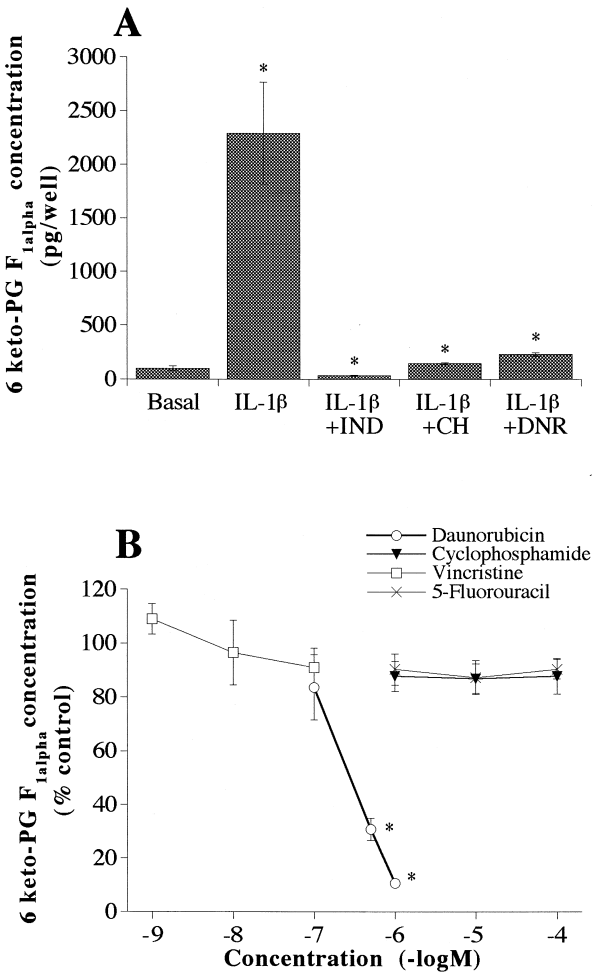

Fig. 1. (A) Effects of indomethacin (1 μM, IND), cycloheximide (2 μM, CH) and daunorubicin (1 μM, DNR) on interleukin-1β (2.5 ng/ml, IL-1β)-stimulated prostacyclin production in rat aortic smooth muscle cells. *, significantly different ($P < 0.05$) compared with prostacyclin production without stimulation (Basal). **, significantly different ($P < 0.05$) compared with the control incubated with vehicle ($n = 6$). The amount of prostacyclin production is expressed as the absolute value of 6-keto prostaglandin F1α (pg) per well. (B) Effects of daunorubicin (0.1–1 μM), cyclophosphamide (1–100 μM), vincristine (1–100 nM) and 5-fluorouracil (1–100 μM) on interleukin-1β (2.5 ng/ml)-stimulated prostacyclin production in rat aortic smooth muscle cells. *, significantly different ($P < 0.05$) compared with the control incubated with vehicle ($n = 5$). The amount of prostacyclin production is expressed as the % 6-keto prostaglandin F1α release of the control treated with vehicle. The mean actual value of the controls was 1914 ± 213 pg/well.

2.7. Chemicals

Daunorubicin (Wako, Osaka, Japan), cyclophosphamide (Sigma, St. Louis, MO, USA), vincristine (Wako), 5-fluorouracil (Sigma) and cycloheximide (Sigma) were dissolved in PBS to make stock solutions of 1 mM, 10 mM, 1 mM, 10 mM and 1 μM, respectively, kept at 4°C, and diluted with PBS just before use. Interleukin-1β (Genzyme, Cambridge, MA, USA) was dissolved in PBS to make a stock solution of 1 mg/ml and were kept at −20°C. Indomethacin (Sigma) was dissolved in equimolar solution of Na_2CO_3 to make a solution of 1 mM just before use, and diluted with PBS. The concentration of each drug was expressed as the final concentration in the well.

3. Results

3.1. Effects of antitumor drugs on prostacyclin production of rat aortic smooth muscle cells

Prostacyclin production of rat aortic smooth muscle cells was greatly increased by stimulation with interleukin-1β (2.5 ng/ml) for 24 h and this increase was abolished by coincubation with indomethacin (1 μM), a nonspecific cyclooxygenase inhibitor, or cycloheximide (2 μM), a nonspecific inhibitor of intracellular protein synthesis (Fig. 1A). Among the antitumor agents tested, daunorubicin (0.1–1 μM) significantly inhibited the interleukin-1β-stimulated production of prostacyclin in a concentration-dependent manner (Fig. 1B). However, it was not affected by cyclophosphamide (1–100 μM), 5-fluorouracil (1–100 μM) or vincristine (1–100 nM) (Fig. 1B).

3.2. Effects of antitumor drugs on LDH release from rat aortic smooth muscle cells

In order to examine the effects of these drugs on nonspecific toxicity to rat aortic smooth muscle cells, LDH levels in the supernatants of the cells were measured. None

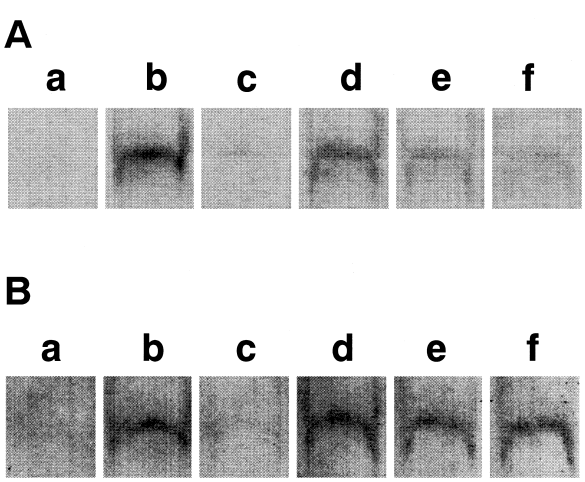

Fig. 2. Western blot analysis of cyclooxygenase-2 in rat aortic smooth muscle cells. The supernatant fraction of cell lysate was separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane and blotted with cyclooxygenase-2 antibody. (A) Rat aortic smooth muscle cells were incubated for 24 h with vehicle (lane a), 2.5 ng/ml interleukin-1β (lane b), interleukin-1β plus 1 μM cycloheximide (lane c), interleukin-1β plus daunorubicin at a concentration of 0.1 μM (lane d), 0.5 μM (lane e) or 1 μM (lane f). (B) Rat aortic smooth muscle cells were incubated for 24 h with vehicle (lane a), 2.5 ng/ml interleukin-1β (lane b), interleukin-1β plus 1 μM daunorubicin (lane c), interleukin-1β plus 100 μM cyclophosphamide (lane d), interleukin-1β plus 100 μM 5-fluorouracil (lane e) and interleukin-1β plus 100 nM vincristine (lane f).

of the drugs at the above concentrations induced the release of LDH [LDH level in the supernatant, not detected (<14 IU/ml)]. Thus, the inhibitory effect of daunorubicin on inducible prostacyclin production is not due to its cytotoxic action. We did not use higher concentrations (>100 nM) of vincristine, since they induced aortic smooth-muscle cell damage, as evaluated by LDH levels in the cell supernatants [100 nM vincristine-treated, <14 IU/L (not detected); 500 nM vincristine-treated, 35.2 ± 2.2 IU/L].

3.3. Effects of antitumor drugs on cyclooxygenase-2 expression in rat aortic smooth muscle cells

Stimulation of rat aortic smooth muscle cells with interleukin-1β (2.5 ng/ml) revealed immunoblot of cyclooxygenase-2, which was indeed abolished in the presence of cycloheximide (1 μM) (Fig. 2A, a–c). Protein expression of cyclooxygenase-2 was inhibited by daunorubicin (0.1–1 μM) concentration-dependently (Fig. 2A, d–f), but was not affected by cyclophosphamide (100 μM), 5-fluorouracil (100 μM) or vincristine (100 nM) (Fig. 2B). Therefore, daunorubicin disrupted the expression of cyclooxygenase-2 protein to result in decrease of prostacyclin sysnthesis.

4. Discussion

Based on clinically attainable blood concentrations of the drugs, we decided on drug concentrations used for the experiments in this study. Daunorubicin is known to show antineoplastic action by direct damage to DNA resulting from superoxide anion radical formation, whereas 5-fluorouracil and vincristine act by inhibiting synthesis of thymidine and inducing microtubulus injury, respectively (Chabner et al., 1996). Thus, it is likely that daunorubicin inhibits cyclooxygenase-2 induction by inhibiting gene transcription. Further studies are required to investigate the detailed mechanism of daunorubicin action on prostacyclin production at the gene level and whether other mechanisms (e.g., direct effect on cyclooxygenase-2 activity) are involved in this action. 5-Fluorouracil blocks thymidylate synthase, resulting in the inhibition of DNA and RNA synthesis. The reason for the lack of inhibition of cyclooxygenase-2 expression by 5-fluorouracil in the present study may be the rather short time (24 h) of drug incubation. Moreover, the intracellular storage of thymidine might be available for interleukin-1β-stimulated cyclooxygenase-2 gene expression in the present experimental condition. In the previous study, incubation of bovine aortic endothelial cells with 5-fluorouracil (5 μg/ml ≈ 38 μM) caused a release of prostacyclin from the cells (Cwikiel et al., 1996). However, in the present study, 5-fluorouracil up to 100 μM did not affect the prostacyclin levels in the supernatants. This may be due to the differences in the kind of cell (vascular smooth-muscle cells vs. endothelial cells) and/or the duration of the drug incubation (24 h vs. 48 h). On the other hand, cyclophosphamide, which induces DNA injury due to guanine alkylation (Chabner et al., 1996), did not show inhibitory action on prostacyclin production even at a high concentration such as 100 μM in rat aortic smooth muscle cells. The difference in the actions on prostacyclin production between daunorubicin and cyclophosphamide, both of which cause DNA damage, may be explained as follows. Cyclophosphamide requires phosphatase or phosphamidase activity in cells to produce an activated nitrogen mustard (Chabner et al., 1996), and the capability of this conversion might be not sufficient or be lacking in rat aortic smooth muscle cells. Moreover, there may be a difference in the sensitivity of the cells to these drugs.

Our previous study showed that daunorubicin at concentrations of 35.5 μM and over causes vasocontraction of rat aortic smooth muscle in vitro (Wakabayashi et al., 1989). However, eicosanoids including prostacyclin from vascular wall are thought to not be involved in this action, since the vasocontraction induced by daunorubicin was affected by neither bromophenacyl bromide, a phospholipase A2 inhibitor, nor mechanical disruption of the endothelium (Wakabayashi et al., 1989). Submicromolar concentrations of daunorubicin which inhibit cyclooxygenase-2 induction in rat aortic smooth muscle cells are much lower than those which cause vasocontraction. The peak blood concentration of daunorubicin during antileukemic chemotherapy was reported to be about 1 μM (Ogawa et al., 1987), which is the concentration inducing almost maximum inhibition of prostacyclin production in the present study. Therefore, the inhibition of prostacyclin production by daunorubicin may affect circulation and coagulability during the therapy at loci where inflammatory cytokines are produced.

The clinical significance of the present finding that daunorubicin inhibits prostacyclin synthesis induced by the inflammatory cytokine can be explained as follows. (1) Thrombotic events following disseminated intravascular coagulation sometimes occur during combined chemotherapy including daunorubicin against blood malignancies (DeLoughery and Goodnight, 1996). Moreover, there has been a previous report that thrombotic thrombocytopenic purpura occurred following acute myelocytic leukemia chemotherapy with daunorubicin (Byrnes et al., 1986). Thus, daunorubicin may affect blood flow and coagulability in the pathophysiology of the vascular complications through inhibitory action on prostacyclin synthesis. (2) Prostacyclin and its stable analogues were shown to interfere with the spread of metastatic tumor (Schirner and Schneider, 1991). Metastatic lesion of blood vessels involves inflammatory processes, resulting in cyclooxygenase-2 induction. Thus, there is a possibility that daunorubicin modulates tumor metastasis by decreasing prostacyclin production in the vascular wall. However, more recent investigations have shown cyclooxygenase-2

activity to be related to metastasis and angiogenesis of tumor, which were suppressed by cyclooxygenase inhibitors such as indomethacin (Lala et al., 1997; Watson, 1998). Therefore, it is difficult at present to come to any conclusion about the significance of cyclooxygenase-2 inhibition by daunorubicin on tumor progression. (3) Cardiotoxicity is a typical side effect during therapy with anthracycline antibiotics and often restricts the dose used. Cardiomyopathy, the primary lesion, is known to be caused mainly by oxygen radicals generated intracellularly from the drugs, but not by coronary ischemia (Shan et al., 1996). However, inducible synthesis of prostacyclin, an important factor preventing generation of thrombosis and preserving blood flow in coronary circulation (Ruschitzka et al., 1997), is reduced by daunorubicin. Moreover, our previous studies have shown that daunorubicin increases vascular tone (Wakabayashi et al., 1989) and inhibits endothelium-dependent vasorelaxation (Wakabayashi et al., 1994). These findings support the postulation that daunorubicin reduces coronary blood flow and aggravates myocardial dysfunction.

In conclusion, submicromolar concentrations of daunorubicin inhibit inducible prostacyclin production in vascular smooth muscle cell by depressing cyclooxygenase-2 induction and this action may be related to the pathogenesis of some cardiovascular complications during antitumor chemotherapy with the drug.

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