A Selective Cyclooxygenase-2 Inhibitor Suppresses Tumor Growth in Nude Mouse Xenografted with Human Head and Neck Squamous Carcinoma Cells

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The anti-tumor effect of a selective cyclooxygenase (COX)-2 inhibitor, JTE-522, was examined with the human head and neck squamous cell carcinoma cell line KB. KB cells do not produce prostaglandin (PG)-E2. *In vitro*, JTE-522 induced an increase of G1 phase-arrested cells, suppression of platelet-derived growth factor (PDGF) production and inhibition of telomerase activity. No cytotoxic effect was detected. *In vivo*, the growth of the tumor xenografted into nude mice was significantly suppressed by JTE-522. Suppression of angiogenesis at the periphery of the tumor, increase of G1-arrested cells and suppression of telomerase activity were observed, together with an increase of apoptotic cell death in the tumor. Immunological enhancement did not play a role. We concluded that the anti-tumor effect of JTE-522 was caused by anti-angiogenesis action, cell cycle arrest and inhibition of telomerase activity of the tumor cells. These combined effects might induce apoptosis.

Key words: Head and neck squamous carcinoma cells — Selective cyclooxgenase-2 inhibitor — Cell cycle arrest — Anti-angiogenesis — Telomerase activity inhibition

Nonsteroidal anti-inflammatory drugs (NSAIDs) are promising candidates for chemopreventive agents active against the development of cancers in the colon, the bladder, the mammary gland, the skin, and the liver.[1–4] NSAIDs exert their pharmacological effects by inhibiting cyclooxygenase (COX) and enzymes responsible for prostaglandin (PG) synthesis. The inhibition of COX and PGs has been postulated to be one of the chemopreventive mechanisms by directly intervening in the signal cascade for cell proliferation or by disturbing immunological surveillance.[2, 5, 6] However, prolonged use of the drugs frequently results in untoward gastrointestinal side effects, which has led to the development of more selective COX inhibitors.[7] Two isoforms of COX, COX-1 and COX-2, have been identified and they are rate-limiting enzymes involved in the conversion of arachidonic acid to prostanoids. *COX-1* is considered to be the product of a housekeeping gene, and to be responsible for various physiological functions, e.g., cytoprotection of the stomach, vasodilation in the kidney and production of a proaggregatory prostanoid, thromboxane, by platelets.[8] *COX-2*, which is encoded by an inducible immediate-early gene, is thought to have various other functions in addition to its well-known role in inflammatory reactions.[8, 9] *COX-2* gene is highly expressed in colon, stomach, skin and mammary gland tumors,[10–14] and its overexpression in rat intestinal epithelial cells renders them resistant to apoptosis.[15] Furthermore, double knockout of the *COX-2* gene suppresses intestinal polyposis in *APC* gene knockout mouse.[16] Consequently, selective COX-2 inhibitors may be more effective and safer cancer chemopreventive agents than classical NSAIDs.

The cancer chemopreventive mechanism of NSAIDs is not fully understood. In cultured human colon adenocarcinoma cells,[17] sulindac, a non selective COX inhibitor, and its metabolite, sulindac sulfide, reduced the cell proliferation rate, changed the cell morphology and induced cellular apoptosis, due to inhibition of prostanoid synthesis.[18] PG-E2, a major product of COX-2, has been shown to inhibit apoptosis and upregulate bcl-2 expression in a human colon cancer cell line.[19] NS-398, a selective COX-2 inhibitor, induced apoptosis in human prostate cancer cell line in a time- and dose-dependent fashion, concomitantly with down-regulation of bcl-2 protein expression.[20] Some studies have shown that a sulfone derivative of sulindac, which essentially lacks prostaglandin synthesis-inhibitory activity,[18] also inhibits chemical carcinogenesis, suggesting an additional mechanism of antineoplastic activity by sulindac and its metabolites.[21] COX-2-overexpressing colon cancer cells produced high levels of angiogenic factors, which stimulated endothelial tube formation, and NS-398 inhibited both the expression of the angiogenic factors and endothelial tube formation.[22] To clarify the mechanism of COX-2 inhibitors in suppressing tumor growth, we investigated the effects of JTE-522, a selective

COX-2 inhibitor, on the growth of a human head and neck squamous cell carcinoma cell line, KB,[23] *in vitro* and *in vivo*. JTE-522 inhibits sheep COX-2 with an IC_{50} of 6.4×10^{-7} *M* and human recombinant COX-2 with an IC_{50} of 8.5×10^{-8} *M*, but does not inhibit sheep COX-1 or human platelet COX-1 at concentrations up to 10^{-4} *M*.[24, 25]

MATERIALS AND METHODS

Materials JTE-522, a selective COX-2 inhibitor, was a kind gift from Japan Tobacco Inc. (Tokyo). The chemical structure of JTE-522 is shown in Fig. 1. Female 5-week-old athymic nude mice (BALB/c *nu/nu*) were purchased from Charles River Japan Inc. (Atsugi). The animals were housed in a room kept at $24 \pm 2°C$ temperature and 40–70% humidity with a 12 h light/dark cycle.

Cell line KB cells (human oral floor squamous cell carcinoma cell line) were grown in RPMI-1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS), 2 m*M* glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (complete medium) at 37°C in a 5% CO_2 atmosphere. PG-E2 secretion in the supernatant was undetectable with the PG-E2 Monoclonal Enzyme Immunoassay Kit (Cayman Chemical, Ann Arbor, MI), according to the protocol recommended by the manufacturer. The PG-E2 concentration found in the supernatant of KB cells cultured to 80–90% confluence was 50 pg/ml and was equal to the value found in RPMI-1640 containing 10% FCS.

In vitro

Cell proliferation analysis: Cells were seeded on a 96-well plate (Falcon; Becton Dickinson Labware, Lincoln Park, NJ) at 1×10^3 cells/well in complete medium. After 48, 72 and 96 h treatment with JTE-522 (10^{-4}–10^{-8} *M* of medium), the number of cells was quantitated by an assay using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide[26] (MTT; Sigma Chemical Co., St. Louis, MO). The cells were seeded on a ϕ10 cm plastic dish (Falcon) at 5×10^5 cells/well in complete medium and treated with JTE-522 (10^{-4}–10^{-7} *M* in the medium). After 72 h treatment, monolayer cells were washed with cold phosphate-buffered saline (PBS), trypsinized and counted.

Cell viability analysis: Cells were seeded on a ϕ10 cm plastic dish at 1×10^6 cells/well in complete medium. After 48 and 72 h treatment with JTE-522 (10^{-5} and 10^{-6} *M*), cell viability was determined by cell counting after staining with trypan blue. Surviving cells were expressed as a percentage of the total cell population.

Cell cycle analysis: Cells were seeded on a ϕ10 cm plastic dish at 1×10^6 cells/well and were either untreated or treated with ethanol alone or various concentrations of JTE-522 (10^{-5} and 10^{-6} *M* in the medium) in ethanol. After 48 and 72 h treatment, cells were trypsinized and cell pellets were collected. The cells were suspended in
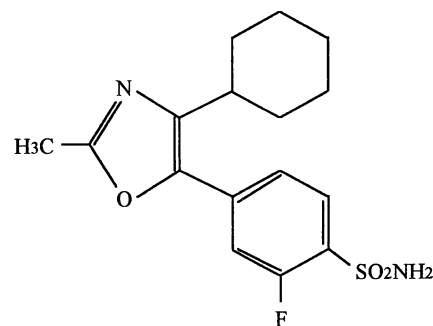

Fig. 1. The chemical structure of JTE-522.

500 μl of PBS, and digested with 20 μg/ml RNase at 37°C for 30 min. The digest was chilled on ice for 10 min, and then cellular DNA was stained with 50 μg/ml propidium iodide by incubation for 1 h at room temperature in the dark. Cell cycle distribution was analyzed by flow cytometry using the Becton Dickinson FACS system.

Immunohistochemistry: Cells were seeded on 4-well LAB-TEK chamber slides (Nalge Nunc, Naperville, IL) at 1×10^3 cells/well in complete medium. After 48 and 72 h treatment with 10^{-4} and 10^{-5} *M* JTE-522, cells were fixed and slides were blocked with acetone for 10 min at $-20°C$. The slides were incubated with 1% *Bandeiraea simplicifolia* agglutinin (BSA) for 20 min and then with an anti-human COX-2 goat monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (1:100 dilution) overnight at 4°C in a humidified chamber. The slides were incubated with biotinylated rabbit antigoat immunoglobulin, followed by incubation with peroxidase-conjugated streptavidin (ABC kit; Nichirei, Tokyo). Signals were visualized by treatment of the slides with diaminobenzidine and examined under a microscope after counterstaining with hematoxylin.

Reverse transcription-polymerase chain reaction (RT-PCR): Total RNA was extracted from KB cells, which had been untreated or treated with 10^{-5} and 10^{-6} *M* JTE-522 for 48 and 72 h, with phenol solution ("ISOGEN"; Nippon Gene Inc., Tokyo) according to the manufacturer's protocol. cDNAs were synthesized by reverse transcriptase (RT) of mRNA using "Superscript" (Gibco BRL Products, Rockville, MD) with random hexamers. The cDNA synthesis was started with 2 μg of total RNA in each sample and 1 μl aliquots of RT products were used for subsequent PCR. The cDNAs were then amplified by PCR for 30 cycles with the *Taq* DNA polymerase ("Taq"; Takara Biomedicals, Tokyo) on a thermal cycler (Takara). The cycle parameters were 94°C for 2 min followed by 72°C for 10 min for the final elongation. The PCR primers used were COX-2 sense: CCGAGGTGTATGTATGAG, COX-2 anti-sense: ATCAGGCACAGGAGGAAG. The COX-2

primers bracketed the insertion site on human COX-2 cDNA and would generate a product of 336 bp. The PCR products were applied to a 2% agarose gel and electrophoresed. The gel was then stained with ethidium bromide and illuminated on a UV table.

Production of angiogenic factors: The detection of angiogenic factors, basic fibroblast growth factor (b-FGF), vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), in the supernatants of KB cells, which had been untreated or treated with JTE-522 (10^{-5} and 10^{-6} M) for 48 and 72 h, was examined by enzyme-linked immunosorbent assay (ELISA). All kits were purchased from R&D Systems (Minneapolis, MN). Immunoassays were performed according to the instructions of the manufacturers. The minimal detectable levels by ELISA are 1.0 pg/ml of b-FGF, 5.0 pg/ml of VEGF and 8.4 pg/ml of PDGF.

DNA fragmentation: Cells at 80–90% confluency were either untreated or treated with ethanol alone or various concentrations of JTE-522 (10^{-5} and 10^{-6} M in the medium) in ethanol for 48 and 72 h. Cells were lysed in lysis buffer [10 mM Tris (pH 7.4), 5 mM EDTA and 1% Triton X-100] for 20 min on ice. Microcentrifugation was performed at 11,000g for 20 min to separate the nuclear DNA precipitate from the fragmented DNA present in the supernatant. The supernatant was treated with 50 μg/ml of RNase A at 37°C for 1 h, and then proteinase K was added at 0.1 mg/ml for another hour. After phenol-chloroform extraction, DNA from the supernatant was precipitated with ethanol and resuspended in 100 μl of TE buffer [10 mM Tris (pH 8.0) and 1 mM EDTA]. Equal amounts of DNA samples (20 μg) were electrophoresed on 1.2% agarose gel and visualized by ethidium bromide staining.

Assay of telomerase: Telomerase activity was assayed by means of hybridization protection assay coupled with a telomeric repeat amplification protocol (TRAP/HPA). Briefly, extracts from KB cells untreated or treated with JTE-522 for 48 and 72 h at the dose of 10^{-5} or 10^{-6} M were prepared according to the protocol of Kim *et al.*[26] by using the detergent 3-[3-cholamidopropyl-dimethylammonio]-1-propanesulfonate. The supernatants were divided into aliquots and quickly frozen at −80°C. Protein concentrations of the tissue supernatants were determined by Coomassie brilliant blue assay. Six micrograms of protein was used for the TRAP assay. The reaction mixture was subjected to polymerase chain reaction (31 cycles at 94°C for 40 s, 50°C for 40 s and 72°C for 60 s). A 5 μl sample of telomerase reaction product was heat-denatured for 5 min at 95°C. Then, 100 μl of acridinium-ester-labeled probe (CCCTAACCCTAACCCTAACTCTGCTCGAC), 3×10^7 relative light units (rlu), in hybridization buffer (0.1 M lithium succinate buffer, pH 4.7, containing 20% dodecyl sulfate, 1.2 M lithium chloride, 20 mM EDTA and 20 mM EGTA) was added to each reaction tube and incuba-

tion was continued for 20 min at 60°C. A 300 μl aliquot of selection buffer (0.6 M sodium tetraborate buffer, pH 8.5, containing 5% Triton X-100) was added to differentially hydrolyze unhybridized probe during incubation for 10 min at 60°C. After the differential hydrolysis, 50 μl of 0.05% phenol red (Gibco BRL, Grand Island, NY) was mixed to quench the chemiluminescence. Chemiluminescence (rlu) was measured (2 s/tube) by a luminometer. To express telomerase activity in tissue, we defined the activity equivalent to that in one KB cell as 1 unit. Telomerase activity of more than 5 units was regarded as positive.

Cytotoxicity assay: Peripheral blood mononuclear cells (PBMC) of healthy volunteers were isolated from heparinized peripheral blood by Ficoll-Hypaque gradient (Becton Dickinson VACUTAINER Systems, Franklin Lakes, NJ) centrifugation. PBMC were cultured in RPMI-1640 supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 10% heat-inactivated human AB serum, and recombinant human interleukin-2 (IL-2, 100 IU/ml; Takeda Pharmaceutical Co., Ltd., Osaka) for 14 days and lymphokine activated killer (LAK) cells were induced. These LAK cells were used as effector cells for the cytotoxicity assay. The cytotoxic activity of effector cells was tested *in vitro* in a 4 h ^{51}Cr release assay using K-562 cells and Daudi cells as targets for natural killer (NK) cell activity and LAK cell activity, respectively. As a control effector, PBMC (2×10^7) were cultured in medium for 72 h (Fig. 2, control). To evaluate the changes of cytotoxicity in the presence of KB cells and/or JTE-522, PBMC were co-cultured with them under various conditions. PBMC were cultured with 10^{-6} M JTE-522 (Fig. 2a), in 10^{-6} M JTE-522 with KB cells (2×10^6) directly in contact with PBMC (Fig. 2b), in normal medium with KB cells separated with Biocoat Cell Culture Inserts (Becton Dickinson Labware; Fig. 2c) and in 10^{-6} M JTE-522 with separated KB cells (Fig. 2d). A total of 1×10^7 effector cells from each dish, in a volume of 1 ml was labeled with 100 μCi of [^{51}Cr]Na 204 for 1 h at 37°C and washed 3 times. Then, 1×10^4 target cells (100 μl: 1×10^5/ml) and 2×10^5 effector cells (100 μl: 2×10^6/ml) were placed (effector-to-target ratio was fixed at 20:1) in 96-well round-bottomed microtiter plates. Supernatants of microtiter plates were harvested after 4 h incuation at 37°C and counted for radioactivity using a γ counter. The percentage cytotoxicity was calculated as follows (all ^{51}Cr values in cpm).

$$\frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})} \times 100$$

Target cells incubated in medium alone and with 1 M HCl were used to determine the spontaneous release and the maximum release of chromium, respectively.

In vivo
Tumor growth: KB cells (5×10^6) were injected s.c. into the left flank of mice. The local s.c. tumor was measured once
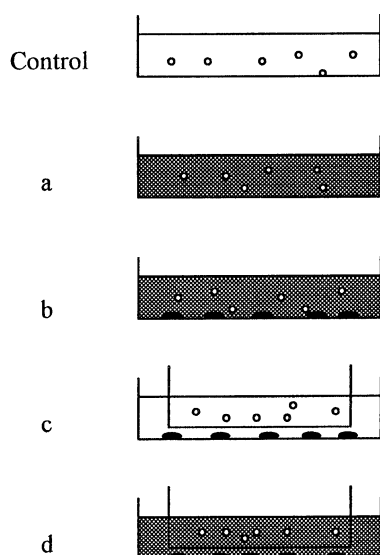
Fig. 2. LAK cells, from PBMC of healthy volunteers, were used as effector cells for the cytotoxicity assay. The cytotoxic activity of effector cells was tested *in vitro* in a 4 h ^{51}Cr release assay using K-562 cells and Daudi cells as targets for NK cell activity and LAK cell activity, respectively. As a control, PBMC (2×10^7) were cultured in medium only for 72 h (control). To evaluate the changes of cytotoxicity in the presence of KB cells and/or JTE-522, PBMC were co-cultured with them in various situations. PBMC were cultured in 10^{-6} *M* JTE-522 (a), in 10^{-6} *M* JTE-522 and KB cells (2×10^6) in direct contact (b), in normal medium with KB cells separated with Biocoat Cell Culture Inserts (c) and in 10^{-6} *M* JTE-522 with separated KB cells (d). A total of 1×10^7 effector cells from each dish was used for the assay. ○, PBMC; ●, KB cell; □, medium only; ■, 10^{-6} *M* JTE-522 in the medium.

a week in two perpendicular dimensions with a caliper. Tumor size in mm^3 was calculated by using the formula $(a\times b^2)/2$, where a is the larger and b is the smaller dimension of the tumor. Two weeks after s.c. inoculation, the tumor had grown to 600 mm^3 in volume. At this point, the mice were randomly divided into three groups of 5 animals, which received 0, 10 or 30 mg/kg of JTE-522, five times per week orally for 4 weeks. To assess whether NK cells cooperate with JTE-522 and show an antitumor effect *in vivo* or not, some mice were treated with 30 mg/kg of JTE-522 and rabbit anti-asialo GM1 (Wako Chemical, Osaka). Cells were injected as described above. Treatment with JTE-522 was started from the 3rd week for 4 weeks and anti-asialo GM1 was injected (50 μl each) into the tail vein at the beginning of the 1st and 3rd treatment weeks. To compare the effects, untreated control mice and JTE-522 (30 mg/kg) alone-treated mice were used. At the termination of treatment, each mouse was killed and the tumor weight was determined.

Immunohistochemistry: Paraffin-embedded tumor sections were deparaffinized in xylene and rinsed in absolute ethanol. After treatment with 0.3% hydrogen peroxide, the sections were incubated in 1% BSA for 20 min and then with an anti-human COX-2 goat monoclonal antibody (Santa Cruz Biotechnology, Inc.), an anti-human factor VIII rabbit monoclonal antibody (Nichirei) and an anti-human Ki 67 rabbit monoclonal antibody (Immunoteck, Marseille, France). The sections were incubated with bitotinylated rabbit antigoat or goat antirabbit immunoglobulin, followed by incubation with peroxidase-conjugated streptavidin (ABC kit; Nichirei). Signals were visualized by treatment of the section with diaminobenzidine and sections were examined under a microscope after counterstaining with hematoxylin.

Apoptosis: Tumors were minced in cold Tris-buffered saline and homogenized. The homogenates were filtered and washed with cold Tris-buffered saline, followed by resuspension in 500 μl of lysis buffer containing 500 m*M* Tris-HCl (pH 9.0), 2 m*M* EDTA, 10 m*M* NaCl, 1% sodium dodecyl sulfate (SDS) and 1 mg/ml proteinase K (Wako Chemical). After incubation at 50°C for 20 h, samples were extracted twice with phenol, once with phenol-chloroform and finally with chloroform, and aliquots (1 μg) were loaded on 1.5% agarose gel and visualized by ethidium bromide staining. Then terminal deoxynucleotidyl transferase-mediated cUDP nick end labeling (TUNEL) was performed using a commercial kit, Apop Taq Plus (Oncor, Gaithersburg, MD) for deparaffinized paraffin-embedded tumor sections. Counting of immunoreactive cells was based on the distribution of apoptotic tumor cells in three different fields within the same section; the apoptotic index was expressed as the percentage of TUNEL-positive cells with respect to the total number of the cells.

Assay of telomerase: Telomerase activity was also assayed by means of hybridization protection assay coupled with a telomeric repeat amplification protocol, but the extracts were obtained from tissues of the mice. Other steps of the *in vitro* assay were the same as described above.

Statistical analysis: One-way ANOVA on Sigma Stat for Windows version 1.0 was used to compare different groups at the 95% confidence level. Student's t test was also used to obtain P and to compare different treatment groups. A P of <0.05 was considered statistically significant.

RESULTS

***In vitro* effects** The effects of JTE-522 on cell proliferation are shown in Fig. 3A. Cell proliferation was suppressed dose-dependently at each treatment time. Cell viability was assessed by trypan blue exclusion, and in each case 97.1–98.7% of cells were viable at the times
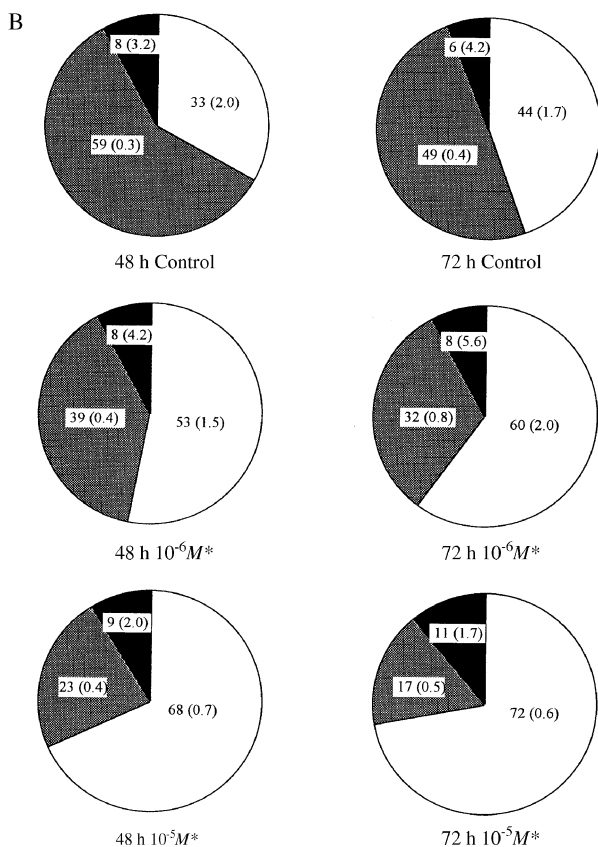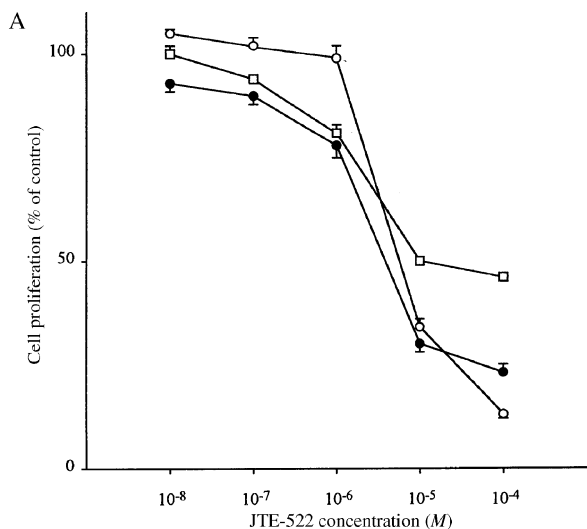
A

B

48 h Control

72 h Control

48 h $10^{-6}M*$

72 h $10^{-6}M*$

48 h $10^{-5}M*$

72 h $10^{-5}M*$

Fig. 4. Telomerase activity tended to be suppressed when KB cells were treated with JTE-522 at 10^{-5} M at each time, although it was unaffected at 10^{-6} M JTE-522.

Fig. 3. The effects of JTE-522 on cell proliferation, determined by using the MTT method, are shown in A. At every treatment time, 48 (□), 72 (●), and 96 h (○), cell proliferation was decreased dose-dependently. Cell cycle analysis with flow cytometry indicated that JTE-522 caused significant arrest in the G1 phase of the cell cycle, dose- and time-dependently (B). *, $P<0.05$; bars, (   ), SD; □, G1; ▨, S; ■, G2+M.
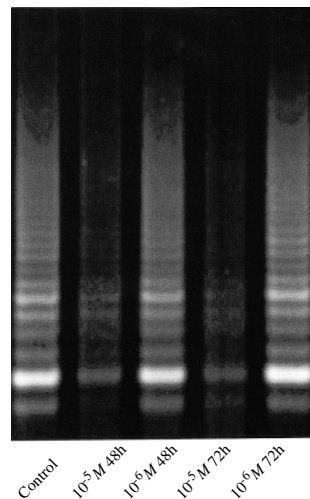
and concentrations described above. Cell cycle analysis with flow cytometry indicated that JTE-522 caused significant arrest in the G1 phase of the cell cycle, both dose- and time-dependently (Fig. 3B). DNA fragmentation was not detected in JTE-522 treated KB cells (data not shown). Telomerase activity was found to be suppressed when KB cells were treated with JTE-522 at 10^{-5} M in each case, although JTE-522 was ineffective at a concentration of 10^{-6} M JTE-522 (Fig. 4). The changes of NK and LAK activity are summarized in Table I. Both NK and LAK activity were significantly suppressed when PBMC were co-cultured with KB cells, regardless of whether they were in direct contact or not, or whether they were treated in JTE-522 mixed medium or in normal medium (Table I, b, c and d). However, when PBMC were cultured in JTE-522-added medium, the activities were not suppressed (Table Ia). JTE-522 did not have any immunological effect *in vitro*. In summary, JTE-522 did not lead to direct cell death and immunological enhancement, but induced cell cycle arrest and suppression of telomerase activity.

COX-2 expression Immunohistochemical staining with anti-human COX-2 antibody was performed. Positive cells were observed whether or not the cells had been treated with JTE-522. Positive cell number rate remained constant at the concentrations and treatment times described above. RT-PCR analysis of KB cells which were either untreated or treated with JTE-522 gave COX-2-positive bands in every case following electrophoresis (Fig. 5).

Angiogenesis The concentrations of angiogenic factors in supernatant of cultured KB cells, determined by using ELISA, are shown in Fig. 6. Basic FGF was not detected

Table I. Changes of NK and LAK Activity of PBMC Cocul-
tured with KB Cells and/or JTE-522

	NK activity (%)	LAK activity (%)
Control	66.5 (1.5)	47.0 (3.0)
a	68.5 (1.5)[*]	49.5 (3.0)[*]
b	2.5 (1.5)[**]	2.5 (1.5)[**]
c	2.5 (0.5)[**]	4.5 (0.5)[**]
d	7.0 (1.0)[**]	3.0 (1.0)[**]

*, NS; **, $P<0.05$; ( ), SD.
Control: 2×10^7 PBMC, medium only.
a: JTE-522.
b: JTE-522 plus KB cells in direct contact.
c: normal medium plus separated KB cells.
d: JTE-522 plus separated KB cells.
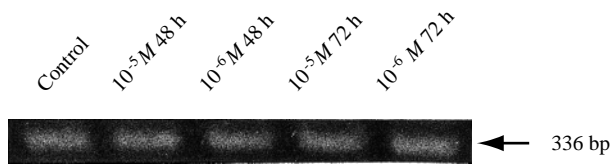For details, see Fig 2.

Fig. 5. Expression of COX-2 in KB cells was examined. RT-
PCR analysis of KB cells which were untreated or treated with
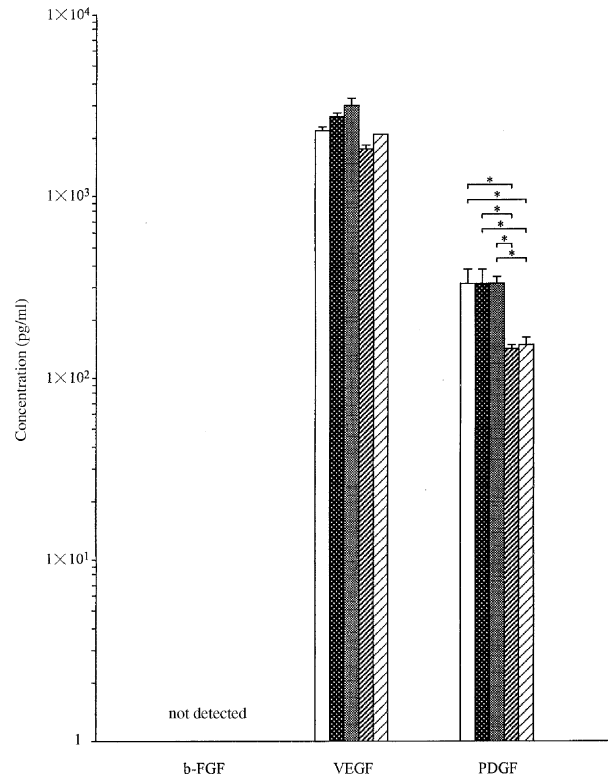JTE-522 showed a COX-2 band (336 bp) in every group.

Fig. 6. The concentrations of angiogenic factors in supernatant
of KB cells, detected with ELISA. Basic FGF was not detected at
any concentration or treatment time, and VEGF concentration
was unaffected by treatment. However, PDGF concentration was
significantly decreased when KB cells were treated for 72 h at
each concentration of JTE-522. No mark, NS; *, $P<0.05$; bars,
SD; □, Control; ■, 10^{-5} M, 48 h; ■, 10^{-6} M, 48 h; ▨, 10^{-5} M,
72 h; ▨, 10^{-6} M, 72 h.

at any concentration or treatment time, and VEGF concen-
tration was unchanged regardless of treatment. PDGF con-
centration was significantly decreased when KB cells were
treated for 72 h at each concentration of JTE-522.

***In vivo* tumor growth** Female nude mice were implanted
with KB cell xenografts on day 0, and JTE-522 was
administered orally from day 14 (5 times/week) at 10 and
30 mg/kg for 4 weeks. Treatment with JTE-522 had no
significant effect on the body weight of the animals (data
not shown). The slight decrease observed for the treated
animals was a result of the decreased tumor weight caused
by the antitumor effect of the drug. The results of the *in
vivo* efficacy trials of JTE-522 in KB cells xenografts
growing in nude mice are shown in Fig. 7A. The tumor
weight was examined at the termination of treatment (Fig.
7B). The results showed that JTE-522 treatment resulted
in significant inhibition of the tumor growth ($P<0.01$).

The tumor size was not different between mice simulta-
neously treated with JTE-522 and anti-asialo GM1 and
mice treated with JTE-522 alone, though it should be
emphasized that the tumor size in both these groups was
significantly smaller than in the untreated control mice.

Angiogenesis To evaluate the anti-angiogenic effect with
JTE-522 *in vivo*, the tumor was immunohistochemically

stained with anti-human factor VIII. The number of
microvessels peripheral to the site of the tumor, positive
for anti-human factor VIII, was significantly less when
mice were given 30 mg/kg of JTE-522 compared to those
given 10 mg/kg, and significantly less than in the control
mice (Fig. 8A). Macroscopic evaluation of tumor angio-
genesis indicated fewer tumor surface vessels in the 30
mg/kg treatment group than in the 10 mg/kg group, and
both were less than in the untreated controls.

Cell cycle analysis To assess the effect on the tumor cell
cycle *in vivo*, immunohistochemical staining with anti-
human Ki 67 (MIB-1) antibody was carried out. Ki 67-
positive cells were observed in each group and the rate of
positive cells showed a slight dose-dependence (Fig. 8B).
It was concluded that JTE-522 increased the proportion of
cells in the G1 phase. Since KB cells are negative for
cyclin D1,[28] immunohistochemical staining with anti-
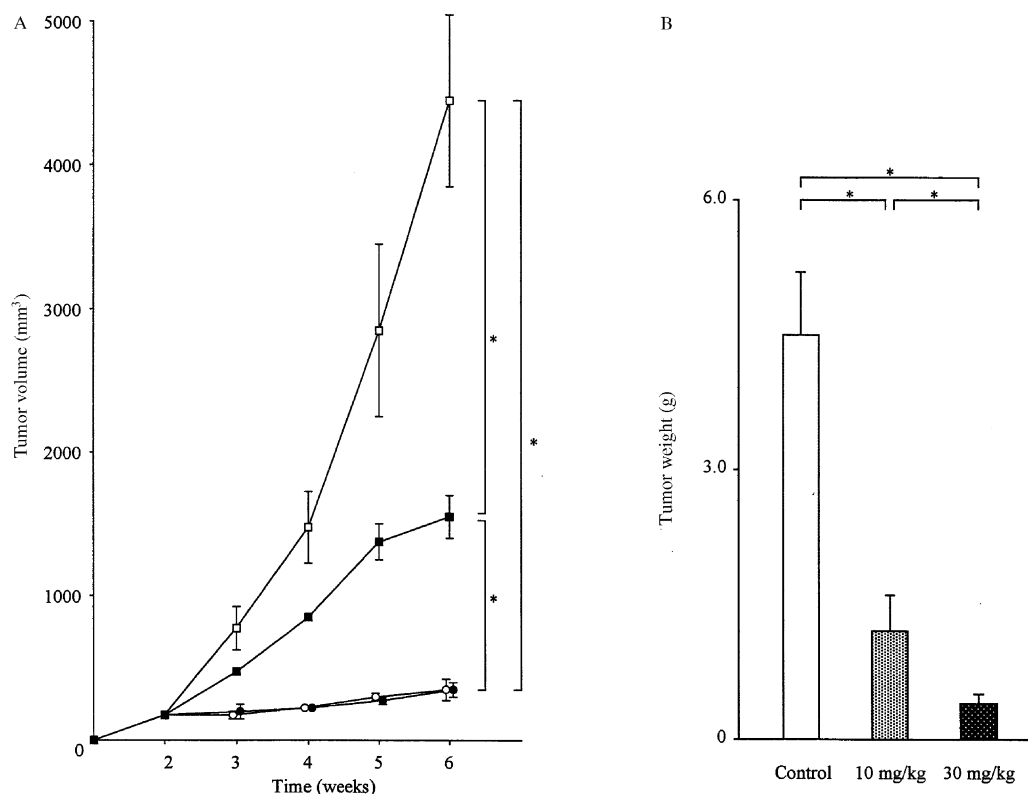cyclin D1 was not carried out.

Fig. 7. Anti-tumor effect on tumor xenografts in nude mice. Female nude mice were implanted with KB cell xenografts on day 0 and JTE-522 was administered p.o. from day 14 (5 times/week) at doses of 0 mg/kg (□), 10 mg/kg (■), or 30 mg/kg (○), or at 30 mg/kg with anti-asialo GM1 (●) for 4 weeks. The results of the *in vivo* efficacy trials of JTE-522 in KB cell xenografts growing in nude mice are shown in A. The tumor weight was examined at the termination of treatment (B). JTE-522 treatment resulted in significant suppression of tumor growth. *, $P<0.01$; bars, SD.

Apoptosis We examined whether the treatment of tumor-bearing mice with JTE-522 was characterized by the induction of apoptotic cell death *in vivo*. JTE-522 treatment resulted in an increased number of TUNEL-positive cells, and a quantitative analysis is summarized in Fig. 9A. As reported, the apoptotic index was significantly higher in tumors from mice treated at a dose of 30 mg/kg with JTE-522 compared to the 10 mg/kg group or untreated control mice. The results of DNA fragmentation analysis are shown in Fig. 9B. DNA was degraded to oligonucleosomal DNA fragments, showing apoptosis-specific laddering on agarose gel electrophoresis, when tumor-bearing mice were treated with 30 mg/kg of JTE-522. Such laddering was not observed with 10 mg/kg treated mice or in untreated control mice.

Telomerase activity We investigated whether the treatment of tumor-bearing mice with JTE-522 was characterized by the inhibition of telomerase activity. As shown in Fig. 10, the telomerase activity of the tumor in the 30 mg/kg group was inhibited, compared with the 10 mg/kg group or untreated mice.

DISCUSSION

COX inhibitors have been used as anti-inflammatory, analgesic, and antipyretic agents for many years, but it was discovered only recently that they could be used to prevent the development of colon cancers. These inhibitors have been reported to lower the incidence of colon tumors induced by chemical carcinogenesis in rat rodent models.[29–32] Moreover, the incidence of cancer appears to be decreased in users of COX inhibitors, such as aspirin, compared with that in control subjects not using these agents.[33, 34] Sulindac, an NSAID, also inhibited the growth of intestinal polyps in patients with familial adenomatous polyposis.[35] These reports suggest the potential usefulness of nonspecific inhibitors of COX in chemoprevention of colon cancers. However, nonspecific inhibitors of COX often cause adverse effects, such as gastrointestinal hemorrhage and ulceration. In contrast, COX-2-specific inhibitors are expected to be nonulcerogenic and less toxic to the subjects. A role of COX-2 in cancer was reported by Tsujii and DuBois,[15] and since then, dramatic progress has
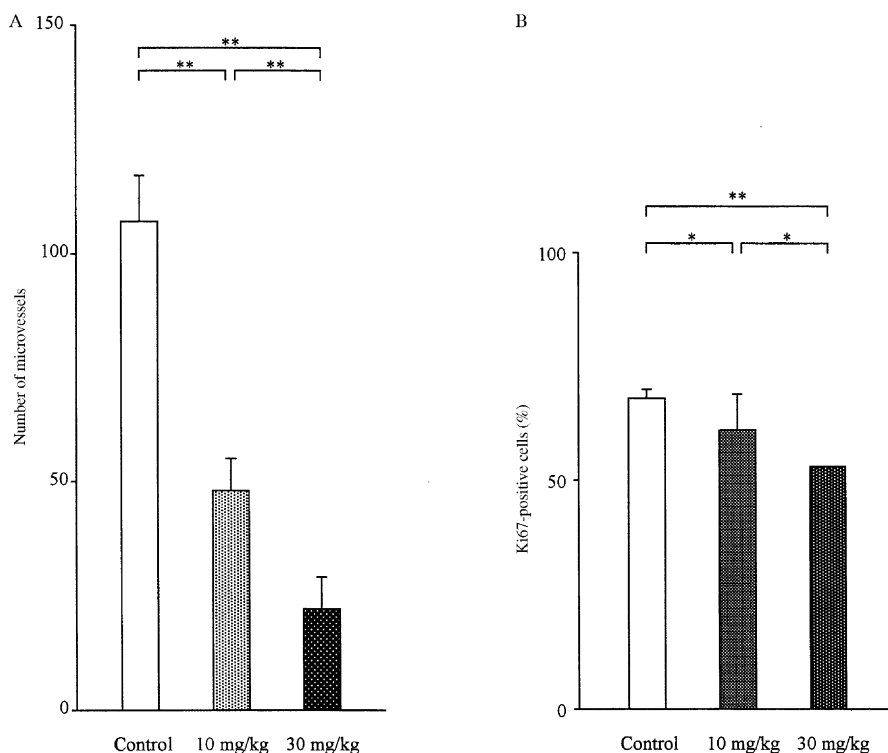
Fig. 8. The number of microvessels in the peripheral part of the tumor, positive for anti-human factor VIII, was significantly reduced in a dose-dependent manner (A). To assess the effect on the tumor cell cycle *in vivo*, immunohistochemical staining with anti-human Ki 67 (MIB-1) antibody was done. Ki 67-positive cells were observed in every group and the rate of positive cells was significantly reduced in a dose-dependent manner (B). *, NS; **, $P<0.05$; bars, SD.
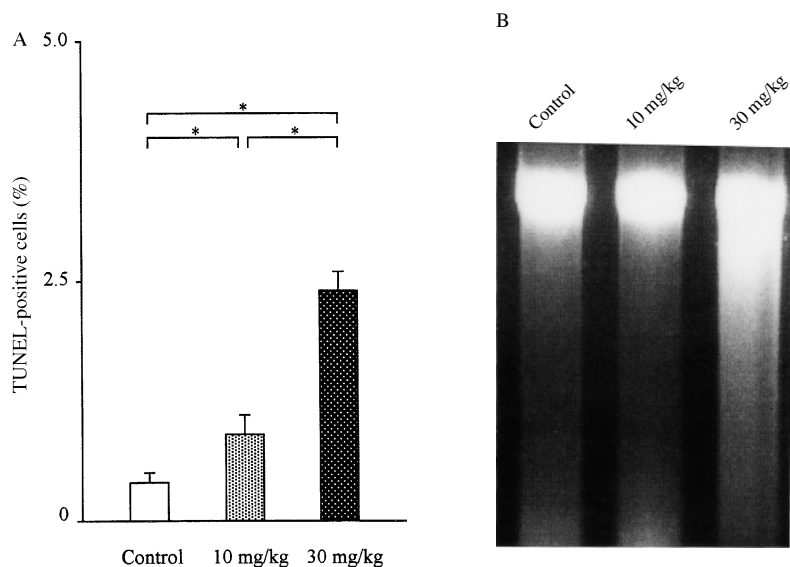

Fig. 9. JTE-522 treatment resulted in an increased number of TUNEL-positive cells and the quantitative analysis is summarized in A. The apoptotic index was significantly higher in tumors from mice that had been treated with JTE-522 at a dose of 30 mg/kg as compared with the 10 mg/kg group or untreated controls. The results of DNA fragmentation analysis are shown in B. Apoptosis-specific laddering was visualized when tumor-bearing mice were treated with 30 mg/kg of JTE-522, whereas such laddering was not observed with 10 mg/kg-treated mice or untreated control mice. *, $P<0.05$; bars, SD.
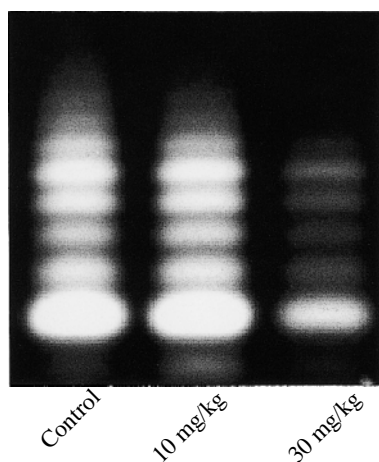
Fig. 10. The telomerase activity of the tumor treated with 30 mg/kg was inhibited compared with that in 10 mg/kg-treated mice or untreated mice.

been made. Several selective COX-2 inhibitors have been tested for their possible utility as chemopreventive agents in colon cancer.[36, 37] However, less is known about the role of COX-2 in other types of human cancer, e.g., gastric and prostatic cancers.[38]

In this study, we investigated whether JTE-522, a selective COX-2 inhibitor, shows antitumor effects on KB cells (a human head and neck squamous carcinoma cell line). We showed that JTE-522 suppressed the growth of KB tumors through mechanisms of cell cycle arrest, anti-angiogenetic effect, suppression of telomerase activity and induction of apoptosis, and the effect did not involve immunological enhancement. Hughes-Fulford *et al.*[38] reported that PG-E2 acted as an autocrine growth factor in the growth of osteoblast cells, and Tjandrawinata *et al.*[39] reported the PG-E2 stimulation of human prostatic carcinoma cell growth. Inhibition of PG-E2 synthesis by COX-2 inhibitors was thought to be one of the chemopreventive mechanisms. However, we used PG-E2 non-producing cells, and their growth was also suppressed with a COX-2 inhibitor. Thus, there seem to be some pathways of chemopreventive action of COX-2 inhibitor, which

do not involve a PG-E2-inhibiting effect. Chinery *et al.*[41] reported that PG-J2 induced proliferation of colorectal cancer cells. We added exogenous PG-E2 and PG-J2 to KB cells, but the proliferative rate was not changed (data not shown). Our present results are consistent with those previously reported,[42] i.e., NSAIDs might exert their anti-neoplastic effect via a PG-independent pathway and the COX-2 enzyme itself might promote cancer development and progression. Zimmermann *et al.*[42] reported that, in esophageal cancer cells producing a large amount of PG-E2, COX-2 inhibitors induced apoptotic cell death and reduced proliferative activity through the inhibition of PG synthesis. In the present study, the COX-2 inhibitor did not induce apoptotic cell death in KB cells not producing PG-E2, but it did inhibit cell proliferation *in vitro*. These results may indicate that there is a correlation between inhibition of PG synthesis and induction of apoptosis by the COX-2 inhibitor, whereas the inhibitor may suppress cell proliferation independently. This study is the first report describing the suppression of telomerase activity by a COX-2 inhibitor. In a study with *cis*-diammine dichloro-platinum or AGM-1470, an angiogenesis inhibitor, Kido *et al.*[43] reported that a decline of telomerase activity of osteosarcoma cells was involved in tumor growth retardation induced by these agents.

We conclude that JTE-522 directly affects KB cells, i.e., it causes significant arrest in the G1 phase of the cell cycle, which induces suppression of tumor proliferation, and inhibition of PDGF causes an anti-angiogenic effect and suppression of telomerase activity. As a result of these various antitumor effects with COX-2 inhibitor, tumor growth seems to be significantly suppressed. Further, the decrease of blood supply, resulting from the anti-angiogenic effect, might also lead to tumor cell apoptosis *in vivo*. These results provide the first evidence that a selective COX-2 inhibitor might be a promising candidate chemopreventive agent against advanced head and neck squamous cell carcinomas, which generally have a poor outcome.

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