Original Papers

Etodolac inhibits interleukin-6 production and improves survival combined with chemotherapy in a colon 26 cachexia model

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Abstract

It has been suggested that inflammatory components, including interleukin (IL)-6 and transforming growth factor (TGF)-β, play an important role in the formation of cancer cachexia. Cyclooxygenase (COX)-2 has been reported to suppress tumor progression and to inhibit production of these inflammatory mediators, and, thus, may not only be effective in preventing cachexia, but also beneficial for survival. Etodolac, one of the selective COX-2 inhibitors, was administered to mice bearing cachexigenic clones of colon 26, together with either one of two doses (5 and 10 mg/kg/day) of 5-fluorouracil. Serum concentrations of IL-6 were measured in each treatment group. In the groups treated with 5-fluorouracil alone, survival and tumor growth were significantly improved with 10 mg/kg/day. In the group with etodolac alone, survival improved, however, tumor growth was enhanced. Combination of 5-fluorouracil (5 mg/kg/day) and etodolac improved survival, and the enhancement of tumor growth seen in the etodolac only group, disappeared. By combining 5-fluorouracil (10 mg/kg/day) with etodolac, positive effects on survival and tumor growth were obtained. Serum concentrations of IL-6 were elevated in tumor-bearing mice and were significantly suppressed by treatment with etodolac. In the group with combined treatment, the suppressive effect on IL-6 was synergistic. Our findings suggest that the potential use of COX-2 inhibitors in combination with chemotherapy might be therapeutically advantageous especially in patients with terminal stages of cancer.

(Received April 20, 2010; Accepted July 4, 2010)

Introduction

A large fraction of cancer patients show weight loss at the time of diagnosis, and nearly all of the patients who die from cancer exhibit wasting. Cachexia, progressive weight loss in neoplastic diseases, is characterized by the loss of muscle and adipose tissue, anemia, anorexia, and asthenia. Moreover, patients with cachexia have a reduced response to chemotherapy and a poorer prognosis. Although malnutrition is generally accepted as being prevalent in patients with cancer, a great deal of controversy surrounds the guidelines for nutritional support. Several studies suggest essential roles of interleukin (IL)-6, IL-1β, and tumor necrosis factor (TNF)-α in the development of cachexia. Some other factors, such as leukemia inhibitory factor (LIF), interferon-γ, and parathyroid hormone-related protein (PTHrP), cause cachectic symptoms in animal models.

Cyclooxygenase (COX) is the rate-limiting step in the conversion of arachidonic acid into prostaglandin endoperoxide. Two forms of COX have been cloned from various animal cells: COX-1, which is constitutively expressed, and COX-2, which is inducible with stimulants. The expression of COX-2 is increased in cancer tissues of the colon, pancreas, head and neck, breast, and lung. Overexpression of COX-2 inhibits apoptosis and increases invasiveness of tumor cells.

Epidemiological studies have shown that chronic intake of nonsteroidal anti-inflammatory drugs (NSAIDs) reduces the incidence of colon and breast cancer, and selective inhibition of COX-2 has been suggested for chemopreventive therapy. This approach would also avoid the gastrointestinal toxicity associated with currently available NSAIDs because inhibition of COX-1 may induce injury of the gastrointestinal mucosa. Thus, several mechanisms by which COX-2 contributes to the progression of cancer have been reported, including stimulation of proliferation and inhibition of apoptosis in cancer cells, stimulation of cancer invasion and angiogenesis, and suppression of immune responses. In addition to an involvement in tumor growth, prostanoids may also play an important role in muscle protein degradation associated with cancer cachexia symptoms. Etodolac, 10 times more selective for inhibition COX-2 than COX-1, is one of the major, clinically available COX-2 inhibitors.

In this study, the effects of etodolac on the survival and growth inhibition of cachexigenic colon 26 cells have been evaluated in a cachexia murine model. The
effects of 5-fluorouracil alone and in combination with etodolac, have also been investigated.

**Materials and Methods**

**Mice**
Pathogen-free male BALB/c mice (4 to 5 weeks of age) were obtained from Japan Charles River Laboratories (Yokohama, Japan). Mice weighing between 20 and 25 g were used for the experiments. All experiments were performed at Tsukuba Animal Research Laboratory. Mice were treated in accordance with the policies of the Bioethics Committee of Tsukuba Animal Laboratory.

**Cells and Inoculation of Tumors**
Cachexigenic clones of colon 26 cells, a gift from Dr. Yutaka Tanaka of Chugai Pharmaceutical Co. Ltd, were maintained in RPMI1640 (Gibco, Frederick, MD, USA) with 10% FCS (Difco, Detroit, MI, USA). Subconfluent cells were treated with trypsin and resuspended at the concentration of $5 \times 10^6$ cells in PBS. The cell suspension (200 μl) was used to subcutaneously inoculate the back of each mouse. Tumor diameter was measured once daily. When the diameter of the tumor nodule reached 10 mm, treatment with 5-fluorouracil and etodolac commenced.

**Treatment protocol**
5-fluorouracil and etodolac were both obtained from Sigma-Aldrich (St. Louis, MO, USA). Etodolac (0.8 mg/day) was administered orally by gavage once daily until death. 5-fluorouracil was injected intravenously with one of two doses (5 and 10 mg/kg/day) once daily for 15 days. Control animals received vehicle alone. Estimated tumor volume was calculated from the formula:

$$EVT = \frac{\text{Length} \times \text{L (width)}^2}{2}$$

**Determination of IL-6 levels**
Blood IL-6 levels were determined using a mouse ELISA kit (Pierce, Rockford, IL, USA).

**Statistical analysis**
Statistical analyses were carried out using the SAS package (SAS Institute, Cary, NC, USA). Results were compared using Student’s t-test and expressed as the mean values ± SE. Survival curves in the series were depicted using the Kaplan-Meier method. Differences were considered statistically significant at p < 0.05. All statistical tests were two-sided.

**Results**
Colon 26 cells successfully grew after inoculation and all mice of the control group died within 21 days of treatment. In the groups treated with 5 or 10 mg/kg/day of 5-fluorouracil alone, no differences in survival or tumor growth were obtained with 5 mg/kg/day, yet survival and tumor growth were significantly improved with 10 mg/kg/day (p < 0.005 and 0.01, respectively, Fig. 1). In the group with etodolac alone, survival improved (p < 0.05); however, tumor growth was enhanced more than that in the control group (p < 0.01, Fig. 2). In the groups with a combination of 5-fluorouracil (5 mg/kg/day) and etodolac, survival improved (p < 0.05), and the enhancement of tumor growth seen in the group with etodolac alone disappeared (Fig. 2). Combining 5-fluorouracil (10 mg/kg/day) and etodolac, significant effects on survival and tumor growth were achieved when compared to the control group; however, there were no differences with...
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The concentration of IL-6 in the control group was extremely high, whereas the levels in normal mice were low, at around the detection level. The levels in the group receiving etodolac alone were significantly lower than those in the control group. Although the effects of 5-fluorouracil were not significant, when in combination with etodolac, the lowest levels of IL-6 were achieved and the effect was synergistic (Fig. 4).

Fig. 3 Effects of 5-fluorouracil (10 mg/kg/day) alone (Fig. 3). The improvement in survival was more clearly seen in the later period of the course.

Cancer cachexia is characterized by severe weight loss that occurs even at the earliest stages of malignant disease, reducing the quality of life of the patients as well as the responsiveness to chemotherapy. Thus, clarification of the cellular and molecular biological mechanisms of cancer cachexia is necessary to develop improved cancer therapies for patients with cachexia. Various investigations suggest that several cytokines are involved in the

Discussion

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Fig. 2 Effects of 5-fluorouracil (5 mg/kg/day) combined with etodolac, (a) Growth curve of SC tumors, (b) Survival curves of mice

* : p < 0.01, ** : p < 0.05

Fig. 3 Effects of 5-fluorouracil (10 mg/kg/day) combined with etodolac, (a) Growth curve of SC tumors, (b) Survival curves of mice

* : p < 0.01, ** : p < 0.0005, *** : p < 0.001

ETV: estimated tumor volume
development of cancer cachexia. Among them, IL-6 has been reported to be a key mediator of cachexia. In the present study, cachexigenic colon 26, which reportedly produces IL-6, was utilized in a cachexia murine model. NSAIDs and COX-2 inhibitors have been widely studied in colorectal cancer prevention, in animal models, and in the treatment of human patients. The bulk of information suggests that COX-2 inhibitors are beneficial in cancer treatment.

Most research has focused on specific COX-2 inhibition as it has been shown to be the main isoform upregulated in a large number of cancers other than colorectal tumors. Our rationale for using COX-2 inhibitors was to control the production of cachexigenic cytokines, such as IL-6. This would result in effective therapy against tumor-associated syndromes that correlate with inflammatory components that present during tumor progression, and also to prolong survival in cachetic cancer patients.

Previous studies have shown that several selective COX-2 inhibitors, including etodolac, inhibit tumor progression in various tumor models. The mechanisms underlying this are suggested to involve motility of tumor cells or release of MMPs (matrix metalloproteinases), inhibition of tumor angiogenesis and improvement of immune responses in cancer-bearing hosts by modulating immune suppression that is, at least partly, driven by inflammatory components. In the present study, etodolac was used to treat transplanted colon 26 cells in combination with 5-fluorouracil. A paradoxical result was observed where tumor growth was enhanced and the survival was prolonged when using etodolac alone. Currently, our data are unable to clarify the mechanism behind this phenomenon. However, the enhancement of tumor growth by etodolac disappeared with combined 5-fluorouracil even at the lower dose (5 mg/kg/day) at which there were no effects on tumor growth or prognosis. Etodolac in combination with 5-fluorouracil at the higher dose improved the survival especially in the late period of the course. Colon 26 cells, reported to produce IL-6, a cachexigenic cytokine, were used in this study and their production of IL-6 was successfully suppressed with etodolac. This suppressing effect was synergistically strengthened by the combined use of 5-fluorouracil. Collectively, these results suggest that etodolac is effective for overall survival and, when combined with chemotherapy even at a low dose, is effective at inhibiting tumor progression by controlling the production of IL-6. Although numerous features of cachexia, such as reduced body weight, malnutrition, anorexia, and anemia, were not evaluated in this study, the results that were obtained, which are believed to be based on the regulation of IL-6 production, seem to be the outcome of inhibiting cachexia induction. Treatments that focus on alleviating the symptoms of cachexia, such as general wasting and malaise, are sometimes much more important than shrinking the tumor, especially in patients with terminal stages of cancer. Therefore, COX-2 inhibitors, such as etodolac, might be desirable for such patients. We believe that our findings may provide a potential use of COX-2 inhibitors in combination with low-dose chemotherapy in cancer treatment. Future clinical studies are therefore required to evaluate this therapeutic model.

References
Endocet inhibits interleukin-6 production and improves survival combined with chemotherapy in a colon 26 cachexia model.