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Peroral TAS-202 reduced vessel density in rats with adjuvant-induced arthritis

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Abstract

The present study was designed to investigate blood vessel density interpreted as an indirect measurement of angiogenesis following 4-(3,4,5-trimethoxyphenyl)-6-(2,4,5-trimethoxyphenyl)-2-diethylamino-pyrimidine (TAS-202) treatment in a rat model of arthritis. Male Lewis rats were inoculated intradermally with *Mycobacterium butyricum* into the hind paw and the arthritic responses were evaluated by measuring the changes in paw volume. Both peroral TAS-202 (10 or 30 mg/kg/day) and indomethacin (1 mg/kg/day) inhibited the autoimmune phase of the arthritic response. However, while the increase in blood vessel density in the synovial tissue was significantly inhibited by TAS-202 (10 and 30 mg/kg/day), indomethacin did not exert this effect (1 mg/kg/day). These results, together with the observation that TAS-202 in combination with indomethacin or prednisolone maintained its ability to exert an antiangiogenic effect, indicate that TAS-202 may offer promise as an oral pro-drug for the treatment of rheumatoid arthritis, through its inhibitory effect on angiogenesis at the inflammation site.

Keywords: TAS-202; Angiogenesis inhibitor; Adjuvant-induced arthritis

1. Introduction

Rheumatoid arthritis is a complex chronic inflammatory and autoimmune disease. Recently, it has also been claimed that significant new blood vessel formation in the synovial tissues of patients with rheumatoid arthritis may contribute to the hyperplasia and proliferation of the synovial membrane observed in these patients. New blood vessels would transport inflammatory cells and supply nutrients and oxygen to the site. Moreover, activated vascular endothelial cells have the potential to produce proinflammatory cytokines, chemokines and small molecules that are known to accelerate and prolong the process of inflammation (Folkman, 1995; Koch, 1998, 2000; Stupack et al., 1999; Brenchley, 2000; Firestein, 1999).

These findings have motivated the use of new approaches to the management of patients with antiangiogenic agents. For instance, *O*-(chloroacetyl-carbamoyl)-fumagillol, TNP-470 (also known as AGM-1470 derived from the naturally occurring fungal product fumagillin), inhibits the proliferation of vascular endothelial cells (Ingber et al., 1990; Kusaka et al., 1994). When administered subcutaneously, it was shown to inhibit collagen- or adjuvant-induced arthritis in rats (Peacock et al., 1992, 1995). We have also reported that magnosalin, a natural compound derived from ‘‘Shin-I’’ (*Flos Magnoliae*), inhibits angiogenesis in adjuvant-induced inflammation models of mice (Kimura et al., 1990). More recently, we have reported that 4-(3,4,5-trimethoxyphenyl)-6-(2,4,5-

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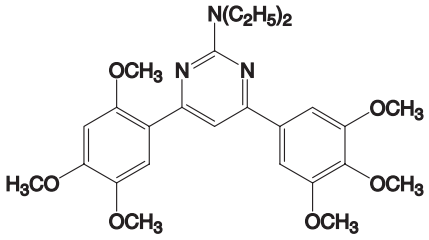

Fig. 1. Chemical structure of TAS-202: 4-(3,4,5-trimethoxyphenyl)-6-(2,4,5-trimethoxyphenyl)-2-dimethylaminopyrimidine.

trimethoxyphenyl)-2-diethylaminopyrimidine (TAS-202) (Fig. 1), a magnosalin derivative, selectively inhibits the proliferation of vascular endothelial cells, and have shown that it can be used as an orally active antiangiogenic and antiarthritic agent on basic fibroblast growth factor-induced angiogenesis and collagen-induced arthritis models of mice (Tanaka et al., 2002). These findings strongly prompted us to investigate the antiangiogenic actions of TAS-202 at the site of inflammation in arthritic joints.

Models of adjuvant-induced arthritis in rats are among the more well-established and validated models for rheumatoid arthritis (Perper et al., 1971). The arthritic response is biphasic; (first phase) the acute inflammatory phase starting with induction of the disease and (secondary phase) the autoimmune phase characterized by the systemic spread of the disease (Theisen-Popp and Müller-Peddinghaus, 1994; Bartlett and Schleyerbach, 1985). It has been reported from many studies that angiogenesis occurs in the synovial tissues in this model (Halloran et al., 1996; Zinn et al., 1999). We now quantitatively evaluated blood vessel density interpreted as an indirect measurement of angiogenesis following TAS-202 treatment of adjuvant-induced arthritis in rats. We demonstrated that TAS-202 modulates the autoimmune phase of the arthritic response through its suppressive effect on angiogenesis in the synovial tissue. Thus, it is proposed that TAS-202 may be advantageous in the therapy of rheumatoid arthritis.

2. Materials and methods

2.1. Animals

Male Lewis rats (6–7 weeks old) were purchased from Charles River Japan (Yokohama, Japan). The rats were housed in a room maintained at a temperature of 23 ± 3 °C with a humidity of $50 \pm 20\%$; the animals were allowed free access to food and water, and a 12-h light/dark schedule was maintained. The present study was performed in accordance with the guidelines approved by our Institutional Animal Care and Use Committee.

2.2. Induction of arthritis and measurement of the arthritic response

Under ether anesthesia, the 8-week-old Lewis rats were inoculated on day 0 with 0.1 mg *Mycobacterium butyricum* suspension in liquid paraffin, into the plantar skin of the right hind-paw (Difco, Detroit, MI, USA). The hind-paw volumes were measured by a water displacement method with a plethysmometer (TK-101; UNICOM, Chiba, Japan). To evaluate the effects, the agents were administered daily from day 0 to the day before the final day of the study.

2.3. Histological examination

The rats were killed with ether so as to avoid any stress to the animals and the hind-paws were harvested for analysis. Paw specimens were fixed in phosphate-buffered saline containing 10% formaldehyde at 4 °C for 7 days, defatted in 50% v/v chloroform/methanol for 1 h and decalcified in phosphate-buffered saline containing 10% w/v of ethylenediaminetetraacetic acid at 37 °C for 7 days. The ankle joint was cut longitudinally through the middle of the tibia-talus line and then embedded in paraffin. Paraffin sections of 3–4 μm thickness were stained with hematoxylin-eosin for identification of synovitis, or with anti-human von Willebrand factor (vWF) polyclonal antibody for immunohistologic evaluation of blood vessel density.

2.4. Immunostaining for von Willebrand factor

To visualize blood vessels in synovial tissue, the sections were preliminarily stained with rabbit anti-human vWF antibody (Dako Japan, Kyoto, Japan). Although the antibodies are for staining human tissue, they are also used to stain tissues from rats and mice (Otsuki et al., 1990; Yamashita et al., 2002; Amano et al., 2003). However, while large vessels stained well, small vessels only stained very slightly and were not visualized (data not shown), suggesting that antigenic reactivity might decrease during the decalcification procedure now used. We found the antibody for enhanced polymer one-step staining with multiple rabbit anti-human vWF antibodies and horseradish peroxidases coupled to an inert polymer backbone (Dako Japan) to be the optimal conditions for immunostaining and visualizing small vessels. Staining with this antibody was performed mostly according to the procedure recommended by the manufacturer. Briefly, after the sections had been deparaffinized and rehydrated, endogenous peroxidase activity was quenched by incubating with 3% hydrogen peroxide in distilled water for 5 min and then the sections were placed in Tris-buffered saline (TBS). Antigen was unmasked by incubating the section with 0.4 mg/ml proteinase K (Dako Japan) in TBS for 6 min. After rinsing, the sections were incubated with the antibody solution for 60 min at room temperature. The sections were then incubated with 3,3′ -diaminobenzidine tetra-hydrochloride solution

(Dako Japan) for about 2 min and the reaction was stopped with distilled water. The sections were counterstained with hematoxylin.

2.5. Measurement of blood vessel density in synovial tissue

We carried out histological examination to observe angiogenesis during the autoimmune phase. To avoid any influence of the acute inflammatory response, synovial tissue from the non-inoculated paws was examined. The number of blood vessels appeared to coincide with the appearance and severity of the inflammation and with the increase in paw volume. In the normal rat (before inoculation, paw volume = 1.82 ml), the synovial tissue consisted of the surface synovial membrane with one or two layers of lining cells and adipose tissue, and few blood vessels were observed. On day 10 (paw volume = 2.25 ml), when the swelling started, hyperplasia, angiogenesis and infiltration of inflammatory cells were observed in the sublining layer. On day 12 (paw volume = 2.83 ml), in the region of the swelling, the surface structure was destroyed and there were more vessels and inflammatory cells than on day 10. On day 14 (paw volume = 3.43 ml), at the time of maximal swelling, a marked increase in the number of vessels and inflammatory cells was observed, and the surface structure and adipose cells had almost disappeared.

Twelve days after the immunization, the non-inoculated hind-paws were collected and examined histologically (see Sections 2.3 and 2.4). A series of photomicrographs at a magnification (266 × on a photograph), that allowed observation of microvessels, were taken of a part of synovial tissue from synovial surface to ligament in the tibia-talus joint, not including any area of bone. In all photomicrographs and non-overlapping fields, the lumens with vWF-positive cells and morphologic features of a blood vessel were identified blind by one pathologist (MW). All lumens identified were counted as blood vessels. The observed areas were measured using a digital analyzer (Digitalizer, Wacom, Tokyo, Japan) by other observers (KT and TS). The total number of vessels per total area of tissue in all the sites examined was determined. Vehicle or drug was administered daily for 12 days from day 0.

2.6. Agents

TAS-202 was synthesized by the Chemistry Laboratory, Taiho Pharmaceutical. Indomethacin. was obtained from Sigma (St. Louis, MO, USA). Prednisolone was obtained from Nacalai Tesque (Kyoto, Japan). TAS-202, indomethacin and prednisolone were all suspended in soy-bean oil and administered orally.

2.7. Statistics

The data are expressed as means ± S.E.M. Welch's *t*-test or Dunnett's test was used to evaluate the significance of differences between the groups. *P*-values less than 0.05 were considered to denote significance.

3. Results

3.1. Inhibitory effects of TAS-202 on the arthritic response

TAS-202, an antiangiogenic agent, and indomethacin, an antiinflammatory agent, were examined for their effects on the development of the arthritic response by measuring the paw volume for 21 days at 3- to 4-day intervals. Inoculation of adjuvant resulted in an acute swelling of the inoculated paw, and an autoimmune phase of swelling in both the inoculated and non-inoculated paw 10 days after the inoculation (data not shown). Test agents were administered daily from days 0 to 20 and their effects in the inoculated or non-inoculated paws are summarized in Table 1. TAS-202 given orally at the dose of 10 and 30 mg/kg/day significantly inhibited the autoimmune phase of the arthritic response on day 21 in both the inoculated and the non-inoculated paws. Orally administered indomethacin (1 mg/kg/day) also significantly inhibited the autoimmune phase of the responses in both the inoculated and the non-inoculated paws. These results show that orally administered TAS-202 inhibited the autoimmune phase of the adjuvant-induced arthritic response as effectively as did the antiinflammatory agent.

3.2. Effect of TAS-202 on blood vessel density in synovial tissues

We evaluated the effects of agents on blood vessel density in the synovial tissue on day 12, the mid-period of the progression of inflammation. TAS-202 (30 mg/kg/day) suppressed the increase in the number of blood vessels in the synovial tissues of the arthritic rats (Fig. 2). To evaluate the effects of the test agents quantitatively, the blood vessels

Table 1
Inhibitory effects of peroral TAS-202 and indomethacin on adjuvant-induced arthritis in rats

	Dose (mg/kg/day)	*N*	Hind paw volume on day 21 (ml)	
			Inoculated side	Non-inoculated side
Normal		5	1.94 ± 0.03	1.91 ± 0.04
Control		10	4.28 ± 0.07	3.28 ± 0.12
TAS-202	3	10	3.91 ± 0.09[a]	3.19 ± 0.10
	10	10	3.69 ± 0.08[b]	2.65 ± 0.12[b]
	30	10	3.08 ± 0.06[b]	2.57 ± 0.08[b]
Indomethacin	1	10	2.49 ± 0.06[b]	2.13 ± 0.04[b]

Arthritis was induced by injection of *M. butyricum* into the plantar skin of the right hind-paw on day 0. Each agent was successively administered daily from days 0 to 20. The rats of normal and control group were given vehicle only. Paw volumes represent the means ± S.E.M. *N*: the number of observations.

[a] $P < 0.05$ vs. control (Dunnett's test).

[b] $P < 0.01$ vs. control (Dunnett's test).

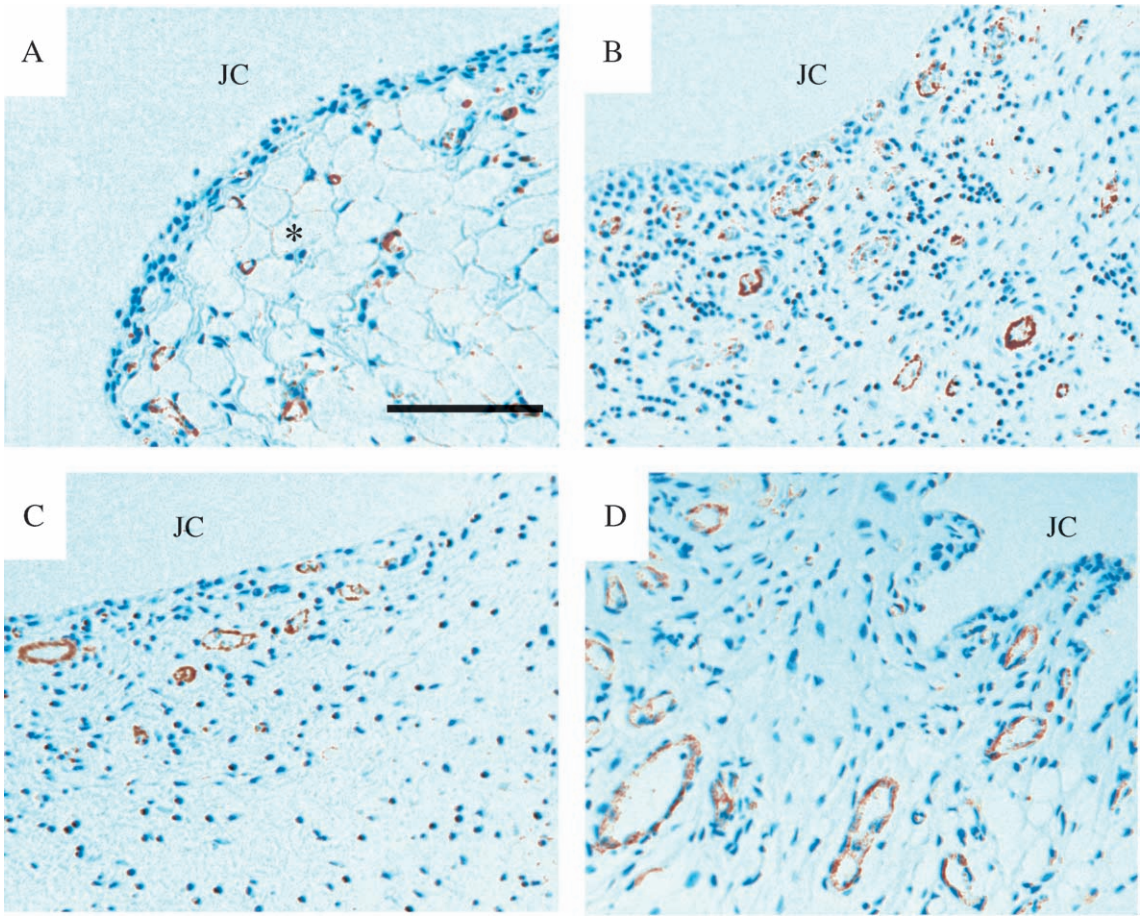

Fig. 2. Blood vessels in the synovial tissue of the tibia-talus joint in non-inoculated paws on day 12. Representative sections are synovial tissues stained with anti-von Willebrand factor antibody: obtained from normal rats given vehicle only (A) and arthritic rats given vehicle only (B), TAS-202 (30 mg/kg/day) (C) or indomethacin (1 mg/kg/day) (D) administered daily from days 0 to 11. TAS-202 suppressed the increase in the number of blood vessels (brown) in the sublining layer of the synovial membrane. JC: joint cavity. Asterisk: adipose tissue. Calibration bar in A indicates 100 μm and is common to all photographs.

were counted and the results are shown in Fig. 3. TAS-202 (10 and 30 mg/kg/day) significantly suppressed the increase in the number of blood vessels per unit area (10 mg/kg/day: $P<0.05$, 30 mg/kg/day: $P<0.01$, vs. control rats), whereas indomethacin (1 mg/kg/day) had no significant effect.

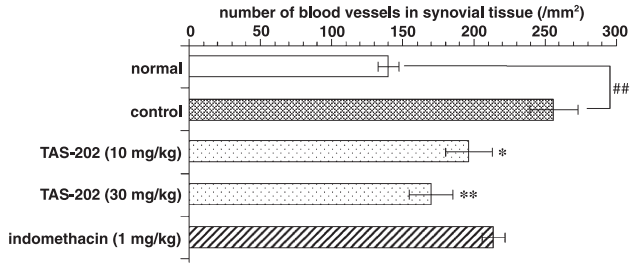

Fig. 3. Effects of TAS-202 and indomethacin on blood vessel density in the synovial tissue. The sections, obtained from non-inoculated paws 12 days after inoculation with adjuvant, were stained with anti-vWF antibody and then photographs were taken of the synovial tissue in the tibia-talus joint. The lumens with vWF-positive cells and with morphologic features were counted as blood vessels and the areas of the synovial tissues were measured. The data are the total number of vessels per total area of tissue in all sites examined. Each agent was administered daily from days 0 to 11. Values are means ± S.E.M. for six rats. *$P<0.05$, **$P<0.01$ vs. control (Dunnett's test). $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. normal (Welch's t-test).

3.3. Effect of TAS-202 in combination with indomethacin or prednisolone

Since the effect of TAS-202 was apparently distinct from that of the antiinflammatory agents, the effects of combined treatment with TAS-202 and indomethacin, or TAS-202 and prednisolone during the autoimmune phase were examined following oral administration of the drugs for 21 days. TAS-202 (30 mg/kg/day) inhibited the increase in paw volume by 52% when administered alone as compared with the volume in the controls ($P<0.01$, Table 1). Table 2 shows the influence of TAS-202 on the antiinflammatory effects of indomethacin or prednisolone. TAS-202 in combination with 0.01 and 0.1 mg/kg/day of indomethacin inhibited the increase in paw volume by 56% and 50%, respectively ($P<0.01$, as compared with indomethacin treatment alone). Furthermore, as Table 2 shows, the results for the administration of TAS-202 in combination with prednisolone are similar to those for the combination with indomethacin.

Table 2
Influence of TAS-202 on the anti-inflammatory effects of indomethacin or prednisolone on adjuvant-induced arthritis in rats

	Dose (mg/kg per day)		*N*	Non-inoculated side volume[a] (ml)	inhibition[b] (%)
Exp. 1					
normal			5	1.82 ± 0.02	
control			10	3.47 ± 0.09	
indomethacin	0.01	alone	10	3.41 ± 0.14 [f]	-
		+ TAS-202	10	2.52 ± 0.07[d] [f]	56
	0.1	alone	10	2.64 ± 0.08[d] [f]	-
		+ TAS-202	10	2.24 ± 0.04[d] [f]	50
Exp. 2					
normal			5	1.87 ± 0.06	
control			10	3.45 ± 0.13	
prednisolone	0.1	alone	10	3.32 ± 0.09 [f]	-
		+ TAS-202	10	2.67 ± 0.08[d] [f]	45
	1	alone	10	2.70 ± 0.12[d] [f]	-
		+ TAS-202	10	2.20 ± 0.04[d] [f]	60

Arthritis was induced by injection of *M. butyricum* into the plantar skin of the right hind-paw on day 0. Each agent was administered daily from days 0 to 20. The dose of TAS-202 was 30 mg/kg/day. The rats from the normal and the control group were given vehicle only. *N*: the number of observations.
[a] Volumes of paws on day 21 after the inoculation represent the means ± S.E.M.
[b] [1 − (combination − normal) ÷ (alone − normal)] × 100.
[c] $P<0.05$ vs. control (Dunnett's test).
[d] $P<0.01$ vs. control (Dunnett's test).
[e] $P<0.05$ vs. alone (Welch's *t*-test).
[f] $P<0.01$ vs. alone (Welch's *t*-test).

TAS-202 in combination with 0.1 and 1 mg/kg/day of prednisolone inhibited the increase in paw volume by 45% and 60%, respectively ($P<0.01$, as compared with prednisolone treatment alone).

4. Discussion

The present study was designed to investigate the effect of the newly synthesized antiangiogenic agent, TAS-202, on blood vessel density in adjuvant-induced arthritis of rats. There is accumulating evidence from both human and rat studies that neovascularization is involved in the pathogenesis of arthritis in rheumatoid arthritis. The endothelium of the synovium is activated before significant joint swelling is observed in adjuvant-induced arthritis in rats (Zinn et al., 1999) and the increase in the number of vessels begins soon after inoculation (Halloran et al., 1996). In conformity with these observations, our histological examination also showed that angiogenesis coincides with the appearance and the progression of inflammation in the synovial tissue and that inflammation in the tissue at day 12 is observed in all rats but not established (see Section 2). Therefore, angiogenesis in the synovial tissue was evaluated at day 12. There is no standard method for quantitative evaluation of angiogenesis in inflammatory synovial tissue. Weidner (1995) found a procedure for counting intratumoral microvessels useful for predicting the prognosis of patients. This was based on the previous report of a count of microvessels per 200× field in a 0.74-mm^2 field, 'hot spot', of most intensive neovascularization in an invasive breast carcinoma (Weidner et al., 1991). The most important aspects in this procedure seem to be: (i) the selection of area for assesment, (ii) methods of assessment and (iii) the cut-off used for correlation with other reports (Fox, 1997). Our purpose was mainly to evaluate differences between the groups, but not to predict the prognosis for an individual. In our case, it was important that all samples were observed under as similar conditions as possible. Our methodology was to express the density as the number of vessels counted in the main part of the angiogenesis in sections stained using an effective antibody at the same time after immunization (see Section 2). The present immunohistochemical study demonstrated that the number of blood vessels per unit area of synovial tissue in the region of the swelling in the paw in arthritic rats was significantly greater than that in normal rats.

However, it is claimed that the efficacy of antiangiogenic agents cannot be judged simply by visualizing alterations in microvessel density during treatment (Hlatky et al., 2002). In our preliminary examinations, subcutaneous TNP-470 (a selective angiogenesis inhibitor, 10 mg/kg/day) completely suppressed the increase in paw volume on the non-inoculated side at day 21 and the same dose of TNP-470 also completely suppressed the increase in the number of blood vessels in synovial tissues at day 12 (data not shown). TNP-470 inhibits selectively proliferation of endothelial cells (Kusaka et al., 1994) similarly to TAS-202 (Tanaka et al., 2002) and the production of vascular endothelial growth factor in a human pancreatic carcinoma line (Shishido et al., 1998). The main effects of TNP-470 on arthritis model seemed related to the inhibition of angiogenesis rather than to modulation of immune cell functions (Peacock et al.,

1992; De Bandt et al., 2000), though the agent has been reported to affect the immune system (Berger et al., 1993; Locigno et al., 2000). Therefore, the results suggest that the difference between control and normal for the number of vessels per unit area may reflect the number of new vessels.

This finding prompted us to compare the mechanisms of the antiarthritic actions of TAS-202 and indomethacin. We found that TAS-202 administered from day 0 significantly inhibited the increase in paw volume observed after inoculation with an adjuvant. As in rheumatoid arthritis, the inflammation seen during the autoimmune phase of rat adjuvant-induced arthritis is a T cell-mediated disease, because the primed T cells in the induction phase produce cytokines, are activated and migrate as do other leukocytes (Pelegrí et al., 1996; Santos et al., 1997; Schmidt-Weber et al., 1999; Bush et al., 2001). TAS-202 up to 100 mg/kg/day, even if administered daily from the day immunization started, did not affect any immune response, such as delayed-type hypersensitivity or immunoglobulin G production in mice (data not shown). The histologic data for day 21 did not indicate a significant reduction of cell influx by TAS-202 (data not shown). Therefore, TAS-202 administered from day 0 was not considered to interfere with T cell immunity in this model. More importantly, TAS-202 significantly inhibited the increase in the density of the blood vessels in the synovial tissue on day 12. TAS-202 did not inhibit the production of an angiogenic factor, vascular endothelial growth factor, or of a proinflammatory cytokine, interleukin-6, in rheumatoid arthritis synoviocytes stimulated with interleukin-1β or fetal bovine serum (data not shown). Therefore, the inhibitory effect of TAS-202 may be mainly due to selective inhibition of vascular endothelial cell growth as we have previously reported (Tanaka et al., 2002). When compared with the mechanism of TNP-470 action, suppression of proliferation of endothelial cells may be enough to prevent the increase in blood vessel density in synovial tissue of adjuvant-induced arthritis rats. Otherwise, there may be the other possible mechanisms relating to down-regulation of angiogenic mediators or up-regulation of antiangiogenic factors by TAS-202.

In contrast to TAS-202, indomethacin showed little effect on the increase in blood vessel density in adjuvant-induced arthritis in rats at day 12 at a dose that strongly suppressed the increase in paw volume. This observation seems not to be consistent with that of others (Leahy et al., 2000; Majima et al., 2000). These latter authors demonstrated that indomethacin inhibits basic fibroblast growth factor-induced angiogenesis in rats. The mechanism is that indomethacin inhibits the prostaglandin-mediated expression of vascular endothelial growth factor via suppression of cyclooxygenase-2. Indeed, basic fibroblast growth factor is elevated in the joints of rats with adjuvant-induced arthritis (Yamashita et al., 2002). Also, adjuvant-induced arthritis is a model of inflammation in which cyclooxygenase-2 plays a prominent role by inducing the production of cytokines, increasing vascular permeability and promoting infiltration by inflammatory cells (Anderson et al., 1996). These functions related with cyclooxygenase-2 are involved in the induction of angiogenesis. In the present study, as indomethacin (1 mg/kg/day) significantly inhibited the arthritic response, it is expected that cyclooxygenase-2 is suppressed by indomethacin. Therefore, our methodology may evaluate mostly the inhibitory effect of an agent against activated endothelial cells during the effector phase of angiogenesis, but not all the antiangiogenic effects. In this study, we demonstrated the difference between TAS-202 and indomethacin as to their mode of antiangiogenic action.

One important observation was that TAS-202 administered in combination with indomethacin or prednisolone had additive effects. We previously reported that TAS-202 administered in combination with indomethacin showed a synergistic effect on collagen-induced arthritis in mice, when arthritic severity was scored by the appearance of arthritis in the limb joints (Tanaka et al., 2002). These observations suggest that the strong effects of these agents administered in combination on the swelling in cases of arthritis may be of great benefit in these cases. As rheumatoid arthritis is a complex disease, a combination therapy may be both useful and necessary to improve the quality of life of the patients. Thus, since TAS-202 appears to be effective when administered orally, its addition to the therapeutic armamentarium for rheumatoid arthritis can be expected.

In conclusion, TAS-202 appears promising as a pro-drug for rheumatoid arthritis, owing to its inhibitory effect on angiogenesis at the site of inflammation in the joints in these cases.

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