ELISA kit (Pierce Biotechnology Rockford, IL) according to the manufacturer's instructions.

Inhibitory effect of canolol on activation of macrophages from the BALB/c mouse

Macrophages were obtained from the peritoneal fluid of mice stimulated with casein. In brief, 1 ml of 5% casein sodium (Wako Pure Chemical) in phosphate-buffered saline was injected intraperitoneally into BALB/c mice. After 3 days, mice were killed, and 5 ml of cold phosphate-buffered saline was injected into the peritoneal cavity, after which peritoneal lavage fluid (~5 ml) was collected, followed by centrifugation of the fluid (1000 r.p.m., 5 min) at 4°C. The macrophages were washed with phosphate-buffered saline three times by centrifugation, and then 15 ml of Roswell Park Memorial Institute 1640 medium (Invitrogen) with 10% FBS was added and macrophages were cultured in a plastic petri dish (100×26 mm; Nunc A/S, Roskilde, Denmark).

To activate the macrophages in culture, lipopolysaccharide (LPS) (1.0 μg/ml) and interferon-γ (0.1 μg/ml) (Sigma) were added to the cells for 24 h. Culture medium was then collected for measurement of the concentration of nitrite, which is formed from nitric oxide (NO). A significantly high amount of NO was generated by activated macrophages, which was attributable to the action of inducible NO synthase (iNOS). The nitrite concentration was quantified by using a Griess reagent kit (NO_2/NO_3 Assay Kit-C II; Dojindo Laboratories), according to the manufacturer's instructions. The production of inflammatory cytokines, i.e. TNF-α and IL-12, was also measured in culture media by using ELISA as mentioned above.

Protective effect of canolol against $ONOO^-$-induced cytotoxicity

HEK293 cells were plated at 3000 cells/well in a 96-well plate (Nunc A/S). After overnight preincubation, 1 mM or 2 mM 3-(4-morpholinyl)sydnonimine hydrochloride [SIN-1 (Dojindo Laboratories)], from which $ONOO^-$ was produced, was added to the cells. Canolol at various concentrations was then added. After an additional 48 h of incubation, cell viability was determined by using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay.

Expression of COX-2, TNF-α, iNOS and heme oxygenase-1 in colon tissues of AOM/DSS-induced carcinogenesis mice with/without feeding canolol

To examine the antioxidative, anti-inflammatory mechanisms of canolol in chemoprevention against AOM/DSS-induced colon carcinogenesis, mRNA expressions of representative oxidative inflammatory molecules [i.e. COX-2, TNF-α, heme oxygenase-1 (HO-1) and iNOS] were detected by reverse transcription–polymerase chain reaction. Briefly, after the protocol of AOM/DSS-induced carcinogenesis, total RNA from colon tissues of distal quarter in which most tumors were observed was extracted by using Sepasol®-RNA I Super reagent (NACALAI TESQUE, Kyoto, Japan), according to the manufacturer's instruction. The nucleotide sequences of the oligonucleotide primers and cycle conditions of PCR are as follows: COX-2: forward, 5′-ACA CAC TCT ATC ACT GGC ACC-3′; reverse, 5′-TTC AGG GAG AAG CGT TTG C-3′; 35 cycles of 15 s at 94°C, 15 s at 55°C and 1 min at 72°C to obtain a 274-bp cDNA; TNF-α: forward, 5′-CTA TGT CTC AGC CTC TTC TC-3′; reverse, 5′- CAG CCT TGT CCC TTG AAG AG -3′; 40 cycles of 15 s at 94°C, 30 s at 56°C and 30 s at 68°C to obtain a 353-bp cDNA; HO-1: forward, 5′-GGC CCT GGA AGA GGA GAT AG-3′; reverse, 5′-GCT GGA TGT GCT TTT GGT G-3′; 30 cycles of 30 s at 94°C, 30 s at 56°C and 30 s at 72°C to obtain a 888-bp cDNA; iNOS: forward, 5′-CCC TTC CGA AGT TTC TGG CAG CAG C-3′; reverse, 5′-GGC TGT CAG AGC CTC GTG GCT TTG G-3′; 35 cycles of 1 min at 95°C, 1 min at 65°C and 2 min at 72°C to obtain a 496-bp cDNA; GAPDH (inner control): forward, 5′-CAT GTG GGC CAT GAG GTC CAC CAC-3′; reverse, 5′-TGA AGG TCG GAG TCA ACG GAT TTG GT-3′; 30 cycles of 1 min at 94°C, 1 min at 56°C and 1 min at 72°C to obtain a 983-bp cDNA. PCR products then underwent electrophoresis on ethidium bromide-stained 1.2% agarose gels.

Safety of canolol

Female ICR mice, beginning 6 weeks of age, were fed normal diet (untreated control) or 0.3% canolol for 6 weeks. Then, mice were killed, and blood samples were obtained. Red blood cell count, white blood cell count and hemoglobin levels were determined using an automated blood counter (F-800 Microcell Counter, Toa Medical Electronics, Kobe, Japan). Plasma obtained by centrifugation was used for measurement of the liver and the kidney functions including alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, blood urea nitrogen and total creatine using a sequential multiple Auto Analyzer system (Hitachi Ltd., Tokyo, Japan).

Statistical analyses

Data were analyzed by one-way analysis of variance followed by the Bonferroni *t*-test. Some studies with two experiments were analyzed by Mann–Whitney *U*-test, and a Fisher's exact test was used to analyze the data of tumor incidence. A difference was considered statistically significant when $P < 0.05$.

Results

Protective effect of canolol against DSS-induced colitis

Severe diarrhea accompanied by hematochezia, characterized by significant increase in DAI, was observed on day 7 in the DSS-induced colitis group without canolol; these DSS-treated mice showed a decrease in body weight though no statistical significance was found (Table I). These symptoms were markedly improved with significantly decreased DAI when canolol was added to the diet in a dose-dependent manner, and these mice showed no apparent loss of body weight (Table I). Moreover, mice with DSS-induced colitis demonstrated shortening of the large bowel, which is one of the indexes of colitis, and this pathological change was significantly improved by canolol dose dependently (Table I). No significant differences in DAI and in length of large bowel were observed in mice receiving 1% of canolol compared with normal mice (Table I), suggesting an almost complete cure of colitis. However, 1% of canolol becomes impracticable as a chemopreventive agent or supplement especially for long-term application, i.e. canolol

Table I. Protective effect of canolol against DSS-induced colitis (on day 7) and AOM/DSS-induced colon carcinogenesis (at 6 weeks)

Group	Body weight (g)	Liver weight (g)	Length of large bowel (cm)	DAI[a]
Normal	29.7±2.0	3.1±0.7	15.0±1.5**	0
DSS	26.9±4.4	2.4±0.6	9.6±2.1	9.6±0.3
DSS + 0.1% canolol	27.6±2.2	2.8±0.5	12.8±1.2*	5.2±0.7**
DSS + 0.3% canolol	28.8±3.3	2.7±0.5	13.3±2.1**#	3.7±0.6**
DSS + 1% canolol	29.3±1.5	2.9±0.1	13.7±1.7**#	1.2±1.8**#

Group	Body weight (g)	Liver weight (g)	Length of large bowel (cm)[#]	Incidence and multiplicity of tumors	
				Tumor incidence (%)[b]	Tumor multiplicity (no. of tumors/animal)
Normal	48.0±4.7	2.5±0.4	13.0±0.9**	0**	0
AOM/DSS	44.0±3.3	2.0±0.2	8.7±0.6	100	10.8±4.2
AOM/DSS + 0.1% canolol	44.6±6.1	2.1±0.1	10.9±0.9*	60**	5.3±2.7*
AOM/DSS + 0.3% canolol	44.7±1.9	2.0±0.1	10.7±0.3*	57**	5.6±2.7*

Data are means ± SD, n = 5–14 for DSS-induced colitis experiments, and n = 10–20 for AOM/DSS-induced colon carcinogenesis experiments.
[a]See text for details.
[b]Statistical significance was analyzed by Fisher's exact test.
*$P < 0.05$, **$P < 0.01$, versus the DSS control group, or versus the AOM/DSS control group.
#No significant difference ($P > 0.05$), versus normal group.

needs to be given more than 10 g a day in humans. Canolol at higher than 0.3% was not pursued in the carcinogenesis study and was not subjected for further investigations, i.e. pathological studies and examination of inflammatory cytokines.

Regarding the histopathology of the colon, as Figure 1 shows, the canolol-treated groups (Figure 1C and D) had much less tissue damage compared with the DSS control (Figure 1B) showing severe inflammation and erosion. Inflammation looks more alleviated in the higher dose (0.3%) group (Figure 1D) resembling normal mucosa (Figure 1A) than in the lower dose (0.1%) group (Figure 1C). After 7 days of consumption of the canolol diet, mice in both canolol groups showed significantly suppressed formation of ulcers in the colonic mucosa compared with the DSS-induced colitis group without canolol (Figure 1E).

Canolol exhibits anti-inflammatory and antioxidative activity in DSS-induced colitis

COX-2-specific immunostaining confirmed that DSS-induced colitis clearly associated with colon inflammation (Figure 2A). The COX-2 expressions in DSS control mice were significantly higher than that in normal ICR mice. However, we found the scores of COX-2 in the canolol-treated groups were reduced compared with the DSS control, though no significance was observed ($P = 0.063$).

Consistent with these findings, amount of free 8-OHdG in the plasma, that is a common index for oxidative injury of DNA, was significantly increased after DSS treatment, whereas it was suppressed dose dependently by canolol; a significant difference was observed between DSS group and DSS + 0.3% canolol group (Figure 2B).

Suppression of inflammatory cytokine production in vivo *by canolol treatment in the DSS-induced colitis model*

The anti-inflammatory tissue protective effect of canolol was further confirmed by measuring the IL-12 and TNF-α levels, which are major cytokines involved in cell killing, in the serum of mice with DSS-induced colitis. As seen in Figure 2C and D, DSS-treated mice had significantly elevated levels of both cytokines, whereas these levels decreased after treatment with canolol in a dose-dependent manner, though no significance was observed for 0.1% canolol group compared with DSS control group. This finding is consistent with the improved symptoms and pathology of colitis as noted in Table I.

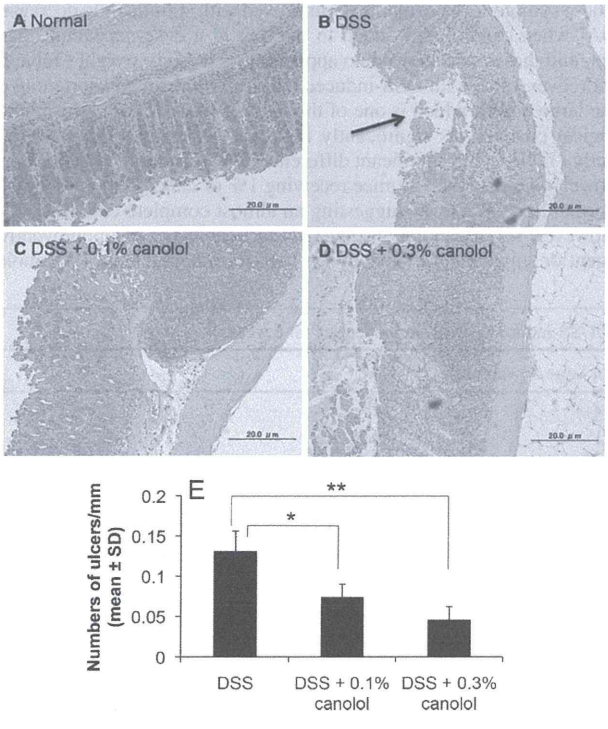

Fig. 1. Histological examination of the large bowel in DSS-induced colitis, with and without canolol treatment. (**A–D**) Hematoxylin and eosin staining of colon tissue from each experimental group. The arrow indicates the ulcer (necrosis) in the colonic mucosa. (**E**) Quantification and summary of the numbers of ulcers in each experimental group. See text for details. Data are means ± SD; $n = 6–10$. $^*P < 0.05$, $^{**}P < 0.01$.

Suppression of macrophage activation and cytokine production by canolol in vitro

The effect of canolol on the progression of inflammation as manifested by macrophage activation was investigated *in vitro* with macrophages from BALB/c mice. Canolol, at concentrations up to 200 μM, showed no apparent cytotoxicity in macrophages and human colon cancer Caco-2 cells (Supplementary Figure 2A and B, available at *Carcinogenesis* Online). Activation of macrophages was induced by simultaneously adding LPS and interferon-γ, and activation was assessed by measuring the generation of NO as nitrite (Figure 3A). Under the same conditions, when canolol was added to the cells, macrophage activation was significantly inhibited in a dose-dependent manner (Figure 3A). Moreover, canolol treatment significantly suppressed generation of inflammatory cytokines (i.e. IL-12 and TNF-α) by the macrophages (Figure 3B and C). These data clearly indicate the anti-inflammatory effect of canolol.

Protective effect of canolol against $ONOO^-$-induced cytotoxicity

Canolol is known as a compound with potent antioxidative activity, which is thought to contribute to its anti-inflammatory and cancer-preventive effects. To evaluate this, we investigated the cytoprotective effect of canolol against $ONOO^-$, which is highly cytotoxic to many cells including bacteria (14,15,20,21). $ONOO^-$ is an endogenous product of NO plus superoxide anion radical (O_2^-) in inflammatory reactions (22), and it can damage DNA, RNA, proteins and other critical molecules by means of oxidation, nitration and hydroxylation (23,24). To investigate the cytotoxicity of $ONOO^-$ and the antioxidative cytoprotection of canolol, we selected a normal cell line, human embryonic kidney cells HEK293. In this *in vitro* system, we found that ~50–60% of cells died after treatment with $ONOO^-$, which was supplied by means of a donor, SIN-1, at 1 mM (Figure 3D). Because of the short half-lives of SIN-1 (1–2 h in plasma) and $ONOO^-$ (2–3 s at physiological pH), the cytotoxicity of $ONOO^-$ in this *in vitro* culture study may be underestimated. However, the important finding is the significant inhibition of $ONOO^-$-induced cytotoxicity by canolol. In addition, a dose-dependent effect of canolol observed in HEK293 cells and the cytotoxicity of 1 mM SIN-1 was completely inhibited by 50 μM canolol (Figure 3D). In addition, canolol itself had no apparent cytotoxicity for this cell line, at least up to 100 μM (Supplementary Figure 2C, available at *Carcinogenesis* Online), which suggests that canolol is safe.

Preventive effect of canolol on AOM/DSS-induced colon carcinogenesis

Inflammatory colitis is believed to be closely associated with the occurrence of colon cancer (1–4). We, thus, investigated the preventive effect of canolol in the AOM/DSS-induced colon carcinogenesis model. The results, as shown in Table I, clearly indicated the suppressive effect of canolol on the occurrence of colon cancer. Compared with AOM/DSS control mice, 100% of which had colon tumors, ~40% of canolol-treated mice did not have these tumors. In addition, the multiplicity was significantly reduced by ~50% in the canolol-treated group compared with the untreated control group (Table I). This effect showed no clear dose dependence, as 0.1 and 0.3% canolol produced similar effect.

Suppression of COX-2, TNF-α, iNOS and HO-1 expression by canolol in AOM/DSS-induced colon carcinogenesis

To investigate the chemopreventive mechanisms of canolol, we measured mRNA expression of proinflammatory cytokines, i.e. COX-2,

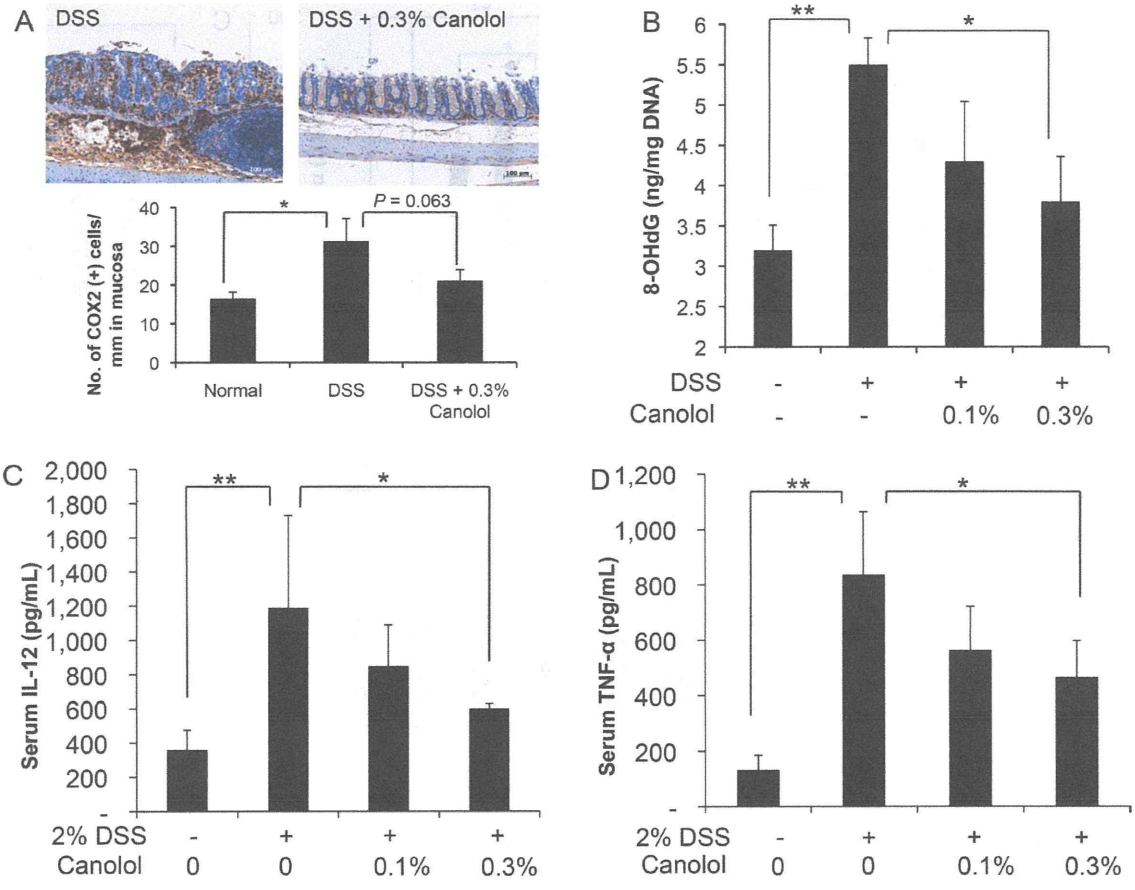

Fig. 2. Immunohistochemistry of COX-2 (**A**) in colorectal lesions, and plasma levels of 8-OHdG (**B**), as well as production of IL-12 (**C**) and TNF-α (**D**) in DSS-induced colitis and the protective effect of canolol. The protocols of DSS-induced colitis and canolol treatment are presented in Figure 1. Seven days after the start of DSS administration, mice were killed and serum samples were collected for measuring IL-12 and TNF-α by means of ELISA. See text for details. Data are means ± SD ($n = 6$–10). $^*P < 0.05$, $^{**}P < 0.01$.

TNF-α and iNOS in AOM/DSS-induced colon carcinogenesis. Similar to the findings in DSS colitis experiments (Figure 2A and D), significant decreases of TNF-α and iNOS expression were observed (Figure 4B and C). As to COX-2, though no significance was observed ($P = 0.054$), apparent lowered expression was found after feeding 0.3% of canolol (Figure 4A), and further immunohistochemical staining of COX-2 in colon mucosa also showed that average number of COX-2-positive cells in unit length tended to be lower at 0.46±0.31/mm in canolol group compared with 0.89±0.39/mm in AOM/DSS control group, although without statistical significance (data are mean ± SD; $P = 0.082$, Mann–Whitney U-test) (Figure 4E).

Moreover, when we examined the expression of HO-1, a major antioxidative antiapoptotic molecule in various tumors reflecting oxidative and other cellular stresses (25), a significantly decreased expression was observed in canolol group compared with AOM/DSS control group (Figure 4D), which in part supported the antioxidative effect of canolol, i.e. higher oxidative levels in AOM/DSS group inducing higher expression of HO-1, whereas suppressed oxidative stress by canolol resulted in lower expression of HO-1.

Effect of canolol on colon 26 solid tumor model

To further examine the effect of canolol on tumor growth, a syngeneic mouse colon tumor model (colon 26) was used. After oral administration of canolol (100 mg/kg) for three times, COX-2 expressions in tumors were significantly lowered (Figure 4F): average area of COX-2 was 1.42±0.47% in the control (no canolol) group, whereas 0.215±0.072% in the group fed with 0.3% canolol (data are mean ± SD; $P < 0.002$, Mann–Whitney U-test). However, only a slight but no significant suppression of tumor growth was found (Supplementary Figure 3, available at *Carcinogenesis* Online).

Safety of canolol

As summarized in Table II, no significant adverse effects such as decreases in red blood cell and white blood cell counts and hemoglobin levels were found in ICR mice after feeding 0.3% canolol for 6 weeks, which is the same dose for preventing AOM/DSS-induced colon carcinogenesis. Also, no significant changes in the liver enzymes and kidney functions were found under the same conditions.

Discussion

In this study, we demonstrated the protective effect of canolol, a potent antioxidant that was recently isolated from canola (rapeseed) oil (12), on IBD in a DSS-induced mouse model. Oral administration of a diet containing canolol to the mice significantly reduced the symptoms and suppressed the progression of this disease, as supported by the lengthening of the large bowel (Table I), as well as by reduced severity and numbers of ulcers in the colonic mucosa (Figure 1) and lower levels of COX-2 expression and inflammatory cytokines (Figure 2A, C and D). These findings were associated with a decreased occurrence of colon carcinogenesis induced by AOM/DSS (Table I). However, though suppression of COX-2 expression by canolol was also found in colon 26 solid tumor (Figure 4F), no significant delay of tumor growth was observed (Supplementary Figure 3,

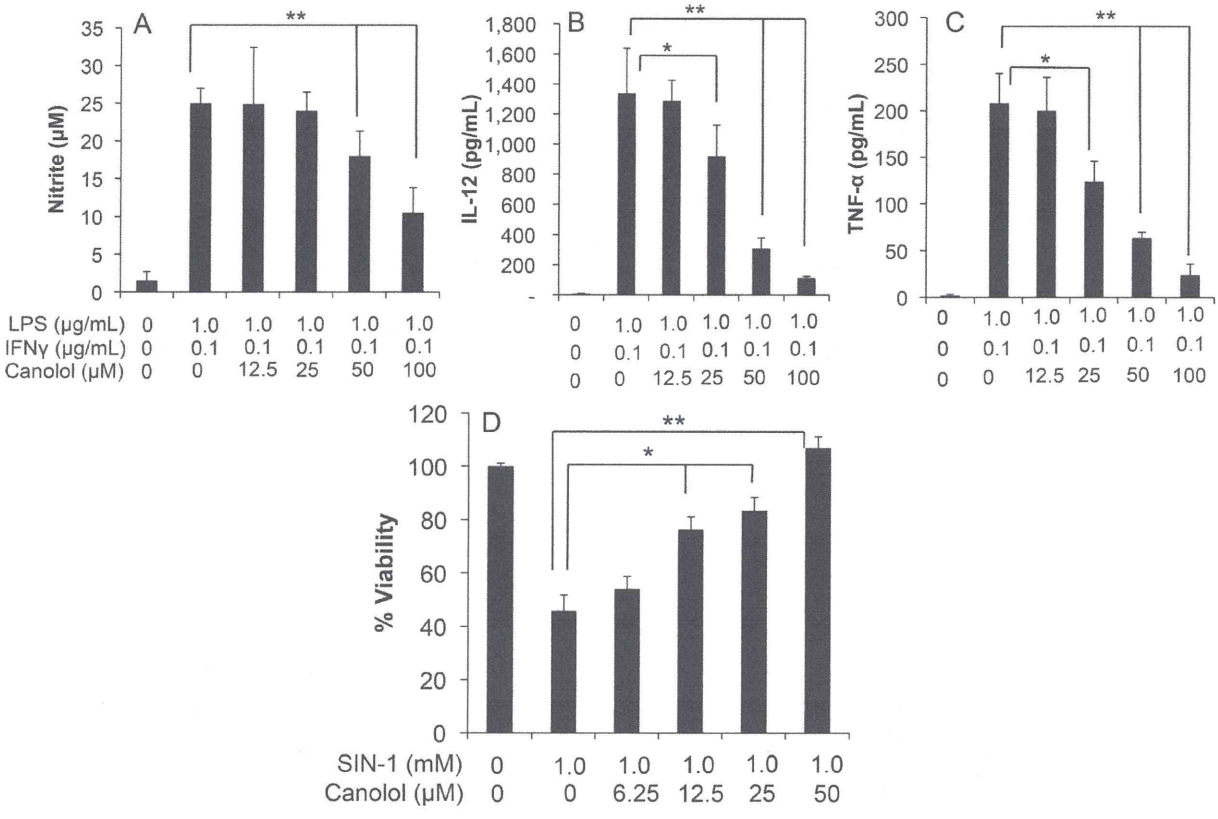

Fig. 3. Suppression of macrophage activation (**A**) and subsequent generation of IL-12 (**B**) and TNF-α (**C**), and cytoprotective effect against the toxicity of peroxynitrite ($ONOO^-$) in HEK293 (**D**) by canolol. Mouse macrophages were obtained from BALB/c mice. Macrophage activation, as generation of NO, was evaluated by using a Griess Reagent kit. IL-12 and TNF-α were measured using ELISA. See text for details. For cytotoxicity study, 3000 cells/well were plated in a 96-well plate. After overnight preincubation, 1 mM SIN-1 ($ONOO^-$ donor) was added to the cells. Different concentrations of canolol were administered. After an additional 48 h of incubation, cell viability was determined by using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Data are means ± SD ($n = 6–8$). $^*P < 0.05$, $^{**}P < 0.01$.

available at *Carcinogenesis* Online). Partly consistent with these findings, canolol does not show apparent cytotoxicity against cultured cells including colon cancer cells Caco-2 (Supplementary Figure 2, available at *Carcinogenesis* Online). These data suggested that canolol might not exhibit chemotherapeutic/cytotoxic effect against the growing tumors, whereas it exhibited significant chemopreventive effect probably during the stages of initiation and/or promotion via its antioxidative and anti-inflammatory activities.

Part of this anti-inflammatory effect of canolol may be attributable to its antioxidative or scavenging activity against the excess ROS that are produced during inflammation. ROS are known to be involved in many diseases including inflammation, infections, ischemia/reperfusion injury, neurological disorders, Parkinson's disease, hypertension and cancer (26–28). During the process of inflammation, O_2^- is extensively produced in infiltrated neutrophils and activated macrophages by means of reduced nicotinamide adenine dinucleotide phosphate oxidase and probably even more by xanthine oxidase, which is highly expressed in inflamed tissues (27–30). We described similar results in our previous study with an influenza virus infection model (29,31) and in our more recent study with a xanthine oxidase inhibitor in a rat liver ischemia/reperfusion model (30). Excess generation of ROS was also observed in DSS-induced colitis model and could be suppressed by xanthine oxidase inhibitor (Fang,J., Yin,H.Z., Liao,L., Qin,H.B., Ueda,F., Uemura,K., Eguchi,K., Bharate,G.Y., Nakamura,H., and Maeda,H., unpublished data). O_2^- is then converted to hydrogen peroxide by superoxide dismutase and/or glutathione peroxide, after which the hydrogen peroxide is converted to hydroxyl radicals in the presence of transition metals (e.g. Fe^{2+}). A massive amount of NO is also generated by iNOS that is upregulated in activated macrophages (22,28), and NO can react rapidly with O_2^- to form the more toxic species $ONOO^-$. All of these highly reactive biological radicals readily cross cell membranes and react with proteins, DNA and lipids (23,24,32–34), which results in cell damage. Furthermore, removal of NO by reaction with O_2^- on the vascular endothelial surface causes vasoconstriction and triggers neutrophil adherence and accumulation, which will promote the pathological process of inflammation (20,21). This notion is supported by results of the present study, in which canolol treatment significantly protected cells against the toxicity of $ONOO^-$ (Figure 4D and E), and moreover it also significantly decreased the levels of 8-OHdG, one of the major indicators of oxidative stress, in DSS-induced colitis model (Figure 2B).

Moreover, it was also reported that the antioxidative and cytoprotective effect of canolol is probably partly through upregulating antioxidative molecules such as NF-E2-related factor, HO-1, catalase and glutathione S-transferase-pi via an extracellular signal-regulated kinase-mediated pathway (35). However, in this study we found the decrease of HO-1 expression in the distal quarter of the colon where tumors occurred most frequently (Figure 4D). This finding may indicate the different expression profile of HO-1 in normal tissues and tumor tissues. In normal tissues, upregulated HO-1 protects against oxidative stress and other damages, whereas many tumors highly express HO-1 to support their rapid growth and protect against various oxidative stresses (25). Moreover, these findings partly agreed with a recent report showing that HO-1 may protect healthy tissues against carcinogen-induced injury, but in already growing tumors, it seems to favor their progression toward more malignant forms (36).

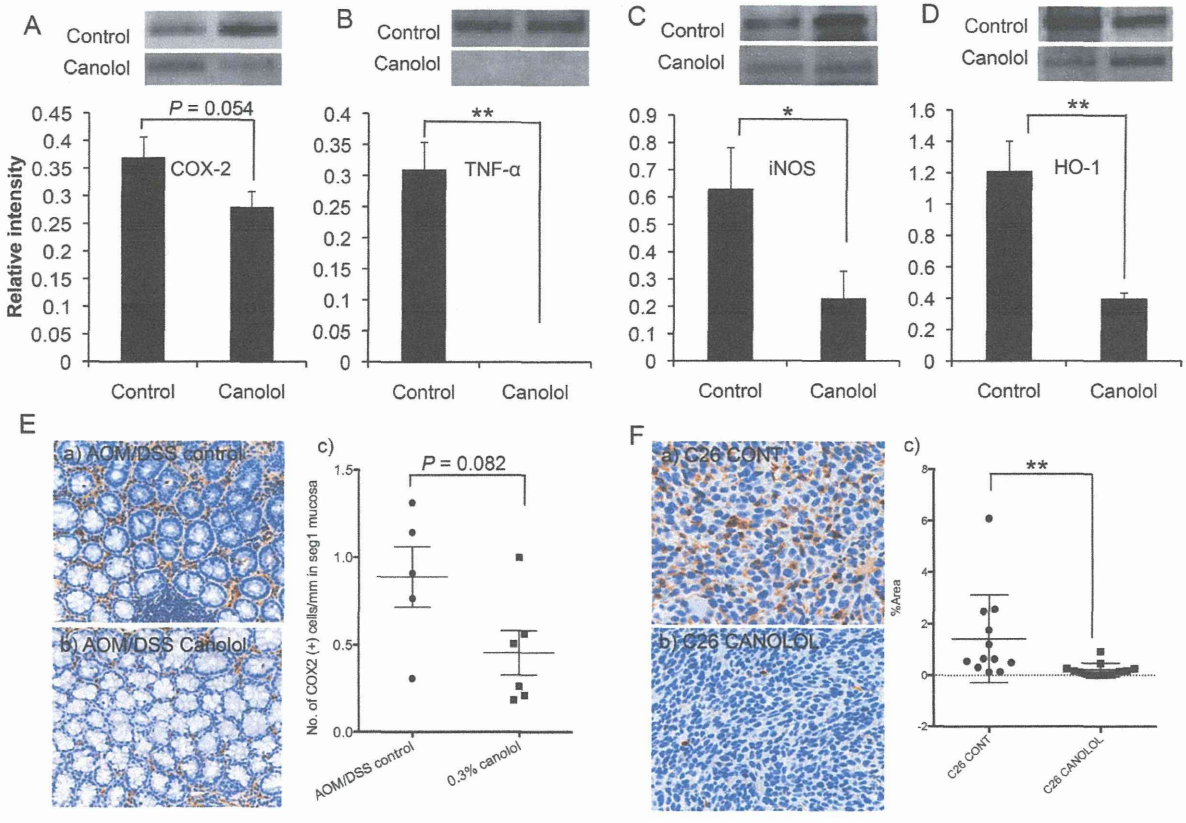

Fig. 4. Suppression of COX-2 (**A**), TNF-α (**B**) and iNOS (**C**) and upregulation of HO-1 by canolol as evaluated by reverse transcription–polymerase chain reaction, and the decreased COX-2 expression in colon tissue of AOM/DSS-treated ICR mice (**E**) and colon 26 (C26) mouse tumor (**F**) *in vivo* after canolol treatment. In (**A**–**D**), two representative DNA bands of each group (control and canolol group) are shown, and the results were semiquantitated as relative intensity compared with the intrinsic DNA expression of GAPDH as control. Immunohistochemical staining of COX-2 in colon tissue of AOM/DSS control mice (E-a) and canolol-treated mice (E-b) is shown in (E), and that in C26 tumor tissues without and with canolol treatment is shown in (F-a) and (F-b), respectively; in both cases, the COX-2-positive (brown colored) area was quantitated (E-c and F-c). See text for details. Data are means ± SD (n = 5–12). $^{*}P < 0.05$, $^{**}P < 0.01$ by Mann–Whitney U-test.

Table II. Change in RBC, WBC, hemoglobin and plasma liver enzyme levels and kidney function after feeding canolol (0.3% for 6 weeks) in ICR mice[a]

	RBC (10^4/μl)		WBC (10^2/μl)		Hb (g/dl)
Normal	992.2±35.9		30.4±4.2		16.0±0.6
Canolol[b]	953.0±20.2		28.8±1.9		15.1±0.4
	BUN (mg/dl)	Cr (mg/dl)	AST (IU/l)	ALT (IU/l)	LDH (IU/l)
Normal	20.7±0.8	0.15±0.02	67.3±2.8	28.4±2.2	1383.0±54.7
Canolol[b]	21.5±1.3	0.13±0.01	65.1±4.5	37.6±4.2	1036.4±149.3

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; Cr, creatinine; Hb, hemoglobin; LDH, lactate dehydrogenase; RBC, red blood cells; WBC, white blood cells.
[a]No significant difference was found between canolol feeding mice and normal mice in all selected indices. Values are presented as means ± SE, n = 5–8.
[b]Canolol was administered at 0.3% (w/w) in diet. Assays were carried out at 6 weeks after feeding canolol.

Taken together, the association of canolol with HO-1 in AOM/DSS colon carcinogenesis seems to be different from that in normal tissues during stresses and damages as described earlier (35); further investigations are thus warranted to make clear the mechanisms involved in the effect of canolol in different conditions.

COX-2 is the enzyme that catalyzes the conversion of arachidonic acid to prostaglandins, it is unexpressed under normal conditions in most cells, but elevated levels are found under inflammatory condition and is thus largely responsible for causing inflammation (37). Many studies revealed that the products of COX-2 prostaglandins are highly involved in the carcinogenesis of many tumors including colorectal cancer metastasis and tongue and esophageal cancers (38,39). In this study, we found a decrease in COX-2 expression (both in mRNA and protein levels) after canolol treatment, though not statistically significant, in DSS-induced colitis (Figure 2A) and in AOM/DSS-induced colon carcinogenesis (Figure 4A). These findings at least partly suggested that suppression of COX-2 expression is probably involved in the effect of canolol on DSS-induced colitis and colon carcinogenesis.

Both the present study and our previous study (16) of canolol *in vivo* showed suppression of a number of inflammatory mediators

such as TNF-α, IL-12, IL-1β, iNOS and COX-2 (Figure 2A, C, and D, Figure 4A–C and ref. 16), which confirms that this suppression will contribute to the anti-inflammatory activity and the antioxidative effect of canolol against IBD and the subsequent colon carcinogenesis. Infiltrated neutrophils and activated macrophages are major producers of these inflammatory cytokines during inflammatory diseases including IBD (40,41). ROS play an important initial role in both the activation of macrophages and the induction of inflammatory cytokines. Bulua et al. (42) recently reported that ROS are crucial in LPS-stimulated macrophages for inducing production of several proinflammatory cytokines through an mitogen-activated protein kinase signaling pathway, as an essential feature of innate immunity. Apoptosis signal-regulating kinase 1 is also involved in this immune response (43). Consistent with this result, we found in this study that the ROS scavenger canolol suppressed activation of macrophages stimulated by LPS and interferon-γ, as evidenced by reduced NO generation (Figure 3A) and lower levels of IL-12 and TNF-α (Figure 3B and C). Also, certain cytokines, i.e. TNF-α and IL-12, secreted by activated phagocytic cells, can enhance ROS generation (44,45), which explains the important role of cytokines in the pathogenic process of inflammation. These findings suggested that the chemopreventive effect of canolol is mostly via its antioxidative and anti-inflammatory effect to inhibit the oxidative stress and inflammation, thus inhibiting the carcinogenesis cascades.

Canolol is extracted from crude canola oil after roasting the rape seeds and is a naturally occurring compound in this edible oil whose concentration is estimated to be ~220–1200 ppm (12), which could provide doses similar to that used in our study. The amounts of canolol administered orally in the diet in this study were 0.1% and 0.3%, or equivalent to 1–3 g/kg (dry weight) of feed for humans, which is a reasonable range for supplement diet. In our previous study, we also applied the 0.1% concentration in an *H.pylori*-induced gastric carcinogenesis model and showed a significant cancer-preventive effect (15). Because the present colon carcinogenesis prevention study revealed no dose dependency (Table I), the 0.1% canolol concentration may be the level of saturation. That is, 0.1% canolol may be sufficient to prevent colon carcinogenesis.

Furthermore, canolol showed very little cytotoxicity to cells in culture: it had no apparent toxicity in human HEK293 cells at least up to 100 μM, in human Caco-2 cells up to 200 μM (Supplementary Figure 2B and C, available at *Carcinogenesis* Online) or in macrophages up to 300 μM (0.054 mg/ml), as described previously (16) and in present study (Supplementary Figure 2A, available at *Carcinogenesis* Online), which is a far higher concentration than the concentration for effective scavenging of ROS (i.e. 1–20 μM) (12,46). Similar results were obtained in our *in vivo* study. Mice receiving a diet containing canolol up to 0.3% for 6 weeks showed no apparent change in body weight (Table II) and no apparent toxicity as reflected by blood cell count and biochemistry of liver and kidney functions (Table II). This safety profile suggests that canolol has the potential to be not only a drug but also a food supplement for disease prevention.

Colon cancer is the most common type of cancer in developed countries, with the highest incidence and mortality rates (47). With regard to the mechanisms of colon carcinogenesis, genetic factors seem to play an important role, as in familial adenomatous polyposis (48). However, ROS were recently found to be one of the critical factors in colon carcinogenesis and in familial adenomatous polyposis (49). Dietary habits are known to be highly associated with the occurrence of colon cancer (50,51). For example, oxidized oils in high-fat diets, which are a risk factor for colon cancer, generate lipid peroxyl radicals in the presence of heme or iron, damage DNA and consequently induce colon cancer (50). Also, ROS contribute to many conditions other than inflammation, such as virus infections and ischemia/reperfusion injury, as described above.

Moreover, an unhealthy diet, with a low consumption of green vegetables and thus less antioxidants, may lead to the adverse consequences of these ROS-related diseases. It should be noted that purified canola oil that is available in large supermarkets does not contain canolol because the refining process removes it (12). It should be also noted that the content of canolol increases dramatically by roasting process as used in traditional oil refining process (12). Thus, the refining process should be modified so that canolol is retained. The canolol used in this study was synthesized, so synthetic canolol may be used as a preventive agent for these diseases.

For IBD treatment, drugs commonly used in clinical settings provide symptomatic or palliative relief. Canolol treatment, however, aims more at the cause of the disease, i.e. ROS. All these data therefore suggest that canolol may be effective not only for IBD-associated colon cancer but also for ROS-dependent carcinogenesis, as described for gastric cancer involving *H.pylori* infection (16). Canolol thus holds promise as a preventive agent or supplement for both IBD and colon cancer.

Supplementary material

Supplementary Figures 1–3 can be found at http://carcin.oxfordjournals.org/

Funding

Grants-in-Aid from the Ministry of Education, Science, Culture, Sports and Technology of Japan (No. 08011717); Cancer Speciality grant from the Ministry of Health, Welfare and Labour (H23-3rd Cancer Project-General-001); research funds of the Faculty of Pharmaceutical Sciences at Sojo University (RW15000025).

Acknowledgements

The authors thank Ms Masayo Idenoue for her excellent technical assistance.

Conflict of Interest Statement: None declared.

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Received December 13, 2012; revised July 8, 2013; accepted July 23, 2013

articles

がん治療におけるナノテクノロジー

ジェニファー・グロスマン，スコット・マクニール

腫瘍血管の毛細血管レベルでの微細構造はこれまでほとんど未開拓の分野であり，それががん研究の弱点であった。ナノマテリアルの登場によって正常組織に傷害を与えることなくがん治療の道が開けつつある。腫瘍の弱点は，腫瘍（細胞）そのもののもつ弱点ではなくて，腫瘍に栄養を補給している腫瘍の血管そのものにあり，本論文はその正常血管との違いを利用することを可能にするナノメディシンの開発と関連づけて論じるものである。

前田　浩 訳

Nanotechnology in cancer medicine

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Physics Today Vol. 65 No. 8

がんは生物の生まれながらの宿命的病気であり，生命現象の象徴でもある。また，がんは細胞複製の制御が逸脱した状態である。歴史的にがん治療において外科手術は有効であるが，化学的手法（いわゆる化学療法）はもっとも効果的な方法の1つである。しかし，細胞に毒性のある化学物質を用いるこの手法はがん細胞と同時に健全な正常細胞も殺してしまう。したがって，このような化学療法剤を用いるかぎり，正常細胞には無毒でがん細胞のみを選択的に殺すことは至難の業である。それがもしできるとしてもほんの短時間のみで例外的な場合である。一方，がん細胞の増殖速度はたいへん早く，また，同時に薬剤耐性の能力をいち早く獲得することもがん治療が困難な理由である。

ナノサイズ分子の新薬の出現によって，研究者の対がん作戦の範囲はいまや物理学的な問題として，たとえば物質輸送や流体力学の手法を用いて挑戦できるようになっている。これらのナノマテリアル型の薬剤（ナノメディシン）を開発することによりがん研究者はいくつかの成功をみつつあるが，同時に一連の物理学上の問題も提起している。

ナノメディシンの原理・原則

腫瘍形成のごく初期においては，腫瘍固有の血管はなく，腫瘍は酸素や栄養素を周辺の正常組織の血管から摂取している。その腫瘍細胞が増殖し，腫瘍（塊）となると，その腫瘍塊の周辺部の腫瘍細胞は，その中心部の腫瘍細胞と比べ外部に接しているため，より容易に栄養を摂取できるので，増殖は盛んである。すなわち，腫瘍の中心部の細胞は栄養が乏しく，また酸素も欠乏状態になっており，低酸素下にある旨のシグナルタンパク質を放出する。そのタンパク質は正常組織の血管にまで拡散により到達し，それによってそこから新しい引き込み線としての血管[*1]の新生を促し，酸素や諸々の栄養の供給を確保し，旺盛な腫瘍細胞全体の増殖を保持している。

上述のように血管新生，つまり新生血管の成長（増殖）は固型がんの特徴の最たるものであり[1]，これによってがんに栄養を供給し，旺盛な増殖を支える。その血管は正常血管のような規則性はなく，不規則で粗雑であり，さらに，血管壁を形成する血管内皮細胞間の間隙が大きく，穴あき状態となり，血液成分は漏出しやすい。この血管内皮細胞間の間隙の大きさは，その腫瘍組織の種類やステージ（進行度）によって異なるが，一般に数百nmから3～4 μmまであるといわれている[2]。これに対して，正常の血管の内皮細胞間の間隙のサイズはわずか2～6 nmである。したがって，直径10～300 nmのナノ粒子のサイズの粒子であれば，腫瘍に栄養を供給する新生血管の内腔側から外側へ漏出するのに好都合な間隙である。ここで重要なのは正常の血管では，このサイズのナノ粒子の薬物（制がん剤）は透過・漏出しないことである。したがって，ナノ粒子型薬物による正常組織に対する傷害はない。このように原理的にはこのようなナノ粒子を化学療法剤として用いれば，がんに対して選択的に薬物を送達できるが，正常の細胞に対しては毒が届かないので，傷害（副作用）がない。〈図1〉にこのことを示している。

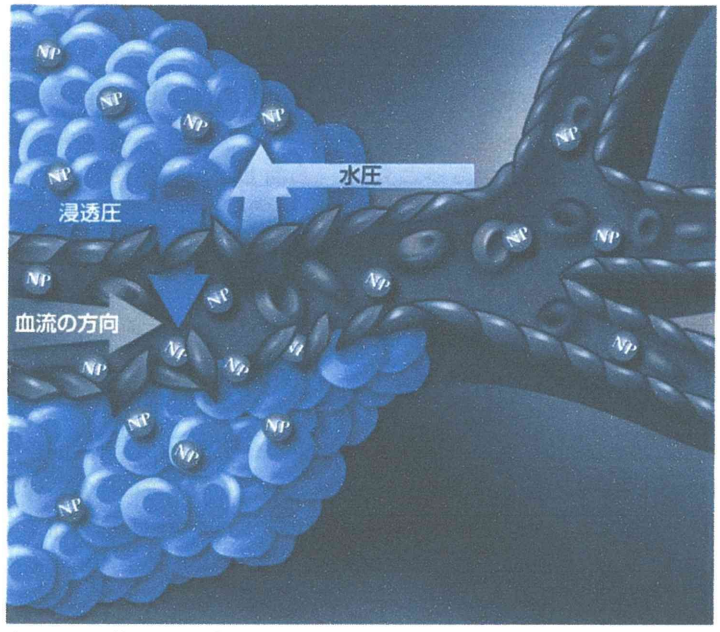
事実，ナノ粒子は選択的にがん組

＊1　［訳注］新生血管＝腫瘍血管。

＊2　［訳注］この現象を前田らはEPR効果と命名した[3]。

＊3　［訳注］分子量では約5万以上。

＊4　［訳注］すなわち，静脈注射により血中に入り，血液循環の途中の血管内皮細胞などに吸着してしまうため，静脈内投与後すぐに血中から消失し，腫瘍部までに到達しない。



（NP：ナノ粒子のこと）

〈図1〉固型腫瘍の血管
固型腫瘍の血管は正常の血管に比べ，その内皮細胞間の間隙が大きいこと，あるいは，その血管の走行が不規則なこと，血流の流れに恒常性がないことなどが特徴である。直径が300 nm以下のナノ粒子はその間隙を容易に純物理的に透過漏出し（permeability），その腫瘍部局所に蓄積（retention）する＊2。（図の縮尺は実際とは異なる）。

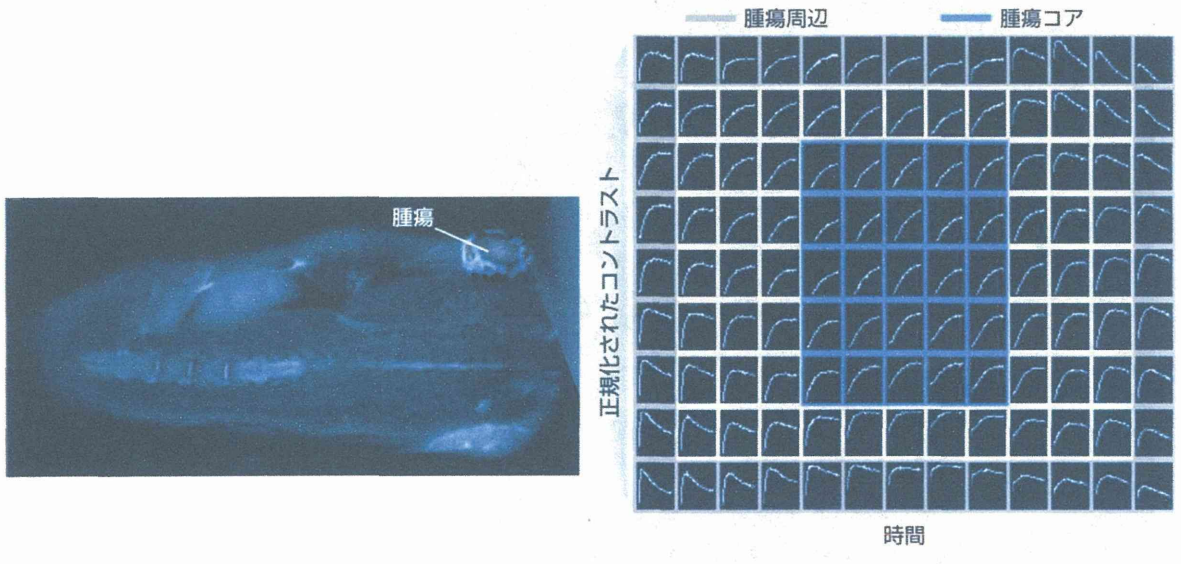
織に純物理的理由により集積する。この現象はがん組織におけるEPR効果（enhanced permeability and retention effect：高分子の血管透過性亢進と滞留性）とよばれている[3]（コラム参照）。〈図2〉に，ナノ粒子ではない低分子性の酸化鉄を静脈注射し，マウス大腿背部に移植した腫瘍における動きを観察した様子を示している。すなわち，この薬剤の腫瘍内部への移動の様子（漏出）を45分間にわたって，追跡，可視化したものである。それによれば，低分子の薬物はそれがんの中心部に到達する頃には，腫瘍周辺からは拡散により消失している。これに対し，〈図3〉に示すように，そのマウスに酸化鉄のナノ粒子を注射すると，今度はそれとは異なり，時間の経過につれてEPR効果により腫瘍全体に集積するのが見られる。投与24時間後でもナノ粒子の腫瘍への集積はまだ増加が続いている（コラム参照）。

ナノ粒子の薬剤の使用にあたって1つ困ったことがある。動物には本来，生体防衛のための免疫系が存在する。ウイルス（や細菌）のような外来性の微粒子体は，白血球の一種，マクロファージ（単核喰食細胞系システム，mononuclear phagocyte system，MPS）によって血中から急速に捕捉除去されてしまう。これは外来性の感染性病原体に対する正常の生体防御の大切な反応の1つである。その結果，静脈内に投与されたナノ粒子はこのMPS系細胞により除去され，さらにMPSにより肝臓や脾臓に運ばれるため，目的のがん組織には集まらないことが多い。

EPR効果を発現しつつ，がん治療を行うためにはナノ粒子はサイズの大きさ＊3以外に，表面の性質を考慮しなければならない。表面の特性はナノレベルの粒子では表面積/体積比が大きいため，とくに重要となる。ナノ粒子を考えるにあたって，次の基本的な2つの要素に限って考えてみると便利である。すなわち，環境に接触しないナノ粒子の中心部と，環境に接するその表面層部である。

すべての細胞の表面にある細胞膜はほとんど陰性荷電を有しており，したがって細胞に対して表面が陽性荷電をもつナノ粒子は細胞に吸着して内容物（薬剤）を細胞内へとり込みやすいといえるが，がん細胞に到達する前に正常組織の細胞に吸着するかもしれない＊4。そのため，一般に中性のポリエチレングリコール（PEG）で被覆（コーティング）し，ステルス化することにより，細胞との相互作用を抑え

＊5　［訳注］浮腫状態という



〈図2〉腫瘍を移植したマウス

腫瘍を移植したマウスにおいて低分子性の造影剤（非ナノ粒子）を静脈内に注射したときのMRI装置で得た画像を示す。右側の各格子は画素（ピクセル）ごとの45分間にわたる画像の経時変化を呈示している。この低分子（造影剤）はまず腫瘍の辺縁に浸透するが，すばやく（拡散により）消失する。中心部へは時間とともに浸透するものの，低分子造影剤は次第に拡散し消失する。左側の図右上部と下部は腫瘍部である。（Courtesy of Marcelino Bernardo and Lilia Ileva.）

るとともにMPSによる捕捉から回避することが可能になることが知られている。その結果，血液の循環中の滞留性が高くなり，EPR効果により，標的腫瘍により多く集まるようになる。PEG鎖の長さとその被覆密度を上げることによって標的腫瘍への到達性を上げることができる[4]（コラム参照）。

ナノメディシン製剤の腫瘍標的へのデリバリー（送達）は腫瘍細胞に至るまでのいくつかの物理的な障壁があり複雑なものである。多くのがん組織ではがん細胞をとり巻き，保護しているがんの間質（組織のこと）といわれる組織に囲まれている。このがんの間質には，線維芽細胞，内皮細胞および免疫系細胞，血管周皮細胞，分泌された増殖因子（タンパク質）などに加えて，細胞外のマトリックス物質（コラーゲン）などが存在する。ある腫瘍では細胞外の高密度に相互架橋したコラーゲン線維でつながっており，これがナノ製剤その他の薬剤の浸透性の障害にもなっている。たとえば，膵臓のように間質が強固で密な場合は薬剤がほとんど入っていかない。そのようながんをもつ患者にとっては化学療法を行っても数か月以上の生存は困難である（コラム参照）。

もう1つのがんへの薬剤の到達性に対する物理的障壁は腫瘍中心部における高い水圧（浸透圧）である。この圧力＊5の高まりが脈管系を圧迫し，とくに固型腫瘍におけるリンパ系回収システムの機能が抑えられる。中心部の圧は周辺部の圧より高く，大きい腫瘍ではその差がとくに大きい。したがって，主たる物質の動きは拡散によることとなり，ナノ粒子の運動も限られてしまう。

近年，抗がん剤の腫瘍へのデリバリーの研究は盛んであるが，その方向の1つは物質輸送の腫瘍物理学ともいえるものである。そこでは，物質輸送の物理的障壁の諸特性ならびに時間的動力学の2つの要因を問題にしている[5]。ハーバード大学医学部（マサチューセッツ総合病院）のラキャシュ・ジェイン（Rakesh Jain）教授はこの基本的問題を研究している1人である。彼によると，このような腫瘍組織内の障壁はナノメディシンの到達性を減弱し，問題であるという。その理由は，周辺部へはEPR効果により効率よく