did not affect the expression of COX-1 and COX-2, suggesting that expression of HSP27 protects gastric mucosa against NSAID-induced lesions through cytoprotection rather than modulation of inhibition of COX by NSAIDs [22].

In order to evaluate the contribution of the HSP-inducing activity of GGA to its anti-ulcer activity, we investigated the effect of GGA in HSF1-null mice. First, we examined the effect of GGA and/or ethanol on gastric mucosal HSP70 expression in wild-type mice, revealing a potent expression induced by ethanol, and a lower level of expression in response to GGA (Fig. 2A). Interestingly, pre-administration of GGA enhanced the ethanol-dependent HSP70 response (Fig. 2A). We confirmed that administration of GGA and/or 40% ethanol did not induce HSP70 (Fig. 2B). Figure 2C shows the effect of pre-administration of GGA on ethanol-produced gastric lesions in wild-type and HSF1-null mice. In order to obtain similar levels of gastric lesions, 100% and 40% ethanol administration were administered to wild-type and HSF1-null mice, respectively. In fact, 40% ethanol administration in HSF1-null mice caused the comparable lesion score as 100% ethanol administration in wild-type mice (Fig. 2C). Pre-administration of GGA significantly suppressed the ethanol-dependent production

of gastric lesions in wild-type mice (Fig. 2C). In contrast, no significant effect was recorded in the HSF1-null mice (Fig. 2C). This result shows that HSF1 is required for the efficacy of the anti-ulcer activity of GGA against ethanol. Overall, the results in Fig. 2 suggest that the loss of the protective effect of GGA in HSF1-null mice is due to the lack of expression of HSPs (such as HSP70); in other words, the HSP-inducing activity of GGA contributes to its anti-ulcer activity.

**The Role of HSF1 and HSPs in IBD-related Colitis.** We examined the role of HSF1 and HSP70 in DSS-induced colitis by use of HSF1-null mice and transgenic mice expressing HSP70 [23]. The severity of DSS-induced colitis can be monitored by various indexes, such as body weight, DAI, length of colon and MPO activity. Administration of 3% DSS caused a mild increase in the DAI but did not affect the body weight of the wild-type mice. In contrast, administration of 3% DSS resulted in a higher DAI score and loss of body weight in HSF1-null mice [23]. DSS-induced colon shortening, used as a morphometric measure for the degree of inflammation, was more severe in HSF1-null mice than in the wild-type mice [23]. Colonic MPO activity, an

indicator of infiltration of leukocytes, was much higher in DSS-administered HSF1-null mice than the wild-type mice [23]. The results show that HSF1-null mice are more sensitive to DSS-induced colitis than their respective wild-type mice. We monitored expression of *hsp* mRNAs in colonic tissues of DSS-administered and untreated HSF1-null mice and wild-type mice by real-time RT-PCR. The expression of *hsp70* but not *hsp25* and *hsp60* mRNAs was significantly lower in the DSS-treated HSF1-null mice than in the wild-type mice [23]. Development of DSS-induced colitis was compared in transgenic mice expressing HSP70 and their respective wild-type mice. DSS-dependent increase in DAI was clearly suppressed in transgenic mice expressing HSP70 compared to the wild-type mice [23]. All of the other indexes of colitis that were tested (colon length and colonic MPO activity) showed that transgenic mice expressing HSP70 are more resistant than the wild-type mice to DSS-induced colitis [23]. The results suggest that HSP70 expression somehow suppresses DSS-induced colitis.

Another group addressed this issue by use of GGA and mice. Ohkawara *et al.* reported that oral administration of GGA (300 – 500 mg/kg) suppressed DSS-induced increase in DAI, colon shortening, increase in colonic MPO activity and colonic

mucosal damage. They also showed that administration of GGA lowered the colonic level of pro-inflammatory cytokines. Furthermore, they showed that GGA up-regulated the expression of HSP70 and HSP40 but not other HSPs in the colons [24]. They also examined the effect of GGA on trinitrobenzene sulfonic acid (TNBS)-induced colitis, another animal model of IBD. Oral administration of GGA (300 mg/kg) suppressed TNBS-induced decrease in body weight, increase in DAI, increase in colonic MPO activity and colonic mucosal damage. They also showed that the survival rate of mice treated with TNBS significantly increased by GGA administration [25]. These results support the idea that HSPs are protective against IBD-related colitis and suggest that non-toxic inducers of HSP expression are therapeutically beneficial for IBD.

Tao *et al.* reported that HSP70-null mice is susceptible to azoxymethane (AOM)/DSS-induced colitis (such as mucosal damage and inflammation). They also showed that AOM/DSS-induced colonic tumors was promoted in HSP70-null mice, compared to wild-type mice [26].

**Mechanism for Protective Role of HSP70 against Colitis.** In order to understand the mechanism governing the decreased susceptibility of transgenic mice expressing HSP70 to DSS-induced colitis, we compared the mRNA expression of various inflammation-related proteins in the colonic tissues [23]. As shown in Fig. 3A, the mRNA expression of *tnf*-α, *il-1*β and *il-6* in colonic tissues was significantly lower in DSS-administered transgenic mice expressing HSP70 than in the wild-type mice, suggestting that HSP70 negatively regulate the expression of the selected pro-inflammatory cytokines under inflammatory conditions. To test this idea *in vitro*, we compared the LPS-stimulated production of the pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) in peritoneal macrophages prepared from the transgenic mice and their wild-type counterparts. LPS stimulated the production of all of these pro-inflammatory cytokines and the levels of not only TNF-α but also of IL-1β and IL-6 were much lower in the medium of the LPS-treated macrophages prepared from transgenic mice expressing HSP70 than from wild-type mice [23]. These results suggest that expression of HSP70 may suppress the production of these pro-inflammatory cytokines under inflammatory conditions.

We compared mRNA expression of *vcam-1, icam-1* and *madcam-1* in the colonic tissues of transgenic mice expressing HSP70 and wild-type mice. The mRNA expression of these CAMs was much lower in the DSS-administered transgenic mice than the wild-type mice (Fig. 3A), suggesting that expression of CAMs is negatively regulated by HSP70 under inflammatory conditions. To test this idea *in vitro*, we examined the effect of siRNA specific for HSF1 or HSP70 on the LPS-induced mRNA expression of the CAMs in bEnd.3 (mouse brain endothelioma) cells. Transfection with HSF1 siRNA up-regulated the mRNA expression of *vcam-1* and *icam-1* but down-regulated that of *madcam-1* in the presence of LPS. Transfection of the cells with siRNA specific for HSP70 did not significantly up-regulate the mRNA expression of the CAMs in the presence of LPS, suggesting that, at least *in vitro*, HSP70 does not negatively regulate the mRNA expression of these CAMs under inflammatory conditions [23].

We compared the level of cell death in the colonic mucosa of DSS-administered transgenic mice expressing HSP70 and the respective wild-type mice by use of the TUNEL assay. Less TUNEL-positive cells were observed in the colonic mucosa of

DSS-administered transgenic mice expressing HSP70 than the wild-type mice (Fig. 3B). The results suggest that ROS-induced cell death associated with DSS-induced colitis is suppressed in transgenic mice expressing HSP70, respectively.

To test the role of HSP70 in ROS-induced cell death *in vitro*, we examined the effect of siRNA specific for HSP70 on cell death induced by menadione, a superoxide anion (a representative ROS) releasing drug, in a colonic cancer cell line (HCT-15). Transfection of cells with siRNA for HSP70 clearly stimulated cell death induced by menadione [23]. The results suggest that HSP70 protects colonic cells from ROS-induced cell death and that this effect may be involved in the improved resistance to DSS-induced colitis that is observed in transgenic mice expressing HSP70.

## DISCUSSION

A number of previous observations have suggested that HSPs and their up-regulation by gastric irritants play an important role in protecting the gastric mucosa against lesion development [4, 6-9, 27]. We found that HSF1-null mice are more susceptible to irritant-induced gastric lesions, providing direct genetic evidence for the

significance of HSPs in ameliorating the outcome of irritant-induced gastric insults (Fig. 4A).

GGA has attracted considerable attention as an HSP-inducer, largely due to its clinical value as an anti-ulcer drug and because it can induce HSPs without affecting cell viability [4]. GGA has been suggested to play a protective role through HSP-induction in a variety of disease states [5, 28]. However, no previous reports have shown that the HSP-inducing activity of GGA contributes to these clinically beneficial outcomes, including its anti-ulcer effects. Using immunohistochemical analysis, we have demonstrated that oral administration of GGA alone up-regulates gastric mucosal HSP70, and that pre-administration of GGA stimulates the ethanol-induced up-regulation of HSP70. Furthermore, we have revealed that pre-administration of GGA suppresses gastric lesions in wild-type mice but not in HSF1-null mice [21]. These results argue strongly in favour of the HSP-inducing activity of GGA contributing to its anti-ulcer effects, providing the first direct genetic link between the pharmacological behaviour of the drug and the resultant clinical outcome.

Because some HSPs have been reported to be over-expressed in the intestinal tissues of IBD patients [18, 29, 30], HSF1 and HSPs are thought to be involved in the pathogenesis of IBD. We have gathered evidence that HSF1 and HSPs have negative roles in the development of IBD (protective roles against IBD) by demonstrating the sensitive phenotype of HSF1-null mice and the resistant phenotype of transgenic mice expressing HSP70 (or HSF1) against DSS-induced colitis, an animal model for IBD [23]. Furthermore, we have examined the molecular mechanisms governing the susceptibility of HSF1-null mice and resistance of transgenic mice expressing HSP70 to DSS-induced colitis, focusing on the expression of pro-inflammatory cytokines and CAMs and ROS-induced cell death both *in vivo* and *in vitro* (Fig. 4B).

Pro-inflammatory cytokines, in particular TNF-α, positively contribute to the progression of IBD [31, 32]. DSS-induced mRNA expression of various pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) in colonic tissues was inhibited in transgenic mice expressing HSP70 [23]. We consider that this inhibition of cytokine mRNA expression is responsible for the DSS-induced colitis phenotypes exhibited by the mice. The LPS-induced production of not only TNF-α but also IL-1β and IL-6 was

inhibited in peritoneal macrophages prepared from transgenic mice expressing HSP70 [23], suggesting that HSP70 suppresses the production of these pro-inflammatory cytokines under inflammatory conditions (Fig. 4B). We speculate that this suppression is mediated by HSP70-dependent inhibition of nuclear factor kappa B (NF-κB), which plays an important role in the induction of inflammation. It is known that NF-κB positively regulates expression of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6. Furthermore, it is also known that up-regulation of HSP70 expression by heat shock inhibits the inflammatory stimuli-dependent activation of NF-κB through various mechanisms [33-39].

CAMs also positively contribute to the progression of IBD hrough recruitment of blood circulating leukocytes into inflamed intestinal tissues [17]. DSS-administration induced mRNA expression of CAMs that are mainly expressed on vascular endothelial cells and of those on leukocytes. However, HSF1 deficiency only affected mRNA expression of CAMs on vascular endothelial cells [23], suggesting that these CAMs rather than those on leukocytes contribute to the greater sensitivity of HSF1 null mice, relative to their wild-type controls, to DSS-induced colitis. The DSS-induced mRNA

expression of ICAM-1, VCAM-1 and MAdCAM-1 was suppressed in transgenic mice expressing HSP70 [23], and this may be involved in conferring resistance to DSS-induced colitis (Fig. 4B).

Colonic mucosal cell death induced by ROS released from activated leukocytes is thought to be directly responsible for the pathogenesis of human IBD [14]. Analysis using the TUNEL assay revealed that cell death in colonic mucosa was inhibited in transgenic mice expressing HSP70, respectively. This correlates with other parameters for DSS-induced colitis, however, it was not clear whether these alterations to cell death cause or result from the progression of DSS-induced colitis. Given that transfection with siRNA for HSP70 stimulated ROS-induced cell death *in vitro* [23], this result suggests that HSP70 protects colonic mucosal cells from ROS-induced cell death, which seems to contribute to the lower level of cell death seen in the colonic mucosa of DSS-administered transgenic mice expressing HSP70 (Fig. 4B).

The results of this study suggest that non-toxic inducers of HSP expression are therapeutically beneficial for IBD. Supporting this notion, GGA suppresses both DSS- and TNBS-induced colitis [24, 25]. However, the ability of GGA to induce HSP

expression is not strong which may explain its relatively weak effect on these types of colitis [24, 25]. Therefore, we propose that more potent non-toxic HSP inducers would be therapeutically beneficial for IBD.

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## FIGURE LEGENDS

**Fig. 1.**

Production of gastric lesions and expression of HSPs in wild-type and HSF1-null mice. Wild-type (WT) and HSF1-null mice (HSF1-KO) were orally administered the indicated doses of ethanol (A, C) or hydrochloric acid (B). After 4 h, the stomach was removed and scored for hemorrhagic damage. Values are mean ± S.E.M. (n=4-6). *$P<0.05$ (A, B). After 4 h, the gastric mucosa was removed, and protein extracts were prepared and analysed by immuno-blotting with an antibody against HSP25, HSP60, HSP70, HSP90, or actin (C). This figures was published previously and is reprinted here with permission of the journal [21].

**Fig. 2.**

Effect of ethanol and/or GGA on expression of HSP70 and production of gastric lesions. Wild-type (WT) (A, C) and HSF1-null (HSF1-KO) (B, C) mice were orally pre-administered 200 mg/kg GGA (10 ml/kg as emulsion with 5% gum arabic), 1 h after

which they were orally administered with the indicated doses of ethanol. After 4 h, sections of gastric tissues were prepared and subjected to histological examination (H & E) and immunohistochemical analysis with an antibody against HSP70 (A, B). After 4 h, the stomach was removed and scored for hemorrhagic damage. Values are mean ± S.E.M. (n=3-6). $^*P<0.05$. n.s., not significant (C). This figures was published previously and is reprinted here with permission of the journal [21].

**Fig. 3.**

The mRNA expression of various genes and levels of cell death in colonic mucosa. Transgenic mice expressing HSP70 (HSP70 Tg) and wild-type mice (WT) were treated with or without 3% DSS for 7 days (A, B). Relative mRNA expression of each gene in colonic tissues was monitored and expressed. Values are mean ± S.E.M. (n=3-6). $^{**}P<0.01$; $^*P<0.05$ (A). Sections of colonic tissues were prepared and subjected to TUNEL assay and DAPI staining (B). This figures was published previously and is reprinted here with permission of the journal [23].

**Fig. 4.**

The proposed mechanism for protective role of HSF1 and HSP70 against gastric ulcer (A) and IBD (B).

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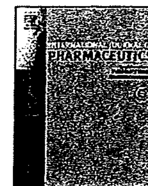

Pharmaceutical Nanotechnology

# Preparation and characterization of a nanoparticulate formulation composed of PEG-PLA and PLA as anti-inflammatory agents

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ABSTRACT

We have prepared polymeric nanoparticles using a blend of poly(lactic acid) and monomethoxy-polyethyleneglycol(PEG)-polylactide block copolymer along with betamethasone disodium phosphate (BP). Nanoparticles have been screened for anti-inflammatory activity using experimental rat models of inflammation. In the present study, we examined the degradation of nanoparticles in vitro during incubation. We found that the nanoparticles lost the PEG chains present on their surfaces within a few days, and subsequently gradually released BP. Furthermore, we found that these nanoparticles preferentially accumulated in the inflammatory lesion in adjuvant arthritis rat models, and that the amount of BP gradually depleted from the lesion over 14 days. These results suggested the mechanism underlying the anti-inflammatory effect of the nanoparticles in vivo: the initial accumulation of BP in the lesion due to the enhanced permeability and retention effect, the subsequent internalization in inflammatory macrophages due to the loss of PEG, and the release of BP in cells during the hydrolysis of polymers. The nanoparticles were successfully prepared on a large-scale and stably stored in the form of a freeze-dried formulation for at least 69 weeks below 25 °C. These results suggest that the nanoparticles can be used as an anti-inflammatory pharmaceutical formulation in a clinical setting.

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## 1. Introduction

Great efforts have been made to develop colloidal carriers such as liposomes, solid nanoparticles, polymeric micelles, and lipid emulsions so that they can be used as pharmaceutical agents for expanding the utility of drugs in a clinical setting (Yih and Al-Fandi, 2006; Peer et al., 2007). Since the last 2 decades, studies have particularly focused on the stealth-type carriers (Moghimi et al., 2001). Polyethyleneglycol (PEG) has been widely used for modifying the surface of the stealth-type carriers because the PEG chains on the surfaces of these carriers act as a steric barrier and reduce interaction with opsonins and cells of the mononuclear phagocyte system; as a result, the carriers remain in the blood circulation for a prolonged duration. These stealth-type (long-circulating) carriers show preferential accumulation in tumors and sites of inflammation because of the enhanced permeability and retention (EPR) effect (Maeda et al., 2000).

In our previous report, we described the preparation of polymeric nanoparticles from a blend of poly(D,L-lactic acid-co-glycolic acid)/poly(D,L-lactic acid) (PLGA/PLA) homopolymers and PEG-poly(D,L-lactide-co-glycolide)/poly(D,L-lactide) (PEG-PLGA/PLA) block copolymers (Ishihara et al., 2009a,b). Although the efficient encapsulation of drugs in these solid nanoparticles remains a challenging task, water-soluble betamethasone disodium 21-phosphate (BP) could be efficiently encapsulated in the nanoparticles by a unique technique involving the use of zinc ions (Ishihara et al., 2009a). Furthermore, by controlling the blend ratio and the compositions or molecular weights of the polymers, various types of nanoparticles with different drug loading capacities and diameters and PEG chains of different lengths or densities at the surfaces of the nanoparticles could be easily prepared (Ishihara et al., 2009b). It has been found that these parameters significantly influence the stealthiness of the nanoparticles and the release of BP in vitro. It is assumed that the therapeutic efficiency of these nanoparticles in vivo is influenced by multiple complex factors such as the systemic distribution, local distribution, and release behavior of BP. Therefore, nanoparticles with the highest anti-inflammatory activity were finally screened using experimental rat models of inflammation (Ishihara et al., 2009c). The nanoparticles (diameter, approximately 120 nm) that were composed of the PLA (Mw: 6170) homopolymer and a block copolymer of PEG (Mw: 5580) and PLA (Mw: 9430) (PEG content in the polymer blend: 10 wt.%) exhibited the highest anti-inflammatory activity.

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