CHAPTER 4

DISCUSSION

The present experiments were designed to validate the use of curcumin in various gastroinflammatory diseases: reflux esophagitis, gastritis, peptic ulcer and ulcerative colitis. The therapeutic dosage level of curcumin for gastrointestinal inflammatory diseases is 20-80 mg/kg. Systemic administration of curcumin, at the dose of 20 mg/kg, definitely inhibited the appearance of acute acid and mixed reflux esophagitis. Although curcumin had a less potent antiulcer effect than did lansoprazole against acid reflux esophagitis, it was more potent against mixed reflux esophagitis. Systemic administration of curcumin at doses between 20 and 80 mg/kg/d effectively inhibited the development of acute gastritis in different rat models and also accelerated the healing of chronic gastric ulcer induced by acetic acid in rats. The prevention of acute gastric damage by curcumin is effective at doses lower than 40 mg/kg probably by involvement of its increase in mucin secretion, antioxidant property, and inhibition of iNOS. On the other hand, the acceleration of healing of chronic gastric ulcer by curcumin is effective even at higher doses than 40 mg/kg because of its inhibition of iNOS and cytokines controlled inflammatory mechanism. However, oral administration of curcumin, at the dose of 20-80 mg/kg, had no preventive effect against the development of colon inflammation in DSS-induced colitis.

In the pylorus-ligated rat model, curcumin reduced the basal gastric acid secretion. It is known that gastric mucosal constitutive nitric oxide synthase (cNOS) regulates gastric mucus synthesis and secretion as well as gastric mucosal microcirculation. However, the overproduction of nitric oxide by leukocytes through the induction of iNOS activity has been recently found to play an important role in depressing gastric acid secretion and inducing gastric lesions on ischemia-reperfusion in the pylorus-ligated rat (Rastogi, *et al.*, 1998; Tanaka, *et al.*, 2001). It has been also found that pretreatment with aminoguanidine, a selective iNOS inhibitor, prevents lesion formation in rats, although it augments the

gastric acid output that is depressed by ischemia-reperfusion (Tanaka, *et al.*, 2001). These findings, with the present results, suggest that curcumin at doses of more than 40 mg/kg partially attenuates the antiulcerogenic effect through the increase in acid secretion.

Acid reflux esophagitis induced by the 6-hour simultaneous ligation of the pylorus and the limiting ridge was limited to the erosive and/or ulcerative type, and was used as an experimental model for human acute esophagitis (Nakamura, *et al.*, 1982). The reflux of gastric contents containing enough pepsin activity has been implicated as the principal causative factor for the ulcer formation, while the presence of acid aggravates the ulcer formation by its corrosive property and by keeping an optimum environment for pepsin activity. Agents that neutralize or make the gastric contents definitely alkaline or those that reduce pepsin activity or have mucosal protecting action were found to prevent the ulcer formation (Nakamura, *et al.*, 1982). There is still much controversy about the identities of the main pathophysiologic factors that induce esophagitis. Chronic acid reflux esophagitis induced by ligating the limiting ridge and covering the duodenum near the pylorus ring with a suitable catheter, produces the long-term survival rate of the animals with histologic features of esophagitis that correspond to those of typical human chronic reflux esophagitis (Omura, *et al.*, 1999). The regurgitation of the gastric acid has also been found to be associated with ulcer formation, however, the exact pathophysiological mechanisms of esophageal damage during continuous exposure to gastric contents needs to be further investigated. Lansoprazole, at the dose of 1 mg/kg/day, that is nearly the same as the normal clinical dose, has been reported to exert potent antiulcer activities by suppressing acid secretion through an inhibition of a proton pump in gastric parietal cells without any effect on pepsin secretion (Satoh, *et al.*, 1989). It has also been reported to exert a gastroprotective activity by scavenging hydroxyl radical but not by acting through prostaglandins or the endogenous nitric oxide mediated pathway (Biswas, *et al.*, 2003; Chandranath, *et al.*, 2002). DMSO, at the dose of 1 ml/kg/day, acts as an effective neuroprotectant in focal cerebral ischemia in rats (Shimizu, *et al.*, 1997) and is safe for long-term administration (Devasena, *et al.*, 2003). It has been recently implicated as a potent antioxidative agent acting as a specific hydroxyl radical scavenger (Wood and Wood, 1975), and as an inhibitor of NF-κB activation (Chang, *et al.*, 1999). Furthermore, it

has been found to exert a potent antiulcerogenic activity against stress- or indomethacin-induced gastric lesions without affecting the luminal acid content (Biswas, *et al.*, 2003). As lansoprazole has a more potent potential to prevent acute or chronic acid esophagitis than does DMSO or curcumin, this indicates that hydroxyl radicals as well as gastric acid, play an important role in the pathophysiological mechanisms of acid reflux esophagitis. Thus, the similar mechanisms are considered to occur by the co-administration of curcumin and DMSO through antisecretory or mucus stimulating effect of curcumin and radical scavenging effect of DMSO in the chronic acid reflux esophagitis experiment. The explanations for the failure of iNOS inhibitors to prevent ulcer formation relate to the ability of iNOS-derived NO to attenuate the leukocyte adhesion and the recruitment of leukocytes in postcapillary venules during acute inflammation (Kawachi, *et al.*, 1999), and in promoting the restitution and repair of the gut mucosal barrier (McCafferty, *et al.*, 1997). A dose of 40 mg/kg of curcumin is also considered to produce a significant inhibition of iNOS (Chan, *et al.*, 1998), similarly to aminoguanidine. Hence, a regimen, that combines both a potent hydroxyl radical scavenger and antisecretory drugs, may be beneficial in the prevention and treatment of acid reflux esophagitis, whereas the continuous administration of an inducible nitric oxide synthase inhibitor will aggravate the severity of esophagitis formation. Accordingly, the preventive effect of curcumin against esophagitis formation at a dose of less than 40 mg/kg may be mainly due to its ability to increase mucus secretion (Sinha, *et al.*, 1975), its antioxidant activity through the quenching of singlet oxygen (Das and Das, 2002) and by the inhibition of NF-κB (Chan, 1995; Jobin, *et al.*, 1999; Pan, *et al.*, 2000; Singh and Aggarwal, 1995) which controls the expression of various cytokines and the expression of many genes that are related to inflammatory responses. It has also been confirmed from this study that curcumin is not an effective hydroxyl radical scavenger.

Several experimental studies have provided confirmative evidence that mixed reflux of gastroduodenal contents into the esophagus produces more free radical damage in the esophageal mucosa than pure acid reflux, and antioxidant treatment or anti-neutrophil serum is important in the prevention of esophageal mucosal damage (Kauer, *et al.*, 1995; Lee, *et al.*, 2001; Oh, *et al.*, 2001; Stein, *et al.*, 1992; Tomatsuri, *et al.*, 2001; Wetscher, *et al.*, 1995). From the present study, the suppression of iNOS activity has been found to

have no preventive effect against the esophagitis formation. Although the macroscopical findings such as the presence of hemorrhagic ulcers or lesions could not be clearly observed on the sixth postoperative hour, the histological findings have a great deal in common with those of the histological criteria used to diagnose acute reflux esophagitis in the squamous epithelium. Apart from lysolecithin and trypsin in the duodenal contents, bile acids are the most likely noxious agents and their effects depend on the surrounding pH and their degree of conjugation (Vaezi, *et al.*, 1995). Under the normal duodenal pH of approximately 7, more than 90% of bile acids are conjugated with either taurine or glycine. This results in an increased solubility and ionization of bile acid. With pH ranges of 2 to 7, a lipophilic, nonionized acid is present and can move through the esophageal mucosal barrier into the esophageal mucosal cells. Under the normal physiological acidic gastric environment, bile acids precipitate and have minimal effects. If bile acids move into the esophageal mucosal cells, they will ionize and damage the mitochondria (Krahenbuhl, *et al.*, 1994), activate oxidative stress-related genes (Bernstein, *et al.*, 1999), and activate ornithine decarboxylase (Furihata, *et al.*, 1987) plus cyclooxygenase 2 in an AP1-dependent mechanism through the protein kinase C pathway (Zhang, *et al.*, 1998). Furthermore, a combination of bile acids, hydrochloric acid, and even a low concentration of pepsin, results in further esophageal mucosal damage by increasing the mucosal permeability. This results in back-diffusion of hydrogen ions and an increase in mucosal injury by the proteolytic activity of pepsin, which causes the detachment of the surface cells from the epithelium (Salo, *et al.*, 1983). Clinically, Kauer and colleagues have indicated that the vast majority of duodenal reflux in patients with gastroesophageal reflux disease occurs at a pH of 4 to 7, at which bile acids are in unionized lipophilic form and are capable of damaging the esophageal mucosa (Kauer, *et al.*, 1995). These authors also suggest that the alterations in the gastric pH environment caused by acid-suppressive therapy may actually increase potential esophageal injury. These changes may also encourage metaplasia by increasing the reflux of gastric juice containing nonionized lipophilic form of bile acids and activate duodenal enzymes (Kauer, *et al.*, 1995). This may explain why lansoprazole exacerbates the incidence and the severity of histological changes as shown in the present study. In contrast, the beneficial preventive effect of curcumin on

either the incidence or the severity of histological changes may be due not only to its antioxidant activity and its potent inhibitory activity on NF-κB activation, but also to its anti-inflammatory activity in inhibiting all branches of the arachidonic acid cascade (Ammon, *et al.*, 1993). Consequently, this confirms that curcumin does not reduce the acid output through its direct inhibitory activity of acid secretion. Although it has been reported that the intravenous injection of curcumin (25 mg/kg) exerts a choleretic effect in animals (Deters, *et al.*, 2000), evidence for a similar effect after oral administration has not been determined. In addition, prolonged oral administration of a high dose (100 mg/kg) shows no choleretic effect in cholestasis rats induced by cyclosporine (Deters, *et al.*, 2002). Since chronic duodenal contents refluxing into the esophagus induces severe oxidative damage and this is related to carcinogenesis and a columnar lined esophagus (Barrett's esophagus), curcumin possibly plays a chemopreventive role by preventing the development of esophageal carcinoma and Barrett's esophagus.

Both curcumin and aminoguanidine prevented ethanol–induced gastric mucosal lesions. Cimetidine also showed a marked protection against this model. Acute gastric lesions caused by ethanol have been indicated to be due to not only the gastric acid but also the impairments in defensive factors such as mucin content (Kuwata, *et al.*, 1985), mucosal microcirculation (Trier, *et al.*, 1987), and scavenging of free radicals generated from xanthine oxidase (Mutoh, *et al.*, 1990) and neutrophils (Tepperman and Soper, 1990) in the gastric mucosa. The important free radicals in producing acute gastric lesions are well known to be superoxide radical, hydroxyl radical, and cytotoxic peroxynitrite compound that was formed in the presence of excessive NO generated from the induction of iNOS activity and superoxide radicals. The gastroprotective effect of cimetidine is independent of its action on gastric acid secretion and may be related to its powerful activity in scavenging hydroxyl radical which is more reactive and toxic to tissue than superoxide radical (Uchida and Kawakishi, 1990). The pathogenesis of acute gastric lesions induced by repeated subcutaneous administration of serotonin for 4 days in rats was thought to be through the up-regulation of iNOS/NO system, the formation of oxygen radicals and the generation of cytotoxic peroxynitrite compound (Yasuhiro, *et al.*, 1997). The model of acute gastric mucosal lesions with a single compound 48/80 treatment in rats was indicated as kinds of

ischemia-reperfusion, oxidative stress, and inflammation-induced injury which resemble varioliformed gastritis occurring in humans (Ohta, *et al.*, 1990; 1997). It has also been indicated that acutely released endogenous serotonin at 0.5 hour after compound 48/80 administration caused gastric mucosal lesions, while released endogenous histamine from mast cells mainly contributed to the lesion development by enhancing neutrophil infiltration into the gastric mucosal tissue via histamine H_1 receptor (Ohta, *et al.*, 1990). Moreover, cimetidine has been found to have no preventive effect against the lesion development in both models. The increase in free radical nitric oxide (peroxynitrite) production by iNOS has also been found to be associated with the development of lesions (Yasuhiro, *et al.*, 1997). In the present study, the beneficial effect of aminoguanidine against the development of lesions in both models confirms the important role of iNOS in enhancing the lesion progression. Curcumin, at the oral dose of 20-80 mg/kg, exerted the dose-dependent preventive effect on gastric lesion formation in both models, although it possesses anti-inflammatory activity resulting from a blockade of all branches of the arachidonic acid pathway (Ammon, *et al.*, 1993). This preventive effect of curcumin is consistent with the findings reported by several authors that the inhibition of prostaglandins synthesis is unlikely to be the mechanism responsible for the inflammatory ulceration formation, and curcumin may exert its preventive effect through its antioxidant activity and its potent inhibitor of NFκB activation including its potent suppressive effect on mast cell degranulation (Yano, *et al.*, 2000). Although there is a potential increase of gastric acid secretion with a high dose of curcumin, gastric acid secretion has been found to play little role in the pathogenesis of this model (Ohta, *et al.*, 1999).

The model of chronic gastric ulcer induced by topical application of acetic acid in rat resembles the human chronic gastric ulcer both grossly and histologically (Okabe, *et al.*, 1971). It has been implicated that the proliferation of mucosal cells, neovascularization, adequate mucosal blood flow, and growth factors such as transforming growth factor or epidermal growth factor contribute to the healing process of the produced ulcer (Ohta, *et al.*, 1999; Szabo and Vincze, 2000). Recently, oxygen free radicals derived from infiltrated neutrophil, have been found to exert an inhibitory effect on the healing of ulcer, and that the reduction of neutrophil infiltration into ulcerated tissues or the inhibition of enhanced

lipid peroxidation in ulcerated tissues promotes the ulcer healing process (Motilva, *et al.*, 1996). In the present study, both cimetidine and aminoguanidine were found to possess a significant healing-promoting effect on acetic acid-induced chronic gastric ulcer, suggesting the role of overproduction of nitric oxide by iNOS of activated macrophage in the development of the ulcer and the involvement of gastric acid in aggravating gastric ulcer. Curcumin, at the oral dose of 20-80 mg/kg/day, also exhibited an apparent healing-promoting effect with the enhancement of the regeneration of the mucosal layer on the acetic acid-induced gastric ulcer. This effect was not seen in a dose-related fashion, presumably through some curative mechanisms differently modified by curcumin dosage. The therapeutic effect occurred at doses more than 40 mg/kg which did not show preventive effect against ethanol-induced acute gastric mucosal lesion models. Accordingly, it is conceivable that the preventive and curative effect of curcumin in various gastritis models and gastric ulcer occurs at least partly through its increased mucin secretion, inhibition on cytokine mediated inflammatory mechanism, suppression of iNOS activity, and antioxidant activity as mentioned above. In addition, the curative properties in the healing of the produced ulcer might result from the elevation of EGF and TGF-β1 as shown in acceleration of the cutaneous wound healing in rats, guinea pigs and mice (Sidhu, *et al.*, 1998).

On the other hand, curcumin, at the dose of 20-80 mg/kg, *p.o.*, has not been found to exert any preventive efficacy against the development of colon inflammation in DSS-induced colitis. Neither improvement of mortality rate, body weight loss nor morphological changes was observed in curcumin therapy after the DSS-induced colitis as well. Moreover, curcumin tended to exacerbate colon tissue damage at doses over than 80 mg/kg. DSS-induced colitis in mice is an experimental model in which the morphological changes correspond well to the clinical signs of human ulcerative colitis and which has a number of advantages over other models, such as simplicity of the experimental method, reproducibility of the time course of development and severity of the colitis among individual mice, and relative uniformity of the lesions. Therefore, this model has served as a reliable model for studying the pathogenesis of ulcerative colitis and screening of drugs for use in the treatment of ulcerative colitis (Elson, *et al.*, 1995; Dieleman, *et al.*, 1994;

Murthy, *et al.*, 1993; Okayasu, *et al.*, 1990). Although, the precise mechanism of the induction of acute experimental colitis by DSS remains unclear, there are suggestions that DSS primarily injures epithelial cells and then activates various immune and inflammatory cells through chemical mediators including cytokines, free radicals and nitric oxide in the latter phases of the response. Cytokines that are thought to be important factors in the inflammatory cascade in DSS-induced colitis are TNF-α, IL-1 and IL-18 (Okayasu, *et al.*, 1990; Elson, *et al.*, 1995; Siegmund, *et al.*, 2001). Due to the opposing actions of $O_2^{\cdot}$ and $NO^{\cdot}$, which may result from their ability to chemically react with and decompose each other, it is widely held that conditions which alter the balance between $O_2^{\cdot}$ and $NO^{\cdot}$ level may promote colon inflammation. Nevertheless, a number of studies suggest that reactive oxygen and nitrogen species may not play an important role in initiating and/or perpetuating colitis. Phagocyte-derived reactive oxygen metabolites have also been found later to play little or no role in the presence of iNOS in DSS colitis (Blackburn, *et al.*, 1998). In addition, NADPH oxidase (superoxide anion) is reported not to be involved in the pathophysiology of DSS-induced colitis (Krieglstein, *et al.*, 2001) A beneficial effect of superoxide dismutase treatment in humans has been shown to have limited benefit as well (Emerit, *et al.*, 1991). Similarly, inhibitors of iNOS have been reported to yield mixed results in various experimental models of colitis including beneficial effect (Rachmilewitz, *et al.*, 1995), little effect (Hogaboam, *et al.*, 1995), no effect (Conner, *et al.*, 1995) or exacerbation of experimentally induced inflammation (Pfeiffer and Qui, 1995). Recently, conflicting results have been reported using mice that are genetically deficient in iNOS but have normal levels of cNOS (Zingarelli, *et al.*, 1999). Although peroxynitrate anion ($ONOO^{-}$), formed by the interaction of superoxide with NO, has previously been implicated as a cytotoxic agent, one study has reported that nanomolar concentrations of $ONOO^{-}$ both inhibit leukocyte-endothelial cell interaction and exert cytoprotective effects in myocardial ischemia-reperfusion injury (Lefer, *et al.*, 1997). Later, it has also been reported that NO, including that derived from iNOS, has a protective role against inflammation in Hapten 2,4,6-trinitrobenzene sulphonic acid (TNBS) and DSS induced colitis especially in the early period of inflammation in a similar manner to supplementation with nitric oxide donors (McCafferty, *et al.*, 1999; Yoshida, *et al.*, 2000). It is possible that increased NO

production from iNOS during this early period of acute inflammation may act to limit inflammation by one or more of these mechanisms including reducing neutrophil adhesion (Hickey, *et al.*, 1997), free radical scavenging (Gaboury, *et al.*, 1993; Rubanyi, *et al.*, 1991; Wink, *et al.*, 1993), and stabilizing mast cells (Kanwar, *et al.*, 1994; Kubes, *et al.*, 1993). It is also conceivable that iNOS induced in neutrophil under normal conditions is a normal response to invading organisms and may be a method of maintaining homoeostasis in the intestine. Nevertheless, it has been found that excess of NO may likewise aggravate inflammation in the intestinal mucosa (Yoshida, *et al.*, 2000). Thus, the beneficial efficacy of iNOS inhibitor against ulcerative colitis greatly depends on the dose and the time period of administration and/or type of intestinal inflammation. The present results of curcumin differed considerably from the study using curcumin in TNBS or dinitrobenzene-induced colitis in murine model, where an attenuation of the inflammation was observed (Salh, *et al.*, 2003; Sugimoto, *et al.* 2002; Ukil, *et al.*, 2003). The discrepancy of the study result may be explained by the fundamental differences between the DSS and TNBS model of colitis. Colitis develops within thirty minutes after intrarectal administration of TNBS and healing is continuous after the initial injury with no relapse. In addition, the pathogenesis of TNBS-induced colitis is a Th1 response and immunological more resembles Chron's disease (Fuss, *et al.*, 1996; Parronchi, 1997). Alternatively, the colitis develops gradually in response to continuous administration (7 d) of DSS since neutrophils do not accumulate to a significant degree at an earlier stage. Furthermore, IL-18 and IFN-γ have been consistently suggested to be contributed to the pathology of DSS-induced colitis. Therefore, different pathophysiological mechanisms may be involved in tissue repair after a single insult when compared with a continuous insult.

Further investigation was held to clarify the relationship between the effect of curcumin and various factors which have been known to contribute to the pathogenesis of upper gastrointestinal inflammatory diseases and the healing process of ulcer healing. To investigate whether the *in vivo* curcumin concentration achieved in the inflammatory pathological condition might effectively inhibit important inflammatory mediators, the effect of curcumin on the level of iNOS, COX-2, TNF-α and IL-1β in the gastric ulcerated area of acetic acid-treated rats was evaluated. The period of day 1 to day 3 after ulcer

induction was defined as the inflammatory stage of gastric ulceration characterized with accumulation of neutrophils. The period from day 3 onwards was considered to be the healing stage of gastric ulceration characterized with the intensive proliferation of epithelial cells at the ulcer margin and the development of granulation tissues with angiogenesis. During the healing process, the activated and accumulated inflammatory cells in the lesions are destroyed by apoptosis to promote resolution of acute inflammation (Savill, 1997). Apoptosis also plays an important role in remodeling the inflamed site by destruction of myofibroblasts. Several previous studies have investigated the role of inflammatory proteins and healing-related factors in gastric ulcer induced with acetic acid in rat. It has been observed that, after ulcer induction, cNOS activity is decreased while iNOS activity is increased 6 hours after ulcer induction in the inflammatory cells at the ulcer base and ulcer margin, with the maximal level of expression at day 3 and declining by day 6. Significant level of expression is remained at day 10 after ulcer induction (Akiba, *et al.*, 1998, Ma and Wallace, 2000). In contrast, eNOS expression is found to be relatively stable (Guo, *et al.*, 2003) or slightly increased after ulcer induction and peaks at day 6 (Ma and Wallace, 2000). This suggests that NO generated from iNOS in inflammatory cells participates in the inflammatory process and ulcer formation, however, an amount of NO generated from iNOS is needed to produce a beneficial effect on ulcer healing by producing apoptosis in inflammatory cells in the regenerating mucosa, thereby eliminating iNOS–positive inflammatory cells during ulcer healing (Akiba, *et al.*, 1998). Recent studies have shown that the expression of COX-2 is detected around the ulcer crater on day 3 after ulcer induction and remained at high level during the late ulcer healing stage with marked elevation of enzyme activity on day 15 (Berenguer, *et al.*, 2002; Guo, *et al.*, 2003). It has been reported that non-selective as well as selective COX-2 inhibitor delays ulcer healing and prevents regeneration of the mucosa, maturation and angiogenesis in the ulcer base (Berenguer, *et al.*, 2002; Brzozowski, *et al.*, 2001; Shigeta, *et al.*, 1998; Sun, *et al.*, 2000). Moreover, the expression of COX-2 mRNA and protein is found to be up-regulated at the ulcer margin in a temporal and spatial relation to enhance epithelial cell proliferation and increase expression of growth factors (Halter, *et al.*, 2001). Thus, COX-2 represents (in addition to COX-1) a further line of defense for the gastrointestinal mucosa necessary for

maintenance of mucosal integrity and ulcer healing. High expression of IL-1β has also been observed in fibroblasts and macrophage/monocytes in the upper portion of ulcer base on day 3 after ulcer induction, with the level decreasing thereafter associated with ulcer healing (Takahashi, *et al.*, 1999). Importantly, it was found that an amount of IL-1β is needed since blocking of endogenous IL-1β by administration of IL-1 receptor antagonist caused a significant impairment of ulcer healing accompany with a decrease level of iNOS, COX-2, cytokine-induced neutrophil chemoattractant (CINC)-1, hepatocyte growth factor (HGF) and basic fibroblast growth factor (bFGF). CINC plays a crucial role in activation and increase infiltration of neutrophils into the ulcerated area to eliminate wounded cells, cell debris, and extracellular matrix proteins before tissue regeneration (Fujita, *et al.*, 1998; Watanabe, *et al.*, 1993; Yamada, *et al.*, 1999). HGF involves in mucosal regeneration by stimulating the growth of rat gastric epithelial cells (Kinoshita, *et al.*, 1995), whereas bFGF stimulates gastric fibroblast proliferation (Watanabe, *et al.*, 1995) and promotes angiogenesis (Satoh, *et al.*, 1997). The present study showed that iNOS, COX-2 or TNF-α expression level had still been detected on day 15 after ulcer induction in the vehicle-treated rats. Oral administration of curcumin 80 mg/kg/d significantly reduced the ulcer size. Weaker ulcer curative activity was found in the specific iNOS inhibitor (aminoguanidine)-treated rats. It has been found that oral administration of low dose aminoguanidine (10 mg/kg) reduces ulcer size 3 days after ulcer induction significantly, but does not further reduce ulcer size 1 week after induction, because of its reversible inhibition of iNOS activity without any effect on iNOS expression (Akiba, *et al.*, 1998; Griffiths, *et al.*, 1993.). In addition, it increases iNOS–positive inflammatory cells and epithelial cells in the healing stage of ulceration. The present study showed that daily subcutaneous administration of aminoguanidine (30 mg/kg) inhibited only iNOS protein expression but not iNOS mRNA expression. A significant increase of IL-1β induction was also found, indicating an increase of inflammatory cells in the healing stage by aminoguanidine. It has been reported that dexamethasone (an inhibitor of iNOS expression) exhibited the similar result on gastric ulcer healing as found in aminoguanidine-treated rats, though it reduces the number of inflammatory cells and does not increase the number of apoptotic inflammatory cells. Thus, an agent with only iNOS inhibitory activity is not a

potential agent in treatment of gastric ulcer. In contrast to aminoguanidine, curcumin was found to decrease the expression level of both iNOS and TNF-α. Curcumin significantly increased the expression level of COX-2 and IL-1β at the ulcer area in a dose dependent manner. Thus, there may be an important regulatory link among iNOS, TNF-α, COX-2 and IL-1β during the healing of gastric ulcer. A number of studies have indicated that iNOS and COX-2 can modulate the expression of enzymes responsible for the synthesis of one another (Martin, *et al.*, 2001; Salvemini, *et al.*, 1993, 1996; Swierkosz, *et al.*, 1995). In circumstances in which the production of one of these mediators is suppressed, there are compensatory increases in the production of the other (Martin, *et al.*, 2001). Apart from the effect on pro-inflammatory cytokines, oral administration of curcumin (40 mg/kg/d) is found to accerelate cutaneous wound healing in rats through increasing mRNA transcript of TGF-β1 (Sidhu, *et al.*, 1998). TGF-β1 is known as anti-cytokine in counteracting the effects of pro-inflammatory cytokines or shutting off immune and inflammatory response (Abbas, *et al.*, 1997). Furthermore, TGF-β1 is important in wound healing *in vivo*, as it increases angiogenesis; epithelial migration and proliferation (keratinocyte); production of collagen and fibronectin by fibroblasts; and the rate of formation of granulation tissue. It may be possible that TGF-β1 supports other healing-related factors in enhancement of gastric ulcer healing by curcumin. The study on gastric ulcer induced by acetic acid in rat has also shown that curcumin (40 mg/kg, bid) promoted the greatest enhancement of ulcer healing and mucosal regeneration in the ulcerated portion. An increase of IL-1β production in curcumin-treated rats in spite of an absence or less amount of macrophage-rich infiltrates condition is due to the induction of IL-1β by epithelial cells and endothelial cells (Abbas, *et al.*, 1997). The optimal amount of IL-1β might up-regulate the expression of COX-2 and other healing-related factors as mentioned above. Alternatively, TGF-β1 as well as IL-1β may exert opposite effects between ulcer healing and ulcer relapse. Excessive increase of TGF-β1 or IL-1β in a healed ulcer may cause the relapse of gastric ulcer due to increase of macrophage or neutrophil infiltration into regenerated mucosa which in turn produced oxygen radicals and excess amount of inflammatory cytokines (Takahashi, *et al.*, 1999; Tominaga, *et al.*, 1998). In addition, excessive inhibition of iNOS induction during ulcer healing will increase the accumulation of inflammatory cells by reducing apoptotis of

inflammatory cells and thus delays ulcer healing. Though, curcumin has been reported to exert antioxidant activity, it is not a potent hydroxyl radical scavenger which is more reactive and toxic to tissue than other oxygen radicals (Das and Das, 2002). On the other hand, curcumin can also scavenge nitric oxide radical directly (Sreejayan and Rao, 1997) which will decrease apoptosis in the inflammatory cells, leading to a more accumulative amount of inflammatory cells in the regenerating mucosa. Since the curative potency of curcumin tended to decrease at doses over than 160 mg/kg/d, the appropriate dose of curcumin in treatment of gastric ulcer should not be more than 80 mg/kg/d.

It is known that curcumin exhibits low oral bioavailability and the absorbed curcumin was rapidly biotransformed to tetrahydrocurcumin which is reported to be an active principle of curcumin for its antioxidant effect (Okada, *et al.*, 2001; Osawa, *et al.*, 1995; Pan, *et al.*, 1999; Sugiyama, *et al.*, 1996). In addition, its major degradation product (vanillin) is also a powerful scavenger of oxygen radicals (Wang, *et al.*, 1997). To investigate the possibility that the pharmacological activity of curcumin may be mediated, in part, by its active metabolites or degradation product; curcumin, tetrahydrocurcumin and vanillin were compared in terms of their ability to inhibit LPS-induced iNOS, COX-2, and TNF-α production in the macrophage cell line RAW 264.7. Mouse monocyte/macrophage cell line RAW 264.7 is commonly used in anti-inflammatory studies, since treatment with several inflammatory stimuli such as cytokines and LPS, trigger the cells to express high level of pro-inflammatory cytokines (NO through iNOS, PGE_2 through COX-2, and TNF-α) (Guastadisegni, *et al.*, 2002). Time-course experiments by Northern blot analysis showed that iNOS, COX-2 and TNF-α mRNA were apparent and reached maximum level between a 5-18 h, a 5-20 h and a 5-16 h treatment with LPS respectively (Guastadisegni, *et al.*, 2002; Huss, 2003; Pan, *et al.*, 2000). Therefore, experiments investigating the effects of test compound on iNOS, COX-2 and TNF-α induction were determined at 16 hour after treatment with LPS and the test compound. The results obtained from the present study indicated that, curcumin itself directly inhibited the production of these pro-inflammatory cytokines with the greatest degree of inhibition at a concentration of 10 μM. In contrast, its oxidized form (vanillin) or its major metabolite (tetrahydrocurcumin) which exhibited stronger antioxidative activities had less or no inhibitory effect on LPS-induced production

of iNOS, COX-2 and TNF-α. It was also found that curcumin exhibited great inhibition of iNOS and COX-2 expression but weak inhibition of TNF-α expression. There are several steps in cytokine gene and protein expression: transcription, post transcriptional process (including mRNA transport, control of stability and translation), and protein degradation. Induction of pro-inflammatory cytokines by LPS involves activation of nuclear transcription factors including activating protein (AP)-1 and NFκB (Ahmad, *et al.*, 1998; Jobin, *et al.*, 1999; Kumar, *et al.*, 1998; Satoskar, *et al.*, 1986). These nuclear transcriptional factors bind to specific sequences on promoter regions of the mouse gene encoding iNOS and COX-2 genes initiating gene transcription (Baeuerle and Baltimore, 1996). Since curcumin has been found to inhibit the activation of c-Jun/AP-1 and NFκB in various cell lines (Chan, 1995; Huang, *et al.*, 1991; Jobin, *et al.*, 1999; Kumar, *et al.*, 1998; Pan, *et al.*, 2000; Singh and Aggarwal, 1995), it produces an inhibitory effect on the induction of iNOS and COX-2 through the down-regulation at the transcriptional level. Another possibility is its inhibition on the activation of intracellular signals including protein kinase C and tyrosine protein kinase which have been reported to inhibit NOS induction in macrophage (Dong, *et al.*, 1993; Liu, *et al.*, 1993; Rao, *et al.*, 1993; Severn, *et al.*, 1992). Furthermore, curcumin has been found to down-regulate the arachidonic acid metabolism pathway including cyclooxygenase, phospholipases and lipoxygenases in various cell types (Ammon, *et al.*, 1993; Huang, *et al.*, 1991; Zhang, *et al.*, 1999). Curcumin has also been reported to exhibit a strong inhibition on the production of TNF-α and on TNF-α activity in various cell types (Abe, *et al.*, 1999; Chan, 1995; Kumar, *et al.*, 1998). Though, NFκB up-regulates TNF-α production (Abbas, *et al.*, 1997; Chan, 1995), it was found from the present study that curcumin produced significant inhibition on TNF-α protein expression but minimal inhibition on TNF-α mRNA expression in activated mouse macrophage cell line RAW 264.7 as found in preterm lung inflammatory cells (Literat, *et al.*, 2001). This suggested that curcumin may inhibit TNF-α induction at post-trancriptional level or at protein degradation level. Further mechanistic studies are needed to clarify a precise mechanism in curcumin-induced inhibition of TNF-α production. Whereas NFκB up-regulates TNF-α production, TNF-α activates NFκB reciprocally (Kumar, *et al.*, 1998) as well as the induction of iNOS (Drapier, *et al.*, 1988)

and IL-1β (Abbas, *et al.*, 1997). In addition, IL-1β and TNF-α have been reported to act independently and synergistically to stimulate iNOS expression and enzymatic activity in cultured rat colonic smooth muscle cells (Kuemmerle, 1998). Thus, the possibility that curcumin may down-regulate iNOS, COX-2 and IL-1β induction through the inhibition of TNF-α induction cannot be ruled out. A significant inhibitory effect of curcumin on IL-1β production was also found in our study (data not shown). Of interest, it was shown that the effect of curcumin on COX-2 and IL-1β is controversial to its result obtained from *in vivo* studies. Thus, the experiment model (in *vitro* vs *in vivo*) is crucial in determining the final results of the interaction between curcumin and inflammatory cytokines.