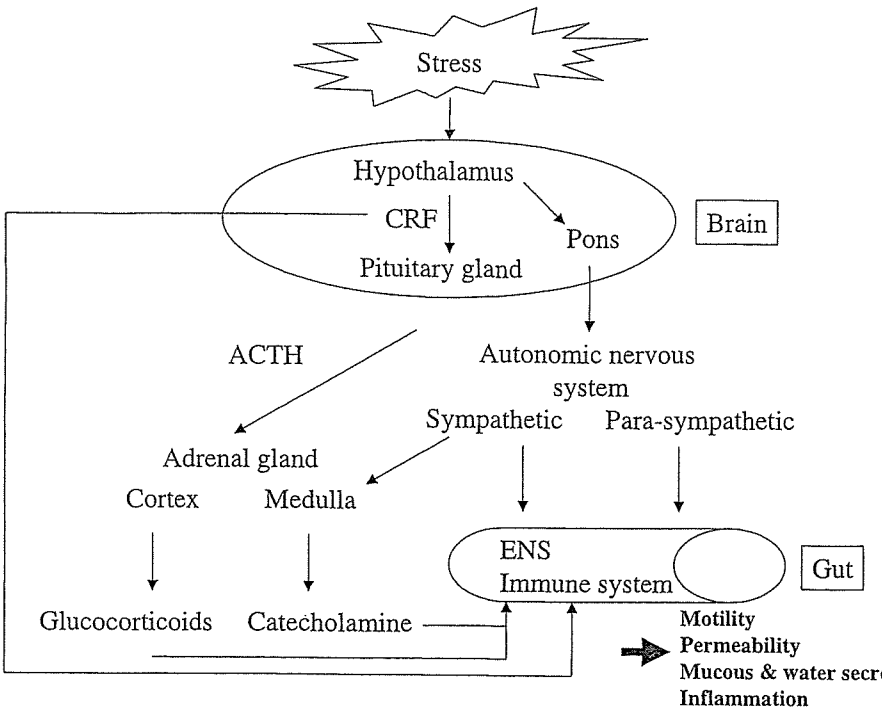

Fig. 1. Schema of the brain–gut axis. *CRF*, corticotrophin-releasing factor; *ENS*, enteric nervous system; *ACTH*, adrenocorticotropic hormone

pathophysiology of human UC. CRF levels are increased in lamina propria mononuclear cells from patients with active UC.[10,11]

Thus, both central and peripheral CRF systems are stimulated by stress and may have the potential to regulate gut homeostasis and so influence IBD pathophysiology.

Psychological stress alters intestinal barrier functions and water secretion

In several experiments using animal models, it has been demonstrated that stress can increase intestinal mucosal permeability and alter bacteria–host interactions. Restraint stress in rats increases jejunal and colonic mucosal permeability,[12,13] possibly by altering the cholinergic nervous system[14] and mucosal mast cell functions.[15] Catecholamine induced by stress also increases bacterial adhesion to intestinal mucosa.[16] Stress in animal models has also been shown to increase water and mucous secretions modulated by the cholinergic nervous system or mast cells, causing increased colonic motility and defecation, similar to observations in human IBS or IBD.[17–20] These observations of increases in stress-induced water and mucous secretions in animal stress models are likely to be consistent with human IBD pathophysiology.

Psychological stress in the course of human IBD

In many clinical trials involving novel therapies for IBD, a high rate (20%–40%) of improvement is often observed in the placebo group. These high placebo effects suggest to us that the IBD course and symptoms are often influenced by psychological conditions. Moser et al.[21] reported that 74% of IBD patients believe that psychological factors contribute to the course of their disease, which is significantly more than is found for other medical outpatients. However, scientific evidence in support of the contribution of psychological stress to IBD pathophysiology is inconsistent. Further, the efficacy of reduction therapy for psychological stress in IBD patients is controversial.

An initial question is, does psychological stress affect the onset of IBD? Li et al.[22] reported a follow-up study in Denmark of parents who had lost a child. They studied the onset of IBD in 21062 parents who had lost a child from 1980 to 1996 in Denmark compared with 293745 family-structure-matched parents. The relative risk of a first hospitalization for Crohn's disease (CD) was 0.97, and 1.01 for UC. Based on these results, they concluded that there was a negative association between psychological stress and the development of IBD in young to middle-aged adults. While studies of the role of psychological stress in the onset of IBD are relatively rare, a number of clinical studies investigate role of psychological stress in the clinical course of IBD, for example, in inducing relapse or worsening. Murray[23] first reported the contribution of psychological stress to

UC in 1930; however, it was not fully shown scientifically. In the 1990s, North et al.[24,25] reviewed the contribution of psychological stress to IBD pathophysiology.[24,25] However, as noted by Maunder,[26] the contribution of psychological stress to human IBD remains controversial. His review assessed several prospective studies of stress, depression, and IBD course. However, the patient populations were not matched in these studies; two studied only pure UC cases,[27,28] two only pure CD cases,[29,30] while five comprised cases of both UC and CD.[32–35] Baselines of disease activity and observation periods also varied. As well, outcomes of studies [relapse, Crohn's Disease Activity Index (CDAI), or symptom diary] differed among studies. Thus, studies that are not cohesively designed can lead to confusing results. In UC, Bitton et al.[28] reported that the odds ratio of relapse by life events was not very high (1.26 per event). Levenstein et al.[27] demonstrated a failure to determine a relationship between life events and relapse.

On the other hand, Mawdsley et al.[36] reported that acute psychological stress induced systemic and mucosal cytokine release in patients with inactive UC.

To assess of the effect of psychological stress for relapse of UC, we administered a multiple-choice questionnaire to 212 patients with UC. To focus on the effect of psychological stress for a relapse of UC and to adjust the baseline, we selected 160 patients with UC who in the past 3 years had had no event of macroscopic bloody diarrhea lasting 1 month (Fig. 2). Among these patients, 138 patients had had episodes of relapse in the most recent 3 years. Twenty-two patients remained in remission. As in previous reports, the most prominent factor involved in relapse by patients was psychological stress (66.2%). Interestingly, we found a seasonal fluctuation of relapse in these patients, suggesting an affect of life events on relapse (Fig. 3). Another question relates to the prognosis of relapsing patients who claim psychological stress as a cause of their relapse. The prognosis of patients with relapse who selected "positive psychological stress" on the questionnaire was not very severe. Indeed, 63.4% of the patients claiming positive psychological stress improved without additional medication or with treatment only involving an enema or a suppository (Fig. 4). As in Maunder's review,[26] it is quite difficult to find direct evidence of a correlation between relapse and psychological stress. Although many patients selected psychological stress as a risk factor for their relapse in this multiple-choice questionnaire, they also selected other factors, such as sleeplessness, physiological distress, and excessive eating or drinking. As in many earlier human studies, it was also hard in this study to assess the effect of pure psychological stress on IBD. Several reasons that studies of the role of stress in IBD are difficult and vague are (1) IBD itself may be a basket disease entity comprising multiple features; (2) there are no adequate methods to measure psychological stress (scoring of life events?); (3) sensitivity to psychological stress may be different among patients; (4)

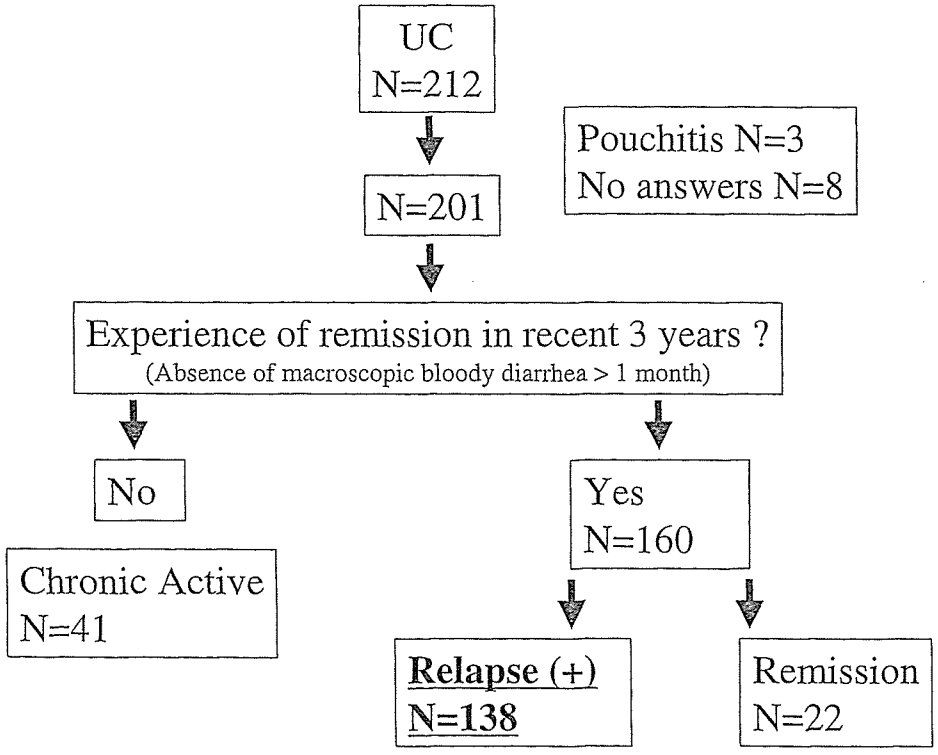

Fig. 2. Multiple-choice questionnaire regarding triggers of a relapse of ulcerative colitis (*UC*). The multiple-choice questionnaire was administered to 212 patients with UC. Of these, 138 patients with UC who in the past 3 years had had no macroscopic bloody diarrhea lasting 1 month were analyzed for the assessment of the role of psychological stress in the relapse of UC

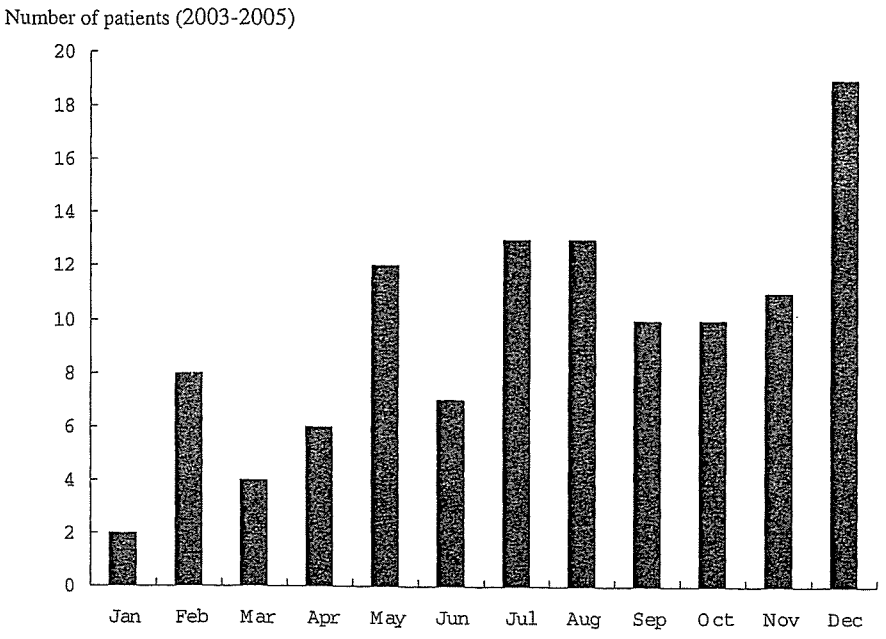

Fig. 3. Seasonal fluctuations of relapses in patients with UC. Results are based on a multiple-choice questionnaire. The *Y* axis shows the number of relapse patients out of 138 patients

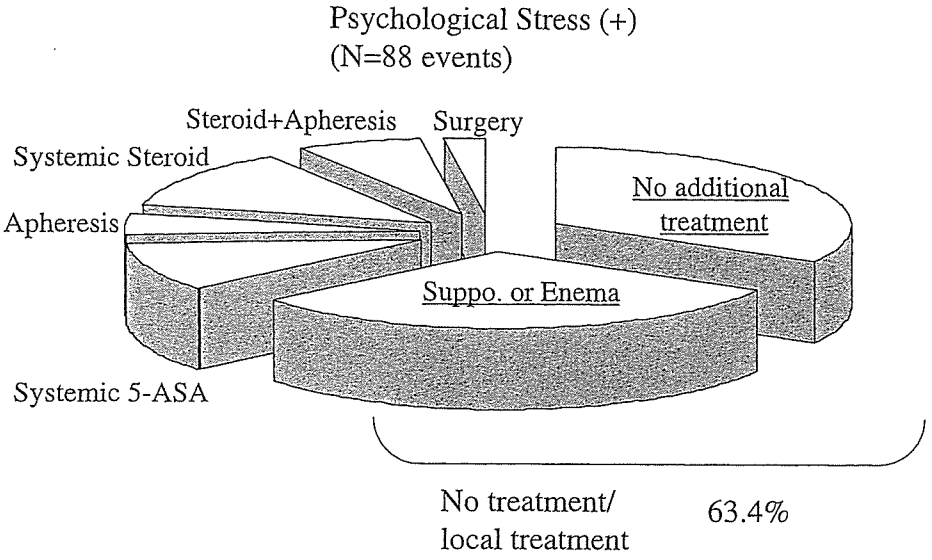

Fig. 4. Prognosis of patients with relapse who implicated psychological stress as a cause of the relapse ("psychological stress positive"). Suppo., suppository; 5-ASA, 5-aminosalicylic acid

there are difficulties in completely neglecting other risk factors, including smoking in CD and use of nonsteroidal anti-inflammatory drugs, in the studies; and (5) it is difficult to design a long-term prospective observation study. Well-organized prospective studies (not a mixture of CD and UC cases, an adjusted baseline, a sufficient observation period, adequate outcomes, and adequate methods for the measurement of stress) are necessary to provide a clearer answer to this historical issue.

Are there overlaps between IBD and IBS in pathophysiology?

IBS is a common chronic functional bowel disorder characterized by intermittent or continuous abdominal pain and alterations in bowel patterns. At this time, IBS is a diagnosis of exclusion, and there are no serological markers or specific pathological findings. For the clinical diagnosis of IBS, the Rome II criteria are widely used.[37,38] It has been reported that the colonic motor response to various psychological and physical stressors is increased in IBS patients.[39–42] While the etiology of

IBS remains unclear, many studies have suggested an overlap between IBS and psychiatric disorders such as anxiety, depression, and somatization disorders.[43]

Thus, while IBS has in recent years been considered to have symptoms caused mainly by unbalanced stress-induced psychological conditions, altered bacteria–host interactions and the mucosal immune system have been suggested to have a role in the mechanisms of action of IBS. Khan and Collins[44] demonstrated a relationship between the immune and motor systems using animal models. They demonstrated that the T helper 2-type immune response is critical for producing alterations in infection-induced intestinal muscle function. Collins[45] presented the case for an immunological basis for IBS. Several other studies have demonstrated activation of the mucosal immune system and active inflammation in IBS.[46,47] Ohman et al.[48] reported that peripheral α4β7-positive T cells, which may be localized to the intestinal mucosa and play an important role as the pathogenic T cells in IBD pathophysiology, are increased in IBS patients. This population of T cells has already been identified as the therapeutic target in CD patients using anti-α4 integrin monoclonal antibody.[49] Furthermore, because IBS is diagnosed by clinical criteria without any lower intestinal findings, patients with microscopic colitis, which is characterized by pathological lymphocyte infiltration into the intestinal mucosa, are often misdiagnosed.

Other current issues regarding IBS include postinfectious IBS and the role of host–bacteria interactions in the pathophysiology of IBS. Since the 1990s, many reports have demonstrated the possibility that an entity called "postinfectious IBS" may exist as a subgroup of IBS. McKendrick et al.[50] reported IBS arising in patients after *Salmonella* infection. Several reports have demonstrated that bacterial gastroenteritis might be a risk factor for the development of IBS.[51–56] Based on these observations, several therapeutic trials for IBS using antibiotics or probiotics have been reported. O'Mahony et al.[57] demonstrate that *Bifidobacterium infantis* 35624 can reduce symptoms in IBS patients. Sharara et al.[58] demonstrate the efficacy of rifaximin in patients with abdominal bloating and flatulence. Because these therapeutic strategies for IBS remain controversial, larger well-designed studies are necessary.

Thus, IBS, as it is now diagnosed, is possibly part of a global disorder; therefore, it is not unreasonable to postulate that there is some overlap of pathophysiology between IBS and IBD. Although several genetic backgrounds have been demonstrated in IBD, including NOD2,[59,60] OCTN,[61] and TNFSF15,[62] they are not correlated with the patterns of IBS. These findings suggest to us that the genetic background is more important in the pathophysiology of IBD than in that of IBS. While psychotherapeutic interventions have increasingly been used to treat refractory IBS, the efficacy of these therapies on IBD has not been scientifically demonstrated, and their effects in IBD treatment remain controversial. In this regard, psychological factors seem to contribute more strongly to IBS than to IBD (Table 1). Thus, although some historical observations suggest an overlap in pathophysiology between IBS and IBD, further studies in human and animal models are necessary to reach a better understanding. In particular, because both IBS and IBD may be part of a global disease entity, these disorders need to be classified into subclasses and the pathophysiological mechanisms in each group analyzed (e.g., high genetic background IBD, psychological factor-dependent IBD, IBS caused by immune disorders, responders in IBS/IBD to antibiotics or probiotics therapy). Such investigations should help in establishing patient-specific therapeutic strategies.

Table 1. Are there any overlap inn pathophysiology between IBD and IBS? (or just overlap of letters "IB"?)

	IBD	IBS
Genetic background	○	?
Immunological dysregulation	○	△
Bacteria–host interaction	○	△
Food	△	?
Dysregulation of motility	△	○
Mucosal permeability	△	△
Psychological stress	?	○

IBD, inflammatory bowel disease; IBS, irritable bowel syndrome
○, strong; △, controversial; ?, unknown

Conclusions

Since the 1930s, psychological stress has been thought to contribute to IBD pathophysiology. However, the scientific evidence is not consistent. Moreover, there is not sufficient scientific evidence to determine whether an overlap between IBD and IBS pathophysiology exists. However, several human studies and studies in animal models have yielded important clues regarding the historical question, "What is the role of psychological stress in IBD." To obtain a definitive answer, further well-organized prospective studies using scientific methodology to measure psychological stress are necessary.

Acknowledgments. This work was supported in part by Grants-in-Aid from the Japanese Ministry of Education, Culture and Science, the Japanese Ministry of Health, Labour and Welfare, Keio University, and the Keio University Medical Fund.

References

1. Measuring stress. A guide for health and social scientists. New York: Oxford; 1997.

2. Chrousos GP, Gold PW. The concepts of stress and stress system disorders. Overview of physical and behavioral homeostasis. JAMA 1992;267:1244–52.
3. Goyal RK, Hirano I. The enteric nervous system. N Engl J Med 1996;334:1106–15.
4. Mawdsley JE, Rampton DS. Psychological stress in IBD: new insights into pathogenic and therapeutic implications. Gut 2005; 54:1481–91.
5. Monnikes H, Tebbe JJ, Hildebrandt M, Arck P, Osmanoglou E, Rose M, et al. Role of stress in functional gastrointestinal disorders. Evidence for stress-induced alterations in gastrointestinal motility and sensitivity. Dig Dis 2001;19:201–11.
6. Tache Y, Perdue MH. Role of peripheral CRF signalling pathways in stress-related alterations of gut motility and mucosal function. Neurogastroenterol Motil 2004;16 Suppl 1:137–42.
7. Leu SJ, Singh VK. Stimulation of interleukin-6 production by corticotropin-releasing factor. Cell Immunol 1992;143:220–7.
8. Singh VK, Leu SJ. Enhancing effect of corticotropin-releasing neurohormone on the production of interleukin-1 and interleukin-2. Neurosci Lett 1990;120:151–4.
9. Leu SJ, Singh VK. Modulation of natural killer cell-mediated lysis by corticotropin-releasing neurohormone. J Neuroimmunol 1991; 33:253–60.
10. Kawahito Y, Sano H, Mukai S, Asai K, Kimura S, Yamamura Y, et al. Corticotropin releasing hormone in colonic mucosa in patients with ulcerative colitis. Gut 1995;37:544–51.
11. Muramatsu Y, Fukushima K, Iino K, Totsune K, Takahashi K, Suzuki T, et al. Urocortin and corticotropin-releasing factor receptor expression in the human colonic mucosa. Peptides 2000; 21:1799–809.
12. Kiliaan AJ, Saunders PR, Bijlsma PB, Berin MC, Taminiau JA, Groot JA, et al. Stress stimulates transepithelial macromolecular uptake in rat jejunum. Am J Physiol 1998;275:G1037–44.
13. Santos J, Saunders PR, Hanssen NP, Yang PC, Yates D, Groot JA, et al. Corticotropin-releasing hormone mimics stress-induced colonic epithelial pathophysiology in the rat. Am J Physiol 1999;277:G391–9.
14. Saunders PR, Hanssen NP, Perdue MH. Cholinergic nerves mediate stress-induced intestinal transport abnormalities in Wistar-Kyoto rats. Am J Physiol 1997;273:G486–90.
15. Santos J, Benjamin M, Yang PC, Prior T, Perdue MH. Chronic stress impairs rat growth and jejunal epithelial barrier function: role of mast cells. Am J Physiol Gastrointest Liver Physiol 2000;278:G847–54.
16. Chen C, Brown DR, Xie Y, Green BT, Lyte M. Catecholamines modulate Escherichia coli O157:H7 adherence to murine cecal mucosa. Shock 2003;20:183–8.
17. Saunders PR, Kosecka U, McKay DM, Perdue MH. Acute stressors stimulate ion secretion and increase epithelial permeability in rat intestine. Am J Physiol 1994;267:G794–9.
18. Castagliuolo I, Lamont JT, Qiu B, Fleming SM, Bhaskar KR, Nikulasson ST, et al. Acute stress causes mucin release from rat colon: role of corticotropin releasing factor and mast cells. Am J Physiol 1996;271:G884–92.
19. Wilson LM, Baldwin AL. Environmental stress causes mast cell degranulation, endothelial and epithelial changes, and edema in the rat intestinal mucosa. Microcirculation 1999;6:189–98.
20. Castagliuolo I, Wershil BK, Karalis K, Pasha A, Nikulasson ST, Pothoulakis C. Colonic mucin release in response to immobilization stress is mast cell dependent. Am J Physiol 1998;274: G1094–100.
21. Moser G, Maeir-Dobersberger T, Vogelsang H, et al. Inflammatory bowel disease: patient's beliefs about the etiology of their disease—a controlled study. Psychosom Med 1993;55:131.
22. Li J, Norgard B, Precht DH, Olsen J. Psychological stress and inflammatory bowel disease: a follow-up study in parents who lost a child in Denmark. Am J Gastroenterol 2004;99:1129–33.
23. Murray CD. Psychologenic factors in the etiology of ulcerative colitis and bloody diarrhea. Am J Med Sci 1930;180239–48.
24. North CS, Clouse RE, Spitznagel EL, Alpers DH. The relation of ulcerative colitis to psychiatric factors: a review of findings and methods. Am J Psychiatry 1990;147:974–81.
25. North CS, Alpers DH. A review of studies of psychiatric factors in Crohn's disease: etiologic implications. Ann Clin Psychiatry 1994;6:117–24.
26. Maunder RG. Evidence that stress contributes to inflammatory bowel disease: evaluation, synthesis, and future directions. Inflamm Bowel Dis 2005;11:600–8.
27. Levenstein S, Prantera C, Varvo V, Scribano ML, Andreoli A, Luzi C, et al. Stress and exacerbation in ulcerative colitis: a prospective study of patients enrolled in remission. Am J Gastroenterol 2000;95:1213–20.
28. Bitton A, Sewitch MJ, Peppercorn MA, de BEMD, Shah S, Ransil B, et al. Psychosocial determinants of relapse in ulcerative colitis: a longitudinal study. Am J Gastroenterol 2003;98:2203–8.
29. Mardini HE, Kip KE, Wilson JW. Crohn's disease: a two-year prospective study of the association between psychological distress and disease activity. Dig Dis Sci 2004;49:492–7.
30. Garrett VD, Brantley PJ, Jones GN, McKnight GT. The relation between daily stress and Crohn's disease. J Behav Med 1991; 14:87–96.
31. Riley SA, Mani V, Goodman MJ, Lucas S. Why do patients with ulcerative colitis relapse? Gut 1990;31:179–83.
32. North CS, Alpers DH, Helzer JE, Spitznagel EL, Clouse RE. Do life events or depression exacerbate inflammatory bowel disease? A prospective study. Ann Intern Med 1991;114:381–6.
33. Duffy LC, Zielezny MA, Marshall JR, Byers TE, Weiser MM, Phillips JF, et al. Relevance of major stress events as an indicator of disease activity prevalence in inflammatory bowel disease. Behav Med 1991;17:101–10.
34. Greene BR, Blanchard EB, Wan CK. Long-term monitoring of psychosocial stress and symptomatology in inflammatory bowel disease. Behav Res Ther 1994;32:217–26.
35. Mittermaier C, Dejaco C, Waldhoer T, Oefferlbauer-Ernst A, Miehsler W, Beier M, et al. Impact of depressive mood on relapse in patients with inflammatory bowel disease: a prospective 18-month follow-up study. Psychosom Med 2004;66:79–84.
36. Mawdsley JE, Macey MG, Feakins RM, Langmead L, Rampton DS. The effect of acute psychologic stress on systemic and rectal mucosal measures of inflammation in ulcerative colitis. Gastroenterology 2006;131:410–9.
37. Thompson WG, Longstreth GF, Drossman DA, Heaton KW, Irvine EJ, Muller-Lissner SA. Functional bowel disorders and functional abdominal pain. Gut 1999;45 Suppl 2:II43–7.
38. Shinozaki M, Kanazawa M, Sagami Y, Endo Y, Hongo M, Drossman DA, et al. Validation of the Japanese version of the Rome II modular questionnaire and irritable bowel syndrome severity index. J Gastroenterol 2006;41:491–4.
39. Narducci F, Snape WJ Jr, Battle WM, London RL, Cohen S. Increased colonic motility during exposure to a stressful situation. Dig Dis Sci 1985;30:40–4.
40. Welgan P, Meshkinpour H, Hoehler F. The effect of stress on colon motor and electrical activity in irritable bowel syndrome. Psychosom Med 1985;47:139–49.
41. Welgan P, Meshkinpour H, Beeler M. Effect of anger on colon motor and myoelectric activity in irritable bowel syndrome. Gastroenterology 1988;94:1150–6.
42. Fukudo S, Nomura T, Muranaka M, Taguchi F. Brain–gut response to stress and cholinergic stimulation in irritable bowel syndrome. A preliminary study. J Clin Gastroenterol 1993;17: 133–41.
43. Whitehead WE, Palsson O, Jones KR. Systematic review of the comorbidity of irritable bowel syndrome with other disorders: what are the causes and implications? Gastroenterology 2002;122: 1140–56.
44. Khan WI, Collins SM. Gut motor function: immunological control in enteric infection and inflammation. Clin Exp Immunol 2006;143:389–97.

45. Collins SM. A case for an immunological basis for irritable bowel syndrome. Gastroenterology 2002;122:2078–80.
46. Chadwick VS, Chen W, Shu D, Paulus B, Bethwaite P, Tie A, et al. Activation of the mucosal immune system in irritable bowel syndrome. Gastroenterology 2002;122:1778–83.
47 Tornblom H, Lindberg G, Nyberg B, Veress B. Full-thickness biopsy of the jejunum reveals inflammation and enteric neuropathy in irritable bowel syndrome. Gastroenterology 2002;123: 1972–9.
48 Ohman L, Isaksson S, Lundgren A, Simren M, Sjovall H. A controlled study of colonic immune activity and beta7+ blood T lymphocytes in patients with irritable bowel syndrome. Clin Gastroenterol Hepatol 2005;3:980–6.
49. Sandborn WJ, Colombel JF, Enns R, Feagan BG, Hanauer SB, Lawrance IC, et al. Natalizumab induction and maintenance therapy for Crohn's disease. N Engl J Med 2005;353:1912–25.
50. McKendrick MW, Read NW. Irritable bowel syndrome—post salmonella infection. J Infect 1994;29:1–3.
51. Neal KR, Hebden J, Spiller R. Prevalence of gastrointestinal symptoms six months after bacterial gastroenteritis and risk factors for development of the irritable bowel syndrome: postal survey of patients. BMJ 1997;314:779–82.
52. Gwee KA, Leong YL, Graham C, McKendrick MW, Collins SM, Walters SJ, et al. The role of psychological and biological factors in postinfective gut dysfunction. Gut 1999;44:400–6.
53. Rodriguez LA, Ruigomez A. Increased risk of irritable bowel syndrome after bacterial gastroenteritis: cohort study. BMJ 1999;318:565–6.
54. Spiller RC. Estimating the importance of infection in IBS. Am J Gastroenterol 2003;98:238–41.
55. Spiller RC, Jenkins D, Thornley JP, Hebden JM, Wright T, Skinner M, et al. Increased rectal mucosal enteroendocrine cells, T lymphocytes, and increased gut permeability following acute *Campylobacter* enteritis and in post-dysenteric irritable bowel syndrome. Gut 2000;47:804–11.
56. Dunlop SP, Jenkins D, Neal KR, Spiller RC. Relative importance of enterochromaffin cell hyperplasia, anxiety, and depression in postinfectious IBS. Gastroenterology 2003;125:1651–9.
57. O'Mahony L, McCarthy J, Kelly P, Hurley G, Luo F, Chen K, et al. Lactobacillus and bifidobacterium in irritable bowel syndrome: symptom responses and relationship to cytokine profiles. Gastroenterology 2005;128:541–51.
58. Sharara AI, Aoun E, Abdul-Baki H, Mounzer R, Sidani S, Elhajj I. A randomized double-blind placebo-controlled trial of rifaximin in patients with abdominal bloating and flatulence. Am J Gastroenterol 2006;101:326–33.
59. Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature 2001;411:603–6.
60. Hugot JP, Chamaillard M, Zouali H, Lesage S, Cezard JP, Belaiche J, et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature 2001;411:599–603.
61. Peltekova VD, Wintle RF, Rubin LA, Amos CI, Huang Q, Gu X, et al. Functional variants of OCTN cation transporter genes are associated with Crohn disease. Nat Genet 2004;36:471–5.
62. Yamazaki K, McGovern D, Ragoussis J, Paolucci M, Butler H, Jewell D, et al. Single nucleotide polymorphisms in TNFSF15 confer susceptibility to Crohn's disease. Hum Mol Genet 2005;14:3499–506.

ELSEVIER

EXPERIMENTAL HEMATOLOGY

Experimental Hematology 34 (2006) 1385–1392

JTE-607, a multiple cytokine production inhibitor, ameliorates disease in a SCID mouse xenograft acute myeloid leukemia model

Naofumi Uesato[a], Kenji Fukui[a], Junji Maruhashi[a], Arinobu Tojo[b], and Nobuyuki Tajima[a]

[a]*Biological and Pharmacological Laboratories, Central Pharmaceutical Research Institute, Japan Tobacco Inc., Osaka, Japan;*
[b]*Division of Molecular Therapy, Advanced Clinical Research Center, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan*

(Received 9 March 2006; revised 16 May 2006; accepted 25 May 2006)

Objective. **Accumulating findings suggest that in acute myeloid leukemia (AML) patients, proinflammatory cytokines and growth factors play important roles in the proliferation and survival of AML cells in an autocrine and paracrine manner, leading to deterioration of AML. JTE-607 is a multiple cytokine inhibitor that potently suppresses production of proinflammatory cytokines. In the present study, we investigated the potency of JTE-607 as an antileukemic agent by exploiting a SCID mouse acute leukemia model.**

Methods. **SCID mice injected with anti-asialo-GM1 antibody were exposed to sublethal total-body irradiation at a dose of 3 Gy and then inoculated intravenously with AML cells. JTE-607 was administered using osmotic minipumps. The effects of JTE-607 on mouse survival time, human interleukin (IL)-8 levels in mouse plasma, and proportion of human CD45$^+$ cells in the bone marrow were studied.**

Results. **The survival time of the mice was strictly dependent on the number of U-937 cells proliferating in vivo. Administration of JTE-607 during the initial 7 days significantly prolonged survival of the mice, suggesting killing activity of JTE-607 against AML cells in vivo. Delayed administration of JTE-607 also prolonged the survival of mice bearing established leukemia with an effect comparable to the maximum tolerable dose of cytarabine. Flow cytometer analysis of bone marrow cells revealed decreased number of human CD45$^+$ cells. Human IL-8 level was also reduced by JTE-607.**

Conclusion. **Our results indicate that JTE-607 has potential to be a new class of antileukemic drug that exerts inhibitory activities against both the proliferation and proinflammatory cytokine production of AML cells.** © **2006 International Society for Experimental Hematology. Published by Elsevier Inc.**

Acute myeloid leukemia (AML) is an aggressive disorder characterized by expansion and accumulation of immature leukemia cells arrested at various stages in the bone marrow [1]. Recent advances in chemotherapy of AML have improved clinical outcome, inducing complete remission in 70 to 80% of AML patients during initial treatment [2,3]. However, in spite of current intensive chemotherapy strategies, disease-free survival remains as low as 30 to 50%, mainly because of relapse after treatment [1–3]. In addition, although allogenic bone marrow transplantation is the most efficacious against relapse, many patients cannot tolerate the high burden of this aggressive therapy [4]. Therefore, novel therapeutic approaches are still required to prevent recurrence and provide beneficial treatment even after relapse.

It has been thought that growth, survival, and resistance to antileukemic agents of AML cells are promoted and sustained by close interaction with stromal cells in the bone marrow microenvironment through local production of cytokines and growth factors (e.g., VEGF, IL-8, and GM-CSF) in an autocrine and paracrine manner [5–10]. In fact, it has been reported that the production of growth factors from AML blasts is upregulated [5,9,11–14] and a strong relationship exists between the circulating levels of growth factors and prognosis of AML [11,15–17]. In addition, increased production of proinflammatory cytokines by leukemic blasts promotes bone marrow neoangiogenesis in a paracrine manner and increased angiogenesis has been demonstrated in the bone marrow of patients with AML

Offprint requests to: Naofumi Uesato, Biological and Pharmacological Laboratories, Central Pharmaceutical Research Institute, Japan Tobacco Inc., 1-1, Murasaki-cho, Takatsuki, Osaka 569-1125, Japan; E-mail: naofumi.uesato@ims.jti.co.jp

0301-472X/06 $–see front matter.
doi: 10.1016/j.exphem.2006.05.016

[18,19]. It was recently demonstrated that thalidomide exerts anti-angiogenesis effect by reducing the release of growth factor by endothelial cells [20] and its clinical efficacy against hematopoietic malignancies, including multiple myeloma (MM), myelodysplastic syndromes (MDS), and AML has been demonstrated [21–24]. Thus, depression of the cytokine / growth factor network is thought to be a new approach to overcome the limitations of current chemotherapy for AML.

JTE-607 [(-)-Ethyl-N-{3,5-dichloro-2-hydroxy-4-[2-(4-methylpiperazin-1-yl)ethoxy]benzoyl}-L-phenylalaninate dihydrochloride] (Fig. 1) was originally discovered as a multiple cytokine inhibitor that broadly suppresses production of proinflammatory cytokines such as IL-1β, IL-6, IL-8, GM-CSF, and TNF-α from human peripheral blood mononuclear cells (PBMCs) stimulated with lipopolysaccharide [25]. In in vivo studies, JTE-607 improved the survival rate in a cecal ligation and puncture-induced mouse septic shock model [26] and showed inhibitory effect on the burn insult–induced mouse lung injury [27]. Because of the presumed importance of cytokine in the pathology of AML, we examined the effect of JTE-607 on AML cell lines and found that JTE-607 suppressed not only spontaneous cytokine production but also proliferation of AML cells in vitro. The cells exposed to JTE-607 showed cell-cycle arrest at S phase and subsequently underwent apoptosis, accompanied by the decrease in c-Myc and the increase in $p21^{waf1/cip1}$ protein levels (manuscript in preparation).

In the present study, we exploited an acute leukemia model established by engraftment of U-937 cells in preconditioned SCID mice, in which the survival of mice was directly correlated with the number of leukemia cells proliferating in vivo. Administration of JTE-607 using osmotic minipumps reduced the number of leukemic cells in the bone marrow and significantly prolonged the mouse survival even after the development of leukemia. Moreover, human interleukin (IL)-8 level in mouse plasma was reduced by injection of JTE-607, indicating both proliferation and cytokine production of AML cells was effectively blocked. Thus, we conclude that JTE-607 is a promising candidate for an antileukemic drug that may bring about a new approach in the therapy of AML.

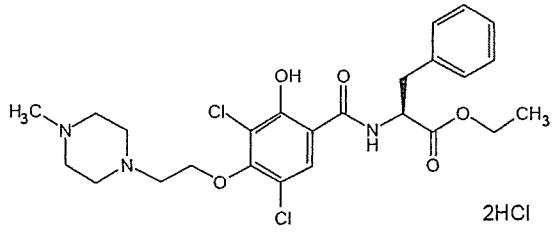

Figure 1. Chemical structure of JTE-607 [(-)-Ethyl-N-{3,5-dichloro-2-hydroxy-4-[2-(4-methylpiperazin-1-yl)ethoxy]benzoyl}-L-phenylalaninate dihydrochloride.

Materials and methods

AML cell lines

Human myeloid leukemia cell lines U-937, HL-60, and THP-1 were obtained from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). The cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), L-glutamine (2 mmol/L), penicillin (100 Units/mL), and streptomycin (100 μg/mL), and maintained at 37°C in a humidified atmosphere that contained 5% CO_2.

Reagents

JTE-607 was chemically synthesized at Central Pharmaceutical Research Institute, Japan Tobacco Inc. (Osaka, Japan). Cytosine-1-β-D(+)-arabinofuranoside (cytarabine) was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). For in vitro studies, these drugs were dissolved in dimethylsulfoxide (DMSO) and diluted with cell culture medium to 1% DMSO. For in vivo studies, JTE-607 was dissolved in 30% hydroxypropyl β-cyclodextrin (HP-β-CD) aqueous solution. Cytarabine was dissolved in saline.

In vitro cell proliferation assay

AML cells were seeded in 96-well flat-bottom microplates at 5×10^2 to 3×10^3 cells/well and incubated in the presence of various concentrations of JTE-607 or cytarabine at 37°C for 3 days (final DMSO concentration: 0.1%). The cells were pulsed with 9.25 kBq/well of [^{3}H]-thymidine during the last 6 hours of culture, and [^{3}H]-thymidine incorporation was determined by liquid scintillation counting. All measurements were performed in triplicate. The 50% inhibitory concentration (IC_{50}) value was calculated in a semilogarithmic proportional manner from the two points enclosing 50% inhibition.

In vitro IL-8 production assay

U-937 cells were seeded in 96-well flat-bottom microplates at 2×10^5 cells/well and incubated with various concentrations of JTE-607 in the presence or absence of LPS (10 μg/mL) at 37°C for 24 hours (final DMSO concentration: 0.1%). Human IL-8 concentrations in the supernatants were measured using a specific ELISA kit (R&D systems, Minneapolis, MN, USA). All measurements were performed in duplicate.

SCID mouse xenograft acute leukemia model

Eight- to 9-week-old female SCID (Fox Chase C.B-17/Icr-scidJcl) mice were purchased from CLEA Japan Inc. (Tokyo, Japan) and housed under specific pathogen-free conditions. Sterile food and water was given ad libitum. Mice were injected intraperitoneally with rabbit anti-asialo-GM1 antibody to deplete natural killer cells. Twenty-four hours after the antibody injection, the mice were exposed to sublethal total-body irradiation at a dose of 3 Gy using a soft x-ray ionization chamber (M-150WE; Softex, Tokyo, Japan) and then inoculated intravenously with AML cells in 200 μL of Hank's Balanced Salt Solution (day 0). For early treatment with JTE-607, Alzet osmotic minipumps (model 2001, Durect Corp., CA, USA) filled with JTE-607 or vehicle solution were implanted under dorsal skin of mice immediately after the U-937 inoculation. In accordance with the pump specifications, concentrations of JTE-607 were adjusted to dosages of 0.2, 0.6, and 1.8 mg/animal/day (10, 30, and 90 mg/kg/day). The nominal duration of pumping was 7 days. For delayed treatment,

minipumps filled with JTE-607 solution for which concentration was adjusted to dosages of 0.6 and 1.8 mg/animal/day (30 and 90 mg/kg/day) were implanted on day 12. Cytarabine was subcutaneously injected at dosages of 3, 30, and 300 mg/kg/day, once a day for 4 days from day 12. General condition, body weight, and survival of the mice were monitored and mean survival days were compared as indication of antileukemic effects. All procedures for animals were reviewed and approved by the Animal Care and Management Committee of Central Pharmaceutical Research Institute, Japan Tobacco Inc.

Detection of human IL-8 in plasma and CD45$^+$ cells in bone marrow

The SCID mice engrafted with U-937 cell were treated with JTE-607 (90 mg/kg/day) for the initial 7 days as described above (early treatment). On day 18, heparinized plasma samples were collected and used for measurement of human IL-8 concentration using a human IL-8 ultra-sensitive ELISA kit (R&D Systems, Minneapolis, MN, USA) that has no cross-reactivity with mouse IL-8. Bone marrow cells of the mice were then recovered by flushing mouse femurs and tibias with ice-cold Hank's Balanced Salt Solution. After removing erythrocytes, the cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-human CD45 (BD Biosciences Pharmingen, San Jose, CA, USA) or isotype control antibody and analyzed by a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, USA). The percentage of human CD45$^+$ cells (U-937 cells) in total bone marrow cells was determined by data analysis using CellQuest software (Becton-Dickinson, San Jose, CA, USA).

In the bolus treatment study, the SCID mice engrafted with U-937 cell were subcutaneously injected once with JTE-607 (100 mg/kg) or vehicle solution on day 18. Three hours after the injection, blood samples and bone marrow cells were collected and analyzed in the same way.

Statistical analysis

Statistical significances between vehicle-treated control group and drug administration groups were determined using the Kaplan-Meier method and the log-rank test for the survival ratio (%) and the Mann-Whitney U test for the mean survival day (MSD) using SAS software (Ver. 8.2; SAS Institute Japan, Tokyo, Japan), respectively. Statistical analysis for CD45$^+$ cell in bone marrow and plasma IL-8 level were performed by Student's t-test. A p value of less than 0.05 was considered to be statistically significant.

Results

Inhibitory activity of JTE-607 on the in vitro proliferation of AML cell lines

In the beginning, we examined the effect of JTE-607 on the spontaneous production of proinflammatory cytokines from AML cell lines, and found that both mRNA levels in the cells and protein levels in the culture supernatant of IL-6 and IL-8 were significantly suppressed by JTE-607 (e.g., % inhibition on IL-6 and IL-8 production from HL-60 cells was 79.4% and 43.7% at the concentration of 1 μmol/L, respectively; manuscript in preparation). This observation led us to hypothesize that JTE-607 might interfere with the constitutively activated cellular responses that were ascribed to proliferation and survival of AML cells. In this line, we next examined the inhibitory effect of JTE-607 on the proliferation of human AML cell lines. As expected, JTE-607 inhibited the proliferation of U-937, HL-60, and THP-1 cells, with IC_{50} values of 0.029, 0.070, and 0.28 μmol/L, respectively (Table 1). The inhibitory activity of JTE-607 appeared to be most potent in U-937 cells, although the activity was 10-fold lower than that of cytarabine, whereas the activities of JTE-607 in HL-60 and THP-1 cells were approximately comparable to those of cytarabine.

Establishment of SCID mouse xenograft acute leukemia model

Engraftment of human leukemic cell lines in SCID mice has been reported to mimic human leukemia and provide suitable conditions for evaluation of antileukemic drugs [28,29]. To establish a well-controllable SCID mice xenograft leukemia model using AML cell lines, we first compared the ability of U-937, HL-60, and THP-1 cells to induce mortal leukemic disorders. The mice receiving anti-asialo GM1 antibody were exposed to sublethal total-body irradiation at a does of 3 Gy and subsequently injected with 1×10^6 AML cells via tail vein. All of the mice showed body weight loss, ruffled fur, and paralysis of hindlimb due to infiltration and expansion of AML cells in lumbar cord, then eventually died. However, duration to death in the mice engrafted with U-937 was much shorter than in the others: the MSD with U-937 was 20 ± 6.7, vs 47 ± 2.6 with HL-60 and 53 ± 1.7 with THP-1 (Fig. 2A). Since the short duration of disease development seemed to mimic aggressive leukemic burden in AML more closely, and as it is also suitable for investigation of in vivo efficacy of drugs, we selected U-937 cells and checked inoculation dose–dependent alteration in MSD. As shown in Figure 2B, the mice inoculated with graded doses of U-937 cell exhibited dose-dependent shortening of survival days, showing 38 ± 4.4, 26 ± 2.6, 22 ± 1.3, and 17 ± 1.2 days for 10^4, 10^5, 10^6, and 10^7 cells of U-937, respectively. A negative correlation between MSD and the number of

Table 1. Inhibitory effects of JTE-607 and cytarabine on proliferation of acute myeloid leukemia cell line in vitro

	IC_{50} (μmol/L)	
AML cell line	JTE-607	Cytarabine
U-937	0.029	0.0032
HL-60	0.070	0.014
THP-1	0.28	0.17

Leukemia cell lines were cultured in 96-well flat-bottom microplates with various concentrations of JTE-607 and cytarabine for 72 hours. The growth inhibitory activities were determined by [^{3}H]-thymidine incorporation assay. Results were shown as the mean of 50% inhibitory concentration (IC_{50}) of two independent experiments.

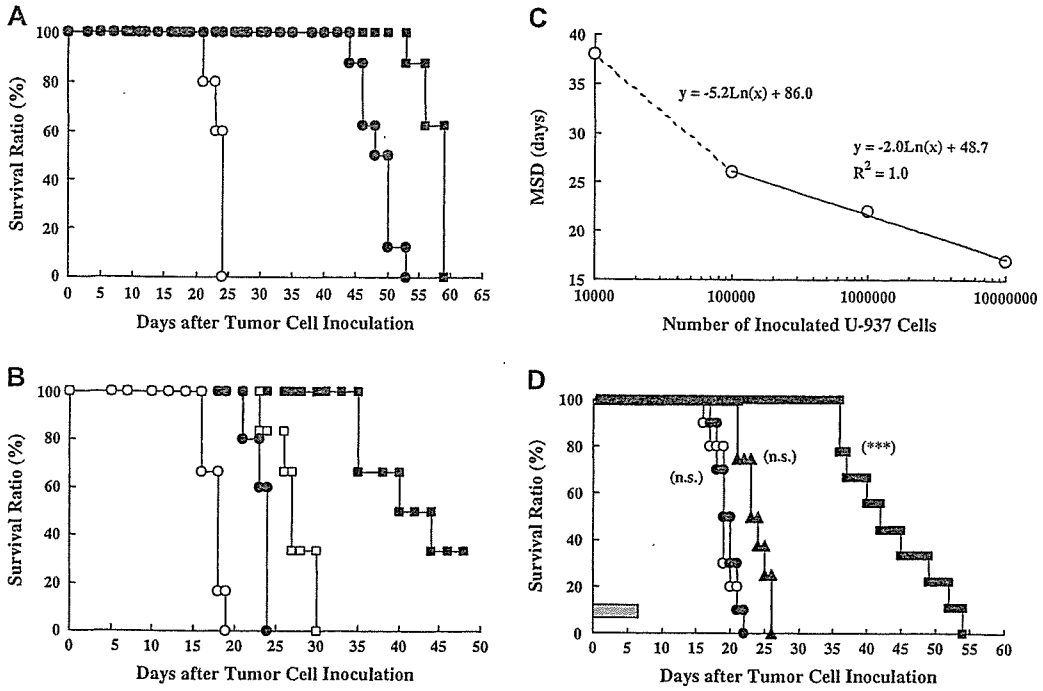

Figure 2. Establishment of a SCID mouse xenograft leukemia model and antileukemic activity of JTE-607. (**A**) Preconditioned SCID mice were inoculated intravenously with 1×10^6 cells of U-937 (○), HL-60 (●), or THP-1 cells (■) on day 0 (n = 8). Mice were observed daily for signs of leukemia and survival. (**B**) Preconditioned SCID mice were inoculated intravenously with 1×10^7 cells (○), 1×10^6 cells (●), 1×10^5 cells (■), or 1×10^4 cells (■) of U-937 on day 0 (n = 6). (**C**) The correlation between mean survival day (MSD) and number of U-937 inoculated. The linear correlation coefficient (R^2) was observed within a range of 10^5 to 10^7 cells. (**D**) Preconditioned SCID mice were inoculated intravenously with 1×10^6 cells of U-937 on day 0. JTE-607 was administered by continuous infusion using osmotic minipumps at dosages of 10 (●), 30 (▲), and 90 mg/kg/day (■) for 7 days, starting on day 0 (n = 10). Control mice (○) were given vehicle in the same way. Mice were observed daily for signs of leukemia and survival. Statistical analysis was performed by the Kaplan-Meier method and the log-rank test. ***: $p < 0.0001$ vs control group. n.s., not significant.

inoculated U-937 cells was observed at a dose of 10^4 to 10^7 cells, and a linear correlation coefficient (R2) was observed in the range of 10^5 to 10^7 cells (Fig. 1C). These results indicate the U-937 xenograft SCID mouse leukemia model examined here was a highly controllable model, which precisely reflects the number of leukemia cells proliferating in vivo and enables sensitive assessment of antileukemic activity of drugs.

Using the U-937 xenograft leukemia model, we first examined the effect of early treatment with JTE-607 on the MSD of mice. Because of its short half-life in vivo ($t_{1/2}\alpha$ = 0.1 hour in human), JTE-607 must be administered by intravenous infusion in clinical use. For this reason, in this study administration of JTE-607 was performed by continuous infusion using osmotic minipumps. Minipumps (nominal pumping duration: 7 days) filled with JTE-607 solution were placed under dorsal skin on day 0, immediately after U-937 inoculation. As a result, prolongation of the survival days was observed by treatment with JTE-607 at a dosage of 30 mg/kg/day and above, and was statistically significant at 90 mg/kg/day (Fig. 2D). The MSD for 10, 30, and 90 mg/kg/day was 20 ± 1.6, 24 ± 2.0, and 43 ± 6.9 days, respectively (Table 2). When proliferation of U-937 is cytostatically suppressed during the initial 7 days, the MSD is estimated to be 26 days from the tight correlation between cell number and MSD. The MSD for the 90 mg/kg/day group was much longer than this estimated MSD; therefore it was likely that JTE-607 exerted killing activity against U-937 cells in vivo.

Table 2. Summary of MSD (mean survival day) in SCID mice treated with JTE-607 and cytarabine

Treatment group	Dosage	MSD ± SD (days)	(p value)
Early treatment (osmotic minipump, S.C., day 0 ± 7, n = 10)			
Control		19 ± 1.7	
JTE-607	10 mg/kg/day	20 ± 1.6	(n.s.)
	30 mg/kg/day	24 ± 2.0	(n.s.)
	90 mg/kg/day	43 ± 6.9	($p < 0.001$)
Delayed treatment (osmotic minipump, S.C., day 12 ± 19, n = 10)			
Control		19 ± 1.5	
JTE-607	30 mg/kg/day	21 ± 1.2	(n.s.)
	90 mg/kg/day	26 ± 1.1	($p < 0.001$)
Delayed treatment (once a day, S.C., day12 ± 15, n = 10)			
Control		20 ± 1.8	
Cytarabine	3 mg/kg/day	22 ± 1.8	(n.s.)
	30 mg/kg/day	29 ± 2.3	($p < 0.001$)
	300 mg/kg/day	19 ± 1.4	(n.s.)

Statistical analysis was performed by Mann-Whitney U test. n.s., not significant vs control group; S.C., subcutaneously; SD, standard deviation.

In vivo therapeutic effect of JTE-607 and cytarabine (delayed treatment)

We next examined the therapeutic effect of JTE-607 on the established leukemia by delayed administration to predict its clinical potency. Continuous infusion of JTE-607 by osmotic minipumps (30 or 90 mg/kg/day, the nominal duration of pumping: 7 days) was started on day 12, at which the mice showed signs of leukemia (paralysis of hindlimb). In parallel, the other leukemia mice were subcutaneously injected with cytarabine at 3, 30, or 300 mg/kg/day for 4 consecutive days starting on day 12. JTE-607 showed its therapeutic potency at 90 mg/kg/day with significant prolongation of the mouse survival (Fig. 3A). The comparable effect was observed with cytarabine at a dosage of 30 mg/kg/day, and this was thought to be close to the maximum effect of cytarabine in this model, because dosage of 300 mg/kg/day cytarabine is apparently toxic (Fig. 3B). The efficacies of JTE-607 and cytarabine are summarized in Table 2, with MSD values.

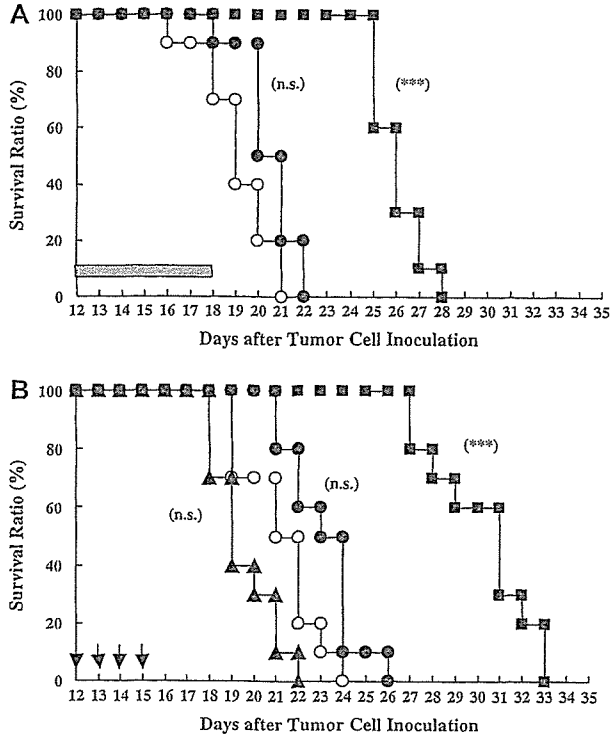

Figure 3. Therapeutic effects of JTE-607 and cytarabine in vivo. (A) Preconditioned SCID mice were inoculated intravenously with 1×10^6 cells of U-937 on day 0. JTE-607 was administered by continuous infusion using osmotic minipumps at dosages of 30 (●) and 90 mg/kg/day (■) for 7 days, starting on day 12 (n = 10). Control mice (○) were given vehicle in the same way. (B) The leukemia mice were subcutaneously injected with cytarabine at dosages of 3 (●), 30 (■), and 300 mg/kg/day (▲), once a day for 4 days from day 12 (n = 10). Control mice (○) were given vehicle in the same way. Mice were observed daily for signs of leukemia and survival. Statistical analysis was performed by the Kaplan-Meier method and the log-rank test. ***: $p < 0.0001$ vs corresponding control group. n.s., not significant.

Inhibitory effects on human IL-8 level in plasma and proportion of human CD45$^+$ cells in bone marrow

In the examination of bone marrow cells of the mice, it was revealed that human CD45$^+$ cells became detectable by flow-cytometer analysis following the appearance of signs of leukemia. The proportion of human CD45$^+$ cells reached 3 to 5% of total bone marrow cells just prior to death of mice (day 18). Human IL-8 also became detectable in the mouse plasma, indicating that lethal infiltration and expansion of U-937 cells in the mouse organs accompanied by massive cytokine production took place. To ascertain the diminishment of AML cells and inhibition of cytokine production by JTE-607, mice injected with U-937 cells were infused with 90 mg/kg/day of JTE-607 for the initial 7 days and sacrificed on day 18, then the proportion of human CD45$^+$ cells in bone marrow and human IL-8 levels in mice plasma were determined. For comparison, the same analysis was conducted with another group of mice that were administered 100 mg/kg of JTE-607 by bolus injection on day 18 and sacrificed 3 hours after the injection. The results showed that JTE-607 administered by infusion significantly reduced both the proportion of human CD45$^+$ cells and the human IL-8 level beyond the limits of detection (Fig. 4). The reduction of IL-8 level was also observed 3 hours after the bolus injection of JTE-607, whereas human CD45$^+$ cells in bone marrow were not altered by the ephemeral treatment (Fig. 4). These results confirm the antileukemic effect of JTE-607 that is exerted through a time-dependent process, and the fast response of AML cells to the activity of JTE-607 on cytokine production in vivo.

Inhibitory effects of JTE-607 on IL-8 production from U-937 cell in vitro

The appearance of human IL-8 in the mouse plasma suggests a large amount is produced from U-937 cells, but this cannot be accounted for by the low level spontaneous production in vitro. It is most likely that U-937 cells were activated in vivo and their cytokine production was upregulated. To examine alterations in the IL-8 production of U-937 cells and in the effect of JTE-607 on it, U-937 cells were exposed to JTE-607 for 24 hours with or without LPS stimulation. The IL-8 protein level in the culture supernatant was 11.9 pg/mL without stimulation, and was markedly increased by LPS to 198.5 pg/mL. JTE-607 inhibited the production of IL-8 concentration-dependently, regardless of LPS stimulation; however, the inhibitory activity against LPS-stimulated production was stronger than for spontaneous production (Fig. 5). These results are in good concordance with the remarkable reducing effect of JTE-607 on the plasma IL-8 level in vivo.

Discussion

AML is an aggressive disorder with disease-free survival of 30 to 50% even for patients treated with intensive

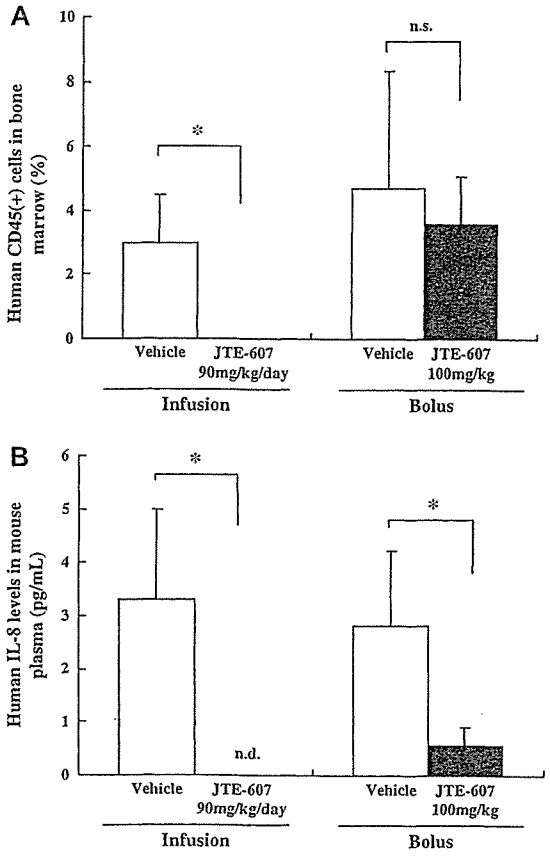

Figure 4. Effects of JTE-607 on human IL-8 level in plasma and proportion of human CD45$^+$ cells in the bone marrow of SCID mice inoculated with U-937 cells. (A) Preconditioned SCID mice were inoculated intravenously with 1 × 10^6 cells of U-937 on day 0. JTE-607 was administered by continuous infusion using osmotic minipumps at a dosage of 90 mg/kg/day for 7 days starting on day 0, or bolus injection at a dose of 100 mg/kg at 3 hours prior to blood collection on day18. Blood samples and bone marrow cells were collected on day18. (A) The proportion of human CD45$^+$ cells in the bone marrow cells was determined by flow-cytometry analysis after staining with FITC-labeled anti-human CD45 and isotype control antibody. Results are expressed as the mean ± standard error (s.e.) (n = 4–5). (B) The concentrations of human IL-8 in plasma were measured by ELISA assay. The detection limit of the assay was 0.5 pg/mL. Results are expressed as the mean ± s.e. (n = 4-5). Statistical analysis was performed using one-way ANOVA by Student's *t*-test *: $p < 0.05$ vs vehicle control group. n.s., not significant.

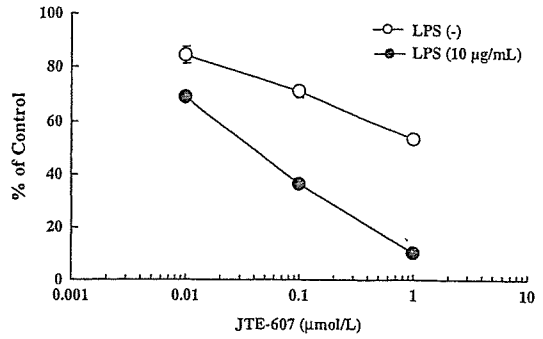

Figure 5. Inhibitory effect of JTE-607 on IL-8 production in U-937 cell in the presence or absence of LPS. U-937 cells (2 × 10^5) were cultured in 24-well flat-bottom microplates for 24 hours in the presence or absence of LPS (10 μg/mL) with indicated concentration of JTE-607 or vehicle. IL-8 concentrations in the culture supernatant were determined by ELISA. Results are expressed as the mean of % of vehicle control in two or three independent experiments.

conventional chemotherapy, because of relapses [1–3]. The crucial roles of inflammatory cytokines and growth factors in the autonomous proliferation of AML blasts are well documented [9]. The majority of leukemic blasts from AML patients produce several inflammatory cytokines and growth factors, such as IL-1, IL-3, IL-6, IL-8, VEGF, GM-CSF, G-CSF, and TNF-α at various levels [11,13,30,31], and a substantial portion of blasts exhibit autonomous proliferation by stimulation with these factors in vitro [7,30]. Inflammatory cytokines produced from AML cells can also stimulate production of growth factors and angiogenic factors from bone marrow stromal cells [5,8,32,33], which support proliferation of AML cells and induce angiogenesis in bone marrow [18,34]. The circulating levels of growth factor in patients with AML is markedly elevated at diagnosis, but decrease to the levels of the normal controls when patients are under chemotherapy or in complete remission [35]. In addition, several lines of evidence suggest that these autocrine and paracrine signalings confer antiapoptotic phenotype and resistance to conventional antileukemic agents in AML cells. In fact, beneficial effects in the clinical application of thalidomide, which inhibits growth factor production, suggest important roles of the autocrine and paracrine signalings in the pathology of hematopoietic malignancies including AML [20,24]. Thus, suppression of cytokine production from AML cells is expected to be a beneficial treatment for AML patients.

JTE-607 has several unique biological activity profiles. JTE-607 inhibits cytokine production from human PBMCs stimulated with LPS, TNF-α, and PMA, with IC_{50} values on the order of 1 nmol/L. The inhibitory activity of JTE-607 emerges as myeloid cell–specific, and no inhibition was observed in the cytokine production from LPS-stimulated fibroblasts or CD3/CD28-stimulated T cells [25]. On the basis of these characteristics, we first examined the effect of JTE-607 on human AML cell lines and found that JTE-607 significantly suppressed cytokine productions that were spontaneously upregulated, such as IL-6 and IL-8. Our work subsequently demonstrated that JTE-607 suppressed proliferation of AML cell lines and AML blasts in patients, by inducing cell-cycle arrest at S phase and apoptosis with accompanying downregulation of c-Myc and upregulation of p21 (manuscript in preparation). Furthermore, unlike conventional antileukemic drugs, the inhibitory effect of JTE-607 on CFU-GM of normal human bone marrow cells was 10 to 100 times weaker than those in the proliferation assay of AML cell lines. Western blot analysis revealed that spontaneous phosphorylation of p38 MAPK and MEK1/2 in U-937 cells was partially reduced

by JTE-607 (data not shown). Although the molecular target of JTE-607 has not yet been identified, these findings suggest that the target of JTE-607 is upstream of both MKK3/6-p38 and MEK1/2-ERK pathways. p38 MAPK is well known to be responsible for inflammatory cytokine production, and ERK is also responsible for cytokine production as well as cell proliferation [36,37]. Thus, in vitro and in vivo effects of JTE-607 are likely to represent concurrent inhibition of constitutively activated p38 and ERK pathways, and ERK inhibition promotes alteration of c-Myc and p21 levels, leading to cell-cycle arrest and apoptosis. It is also noteworthy that JTE-607 has ability to exert these effects without affecting growth of normal bone marrow cells or hematopoietic growth factor production from stromal cells, which are critical for maintenance and recovery of normal bone marrow hematopoiesis after conventional chemotherapy and radiotherapy. With respect to the presumed mechanism of action, sensitivity of AML cells to JTE-607 may be determined by importance of p38 and ERK pathways in cytokine production and autonomous proliferation. However, we have not conducted comparative analysis of MAPK activities in each cell line.

In the present study, we focused on clarifying whether JTE-607 could exert its unique activity on both proliferation and cytokine production in AML cells in vivo. To this end, we employed a SCID xenograft acute leukemia model in which preconditioned SCID mice were engrafted with U-937 cells intravenously. Among the AML cell lines tested, U-937 cells showed the most vigorous proliferation in the mice, which was thought to mimic aggressive leukemic burden in AML and to be suitable for investigation of efficacy of drugs. In addition, the strict correlation between MSD and number of U-937 cells inoculated indicates that MSD directly reflects the number of cells remaining and proliferating in vivo: therefore accurate and sensitive evaluation of antiproliferative effects of drugs can be performed by simple comparison of MSD. In this model, JTE-607 administered at 90 mg/kg/day by continuous infusion for the initial 7 days prolonged MSD to 43 days. Because the mice of control group receiving 10^6 cells showed an MSD of 19 days, if the effect of JTE-607 is cytostatic and the cells restart to grow following the end of administration, then the MSD should be prolonged 7 days and estimated to be 26 days (19 + 7 days). The MSD for the 90 mg/kg/day group was much longer than this estimated MSD, and the prolongation corresponds to a roughly 1/1000 reduction in number of U-937 cells, suggesting killing activity of JTE-607 against U-937 cells in vivo. This was further confirmed by showing the reduced proportion of human $CD45^+$ cells in bone marrow cells even 12 days after the completion of continuous infusion. Moreover, JTE-607 also prolonged the mouse survival in the delayed-treatment study that was conducted to predict the clinical potential, and the effect was comparable with that of cytarabine that was administered with the optimal regimen in this model. Therefore, we conclude that JTE-607 has a potential to exhibit clinically beneficial effect with at least similar extent to that of cytarabine by its antiproliferative, apoptosis-inducing activity.

Among cytokines and growth factors produced from U-937 cells, IL-8 could be detected in the mouse plasma over the detection limit of ELISA after the establishment of leukemia. IL-8 is known as a proangiogenic mediator and is produced by AML blasts at high levels in most patients [12,14]. Although the spontaneous production level of IL-8 is low in U-937 cells in vitro, the production can be ultimately upregulated by LPS stimulation. It is therefore conceivable that preconditioning of the mice caused LPS leakage from gastrointestinal tract and release of cross-reactive inflammatory cytokines from the mouse tissues, and with these proinflammatory stimuli, U-937 cells were activated to produce a large amount of IL-8. We consider that the conditions in this model correspond to the elevated plasma levels of cytokines and growth factors commonly observed in AML patients, which is most likely due to the activation of AML blasts by various stimuli in pathophysiological processes. JTE-607 showed significant inhibition against this upregulated production of IL-8 even after single administration, without affecting human $CD45^+$ cell proportion in the bone marrow cells. Indeed, the inhibitory effect of JTE-607 on IL-8 production was more potent when U-937 cells were stimulated with LPS, probably due to accelerated contribution of the target signal-transduction cascade in the cytokine production. Together, these observations demonstrate the in vivo efficacy of JTE-607 on the cytokine production of AML cells, which may exacerbate the pathology of AML through the autocrine and paracrine signaling.

In conclusion, we demonstrated the unique antileukemic effect of JTE-607 in vivo using a sophisticated acute leukemia model. The results suggest JTE-607 exhibits its antileukemic effect by suppression of both proliferation and inflammatory cytokine production in AML cells. Although further experiments will be required to show the direct involvement of inflammatory cytokines produced from both AML blasts and host cells in the pathology of the leukemia model, the results in the present work revealed that JTE-607 is a promising antileukemic drug candidate that may bring about a new approach in the therapy of AML.

Acknowledgment

We would like to thank Y. Hirata for technical support; Y. Watanabe, K. Wakitani, and K. Iwata for their critical comments; and H. Watanabe for his research coordination over the course of this study.

References

1. Lowenberg B, Downing JR, Burnett A. Acute myeloid leukemia. N Engl J Med. 1999;341:1051–1062.

2. Ohno R, Kobayashi T, Tanimoto M, et al. Randomized study of individualized induction therapy with or without vincristine, and of maintenance-intensification therapy between 4 or 12 courses in adult acute myeloid leukemia. AML-87 Study of the Japan Adult Leukemia Study Group. Cancer. 1993;71:3888–3895.
3. Hann IM, Stevens RF, Goldstone AH, et al. Randomized comparison of DAT versus ADE as induction chemotherapy in children and younger adults with acute myeloid leukemia. Results of the Medical Research Council's 10th AML trial (MRC AML10). Blood. 1997;89:2311–2318.
4. Stone RM. The difficult problem of acute myeloid leukemia in the older adult. CA Cancer J Clin. 2002;52:363–371.
5. Fiedler W, Graeven U, Ergün S, et al. Vascular endothelial growth factor, a possible paracrine growth factor in human acute myeloid leukemia. Blood. 1997;89:1870–1875.
6. Bruserud Ø, Ryningen A, Wergeland L, Glenjen N, Gjertsen BT. Osteoblasts increase proliferation and release of pro-angiogenic interleukin 8 by native human acute myelogenous leukemia blasts. Haematologica. 2004;89:391–402.
7. Ryningen A, Wergeland L, Glenjen N, Gjertsen BT, Bruserud Ø. In vitro crosstalk between fibroblasts and native human acute myelogenous leukemia (AML) blasts via local cytokine networks results in increased proliferation and decreased apoptosis of AML cells as well as increased levels of proangiogenic Interleukin 8. Leuk Res. 2005;29:185–196.
8. Glenjen N, Hatfield K, Bruserud Ø. Coculture of native human acute myelogenous leukemia blasts with fibroblasts and osteoblasts results in an increase of vascular endothelial growth factor levels. Eur J Haematol. 2005;74:24–34.
9. Oster W, Cicco NA, Klein H, Hirano T, Kishimoto T, Lindemann A. Participation of cytokine interleukin 6, tumor necrosis factor-α, and interleukin 1-β secreted by acute myelogenous leukemia blasts in autocrine and paracrine leukemia growth control. J Clin Invest. 1989;84:451–457.
10. Rogers SY, Bradbury D, Kozlowski R, Russell NH. Evidence for internal autocrine regulation of growth in acute myeloblastic leukemia cells. Exp Hematol. 1994;22:593–598.
11. Foss B, Mentzoni L, Bruserud Ø. Effects of vascular endothelial growth factor on acute myelogenous leukemia blasts. J Hematother Stem Cell Res. 2001;10:81–93.
12. Glenjen N, Hovland R, Wergeland L, Wendelbo O, Ernst P, Bruserud Ø. The angioregulatory phenotype of native human acute myelogenous leukemia cells: influence of karyotype, Flt3 abnormalities and differentiation status. Eur J Haematol. 2003;71:163–173.
13. Vinante F, Rigo A, Vincenzi C, et al. IL-8 mRNA expression and IL-8 production by acute myeloid leukemia cells. Leukemia. 1993;7:1552–1556.
14. Tobler A, Moser B, Dewald B, et al. Constitutive expression of interleukin-8 and its receptor in human myeloid and lymphoid leukemia. Blood. 1993;82:2517–2525.
15. Aguayo A, Estey E, Kantarjian H, et al. Cellular vascular endothelial growth factor is a predictor of outcome in patients with acute myeloid leukemia. Blood. 1999;94:3717–3721.
16. Aguayo A, Kantarjian H, Estey E, et al. Plasma vascular endothelial growth factor levels have prognostic significance in patients with acute myeloid leukemia but not in patients with myelodysplastic syndromes. Cancer. 2002;95:1923–1930.
17. Meyers CA, Albitar M, Estey E. Cognitive impairment, fatigue, and cytokine levels in patients with acute myelogenous leukemia or myelodysplastic syndrome. Cancer. 2005;104:788–793.
18. de Bont ES, Vellenga E, Molema G, van Wering E, de Leij LF, Kamps WA. A possible role for spontaneous interleukin-8 production by acute myeloid leukemia cells in angiogenesis related process: work in progress. Med Pediatr Oncol. 2001;37:511–517.
19. Padro T, Ruiz S, Bieker R, et al. Increased angiogenesis in the bone marrow of patients with acute myeloid leukemia. Blood. 2000;95:2637–2644.
20. Komorowski J, Jerczyńska H, Siejka A, et-al. Effect of thalidomide affecting VEGF secretion, cell migration, adhesion and capillary tube formation of human endothelial EA.hy 926 cells. Life Sci. In press.
21. Rosiñol L, Cibeira MT, Segarra M, et al. Response to thalidomide in multiple myeloma: impact of angiogenic factors. Cytokine. 2004;26:145–148.
22. Zorat F, Shetty V, Dutt D, et al. The clinical and biological effects of thalidomide in patients with myelodysplastic syndromes. Br J Haematol. 2001;115:881–894.
23. Greenberg P. Treatment of myelodysplastic syndrome with agents interfering with inhibitory cytokines. Ann Rheum Dis. 2001;60:iii41–iii42.
24. Steins MB, Padro T, Bieker R, et al. Efficacy and safety of thalidomide in patients with acute myeloid leukemia. Blood. 2002;99:834–839.
25. Kakutani M, Takeuchi K, Waga I, Iwamura H, Wakitani K. JTE-607, a novel inflammatory cytokine synthesis inhibitor without immunosuppression, protects from endotoxin shock in mice. Inflamm Res. 1999;48:461–468.
26. Iwamura H, Sato M, Wakitani K. Comparative study of glucocorticoids, cyclosporine A, and JTE-607 [(-)-Ethyl-N[3,5-dichloro-2-hydroxy-4-[2-(4-methylpiperazin-1-yl)ethoxy]benzoyl]-L-phenylalaninate dihydrochloride] in a mouse septic shock model. J Pharmacol Exp Ther. 2004;311:1256–1263.
27. Sasaki J, Fujishima S, Iwamura H, Wakitani K, Aiso S, Aikawa N. Prior burn insult induces lethal acute lung injury in endotoxemic mice: effects of cytokine inhibition. Am J Physiol Lung Cell Mol Physiol. 2003;284:270–278.
28. Sasakawa Y, Naoe Y, Inoue T, et al. Effects of FK228, a novel histone deacetylase inhibitor, on human lymphoma U-937 cells in vitro and in vivo. Biochem Pharmacol. 2002;64:1079–1090.
29. Matsunaga T, Takemoto N, Sato T, et al. Interaction between leukemic-cell VLA-4 and stromal fibronectin is a decisive factor for minimal residual disease of acute myelogenous leukemia. Nat Med. 2003;9:1158–1165.
30. Sugiyama H, Inoue K, Yamagami T, Soma T, Miyake S, Hirata M. The expression of IL-6 and its related genes in acute leukemia. Leuk Lymphoma. 1996;21:49–52.
31. Russell NH. Autocrine growth factors and leukaemic haemopoiesis. Blood Rev. 1992;6:149–156.
32. Oster W, Lindermann A, Horn S, Mertelsmann R, Herrmann F. TNF-α but not TNF-β induces secretion of colony-stimulation factor for macrophages (CSF-1) by human monocytes. Blood. 1987;70:1700–1703.
33. Munker R, Gasson J, Ogawa M, Koeffler HP. Reconbinant human TNF induces production of granulocyte-monocyte colony-stimulation factor. Nature. 1986;323:79–83.
34. de Bont ES, Rosati S, Jacobs S, Kamps WA, Vellenga E. Increased bone marrow vascularization in patients with acute myeloid leukaemia: a possible role for vascular endothelial growth factor. Br J Haematol. 2001;113:296–304.
35. Hsu HC, Lee YM, Tsai WH, et al. Circulating levels of thrombopoietic and inflammatory cytokines in patients with acute myeloblastic leukemia and myelodysplastic syndrome. Oncology. 2002;63:64–69.
36. Dumitru CD, Ceci JD, Tsatsanis C, et al. TNF-α induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. Cell. 2000;103:1071–1083.
37. Kooijman R, Coppens A, Hooghe-Peters E. IGF-I stimulates IL-8 production in the promyelocytic cell line HL-60 through activation of extracellular signal–regulated protein kinase. Cell Signal. 2003;15:1091–1098.

Eur J Haematol 2006: 77: 46–50
doi:10.1111/j.0902-4441.2006.t01-1-EJH2608.x

EUROPEAN JOURNAL OF HAEMATOLOGY

Cord blood transplantation for acute myelogenous leukemia using a conditioning regimen consisting of granulocyte colony-stimulating factor-combined high-dose cytarabine, fludarabine, and total body irradiation

Tomonari A, Takahashi S, Ooi J, Nakaoka T, Takasugi K, Uchiyama M, Tsukada N, Konuma T, Iseki T, Tojo A, Asano S. Cord blood transplantation for acute myelogenous leukemia using a conditioning regimen consisting of granulocyte colony-stimulating factor-combined high-dose cytarabine, fludarabine, and total body irradiation.

Abstract: The cytotoxic effect of cytarabine (Ara-C) on myeloid leukemic cells is enhanced by concomitant use of granulocyte colony-stimulating factor (G-CSF) *in vitro*. The feasibility of a conditioning regimen consisting of G-CSF-combined 24 g/m^2 Ara-C, 90 mg/m^2 fludarabine, and 12 Gy total body irradiation was studied for five patients with acute myelogenous leukemia in cord blood transplantation (CBT). Graft vs. host disease (GVHD) prophylaxis consisted of cyclosporine and methotrexate. After the conditioning regimen, 2.48×10^7/kg (2.28–3.53) of cord blood nucleated cells was infused. Neutrophil counts consistently $> 0.5 \times 10^9$/L was achieved 24 d (22–32) after CBT. Grade I stomatitis and gastrointestinal toxicities occurred in all patients. Grades I and II acute GVHD occurred in one and four patients, respectively, which resolved without steroid therapy. Sepsis and aspergillosis occurred in two and one patients, respectively. All patients were alive without leukemia relapse at a follow up of 15 months (12–43) after CBT. This conditioning regimen could avoid the toxicities of high-dose cyclophosphamide but might enhance the cytotoxic effect of Ara-C. Large-scale studies will be needed to determine the efficacy and safety of the conditioning regimen in CBT.

Akira Tomonari[1], Satoshi Takahashi[1], Jun Ooi[1], Takashi Nakaoka[2], Kashiya Takasugi[1], Michihiro Uchiyama[1], Nobuhiro Tsukada[1], Takaaki Konuma[1], Tohru Iseki[1], Arinobu Tojo[1], Shigetaka Asano[1,3]

[1]Department of Hematology/Oncology, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan; [2]Department of Advanced Medical Science, The Institute of Medical Science, The University of Tokyo, Tokyo; [3]Integrative Bioscience and Biomedical Engineering, School of Science & Engineering, Waseda University, Tokyo, Japan

Key words: granulocyte colony stimulating factor; cytarabine; acute myelogenous leukemia; cord blood, transplantation cardiac dysfunction

Correspondence: Akira Tomonari, MD, PhD, Department of Hematology/Oncology, The Institute of Medical Science, The University of Tokyo, 4–6-1, Shirokanedai, Minato-ku, Tokyo 108–8639, Japan
Tel: +81-3-3443-8111
Fax: +81-3-5449-5429
e-mail: atomonar@ims.u-tokyo.ac.jp

Accepted for publication 5 February 2006

The cytotoxic effect of cell-cycle-dependent agent cytarabine (Ara-C) on myeloid leukemic cells is enhanced by concomitant use of granulocyte colony stimulating factor (G-CSF) *in vitro* (1, 2). The clinical efficacy of a conditioning regimen consisting of G-CSF-combined 24 g/m^2 Ara-C and 12 Gy total body irradiation (TBI) for patients with acute myelogenous leukemia (AML) and myelodysplastic syndrome (MDS) was previously studied in bone marrow transplantation (BMT) from human leukocyte antigen (HLA)-matched sibling donors (1, 3, 4). The results suggested that the regimen was well feasible and might reduce post-transplant relapse in AML and MDS.

Recently, umbilical cord blood transplantation (CBT) from unrelated donors has been performed

increasingly for adult patients (5, 6). For patients with AML and MDS in CBT, we have used a conditioning regimen including G-CSF-combined 12 g/m^2 Ara-C, 120 mg/kg cyclophosphamide (CY), and 12 Gy TBI (7–9). Although this regimen was also shown to be well feasible, high-dose CY has possible adverse effects such as cardiotoxicity, in particular for patients with cardiac dysfunction before CBT (10, 11). In addition, this regimen contained only a half-dose of Ara-C when compared with the regimen for BMT.

To apply the efficacy of G-CSF-combined high-dose Ara-C for myeloid leukemic cells while avoiding toxic effects of high-dose CY, we studied the feasibility of an alternative conditioning regimen consisting of G-CSF-combined high-dose Ara-C, fludarabine, and TBI for patient with AML in CBT. In contrast to BMT from HLA-matched sibling donors, CBT from HLA-mismatched unrelated donors is associated with a high rate of graft failure (12). To enhance the immunosuppressive effect, the conditioning regimen in the present study included fludarabine at a dose of 90 mg/m^2 in addition to the previous regimen used for BMT from HLA-matched sibling donors.

Patients and methods

Patients

From March 2002 to October 2004, 24 patients with AML underwent CBT at our institution. Among them, 19 patients without cardiac dysfunction received a conditioning regimen containing high-dose CY. Because the remaining five patients had cardiac dysfunction at the time of transplantation (Table 1), they were avoided to receive high-dose CY as a conditioning regimen. The patients received a conditioning regimen consisting of G-CSF-combined high-dose Ara-C, fludarabine, and TBI. The patients did not have suitable HLA-matched related and unrelated donors for BMT, and thus were enrolled in our study. Written informed consent was obtained from each patient.

Transplantation procedures

Patients received 12 Gy TBI in four divided fractions on days −8 and −7, Ara-C (3 g/m^2 every 12 h for 4 d) with 5 μg/kg G-CSF (Lenograstim) from 12 h before the first dose of Ara-C to the end of Ara-C, as described previously (1, 3, 4). Administration of Ara-C was initiated at the night on day −6 or the morning on day −5. Fludarabine was administered intravenously at a dose of 30 mg/m^2 on days −5, −4, and −3. Graft vs. host disease (GVHD) prophylaxis consisted of cyclosporine (CSP) (3 mg/kg/d) with a short course of methotrexate (15 mg/m^2 on day +1 and 10 mg/m^2 on days +3 and +6). For bacterial and fungal infection prophylaxis, 450 mg/d tosufloxacin tosilate and 200 mg/d fluconazole or 100 mg/d itraconazole, respectively, were administered from day −14. For herpes simplex virus infection prophylaxis, 1000 mg/d acyclovir was administered orally from day −3 to +35. Cytomegalovirus (CMV) infection was monitored using a CMV antigenemia assay twice a week after engraftment (13). Based on the results of the CMV antigenemia assay, preemptive therapy with intravenous ganciclovir was initiated. To facilitate neutrophil engraftment, G-CSF (Lenograstim) was administered intravenously at a dose of 5 μg/kg/d from day +1 after CBT until sustained neutrophil engraftment was achieved.

Table 1. Characteristics of patients and CB grafts

	Patient number				
	1	2	3	4	5
Age (yr)	47	47	35	43	45
Gender	M	F	M	F	F
Body weight (kg)	76	59	61	45	45
Blood group	A	A	AB	AB	O
FAB classification	M2	M4	M2	M3	M4
Disease status	Rel3	CR1	CR2	CR2	CR2
LVEF (%)	44	53	53	45	45
Medication	ACEI	ACEI	ARB	ARB	ARB
CMV serostatus	P	P	P	P	P
CB graft					
TNC (×10^7/kg)	2.28	2.57	2.48	2.38	3.53
CD34 (×10^5/kg)	0.50	1.11	0.72	0.64	1.35
Gender	F	M	M	M	M
Blood group	A	O	A	A	O
HLA disparity	4	2	3	2	3

M, male; F, female; FAB, French–American–British; Rel3, third relapse; CR1, first complete remission; CR2, second complete remission; LVEF, left ventricular ejection fraction; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; CMV, cytomegalovirus; P, positive; CB, cord blood; TNC, total nucleated cells.

Cord blood graft

The CB unit was chosen according to the number of nucleated cells per recipient's body weight and the HLA compatibility. The matching of HLA-A and B was confirmed by serologic typing methods. The matching of DRB1 was confirmed by genomic typing methods.

Study end points

The primary end points of the study were to evaluate toxicity and engraftment. The secondary end points were assessment of GVHD, day +100 transplantation-related mortality (TRM), relapse, and survival.

Post-transplant evaluation

Neutrophil engraftment was defined as an absolute neutrophil count (ANC) exceeding 0.5×10^9/L for three consecutive days. Platelet engraftment was defined as a platelet count exceeding 20×10^9/L for three consecutive days without platelet transfusion. The chimeric status after CBT was determined either by fluorescence *in situ* hybridization with a Y chromosome probe for sex-mismatched CBT or by polymerase chain reaction (PCR) analyses of polymorphic microsatellite regions for sex-matched CBT. Regimen-related toxicity (RRT) was graded using the Bearman scores (14). Both acute and chronic GVHD were graded according to previously published criteria (15, 16). Data were analyzed as of October 2005.

Results

Patients

Study patients were a median of 45 yr old (35–47) (Table 1). Two patients were males and three patients were females. One patient (no. 1) had AML in relapse and the remaining four patients had AML in complete remission (CR). They all had cardiac dysfunction with left ventricular ejection fraction (LVEF) of <55% as determined by echocardiograph before CBT. Two patients received angiotensin converting enzyme inhibitor (ACEI) therapy and three patients received angiotensin receptor blocker therapy for cardiac dysfunction before CBT.

Engraftment

The median doses of total nucleated cells and CD34-positive cells before freezing were 2.48×10^7/kg (2.28–3.53) and 0.72×10^5/kg (0.50–1.35), respectively (Table 1). An ANC exceeding 0.5×10^9/L was achieved a median of 24 d (22–32) after CBT (Table 2). A platelet count exceeding 20×10^9/L was achieved a median of 31 d (30–75) after CBT. All patients achieved full donor chimerism on the first bone marrow examination after CBT on a median of 41 d (26–47) after CBT. The delayed neutrophil and platelet engraftment in patient no. 2 was probably due to CMV infection and subsequent antiviral treatment before engraftment.

Toxicity

All patients developed grade I stomatitis and gastrointestinal toxicities (Table 2). However, no patients developed grade I or more cardiac, bladder, renal, pulmonary, hepatic, or central nervous system toxicities which were attributable to the conditioning regimen. One patient (no. 4) developed symptomatic congestive heart failure on day +137, which resolved by the administration of diuretics and ACEI, and the discontinuation of CSP. Although the etiology was uncertain, the conditioning regimen seemed not to be associated with the development of this late-onset heart failure. The remaining four patients did not develop symptomatic congestive heart failure after CBT.

Table 2. Results

	Patient number				
	1	2	3	4	5
Engraftment (d)					
Neutrophil	22	32	24	23	25
Platelet	28	75	30	31	39
aGVHD (grade)	I	II	II	II	II
Skin (stage)	2	3	3	3	3
Gut (stage)	0	0	1	0	0
Liver (stage)	0	0	0	0	0
cGVHD	L	L	L	L	No
Toxicity (grade)					
Cardiac	No	No	No	No	No
Bladder	No	No	No	No	No
Renal	No	No	No	No	No
Pulmonary	No	No	No	No	No
Hepatic	No	No	No	No	No
CNS	No	No	No	No	No
Stomatitis	I	I	I	I	I
Gastrointestinal	I	I	I	I	I
Infection (onset day)					
Sepsis	7	7	No	No	No
Aspergillosis	No	No	No	No	18
CMV	31	35	46	36	42
VZV	No	No	No	120	No
HSV	No	144	106	120	No
Survival (months)	43	26	15	12	12
Status	Alive	Alive	Alive	Alive	Alive

aGVHD, acute graft-vs.-host disease; cGVHD, chronic graft-vs.-host disease; L, limited-type; CNS, central nervous system; CMV, cytomegalovirus; VZV, varicella-zoster virus; HSV, herpes simplex virus.

Graft vs. host disease

One patient (no. 1) developed grade I acute GVHD and the remaining four patients developed grade II acute GVHD. However, all patients did not need steroid therapy. Four patients (nos 1–4) developed limited-type chronic GVHD.

Infection

Two patients (nos 1 and 2) developed sepsis by *Enterococcus fecalis* and *fecium*, respectively, both on day +7. Both patients were successfully treated with antibiotics. One patient (no. 5) developed invasive pulmonary aspergillosis on day +18. She was successfully treated with a combination of itraconazole, micafangin, and liposomal ampho-

tericin B. In all patients, CMV infection was documented by an antigenemia assay at a median of 36 d (31–46) after CBT. In one patient (no. 1), CMV antigenemia resolved spontaneously without ganciclovir therapy. In the remaining patients (nos 2–5), CMV antigenemia was successfully treated with pre-emptive therapy with ganciclovir. No patients developed CMV disease. One patient developed localized cutaneous varicella-zoster infection on day +120, and was successfully treated with acyclovir therapy. Two patients (nos 2 and 4) developed oral herpes simplex virus infection on days +144 and +106, respectively, and were successfully treated with acyclovir therapy.

Survival

All patients were alive without leukemia relapse at a median follow up of 15 months (12–43) after CBT.

Discussion

In the present study, we reported five patients with AML who underwent CBT following a conditioning regimen consisting of G-CSF-combined 24 g/m^2 Ara-C, 90 mg/m^2 fludarabine, and 12 Gy TBI. Engraftment and full donor chimerism after CBT were achieved in all patients. The median day of neutrophil engraftment was 24 d after CBT, which seemed to be comparable with 22 and 23 d in our previous studies (7–9). RRT within 100 d after CBT and acute GVHD were well tolerated. All patients were alive without disease progression. The disease status in four of five patients (nos 2–5) was the first or second CR, which would also contribute to the high rate of disease-free survival after CBT. In addition, the graft-vs.-leukemia effect might play a role for preventing leukemia relapse after CBT (5, 6, 9). Although the patient number was too small, these results suggested that this conditioning regimen was feasible. This conditioning regimen could avoid the toxicities of high-dose CY even for patients with cardiac dysfunction but might enhance the cytotoxic effect of Ara-C.

The clinical efficacy of G-CSF-combined high-dose Ara-C for patients with AML and MDS was previously studied by using two conditioning regimens. First, a conditioning regimen consisting of G-CSF-combined 24 g/m^2 Ara-C and 12 Gy TBI was applied to patients with AML and MDS in BMT from HLA-matched sibling donors (1, 3, 4). Next, a conditioning regimen consisting of G-CSF-combined 12 g/m^2 Ara-C, 120 mg/kg CY, and 12 Gy TBI was applied to patients with AML and MDS in CBT from HLA-mismatched unrelated donors (7, 8). These studies showed that the relatively high rates of survival and low rates of relapse and TRM, suggesting that the conditioning regimens with G-CSF-combined high-dose Ara-C were well feasible and might reduce post-transplant relapse in patients with AML and MDS.

High-dose CY used in a conditioning regimen for hematopoietic stem cell transplantation (SCT) can induce significant cardiac toxicity in a dose-dependent manner (10). Thus, we first chose patients with cardiac dysfunction in the present study. In all five patients, LVEF before CBT was reduced to $<55\%$. Fujimaki et al. (11) showed that patients with a reduced LVEF of 55% or less before SCT were at significant risk of severe cardiac toxicity after a CY-containing conditioning regimen. However, it is controversial whether a reduced LVEF before SCT increases the risk of CY-induced cardiac toxicities (17, 18). In our institution, the study on the association between various conditioning regimens and changes of cardiac function after SCT is under way.

One of the major disadvantages in CBT is a high rate of graft failure, particularly in patients with chronic myelogenous leukemia (CML) or severe aplastic anemia (12). Although our results were obtained from only five patients with AML, the conditioning regimen provided sustained engraftment with full donor chimerism and acceptable toxicities. Large-scale studies with a prolonged follow up will be needed to determine the efficacy and safety of this alternative conditioning regimen for CBT.

Acknowledgements

The authors thank Maki Monna-Oiwa for her secretarial assistance. We also thank the Kobayashi Foundation for financial support.

References

1. Takahashi S, Okamoto S, Shirafuji N *et al.* Recombinant human glycosylated granulocyte colony stimulating factor (rhG-CSF)-combined regimen for allogeneic bone marrow transplantation in refractory acute myeloid leukemia. Bone Marrow Transplant 1994;13:239–245.
2. Miyauchi J, Kelleher CA, Wang C, Minkin S, McCulloch EA. Growth factors influence the sensitivity of leukemic stem cells to cytosine arabinoside in culture. Blood 1989;73:1272–1278.
3. Takahashi S, Oshima Y, Okamoto S, Nishiwaki K, Nagayama H, Inoue T, Tojo A, Tani K, Asano S. Recombinant human granulocyte colony-stimulating factor (G-CSF) combined conditioning regimen for allogeneic bone marrow transplantation (BMT) in standard-risk myeloid leukemia. Am J Hematol 1998;57:303–308.
4. Okamoto S, Takahashi S, Wakui M, Ishida A, Tanosaki R, Ikeda Y, Asano S. Treatment of advanced myelodysplastic syndrome with a regimen including recombinant human granulocyte colony-stimulating factor preceding

allogeneic bone marrow transplantation. Br J Haematol 1999;**104**:569–573.
5. Laughlin MJ, Eapen M, Rubinstein P *et al.* Outcomes after transplantation of cord blood or bone marrow from unrelated donors in adults with leukemia. N Engl J Med 2004;**351**:2265–2275.
6. Rocha V, Labopin M, Sanz G *et al.* Acute Leukemia Working Party of European Blood and Marrow Transplant Group; Eurocord-Netcord Registry. Transplants of umbilical-cord blood or bone marrow from unrelated donors in adults with acute leukemia. N Engl J Med 2004;**351**:2276–2285.
7. Ooi J, Iseki T, Takahashi S *et al.* Unrelated cord blood transplantation for adult patients with de novo acute myeloid leukemia. Blood 2004;**103**:489–491.
8. Ooi J, Iseki T, Takahashi S *et al.* Unrelated cord blood transplantation for adult patients with advanced myelodysplastic syndrome. Blood 2003;**101**:4711–4713.
9. Takahashi S, Iseki T, Ooi J *et al.* Single-institute comparative analysis of unrelated bone marrow transplantation and cord blood transplantation for adult patients with hematologic malignancies. Blood 2004;**104**:3813–3820.
10. Goldberg MA, Antin JH, Guinan EC, Rappeport JM. Cyclophosphamide cardiotoxicity: an analysis of dosing as a risk factor. Blood 1986;**68**:1114–1118.
11. Fujimaki K, Maruta A, Yoshida M *et al.* Severe cardiac toxicity in hematological stem cell transplantation: predictive value of reduced left ventricular ejection fraction. Bone Marrow Transplant 2001;**27**:307–310.
12. Rubinstein P, Carrier C, Scaradavou A *et al.* Outcomes among 562 recipients of placental-blood transplants from unrelated donors. N Engl J Med 1998;**339**:1565–1577.
13. Tomonari A, Iseki T, Ooi J *et al.* Cytomegalovirus infection following unrelated cord blood transplantation for adult patients: a single institute experience in Japan. Br J Haematol 2003;**121**:304–311.
14. Bearman SI, Appelbaum FR, Buckner CD, Petersen FB, Fisher LD, Clift RA, Thomas ED. Regimen-related toxicity in patients undergoing bone marrow transplantation. J Clin Oncol 1988;**6**:1562–1568.
15. Przepiorka D, Weisdorf D, Martin P, Klingemann HG, Beatty P, Hows J, Thomas ED. Consensus conference on acute GVHD grading. Bone Marrow Transplant 1995;**15**:825–828.
16. Sullivan KM, Agura E, Anasetti C *et al.* Chronic graft-versus-host disease and other late complications of bone marrow transplantation. Semin Hematol 1991;**28**:250–259.
17. Lehmann S, Isberg B, Ljungman P, Paul C. Cardiac systolic function before and after hematopoietic stem cell transplantation. Bone Marrow Transplant 2000;**26**:187–192.
18. Hertenstein B, Stefanic M, Schmeiser T, Scholz M, Goller V, Clausen M, Bunjes D, Wiesneth M, Novotny J, Kochs M. Cardiac toxicity of bone marrow transplantation: predictive value of cardiologic evaluation before transplant. J Clin Oncol 1994;**12**:998–1004.

In vitro validation of bioluminescent monitoring of disease progression and therapeutic response in leukaemia model animals

Yusuke Inoue[1], Arinobu Tojo[2], Rieko Sekine[2], Yasushi Soda[2], Seiichiro Kobayashi[2], Akiko Nomura[2], Kiyoko Izawa[2], Toshio Kitamura[3], Toshiyuki Okubo[1], Kuni Ohtomo[4]

[1] Department of Radiology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku Tokyo, 108-8639, Japan
[2] Division of Molecular Therapy, Advanced Clinical Research Centre, University of Tokyo, Tokyo, Japan
[3] Division of Cellular Therapy, Advanced Clinical Research Centre, University of Tokyo, Tokyo, Japan
[4] Department of Radiology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

Published online: 24 February 2006

Abstract. *Purpose:* The application of in vivo bioluminescence imaging to non-invasive, quantitative monitoring of tumour models relies on a positive correlation between the intensity of bioluminescence and the tumour burden. We conducted cell culture studies to investigate the relationship between bioluminescent signal intensity and viable cell numbers in murine leukaemia model cells.
Methods: Interleukin-3 (IL-3)-dependent murine pro-B cell line Ba/F3 was transduced with firefly luciferase to generate cells expressing luciferase stably under the control of a retroviral long terminal repeat. The luciferase-expressing cells were transduced with p190 BCR-ABL to give factor-independent proliferation. The cells were cultured under various conditions, and bioluminescent signal intensity was compared with viable cell numbers and the cell cycle stage.
Results: The Ba/F3 cells showed autonomous growth as well as stable luciferase expression following transduction with both luciferase and p190 BCR-ABL, and in vivo bioluminescence imaging permitted external detection of these cells implanted into mice. The bioluminescence intensities tended to reflect cell proliferation and responses to imatinib in cell culture studies. However, the luminescence per viable cell was influenced by the IL-3 concentration in factor-dependent cells and by the stage of proliferation and imatinib concentration in factor-independent cells, thereby impairing the proportionality between viable cell number and bioluminescent signal intensity. Luminescence per cell tended to vary in association with the fraction of proliferating cells.
Conclusion: Although in vivo bioluminescence imaging would allow non-invasive monitoring of leukaemia model animals, environmental factors and therapeutic interventions may cause some discrepancies between tumour burden and bioluminescence intensity.

Keywords: Luciferase – Leukaemia – Retroviruses – Imatinib mesylate – Cell cycle

Eur J Nucl Med Mol Imaging (2006) 33:557–565
DOI 10.1007/s00259-005-0048-4

Yusuke Inoue (✉)
Department of Radiology, Institute of Medical Science,
University of Tokyo,
4-6-1 Shirokanedai, Minato-ku,
Tokyo, 108-8639, Japan
e-mail: inoueys-tky@umin.ac.jp
Tel.: +81-3-34438111, Fax: +81-3-54495746

Introduction

In vivo bioluminescence imaging is used increasingly to evaluate the effects of novel therapeutic strategies against malignant neoplasms in small animal models [1, 2]. For monitoring by bioluminescence imaging, mice are inoculated with tumour cells that stably express luciferase under the control of a constitutive promoter, such as the simian virus 40 (SV40) promoter, the cytomegalovirus (CMV) immediate-early promoter or the long-terminal repeat (LTR) of a retrovirus. Injection of the mice with luciferin, substrate for luciferase, induces light emission from the luciferase-expressing cells, and images that reflect the amount and whole-body distribution of the implanted cells can be acquired using a charge-coupled device (CCD) camera. Quantitative indices of tumour burden can be computed from the images. Owing to the convenient, non-invasive nature of the imaging procedures, measurements can be performed repetitively to assess tumour growth and therapeutic efficacy using each animal as its own control.

The monitoring of tumour models by in vivo bioluminescence imaging relies on a positive correlation between signal intensity on bioluminescence imaging and tumour