cells. GSIs have almost the same effect on progenitor cells of the normal and regenerating crypt, which becomes critically toxic once the epithelia have been damaged. Thus caution is needed with the use of GSIs when intestinal tissue damage is present.

Although studies have revealed various extrinsic factors promoting the regeneration of the intestinal epithelia (2, 3), the intracellular mechanism mediating the regenerative process has not been fully elucidated (15). Our data show that Notch activation maintains the larger number of IECs in the immature state, thereby promoting the proliferation and supporting the rapid recovery of IECs needed to restore proper epithelial structure. Thus we identified Notch signaling as one of the main intracellular pathways mediating the organized regenerative response of the intestinal epithelia. Although we know that several ligands and receptors of the Notch pathway are expressed in the intestine (24, 26), we do not know the precise mechanism by which these ligands activate Notch receptors, in particular IECs. A recent study by Riccio et al. (22) clearly showed that both Notch1 and Notch2 function redundantly in the intestinal epithelia and that they directly regulate the cell cycle progression of crypt progenitor cells. Thus an analysis of the Notch ligand expression is needed to understand the mechanism by which these Notch receptors could be activated during epithelial regeneration and the mechanism by which such activation could be downregulated at the later stage of regeneration.

One of our surprising findings was the upregulation of PLA2G2A in Notch-activated IECs, suggesting that Notch might also modulate immune functions of IECs. PLA2G2A is usually expressed in Paneth cells, and it is known to have an antimicrobial effect (4). The loss of the continuity of the epithelial layer allows various and abundant microorganisms to invade the submucosal area, thereby promoting inflammation and further destruction of the mucosa. Thus the local secretion of PLA2G2A at the damaged mucosal area may be quite beneficial for limiting bacterial invasion and providing a proper environment for regeneration. However, previous reports have shown that PLA2G2A is also expressed by neutrophils and macrophages accumulating at the inflamed mucosa of colitis (29, 39). Consistent with this, we observed an infiltration of PLA2G2A-positive cells in the lamina propria of inflamed mucosa in UC (Fig. 9, *C–F*). An RT-PCR analysis of DSS-colitis showed a significant upregulation of PLA2G2A expression in the inflamed colonic mucosa (Supplemental Fig. S3). However, such an upregulation was not inhibited with LY411,575 treatment, suggesting that the expression of PLA2G2A by neutrophils or macrophages might be less dependent on Notch activation. In those cells, intracellular pathways such as NF-κB might function to promote expression of PLA2G2A in the inflamed colonic mucosa (37). Also, our histological analysis suggested that the upregulation of PLA2G2A secretion was not a general but a partial response in Notch-activated IECs, indicating that an additional condition is required for ectopic expression of PLA2G2A.

Our microarray analysis also revealed a number of genes other than PLA2G2A that are regulated by NICD1 in IECs. Although the results did not show an upregulation of other genes specific to Paneth cells such as lysozyme or α-defensins, our quantitative RT-PCR confirmed that genes such as clusterin or spermidine/spermine N1-acetyltransferase were also upregulated upon Notch1 activation in LS174T cells (data not shown). Trefoil factor-1 may also promote Notch-mediated tissue regeneration because it is known to be a key factor in restitution (11). The group of genes shown in the present analysis was quite distinct from the previous microarray analysis comparing GSI-treated and untreated intestinal tissues (14, 27). Because we used an in vitro IEC-based assay, the group of genes identified can be recognized as candidates of the IEC-specific target genes of Notch. However, because only a limited number of genes were analyzed (up to 10,000 annotated genes) in the present study, a further analysis including a larger group of genes may elucidate additional genes that are regulated downstream of Notch.

In conclusion, Notch signaling acts as an indispensable intracellular signaling pathway in IECs, especially during tissue regeneration. It regulates not only the differentiation, but also the proliferation of IECs, and it also regulates the immune function of IECs. We have shown for the first time that such functions of Notch are also present in the human intestine, both under normal conditions and when tissue damage has occurred. The present study provides a novel molecular basis for the advanced understanding of the regeneration process in the human intestinal epithelia, which may be utilized to establish alternative therapies for refractory ulcers caused by various intestinal diseases.

ACKNOWLEDGMENTS

The authors thank Dr. Tetsuo Sudo for providing the Hes1 antibody, Drs. Ryoichiro Kageyama and Yasuhito Yuasa for providing the plasmids, Drs. Hisao Fukushima, Kazutaka Koganei, Kenichi Sugihara, and Hiroyuki Uetake for providing the tissue samples, Drs. Hideyuki Okano and Takaaki Ito for the helpful discussion, and Dr. Hisanobu Kawamura, Dr. Michio Onizawa, and Ms. Motomi Yamazaki for technical assistance.

GRANTS

This study was supported in part by grants-in-aid for Scientific Research, Scientific Research on Priority Areas, Exploratory Research, and Creative Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology, The Japanese Ministry of Health, Labor and Welfare, The Japanese Society of Gastroenterology, The Foundation for Advancement of International Science, Research Fund of Mitsukoshi Health and Welfare Foundation, and the Research Fund of Japan Intractable Diseases Research Foundation.

REFERENCES

1. **Bjerknes M, Cheng H.** Gastrointestinal stem cells. II. Intestinal stem cells. *Am J Physiol Gastrointest Liver Physiol* 289: G381–G387, 2005.
2. **Bjerknes M, Cheng H.** Modulation of specific intestinal epithelial progenitors by enteric neurons. *Proc Natl Acad Sci USA* 98: 12497–12502, 2001.
3. **Brauchle M, Madlener M, Wagner AD, Angermeyer K, Lauer U, Hofschneider PH, Gregor M, Werner S.** Keratinocyte growth factor is highly overexpressed in inflammatory bowel disease. *Am J Pathol* 149: 521–529, 1996.
4. **Buckland AG, Wilton DC.** The antibacterial properties of secreted phospholipases A(2). *Biochim Biophys Acta* 1488: 71–82, 2000.
5. **Fahlgren A, Hammarstrom S, Danielsson A, Hammarstrom ML.** Increased expression of antimicrobial peptides and lysozyme in colonic epithelial cells of patients with ulcerative colitis. *Clin Exp Immunol* 131: 90–101, 2003.
6. **Fre S, Huyghe M, Mourikis P, Robine S, Louvard D, Artavanis-Tsakonas S.** Notch signals control the fate of immature progenitor cells in the intestine. *Nature* 435: 964–968, 2005.
7. **Haapamaki MM, Gronroos JM, Nurmi H, Alanen K, Kallajoki M, Nevalainen TJ.** Gene expression of group II phospholipase A2 in intestine in ulcerative colitis. *Gut* 40: 95–101, 1997.
8. **Haapamaki MM, Gronroos JM, Nurmi H, Irjala K, Alanen KA, Nevalainen TJ.** Phospholipase A2 in serum and colonic mucosa in ulcerative colitis. *Scand J Clin Lab Invest* 59: 279–287, 1999.

9. **Jensen J, Pedersen EE, Galante P, Hald J, Heller RS, Ishibashi M, Kageyama R, Guillemot F, Serup P, Madsen OD.** Control of endodermal endocrine development by Hes-1. *Nat Genet* 24: 36–44, 2000.
10. **Kopan R.** Notch: a membrane-bound transcription factor. *J Cell Sci* 115: 1095–1097, 2002.
11. **Longman RJ, Douthwaite J, Sylvester PA, Poulsom R, Corfield AP, Thomas MG, Wright NA.** Coordinated localisation of mucins and trefoil peptides in the ulcer associated cell lineage and the gastrointestinal mucosa. *Gut* 47: 792–800, 2000.
12. **Matsumoto T, Okamoto R, Yajima T, Mori T, Okamoto S, Ikeda Y, Mukai M, Yamazaki M, Oshima S, Tsuchiya K, Nakamura T, Kanai T, Okano H, Inazawa J, Hibi T, Watanabe M.** Increase of bone marrow-derived secretory lineage epithelial cells during regeneration in the human intestine. *Gastroenterology* 128: 1851–1867, 2005.
13. **McCormick DA, Horton LW, Mee AS.** Mucin depletion in inflammatory bowel disease. *J Clin Pathol* 43: 143–146, 1990.
14. **Milano J, McKay J, Dagenais C, Foster-Brown L, Pognan F, Gadient R, Jacobs RT, Zacco A, Greenberg B, Ciaccio PJ.** Modulation of notch processing by gamma-secretase inhibitors causes intestinal goblet cell metaplasia and induction of genes known to specify gut secretory lineage differentiation. *Toxicol Sci* 82: 341–358, 2004.
15. **Okamoto R, Watanabe M.** Cellular and molecular mechanisms of the epithelial repair in IBD. *Dig Dis Sci*, 50 *Suppl* 1: S34–S38, 2005.
16. **Okamoto R, Watanabe M.** Molecular and clinical basis for the regeneration of human gastrointestinal epithelia. *J Gastroenterol* 39: 1–6, 2004.
17. **Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R.** A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. *Gastroenterology* 98: 694–702, 1990.
18. **Oshima S, Nakamura T, Namiki S, Okada E, Tsuchiya K, Okamoto R, Yamazaki M, Yokota T, Aida M, Yamaguchi Y, Kanai T, Handa H, Watanabe M.** Interferon regulatory factor 1 (IRF-1) and IRF-2 distinctively upregulate gene expression and production of interleukin-7 in human intestinal epithelial cells. *Mol Cell Biol* 24: 6298–6310, 2004.
19. **Podolsky DK.** Healing the epithelium: solving the problem from two sides. *J Gastroenterol* 32: 122–126, 1997.
20. **Radtke F, Clevers H, Riccio O.** From gut homeostasis to cancer. *Curr Mol Med* 6: 275–289, 2006.
21. **Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R.** Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* 118: 229–241, 2004.
22. **Riccio O, van Gijn ME, Bezdek AC, Pellegrinet L, van Es JH, Zimber-Strobl U, Strobl LJ, Honjo T, Clevers H, Radtke F.** Loss of intestinal crypt progenitor cells owing to inactivation of both Notch1 and Notch2 is accompanied by derepression of CDK inhibitors p27Kip1 and p57Kip2. *EMBO Rep* 9: 377–383, 2008.
23. **Riddle RH.** *Pathology of Idiopathic Inflammatory Bowel Disease.* Orlando, FL: Saunders, 2004.
24. **Sander GR, Powell BC.** Expression of notch receptors and ligands in the adult gut. *J Histochem Cytochem* 52: 509–516, 2004.
25. **Sasai Y, Kageyama R, Tagawa Y, Shigemoto R, Nakanishi S.** Two mammalian helix-loop-helix factors structurally related to Drosophila hairy and Enhancer of split. *Genes Dev* 6: 2620–2634, 1992.
26. **Schroder N, Gossler A.** Expression of Notch pathway components in fetal and adult mouse small intestine. *Gene Expr Patterns* 2: 247–250, 2002.
27. **Searfoss GH, Jordan WH, Calligaro DO, Galbreath EJ, Schirtzinger LM, Berridge BR, Gao H, Higgins MA, May PC, Ryan TP.** Adipsin, a biomarker of gastrointestinal toxicity mediated by a functional gamma-secretase inhibitor. *J Biol Chem* 278: 46107–46116, 2003.
28. **Stanger BZ, Datar R, Murtaugh LC, Melton DA.** Direct regulation of intestinal fate by Notch. *Proc Natl Acad Sci USA* 102: 12443–12448, 2005.
29. **Tomita Y, Jyoyama H, Kobayashi M, Kuwabara K, Furue S, Ueno M, Yamada K, Ono T, Teshirogi I, Nomura K, Arita H, Okayasu I, Hori Y.** Role of group IIA phospholipase A2 in rat colitis induced by dextran sulfate sodium. *Eur J Pharmacol* 472: 147–158, 2003.
30. **Tsuchiya K, Nakamura T, Okamoto R, Kanai T, Watanabe M.** Reciprocal targeting of Hath1 and beta-catenin by Wnt glycogen synthase kinase 3beta in human colon cancer. *Gastroenterology* 132: 208–220, 2007.
31. **van Den Brink GR, de Santa Barbara P, Roberts Development DJ.** Epithelial cell differentiation—a Mather of choice. *Science* 294: 2115–2116, 2001.
32. **van Es JH, Clevers H.** Notch and Wnt inhibitors as potential new drugs for intestinal neoplastic disease. *Trends Mol Med* 11: 496–502, 2005.
33. **van Es JH, van Gijn ME, Riccio O, van den Born M, Vooijs M, Begthel H, Cozijnsen M, Robine S, Winton DJ, Radtke F, Clevers H.** Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. *Nature* 435: 959–963, 2005.
34. **Vooijs M, Ong CT, Hadland B, Huppert S, Liu Z, Korving J, van den Born M, Stappenbeck T, Wu Y, Clevers H, Kopan R.** Mapping the consequence of Notch1 proteolysis in vivo with NIP-CRE. *Development* 134: 535–544, 2007.
35. **Watanabe M, Ueno Y, Yajima T, Okamoto S, Hayashi T, Yamazaki M, Iwao Y, Ishii H, Habu S, Uehira M, Nishimoto H, Ishikawa H, Hata J, Hibi T.** Interleukin 7 transgenic mice develop chronic colitis with decreased interleukin 7 protein accumulation in the colonic mucosa. *J Exp Med* 187: 389–402, 1998.
36. **Wong GT, Manfra D, Poulet FM, Zhang Q, Josien H, Bara T, Engstrom L, Pinzon-Ortiz M, Fine JS, Lee HJ, Zhang L, Higgins GA, Parker EM.** Chronic treatment with the gamma-secretase inhibitor LY-411,575 inhibits beta-amyloid peptide production and alters lymphopoiesis and intestinal cell differentiation. *J Biol Chem* 279: 12876–12882, 2004.
37. **Wu F, Chakravarti S.** Differential expression of inflammatory and fibrogenic genes and their regulation by NF-kappaB inhibition in a mouse model of chronic colitis. *J Immunol* 179: 6988–7000, 2007.
38. **Wu J, Tung JS, Thorsett ED, Pleiss MA, Nissen JS, Neitz J, Latimer JH, John V, Freedman S,** inventors. Cycloalkyl, Lactam, Lactone and related compounds as β-amyloid peptide release inhibitors. US patent WO-1998028268, February 7, 1998.
39. **Yamaguchi O, Sugimura K, Ishizuka K, Suzuki K, Hasegawa K, Ohtsuka K, Honma T, Asakura H.** Correlation between serum phospholipase A(2) IIA levels and histological activity in patients with ulcerative colitis. *Int J Colorectal Dis* 17: 311–316, 2002.
40. **Yamamoto H, Bai YQ, Yuasa Y.** Homeodomain protein CDX2 regulates goblet-specific MUC2 gene expression. *Biochem Biophys Res Commun* 300: 813–818, 2003.
41. **Yamazaki M, Yajima T, Tanabe M, Fukui K, Okada E, Okamoto R, Oshima S, Nakamura T, Kanai T, Uehira M, Takeuchi T, Ishikawa H, Hibi T, Watanabe M.** Mucosal T cells expressing high levels of IL-7 receptor are potential targets for treatment of chronic colitis. *J Immunol* 171: 1556–1563, 2003.
42. **Zecchini V, Domaschenz R, Winton D, Jones P.** Notch signaling regulates the differentiation of post-mitotic intestinal epithelial cells. *Genes Dev* 19: 1686–1691, 2005.

Alimentary Pharmacology & Therapeutics

Clinical trial: comparison of alendronate and alfacalcidol in glucocorticoid-associated osteoporosis in patients with ulcerative colitis

S. KITAZAKI, K. MITSUYAMA, J. MASUDA, K. HARADA, H. YAMASAKI, K. KUWAKI, H. TAKEDATSU, G. SUGIYAMA, O. TSURUTA & M. SATA

Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, Kurume, Japan

Correspondence to:
Dr K. Mitsuyama, Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830-0011, Japan.
E-mail: ibd@med.kurume-u.ac.jp

Publication data
Submitted 9 July 2008
First decision 14 July 2008
Resubmitted 9 November 2008
Accepted 10 November 2008
Epub Accepted Article 25 November 2008

SUMMARY

Background
Bone loss is often observed in patients with ulcerative colitis, particularly if they require glucocorticoids.

Aim
To determine whether the bisphosphonate, alendronate, is safe and effective in preserving bone mass compared to the active vitamin D3, alfacalcidol, in ulcerative colitis patients receiving glucocorticoids.

Methods
Thirty-nine patients with ulcerative colitis and treated with glucocorticoids were randomized to receive alendronate (5 mg/day) or alfacalcidol (1 μg/day) daily for 12 months. Loss of bone mass was evaluated by bone mineral density, bone resorption by urinary *N*-telopeptide for type I collagen, and bone formation by serum bone alkaline phosphatase.

Results
Alendronate, but not alfacalcidol, significantly increased bone mineral density in the lumbar spine. Alendronate decreased serum bone alkaline phosphatase levels, but alfacalcidol did not. Urinary *N*-telopeptide for type I collagen levels decreased in both groups, but were significantly lower in the alendronate group. There were no significant differences in the adverse events in the two groups.

Conclusion
Our study indicates that alendronate is a safe, well-tolerated and more effective therapy than alfacalcidol for preventing glucocorticoid-associated bone loss in patients with ulcerative colitis.

Aliment Pharmacol Ther **29**, 424–430

doi:10.1111/j.1365-2036.2008.03899.x

INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD), collectively referred to as inflammatory bowel disease (IBD), are chronic, aggressive disorders with an increased prevalence of low bone mineral density (BMD).[1–5] Low BMD in IBD occurs in both males and females and may occur at an early age, resulting in significant morbidity due to the increased risk of fragility fracture. Low BMD can be a complication of glucocorticoid use,[6, 7] as glucocorticoids inhibit bone formation by decreasing the number of osteoblasts and hampering their function. Glucocorticoids also increase the rate of bone resorption by stimulating the formation and action of osteoclasts. Furthermore, glucocorticoids decrease intestinal absorption of calcium and increase renal calcium excretion.

There are two primary therapeutic agents for the management of glucocorticoid-associated osteoporosis.[8] The bisphosphonates, including alendronate and risedronate, induce apoptosis of osteoclasts and inhibit bone resorption. Active vitamin D3 analogues, such as alfacalcidol and calcitriol, stimulate the formation and action of osteoblasts leading to increased bone formation. Few trials have tested the effect of bisphosphonates or active vitamin D3 in patients with IBD. Active vitamin D3 and calcium replacement improve BMD in patients with CD and UC.[9–11] Trials limited to patients with CD have examined bisphosphonate therapy.[12, 13] Bisphosphonate was effective for the treatment of osteoporosis in 29 patients with CD and 19 with UC in a double-blind, placebo-controlled trial.[14]

Bisphosphonate and active vitamin D3 show similar efficacy on BMD. In a randomized study of patients with rheumatic disease, alendronate was more effective than alfacalcidol in the treatment of glucocorticoid-associated osteoporosis.[15] In contrast, other studies showed that alendronate and calcitriol were equally effective in the prevention of glucocorticoid-associated osteoporosis in patients with a renal or cardiac transplant.[16, 17] Thus, a comparative study is needed in patients with IBD.

Bisphosphonates can produce adverse effects on the gastrointestinal tract,[18] which is an important consideration for IBD patients. Therefore, clinical trials should incorporate both efficacy and adverse events. Here, we compared the effects of bisphosphonate (alendronate) and active vitamin D3 (alfacalcidol), including the clinical efficacy and adverse events profile, in patients with UC who were either receiving or starting treatment with glucocorticoids.

PATIENTS AND METHODS

Participant selection

Subjects were selected from patients with UC seen at Kurume University Hospital. During this 12-month, randomized study, we enrolled 39 patients between the ages of 17 and 70 years who had UC and were either starting glucocorticoid therapy or had started glucocorticoid therapy at a daily dose of at least 5 mg of prednisone or its equivalent. The diagnosis of UC was based on characteristic clinical, endoscopic, radiological and histological features.

Exclusion criteria included abnormal calcium or bone-related biochemistry, in particular, vitamin D deficiency; concomitant liver and renal disease; pregnancy or breast feeding; the presence of osteoporotic fractures; any hormone replacement therapy and previous bisphosphonate or active vitamin D3 therapy.

Ethical considerations

Before participation in this study, all subjects signed an informed consent document and the study was approved by the Ethics Committee of Kurume University Hospital.

Study design

This is a pilot study conducted in patients with UC. The specific objectives were to determine the efficacy and safety of alendronate treatment in patients with UC in comparison with those treated with alfacalcidol. Patients were randomized to receive alendronate (5 mg/day; Banyu Pharmaceutical, Tokyo, Japan) or alfacalcidol (1 μg/day; Teijin Pharma Limited, Tokyo, Japan) daily for 12 months. Calcium was not supplemented to either group. Patient allocation was undertaken at random by the envelope method. Because of ethical considerations, an untreated control group was not included in this study. The following were obtained every month for 12 months: medical history and physical examination, a haematological screen and urinalysis. Adverse events were determined from a self-report of the patient. Disease activity was determined with the index by Truelove and Witts.[19]

BMD measurements

Bone mineral density was reviewed at baseline and at 6 and 12 months of therapy. BMD was measured by dual energy X-ray absorptiometry using a Hologic DELPHI-C bone densitometer (Hologic Inc., Waltham, MA, USA). Measurements were made at the spine L2–L4 and the bilateral femoral neck sites.

X-ray examinations

Anteroposterior and lateral X-ray films of the lumbar spine were obtained at baseline and at 6 and 12 months of therapy.

Bone metabolism measurements

Bone metabolism markers,[20] serum bone alkaline phosphatase (BAP; a marker of bone formation),[21] urinary type I collagen cross-linked *N*-telopeptides (NTx; a marker of bone resorption)[22] and serum calcium corrected for albumin were determined at baseline and at 3, 6 and 12 months later.

Bone alkaline phosphatase was measured in serum samples using an enzyme-linked immunosorbent assay (ELISA; Osteolinks-BAP high-sensitivity diagnostic ELISA kit; DS Pharma Biomedical, Osaka, Japan). The intra- and inter-assay CV was 2.84% and 2.24% respectively. NTx in urine samples was determined by ELISA (Osteomark; Ostex International, Inc., Seattle, WA, USA). The intra- and inter-assay CV was 4.30% and 2.51% respectively. Serum calcium (mg/dL) was corrected for serum albumin according to the following formula: calcium + [4 − albumin (g/dL)]. The normal range of serum calcium corrected for albumin was 8.7–10.3 mg/dL.

Figure 1. Flow diagram of the patients' progression through the study.

Statistical analysis

Results were expressed as mean ± S.E. All statistical analyses were performed using the SPSS version of 12.0.2J for Windows (SPSS Japan Inc., Tokyo, Japan). Data were compared using paired *t*-tests or unpaired *t*-tests. For all studies, a *P*-value of <0.05 was considered statistically significant.

RESULTS

Study patients

Figure 1 is a flow diagram showing subjects' progression through the study. The first patient started treatment with a study medication in April 2005 and the last patient finished the study in June 2007. Of the 39 patients, 19 were randomized to alendronate and 20 to alfacalcidol, three individuals declined participation, four were lost to follow-up and two withdrew due to concerns over potential side effects. Finally, 14–16 alendronate-treated patients and 18 alfacalcidol-treated patients were analysed in this study.

There were no significant differences in baseline age, gender, body weight, height, body mass index, disease duration, disease activity, daily prednisone doses, cumulative prednisone doses, BMD, serum BAP, urinary NTx or serum calcium between the two groups (Table 1).

At the end of the study, glucocorticoid use was not significantly different in the alendronate vs. the alfacalcidol group; the mean daily prednisone doses

Table 1. Baseline characteristics of patients treated with alendronate or alfacalcidol

	Alendronate	Alfacalcidol	*P*
Age (years)	41.2 ± 12.8	38.1 ± 15.5	0.5199
Gender (male/female)	10/6	12/8	0.8785
Height (cm)	162.7 ± 7.7	163.6 ± 10.3	0.7770
Weight (kg)	59.8 ± 11.6	57.5 ± 11.1	0.5442
Body mass index (kg/m^2)	22.4 ± 2.9	21.5 ± 3.5	0.3806
Disease duration (years)	9.2 ± 9.0	5.6 ± 6.1	0.1777
Disease activity (active/inactive)	10/6	9/10	0.5791
Daily prednisone doses (mg)	9.7 ± 9.7	10.9 ± 6.5	0.6786
Cumulative prednisone doses (mg)	24978 ± 7448	15022 ± 3851	0.2491
Bone mineral density			
Spine (mg/cm^2)	0.926 ± 0.098	0.906 ± 0.125	0.6007
Left femoral neck (mg/cm^2)	0.702 ± 0.118	0.715 ± 0.123	0.7423
Right femoral neck (mg/cm^2)	0.687 ± 0.131	0.706 ± 0.138	0.6752
Bone metabolism markers			
Serum BAP (U/L)	21.7 ± 7.0	24.4 ± 9.6	0.3524
Urinary NTx (nmolBCE/mmol Cre)	42.3 ± 18.6	46.8 ± 22.7	0.5265
Serum calcium (mg/dL)	9.06 ± 0.35	9.11 ± 0.24	0.6801

Values are mean ± S.E.
BAP, bone alkaline phosphatase. NTx, *N*-telopeptide for type I collagen.

during the study were 7.6 ± 1.8 mg in the alendronate group and 5.9 ± 1.0 mg in the alfacalcidol group and the mean cumulative prednisone doses were 2833 ± 743 and 2067 ± 387 mg respectively.

Changes in BMD

Bone mineral density levels at baseline, 6 and 12 months are shown in Figure 2. Alendronate treatment significantly improved spine BMD over baseline at 6 and 12 months ($P < 0.05$ and $P < 0.0005$ respectively). Alfacalcidol treatment did not significantly change spine BMD. Alendronate produced a significantly higher improvement in the spine BMD than alfacalcidol (4.1% vs. 0.9%, $P < 0.05$; Figure 2a). Alendronate also tended to increase BMD in the femoral neck sites (Figure 2b,c).

Fractures

No new fractures or vertebral deformities were diagnosed during the study period in either group.

Changes in bone metabolism markers

Alendronate significantly decreased BAP levels at 3, 6 and 12 months ($P < 0.01$, $P < 0.01$ and $P < 0.0001$ respectively), but alfacalcidol did not (Figure 3). BAP levels were significantly lower at 3, 6 and 12 months ($P < 0.05$, $P < 0.05$ and $P < 0.0005$ respectively) in the alendronate group than the alfacalcidol group (Figure 3a).

Alendronate significantly decreased NTx levels at 3, 6 and 12 months ($P < 0.01$, $P < 0.0005$ and $P < 0.0005$ respectively), but alfacalcidol only at 12 months ($P < 0.05$). NTx levels were significantly lower at 6 and 12 months ($P < 0.005$ and $P < 0.005$ respectively) in the alendronate group than in the alfacalcidol group (Figure 3b).

Alendronate significantly deceased serum calcium levels at 3 months ($P < 0.05$) and alfacalcidol significantly increased levels at 3 and 12 months ($P < 0.05$ and $P < 0.05$ respectively) (Figure 3c). Serum calcium levels were significantly different between the two groups at 1, 3 and 12 months ($P < 0.05$, $P < 0.0005$ and $P < 0.05$ respectively).

Adverse events

Alendronate and alfacalcidol were generally well tolerated by patients. Adverse events were minimal and similar in both groups. One patient in the alendronate group stopped the study because of pruritus and one patient in the alfacalcidol group because of muscle

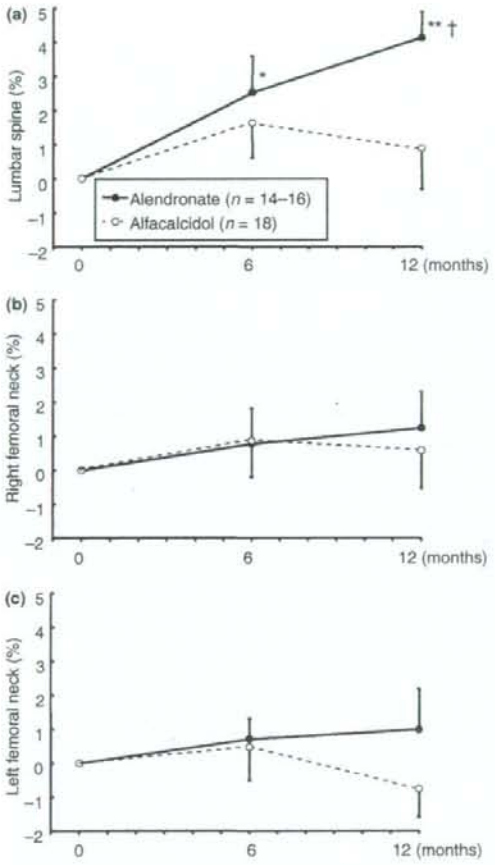

Figure 2. The effects of alendronate and alfacalcidol on bone mineral density at the (a) lumber spine, (b) right femoral neck and (c) left femoral neck over the 12-month period of the study. The bars represent mean ± S.E. * $P < 0.05$, ** $P < 0.0005$ vs. baseline value. † $P < 0.05$ vs. value in the alfacalcidol group.

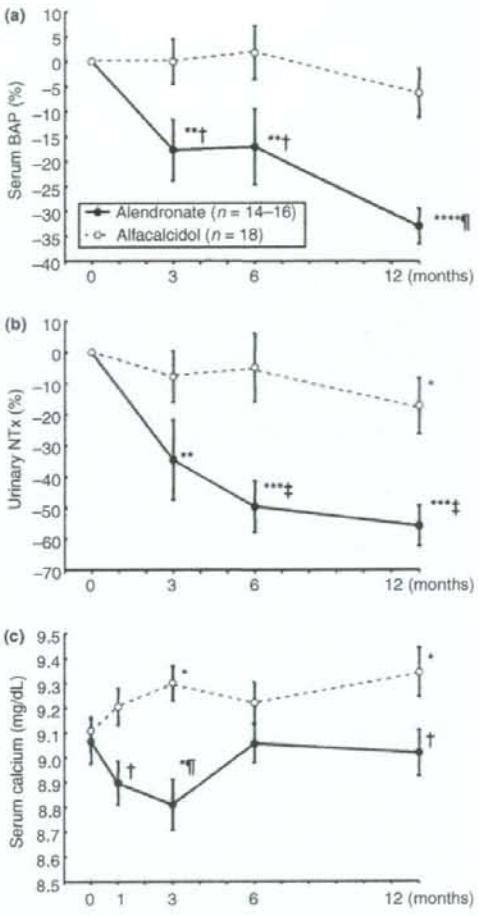

Figure 3. The effects of alendronate and alfacalcidol on (a) serum bone alkaline phosphatase (BAP), (b) urinary type I collagen cross-linked *N*-telopeptide (NTx) and (c) serum calcium over the 12-month period of the study. Corrected [Ca] = measured [Ca (mg/dL)] + (4 − [albumin (g/dL)]). The normal range was 8.7–10.3 mg/dL. The bars represent mean ± S.E. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0005$, **** $P < 0.0001$ vs. baseline value. † $P < 0.05$, ‡ $P < 0.005$, $P < 0.0005$ vs. value in the alfacalcidol group.

pain. In two patients in the alendronate group, gastrointestinal symptoms (one experienced abdominal pain and one diarrhoea) appeared, but treatment was not stopped as symptoms were mild and transient. There were no group differences in full blood counts or routine biochemistry at baseline or during the study. Disease activity was not different between treatment groups at the end of the study (data not shown).

DISCUSSION

To the best of our knowledge, this is the first comparative study between bisphosphonate and active vitamin D3 for the treatment of glucocorticoid-associated

osteoporosis in patients with UC. We found that alendronate treatment for 12 months increased BMD of the lumbar spine more than alfacalcidol in UC patients receiving glucocorticoid therapy. Thus, alendronate may inhibit bone loss in glucocorticoid-associated osteoporosis more effectively than alfacalcidol.

In this study, we chose 5 mg of daily alendronate. The 5 mg/day of alendronate is the maximum dose approved in Japan, which was effective as 10 mg/day in increasing BMD for the Japanese patients with osteoporosis in the 3-year study.[23] The effectiveness of 5 mg/day alendronate was also shown in Japanese patients with primary osteoporosis[24] and in postmenopausal Japanese women[25] where it increased lumber BMD by 4–6% of the baseline value after 12-month treatment. In addition, weekly therapy of alendronate was not available in Japan, when we started conducting the study.

Alendronate increased spinal BMD at the first assessment (6 months), showing that it promotes rapid increases in BMD. Increases in BMD of the femoral neck were smaller than in spinal BMD, similar to previous bisphosphonate studies in patients with CD[12] or other diseases[26, 27] that have shown a strong improvement in BMD at the lumbar spine but mild at the femoral neck. These data indicate that the structural and mechanical properties of the lumbar spine are more sensitive to alendronate than that of the femoral neck. This may reflect that the metabolically active trabecular bone of the spine is more sensitive to changes in the balance of bone resorption and formation during bisphosphonate treatment than the mixed cortical and trabecular bone of the femoral neck region. Longer-term observation may be needed to obtain significant results in the femoral neck region. In contrast, alfacalcidol did not increase BMD at any site indicating that active vitamin D3 prevents bone loss, but does not increase it as much as alendronate in this patient population.

Alendronate also decreased BAP, a marker of bone formation and NTx, a marker of bone resorption, indicating decreased bone resorption and by the coupling of bone resorption and bone formation, decreased bone formation with a net positive result on BMD. In contrast, alfacalcidol decreased NTx, but did not change BAP suggesting that bone formation and resorption are uncoupled during alfacalcidol treatment. Glucocorticoids may therefore inhibit the ability of active vitamin D3 to stimulate bone formation via inhibition of osteoblast function. However, the changes in NTx and BAP show similar patterns even if statistically significant changes occurred only with NTx suggesting that the significance may well be a function of the small numbers of subjects. Active vitamin D3 also enhances intestinal calcium absorption, which may increase circulating calcium levels and produce lower circulating levels of parathyroid hormone to decrease NTx. As alfacalcidol stimulates bone formation and inhibits bone resorption, we expected superior efficacy to alendronate. However, we and others observed the opposite outcome,[15, 28] although other studies on alfacalcidol were promising.[29]

Alendronate was well tolerated with no significant increase in adverse events including gastrointestinal symptoms compared to the alfacalcidol group, as was seen in patients without IBD.[30] Although further studies and surveillance during clinical use will be needed fully to characterize the gastrointestinal safety profile of alendronate, we are encouraged by the results observed in our study. Neither treatment altered disease activity. The safety, efficacy and tolerability data presented in this study support the use of alendronate for patients with UC treated with glucocorticoids.

A potential limitation of this trial is that it included only a small number of patients with UC. Patients were followed-up for 12 months after the initiation of trial and a larger, long-term study is necessary.

In addition, we used alendronate for the Japanese UC patients irrespective of age based on the guidelines of the Japanese Society for Bone and Mineral Research for the management of glucocorticoid-induced osteoporosis.[31] However, the guidelines of the Royal College of Physicians[32] and the British Society of Gastroenterology[33] advise routine co-administration of bisphosphonates only to those who are aged more than 65 years. Therefore, it will be necessary to verify and revise the present Japanese guidelines based on newly collected evidence in the future.

In conclusion, alendronate is safe and well tolerated and prevents the progression of glucocorticoid-associated osteoporosis in patients with UC, whereas alfacalcidol did not. Patients with UC receiving glucocorticoid treatment should be treated with the bisphosphonates rather than alfacalcidol.

ACKNOWLEDGEMENTS

Declaration of personal interests: We thank Prof. Naoto Shiba, Department of Orthopedics, Kurume University for his help during the study. *Declaration of funding interests*: None.

REFERENCES

1 Bernstein CN, Leslie WD. Therapy insight: osteoporosis in inflammatory bowel disease – advances and retreats. *Nat Clin Pract Gastroenterol Hepatol* 2005; 2: 232–9.

2 Bischoff SC, Herrmann A, Goke M, Manns MP, Von zur Muhlen A, Brabant G. Altered bone metabolism in inflammatory bowel disease. *Am J Gastroenterol* 1997; 92: 1157–63.

3 Bjarnason I, Macpherson A, Mackintosh C, Buxton-Thomas M, Forgacs I, Moniz C. Reduced bone density in patients with inflammatory bowel disease. *Gut* 1997; 40: 228–33.

4 Compston JE, Judd D, Crawley EO, *et al.* Osteoporosis in patients with inflammatory bowel disease. *Gut* 1987; 28: 410–5.

5 Dinca M, Fries W, Luisetto G, *et al.* Evolution of osteopenia in inflammatory bowel disease. *Am J Gastroenterol* 1999; 94: 1292–7.

6 Bernstein CN, Seeger LL, Sayre JW, Anton PA, Artinian L, Shanahan F. Decreased bone density in inflammatory bowel disease is related to corticosteroid use and not disease diagnosis. *J Bone Miner Res* 1995; 10: 250–6.

7 Silvennoinen JA, Karttunen TJ, Niemela SE, Manelius JJ, Lehtola JK. A controlled study of bone mineral density in patients with inflammatory bowel disease. *Gut* 1995; 37: 71–6.

8 De Nijs RN. Glucocorticoid-induced osteoporosis: a review on pathophysiology and treatment options. *Minerva Med* 2008; 99: 23–43.

9 Bernstein CN, Seeger LL, Anton PA, *et al.* A randomized, placebo-controlled trial of calcium supplementation for decreased bone density in corticosteroid-using patients with inflammatory bowel disease: a pilot study. *Aliment Pharmacol Ther* 1996; 10: 777–86.

10 Siffledeen JS, Fedorak RN, Siminoski K, *et al.* Randomized trial of etidronate plus calcium and vitamin D for treatment of low bone mineral density in Crohn's disease. *Clin Gastroenterol Hepatol* 2005; 3: 122–32.

11 Vogelsang H, Ferenci P, Resch H, Kiss A, Gangl A. Prevention of bone mineral loss in patients with Crohn's disease by long-term oral vitamin D supplementation. *Eur J Gastroenterol Hepatol* 1995; 7: 609–14.

12 Haderslev KV, Tjellesen L, Sorensen HA, Staun M. Alendronate increases lumbar spine bone mineral density in patients with Crohn's disease. *Gastroenterology* 2000; 119: 639–46.

13 Von Tirpitz C, Klaus J, Steinkamp M, *et al.* Therapy of osteoporosis in patients with Crohn's disease: a randomized study comparing sodium fluoride and ibandronate. *Aliment Pharmacol Ther* 2003; 17: 807–16.

14 Henderson S, Hoffman N, Prince R. A double-blind placebo-controlled study of the effects of the bisphosphonate risedronate on bone mass in patients with inflammatory bowel disease. *Am J Gastroenterol* 2006; 101: 119–23.

15 De Nijs RN, Jacobs JW, Lems WF, *et al.* Alendronate or alfacalcidol in glucocorticoid-induced osteoporosis. *N Engl J Med* 2006; 355: 675–84.

16 Jeffery JR, Leslie WD, Karpinski ME, Nickerson PW, Rush DN. Prevalence and treatment of decreased bone density in renal transplant recipients: a randomized prospective trial of calcitriol versus alendronate. *Transplantation* 2003; 76: 1498–502.

17 Shane E, Addesso V, Namerow PB, *et al.* Alendronate versus calcitriol for the prevention of bone loss after cardiac transplantation. *N Engl J Med* 2004; 350: 767–76.

18 Makins R, Ballinger A. Gastrointestinal side effects of drugs. *Expert Opin Drug Saf* 2003; 2: 421–9.

19 Truelove SC, Witts LJ. Cortisone in ulcerative colitis; final report on a therapeutic trial. *Br Med J* 1955; 2: 1041–8.

20 Watts NB. Clinical utility of biochemical markers of bone remodeling. *Clin Chem* 1999; 2: 1359–68.

21 Gundberg CM. Biochemical markers of bone formation. *Clin Lab Med* 2000; 20: 489–501.

22 Rosen HN, Dresner-Pollak R, Moses AC, *et al.* Specificity of urinary excretion of cross-linked N-telopeptides of type I collagen as a marker of bone turnover. *Calcif Tissue Int* 1994; 54: 26–9.

23 Shiraki M, Kushida K, Fukunaga M, *et al.* A placebo-controlled, single-blind study to determine the appropriate alendronate dosage in postmenopausal Japanese patients with osteoporosis. The Alendronate Research Group. *Endocr J* 1998; 45: 191–201.

24 Shiraki M, Kushida K, Fukunaga M, *et al.* A double-masked multicenter comparative study between alendronate and alfacalcidol in Japanese patients with osteoporosis. The Alendronate Phase III Osteoporosis Treatment Research Group. *Osteoporos Int* 1999; 10: 183–92.

25 Kushida K, Shiraki M, Nakamura T, *et al.* Alendronate reduced vertebral fracture risk in postmenopausal Japanese women with osteoporosis: a 3-year follow-up study. *J Bone Miner Metab* 2004; 22: 462–8.

26 Harris ST, Watts NB, Genant HK, *et al.* Effects of risedronate treatment on vertebral and nonvertebral fractures in women with postmenopausal osteoporosis: a randomized controlled trial. Vertebral Efficacy with Risedronate Therapy (VERT) Study Group. *JAMA* 1999; 282: 1344–52.

27 Reginster J, Minne HW, Sorensen OH, *et al.* Randomized trial of the effects of risedronate on vertebral fractures in women with established postmenopausal osteoporosis. Vertebral Efficacy with Risedronate Therapy (VERT) Study Group. *Osteoporos Int* 2000; 11: 83–91.

28 Sambrook PN, Kotowicz M, Nash P, *et al.* Prevention and treatment of glucocorticoid-induced osteoporosis: a comparison of calcitriol, vitamin D plus calcium, and alendronate plus calcium. *J Bone Miner Res* 2003; 18: 919–24.

29 De Nijs RN, Jacobs JW, Algra A, Lems WF, Bijlsma JW. Prevention and treatment of glucocorticoid-induced osteoporosis with active vitamin D3 analogues: a review with meta-analysis of randomized controlled trials including organ transplantation studies. *Osteoporos Int* 2004; 15: 589–602.

30 Taggart H, Bolognese MA, Lindsay R, *et al.* Upper gastrointestinal tract safety of risedronate: a pooled analysis of 9 clinical trials. *Mayo Clin Proc* 2002; 77: 262–70.

31 Nawata H, Soen S, Takayanagi R, *et al.* Guidelines on the management and treatment of glucocorticoid-induced osteoporosis of the Japanese Society for Bone and Mineral Research (2004). *J Bone Miner Metab* 2005; 23: 105–9.

32 A working group in collaboration with The Royal College of Physicians, The Bone and Tooth Society of Great Britain, and The National Osteoporosis Society. *Glucocorticoid-induced Osteoporosis; Guideline for Prevention and Treatment.* London: Royal College of Physicians, 2002.

33 Scott EM, Gaywood I, Scott BB. Guidelines for osteoporosis in coeliac disease and inflammatory bowel disease British Society of Gastroenterology . *Gut* 2000; 46(Suppl. 1): i1–8.

American Journal of Gastroenterology
© 2008 by Am. Coll. of Gastroenterology
Published by Blackwell Publishing

ISSN 0002-9270
doi: 10.1111/j.1572-0241.2007.01714.x

Adsorptive Depletion of Elevated Proinflammatory $CD14^+CD16^+DR^{++}$ Monocytes in Patients With Inflammatory Bowel Disease

Hiroyuki Hanai, M.D., Ph.D.,[1] Takayuki Iida, M.D.,[1] Ken Takeuchi, M.D.,[1] Fumitoshi Watanabe, M.D.,[1,2] Masami Yamada, M.D.,[3] Masataka Kikuyama, M.D.,[4] Yasushi Maruyama, M.D.,[2] Yasushi Iwaoka, M.D.,[3] Kazuhisa Hirayama, M.D.,[5] Seiji Nagata, M.D.,[6] and Kenji Takai[7]

[1]*Centre for Gastroenterology and Inflammatory Bowel Disease Research, Hamamatsu South Hospital, Hamamatsu, Japan;* [2]*Fujueda General Hospital, Fujieda, Japan;* [3]*Hamamatsu Medical Centre, Hamamatsu, Japan;* [4]*Hamamatsu Rosai Hospital, Hamamatsu, Japan;* [5]*Hamamatsu Insurance Hospital, Hamamatsu, Japan;* [6]*Nagata GI Clinic, Japan; and* [7]*SRL Inc., Tokyo, Japan*

BACKGROUND: In human blood, two monocyte populations exist, $CD14^{++}CD16^-$ classical monocytes and $CD14^+CD16^+$ proinflammatory monocytes, which account for about 10% of total monocytes, but can expand to promote inflammatory conditions. $CD14^+CD16^+$ monocytes produce large amounts of inflammatory cytokines including TNF-α and IL-1. Adacolumn adsorptive carriers adsorb from the blood in the column most of the monocytes/macrophages and granulocytes and this has been associated with clinical efficacy in patients with active inflammatory bowel disease (IBD). This study was to investigate the $CD14^+CD16^+$ monocyte profile in patients with IBD and the impact of Adacolumn on this proinflammatory phenotype.

METHODS: A total of 58 patients with ulcerative colitis (UC, N = 37) or Crohn's disease (CD, N = 21) together with 11 healthy controls were included in this study. Peripheral blood $CD14^+CD16^+$ monocytes were determined by three-color immunofluorescence and flow cytometry.

RESULTS: The percentage of $CD14^+CD16^+$ monocytes in patients with active CD was significantly ($P = 0.0089$) higher than the level in the control group, in patients with quiescent CD ($P = 0.0419$) or quiescent UC ($P = 0.0063$). Further, the percentage of $CD14^+CD16^+$ monocytes in patients with active UC who were on prednisolone (PSL) was less than the level in those not on PSL ($P < 0.0001$), thus PSL might have a suppressive effect on $CD14^+CD16^+$ monocytes. Patients with active IBD were each given up to 10 Adacolumn granulocye/monocyte adsorption (GMA) sessions over an 8-wk period. The percentage of $CD14^+CD16^+$ monocytes decreased dramatically ($P = 0.0077$ in UC and $P = 0.0117$ in CD) compared with entry levels.

CONCLUSIONS: A significant reduction in peripheral $CD14^+CD16^+$ monocytes by GMA should mitigate the inflammatory drive and contribute to the clinical efficacy of this procedure. Reduction of $CD14^+CD16^+$ monocytes by corticosteroids was also seen. Hence, corticosteroids should enhance the efficacy of GMA. This is the first report on $CD14^+CD16^+$ monocytes being decreased by Adacolumn GMA in patients with IBD.

(Am J Gastroenterol 2008;103:1210–1216)

INTRODUCTION

In human blood, two monocyte populations can be distinguished, $CD14^{++}CD16\text{-}DR^+$ classical monocytes and $CD14^+CD16^+DR^{++}$ (or $CD14^+CD16^+$) proinflammatory monocytes, which were first found to be a small subset of the peripheral blood monocyte population (1). These monocytes have been shown to exhibit a distinct phenotype and function; $CD14^+CD16^+$ monocytes are labeled proinflammatory based on a higher expression of inflammatory cytokines and higher potency in antigen presentation (1–5). Currently, it is widely known that within the monocyte population, the $CD14^+CD16^+$ subset is a major pathologic factor in various inflammatory disorders (2–5) due to their high capacity to release inflammatory cytokines, in particular tumor necrosis factor (TNF)-α (2, 4), and are elevated in various inflammatory diseases (6–9). In healthy persons, this minor monocyte subset (approximately 8%) coexpresses CD14 and CD16, a low affinity Fc-gamma type III receptor (5, 6). This monocyte subpopulation shows characteristics of tissue macrophages and expands extensively in acute and chronic inflammatory disorders (6–8), and produces large amounts of interleukin

(IL)-1, TNF-α, and HLA-DR, -DP, and -DQ antigens (2, 5, 10). These findings have led to the impression that serial analysis of monocyte phenotypes might be appropriate for monitoring patients receiving immunosuppressive or anti-inflammatory therapy (5, 10).

Regarding inflammatory bowel disease (IBD), it comprises mainly ulcerative colitis (UC) and Crohn's disease (CD) (11). Factors that initiate and perpetuate IBD are not well understood at present. However, active IBD is often associated with elevated peripheral blood monocytes/macrophages (and also granulocytes) that show activation behavior and increased survival time (12–16). Further, IBD by its very nature is initiated and perpetuated by cytokines like TNF-α, IL-1β, IL-6, IL-12, and IL-23, which are strongly pro-inflammatory (17–20) and are released by $CD14^+CD16^+$ monocytes/macrophages (2, 20–22). These understandings have given rise to the inference that elevated and activated $CD14^+CD16^+$ monocytes should be targets of therapy in IBD (23, 24).

Based on the perception that monocytes (together with granulocytes) are major factors in the immunopathogenesis of IBD, an Adacolumn has been developed recently, which can selectively deplete elevated $CD14^+CD16^+$ monocytes and granulocytes (25, 26). The column is filled with cellulose acetate beads of 2 mm in diameter as the column leukocyte adsorptive carriers; the beads are bathed in physiologic saline (25). The carriers remove from blood in the column most of the monocytes and granulocytes (neutrophils, eosinophils, and basophils) together with some platelets; lymphocytes are spared. In fact, lymphocytes increase (25, 27, 28). Selective removal of granulocytes and monocytes with the Adacolumn has been referred to as GMA (23).

Given that the Adacolumn carriers selectively adsorb from the blood in the column most of the monocytes/macrophages and granulocytes (FcγR and complement receptors bearing leukocytes) and this has been associated with clinical efficacy in patients with IBD (23–25), the precise mechanism(s) of this clinical efficacy needs to be clarified. This study aimed first to investigate the profile of proinflammatory $CD14^+CD16^+$ monocytes in patients with IBD, both during active disease as well as during remission and also look at the impact of GMA on the peripheral $CD14^+CD16^+$ monocyte level.

METHODS

Study Subjects

A total of 58 patients with UC (N = 37) or CD (N = 21), age range 18–59 yr, together with 11 healthy persons of similar age were included in this study. Subject groups did not differ significantly with respect to gender. In patients with active UC (N = 20), the clinical activity index (CAI) ranged from 5 to 17, while a CAI of 0–4 was considered remission (N = 17). CAI was assessed as previously described (29). Similarly, in patients with active CD (N = 10), the CD activity index (CDAI) was $\geq$150 and $\leq$420, while CDAI $\leq$150 was considered clinical remission (N = 11).

Medications

Patients were treated with 5-aminosalicylic acid (5-ASA), 1.5–2.25 g/day, alone or together with prednisolone (PSL), 20–40 mg/day in the active stage or 0–10 mg/day for those in remission. All patients with CD were receiving nutritional therapy with Elental (Ajinomoto, Tokyo, Japan). Immunomodulators like 6-mercaptopurine or azathioprine were not given.

Selective Depletion of $CD14^+CD16^+$ Monocytes in Patients With IBD

The procedures for GMA with the Adacolumn (JIMRO, Takasaki, Japan) have previously been fully described (23, 25, 29). Patients with active UC or with active CD were each given up to 10 GMA sessions over an 8-wk period. The duration of one GMA session was 60 min, at 30 mL/min. Peripheral blood samples were taken at selected time points for measuring the percentage of $CD14^+CD16^+$ monocytes by three-color immunofluorescence and flow cytometry on whole blood samples as recently described by Belge and colleagues (2).

Statistical Analysis

When appropriate, measurements are presented as the mean $\pm$ SD values. As indicated in the figures (or in the figure legends), comparisons were done using the Scheff's test or Wilcoxon's signed test, while correlation of the CAI values with the percentage of $CD14^+CD16^+$ monocytes was done with the Spearman's rank statistic. A significance level of 0.05 was used for all statistical tests, and 2-tailed tests were applied when appropriate.

RESULTS

Detection of $CD14^+CD16^+$ Monocytes

Figure 1 shows typical flow cytomery signals in a patient with active UC before and after Adacolum GMA treatment. In this patient, 18.9% of monocytes were identified as the $CD14^+CD16^+$ phenotype (proinflammatory) in the active UC stage and this was reduced to just 3.5% when the patient achieved remission.

Levels of $CD14^+CD16^+$ Monocytes in Active and Quiescent IBD

Figure 2 shows the expression of $CD14^+CD16^+$ monocytes in healthy controls and in patients with active and quiescent IBD. Elevated $CD14^+CD16^+$ monocytes in some patients with active UC and in all patients with active CD (in particular) are the prominent features in this figure. Clearly there is an overlap between subjects in the normal group and patients with active UC (see below), while patients with active CD consistently showed elevated $CD14^+CD16^+$ monocytes.

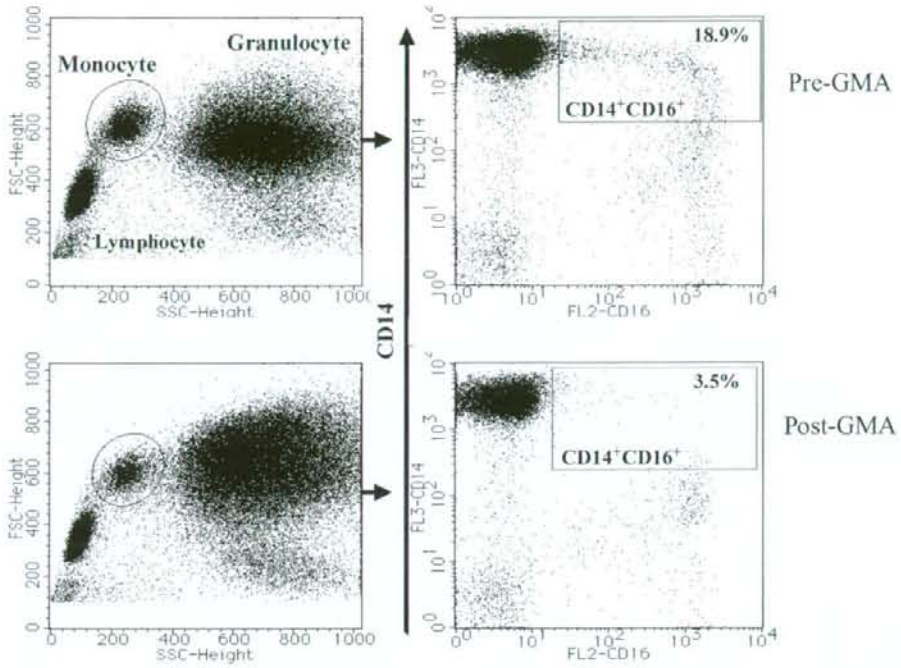

Figure 1. Typical flow cytometry signals in a patient with active UC before and after Adacolumn GMA treatment. As seen, in this patient, 18.9% of monocytes were identified as $CD14^+CD16^+$ monocytes in the active UC stage and this was reduced to just 3.5% when the patient achieved remission. SSC = side scatter; FSC = forward scatter. The percentage calculation was according to reference (2).

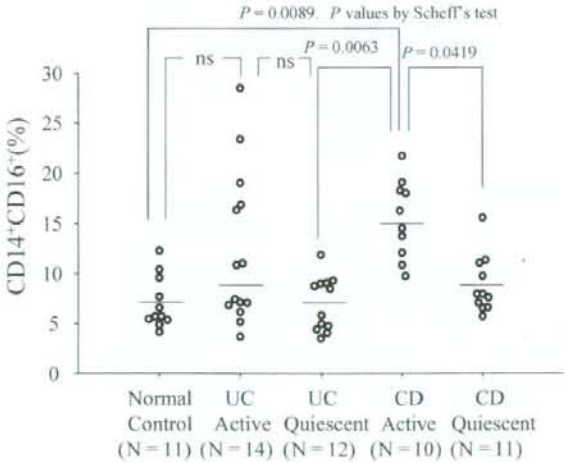

Figure 2. The expression of $CD14^+CD16^+$ monocytes in healthy controls and patients with active and quiescent IBD. Elevated $CD14^+CD16^+$ monocytes in some patients with active UC and in all patients with active CD, in particular, are the prominent features in this figure. As discussed in the text, patients with active UC who were on PSL had lower expression of $CD14^+CD16^+$ monocytes compared with steroid-free patients and this was reduced further by Adacolumn GMA (Fig. 4). Further, PSL appeared to be the main factor for the overlap seen between patients and controls.

Impact of GMA Together With PSL on $CD14^+CD16^+$ Monocyte Levels

In Figure 3, the expression levels of $CD14^+CD16^+$ monocytes in healthy persons and patients with active and quiescent UC, subgrouped into steroid takers (+) or steroid-free (−) are seen. Elevated $CD14^+CD16^+$ monocytes in steroid-free active UC is the prominent feature in this figure and indicates that corticosteroids (PSL) have a suppressive effect on proinflammatory $CD14^+CD16^+$ monocyte expression. Further, Figure 4 shows downmodulation of $CD14^+CD16^+$ monocyte expression in patients with active IBD on steroids (+) or steroid-free (−) by Adacolumn GMA. Elevated $CD14^+CD16^+$ monocytes in steroid-free active IBD is the prominent feature in this figure. Patients on PSL showed lower levels of $CD14^+CD16^+$ monocytes compared with PSL-free patients, and GMA seems to have enhanced the suppressive effect of PSL on $CD14^+CD16^+$ monocytes.

Correlation of CAI With Peripheral Blood $CD14^+CD16^+$ Monocytes

There was strong correlation between CAI and peripheral blood levels of $CD14^+CD16^+$ monocytes in patients with active UC, steroid-free ($r = 0.828$, $P = 0.0002$) as well as steroids takers ($r = 0.633$, $P = 0.0178$). This might indicate

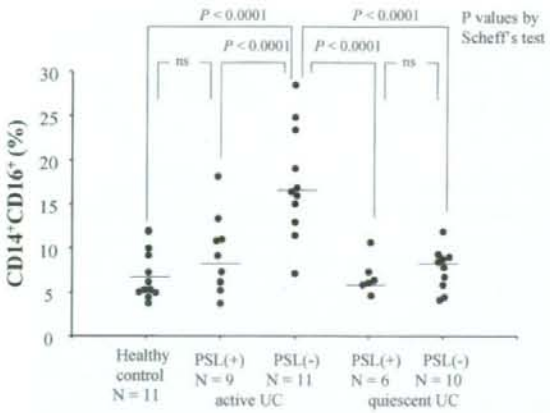

Figure 3. The expression of $CD14^+CD16^+$ monocytes in healthy persons and patients with active and inactive UC, subgrouped in to steroid takers (+) or steroid-free (−) is seen. Elevated $CD14^+CD16^+$ monocytes in steroid-free active UC is the prominent feature in this figure and indicates that corticosteroids (PSL) have a suppressive effect on proinflammatory $CD14^+CD16^+$ monocyte expression.

that $CD14^+CD16^+$ monocytes are associated with the expression of UC symptoms.

DISCUSSION

UC and CD are the major forms of idiopathic inflammatory bowel diseases of the intestine; both are debilitating chronic disorders that afflict millions of individuals throughout the world with symptoms that impair performance and quality of life. Whereas UC is confined to the colon and the rectum, CD may affect any part of the gut from the mouth to the perianal region (11, 30, 31). Currently our understanding of the immunopathogenesis of IBD is inadequate. The overwhelming view is that IBD is exacerbated and perpetuated by inflammatory cytokines, notably TNF-α (17, 19). Based on this thinking, in recent years, anti-TNF antibodies have been developed for the treatment of IBD (32–36). However, the enthusiasm toward biologicals is currently dampened by concerns about their long-term efficacy and safety profiles (reviewed in refs. 23, 24). Further, in patients with active IBD, $CD14^+CD16^+$ monocytes, which have been shown to produce large amounts of TNF (2), were found to be elevated and there was a strong correlation between CAI and the percentage of peripheral blood $CD14^+CD16^+$ monocytes, suggesting that $CD14^+CD16^+$ cells might contribute to the inflammatory drive in patients with active IBD. With this background in mind, we hypothesized that GMA, which has been developed for selective depletion of excess circulating monocytes (25, 26) and which increases lymphocytes (27, 28), should benefit patients with active IBD. The underlying rationale is that selective removal of these TNF-producing $CD14^+CD16^+$ monocytes should alleviate the inflammatory intensity, and allow healing to proceed. In this sense, GMA may be likened to an effective and safe biologic therapy in IBD. Typically, the fraction of proinflammatory $CD14^+CD16^+$ monocytes in our patient populations was about 19% of total monocytes compared with about 8% in healthy controls (5). Further, GMA proved to be very efficient in depleting the $CD14^+CD16^+$ monocytes.

Whereas patients with remission of IBD had $CD14^+CD16^+$ monocyte levels very close to the control group, in patients with active UC, the percentage of $CD14^+CD16^+$ monocytes was very variable. We soon

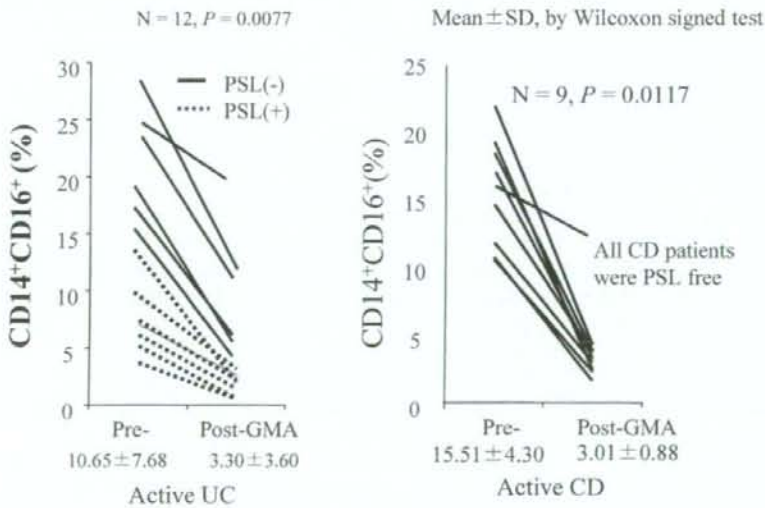

Figure 4. This figure shows the downmodulation of $CD14^+CD16^+$ monocyte expression in patients with active IBD on steroids (+) or steroid-free (−) by Adacolumn GMA. Elevated $CD14^+CD16^+$ monocytes in steroid-free active IBD is the prominent feature in this figure.

realized that this variability was largely attributable to PSL, to say that almost all patients not receiving PSL had elevated $CD14^+CD16^+$ monocytes; this was very striking in patients with active CD, while most patients receiving PSL had lower levels. Accordingly, patients on PSL appeared to be good responders to GMA and had a further fall in $CD14^+CD16^+$ monocytes. Hence, GMA should enhance the efficacy of corticosteroids in patients with IBD.

A decrease in $CD14^+CD16^+$ monocytes has also been seen during extracorporeal blood circulation including hemodialysis (6, 8, 37–39). These decreases are likely to reflect a fall in total leukocyte count, which is common during extracorporeal circulation. In contrast, the Adacolumn GMA used in this study spares and increases lymphocytes (25, 27, 28), while selectively depleting $CD14^+CD16^+$ monocytes. Further, our observation on the effect of PSL on $CD14^+CD16^+$ monocytes is in line with earlier reports seen in the literature. Thus, Fingerle-Rowson and colleagues (40) found that a 5-day course of high-dose glucocorticoid therapy differentially affected the expression of $CD14^{++}$ and the $CD14^+CD16^+$ monocyte subpopulation in 10 patients treated for multiple sclerosis. While the classical ($CD14^{++}$) monocytes exhibited a substantial increase, the proinflammatory $CD14^+CD16^+$ monocytes responded with a pronounced decrease. Similarly, Dayyani and colleagues (41) reported that a high dose of PSL was associated with a significant reduction in peripheral $CD14^+CD16^+$ monocytes in healthy volunteers. However, the doses of corticosteroids (250–500 mg/day) used in these studies (40, 41) are vastly higher than the amount given to patients with IBD, many of whom are on a long-term PSL regimen, and therefore, a direct comparison may not be appropriate. Nonetheless, the fact that PSL and GMA together showed an additive effect on reducing $CD14^+CD16^+$ monocyte was very intriguing to us. Further, whereas depletion in our study involves physical removal of $CD14^+CD16^+$ monocytes, corticosteroid-induced depletion was inhibited by the caspase 3,8 blocker z-Val-Ala-Asp, suggesting that $CD14^+CD16^+$ monocyte apoptosis proceeds in a caspase-dependent manner (41).

As mentioned above, it is widely believed that peripheral blood monocytes have a major pathologic role in various inflammatory diseases (3–6, 10). However, it was not until the publication of the work by Passlick and colleagues (1) that the leukocyte research community learned about what is now known as the proinflammatory $CD14^+CD16^+$ monocyte phenotype. Indeed, most of the best work on this monocyte phenotype, which since then has been appearing in the literature, is published by the same group (2, 3, 40–42). These monocytes are considered to be proinflammatory as they have an increased capacity to produce proinflammatory cytokines, like TNF-α, IL-1, and IL-6, and are elevated in various inflammatory diseases (2, 5–8, 38). Additionally, they readily change to macrophages and dendritic cells, which are inflammatory (5, 39, 42). At the moment, we do not have any obvious explanation as to why the level of $CD14^+CD16^+$ monocytes is high in patients with active CD. However, patients with active IBD show elevated neutrophils as well (25, 29, 43). While, for elevated neutrophils, inflammatory cytokines (44) and corticosteroids (45) have been implicated, for $CD14^+CD16^+$ monocytes, this does not seem to be the case.

As stated above, GMA with the Adacolumn spares lymphocytes and the merits of sparing lymphocytes have been discussed elsewhere (23, 24). Here, let us see how selectivity is achieved with the Adacolumn. Patients with autoimmune diseases have immune complexes (IC) in their plasma (46). Cellulose acetate adsorbs immunoglobulin G (IgG) and IC from the plasma (46, 47). Upon adsorption, the binding sites on IgG and IC become available for the Fcγ receptors (FcγRs) on monocytes/macrophages and neutrophils (26, 46, 47). Further, cellulose acetate with adsorbed IgG and IC generates complement activation fragments including C3a and C5a (46, 47). The opsonins C3b/C3bi and others derived from the activation fragments also adsorb onto the carriers and serve as binding sites for the leukocyte complement receptors (26, 47) CR1, CR2, and CR3 (Mac-1, CD11b/CD18). FcγRs are believed to influence CR3 as well (26, 48). Accordingly, leukocyte adsorption to the carriers is governed by the opsonins, FcγRs, and the leukocyte complement receptors. The expression of these sets of receptors is a common feature of monocytes/macrophages and neutrophils; lymphocytes are not known to express complement receptors except on small subsets of B, T, and NK (natural killer) cells (26, 47, 49). Similarly, FcγRs are not widely expressed on lymphocytes except on small subsets of $CD19^+$ B cells and $CD56^+$ NK cells (26, 47, 50, 51). These basic phenomena proceed well on Adacolumn carriers and lend GMA selectivity.

The current state of knowledge on proinflammatory $CD14^+CD16^+$ monocytes, their impact on IBD, and the role of GMA may be summed up as follows. The monocyte subset expressing the FcγRIII (CD16) receptor expands rapidly and extensively in inflammatory diseases including IBD. Accordingly, the peripheral level of $CD14^+CD16^+$ monocytes showed significant correlation with CAI in patients with active UC. $CD14^+CD16^+$ monocytes are known to be major sources of TNF, IL-1, and other inflammatory cytokines. The FcγRIII receptor on $CD14^+CD16^+$ monocytes renders these cells to become migratory macrophages/dendritic cells, which are intimately associated with mucosal inflammation. The Adacolumn GMA had a dramatic effect on the percentage of $CD14^+CD16^+$ monocytes. Further, the expression of $CD14^+CD16^+$ monocytes was lower in PSL takers compared with PSL-free patients and was reduced further by GMA, indicating that PSL is suppressive on $CD14^+CD16^+$ monocytes and that in this respect, the action of GMA is additive. The elimination of $CD14^+CD16^+$ monocytes by GMA might be viewed as an immunomodulation that should alleviate inflammation and potentially contribute to the clinical efficacy of GMA. This is the first report on $CD14^+CD16^+$ monocytes being reduced by GMA.

STUDY HIGHLIGHTS

What Is Current Knowledge

- Two major monocyte phenotypes are known: $CD14^{++}CD16^-$ (classical) and $CD14^+CD16^+$.
- In healthy persons, less than 10% of monocytes are of the $CD14^+CD16^+$ phenotype.
- $CD14^+CD16^+$ monocytes are proinflammatory and produce tumor necrosis factor (TNF).

What Is New Here

- $CD14^+CD16^+$ monocytes are elevated in patients with active inflammatory bowel disease (IBD).
- Patients with IBD on prednisolone had lower levels of $CD14^+CD16^+$ monocytes.
- Selective leukocytapheresis depleted $CD14^+CD16^+$ monocytes.
- Steroid refractory patients should benefit from selective leukocytapheresis.

Reprint requests and correspondence: Hiroyuki Hanai, M.D., Ph.D., Centre for Gastroenterology and Inflammatory Bowel Disease Research, Hamamatsu South Hospital, 26 Shirowacho, Hamamatsu 430-0846, Japan.

Received September 6, 2007; accepted October 18, 2007.

REFERENCES

1. Passlick B, Flieger D, Ziegler-Heitbrock HW. Identification and characterization of a novel monocyte subpopulation in human peripheral blood. Blood 1989;74:2527–34.
2. Belge KU, Dayyani F, Horelt A, et al. The proinflammatory $CD14^+CD16^+DR^{++}$ monocytes are a major source of TNF. J Immunol 2002;168:3536–42.
3. Ziegler-Heitbrock L. The $CD14^+CD16^+$ blood monocytes: Their role in infection and inflammation. J Leukoc Biol 2006;81:584–92.
4. Schlitt A, Heine GH, Blankenberg S, et al. $CD14^+CD16^+$ monocytes in coronary artery disease and their relationship to serum TNF-alpha levels. Thromb Haemost 2004;92:419–24.
5. Scherberich JE. Proinflammatory blood monocytes: Main effector and target cells in systemic and renal disease; background and therapeutic implications. Int J Clin Pharmacol Ther 2003;41:459–64.
6. Nockher WN, Scherberich JE. Expanded $CD14^+CD16^+$ monocyte subpopulation in patients with acute and chronic infections undergoing hemodialysis. Infect Immun 1998;66:2782–90.
7. Okamoto H, Mizuno K, Horio T. Circulating $CD14^+CD16^+$ monocytes are expanded in sarcoidosis patients. J Dermatol 2003;30:503–9.
8. Saionji K, Ohsaka A. Expansion of $CD14^+CD16^+$ blood monocytes in patients with chronic renal failure undergoing dialysis: Possible involvement of macrophage colony-stimulating factor. Acta Haematol 2001;105:21–6.
9. Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. Immunity 2003;19:2–4.
10. Scherberich JE, Nockher WA. $CD14^{++}$ monocytes, $CD14^+/CD16^+$ subset and soluble CD14 as biological markers of inflammatory systemic diseases and monitoring immunosuppressive therapy. Clin Chem Lab Med 1999;37:209–13.
11. Allison MC, Dhillon AP, Lewis WG, et al., eds. Inflammatory bowel disease. London: Mosby, 1998:9–95.
12. Meuret G, Bitzi A, Hammer B. Macrophage turnover in Crohn's disease and ulcerative colitis. Gastroenterology 1978;74:501–3.
13. Rugtveit J, Brandtzaeg P, Halstensen TS, et al. Increased macrophage subsets in inflammatory bowel disease: Apparent recruitment from peripheral blood monocytes. Gut 1994;35:669–74.
14. Mahida YR. The key role of macrophages in the immunopathogenesis of inflammatory bowel disease. Inflamm Bowel Dis 2000;6:21–33.
15. McCarthy DA, Rampton DS, Liu Y-C. Peripheral blood neutrophils in inflammatory bowel disease: Morphological evidence of in vivo activation in active disease. Clin Exp Immunol 1991;86:489–93.
16. Brannigan AE, O'Connell PR, Hurley H. Neutrophil apoptosis is delayed in patients with inflammatory bowel disease. Shock 2000;13:361–6.
17. Schreaiber S, Nikolaus S, Hampe J. Tumour necrosis factor alpha and interleukin 1beta in relapse of Crohn's disease. Lancet 1999;353:459–61.
18. Kaplanski G, Marin V, Montero-Julian F, et al. IL-6: A regulator of the transition from neutrophil to monocyte recruitment during inflammation. Trends Immunol 2003;24:25–9.
19. Papadakis KA, Targan SR. Role of cytokines in the pathogenesis of inflammatory bowel disease. Annu Rev Med 2000;51:289–98.
20. Neurath MF. IL-23: A master regulator in Crohn's disease. Nat Med 2007;13:26–8.
21. Nikolaus S, Bauditz J, Gionchetti P. Increased secretion of pro-inflammatory cytokines by circulating polymorphonuclear neutrophils and regulation by interleukin-10 during intestinal inflammation. Gut 1998;42:470–6.
22. Cassatella MA. The production of cytokines by polymorphonuclear neutrophils. Immunol Today 1995;16:21–6.
23. Hanai H. Positions of selective leukocytapheresis in the medical therapy of ulcerative colitis. World J Gastroenterol 2006;21:7568–77.
24. Kanai T, Hibi T, Watanabe M. The logics of leukocytapheresis as a natural biological therapy for inflammatory bowel disease. Expert Opin Biol Ther 2006;6:453–66.
25. Saniabadi AR, Hanai H, Takeuchi K, et al. Adacolumn, an adsorptive carrier based granulocyte and monocyte apheresis device for the treatment of inflammatory and refractory diseases associated with leukocytes. Ther Apher Dial 2003;7:48–59.
26. Saniabadi AR, Hanai H, Bjarnason I, et al. Adacolumn for selective leukocytapheresis as a non-pharmacological treatment for patients with disorders of the immune system: An adjunct or an alternative to drug therapy? J Clin Apher 2005;20:171–84.
27. Suzuki Y, Yoshimura N, Saniabadi AR, et al. Selective neutrophil and monocyte adsorptive apheresis as a first line treatment for steroid naïve patients with active ulcerative colitis: A prospective uncontrolled study. Dig Dis Sci 2004;49:565–71.
28. Aoki A, Nakamura K, Yoshimatsu Y, et al. Adacolumn selective leukocyte adsorption apheresis in patients with active ulcerative colitis: Clinical efficacy, effects on plasma IL-8 and the expression of toll like receptor 2 on granulocytes. Dig Dis Sci 2007 [in press].

29. Hanai H, Watanabe F, Takeuchi K, et al. Leukcocyte adsorptive apheresis for the treatment of active ulcerative colitis: A prospective uncontrolled pilot study. Clin Gastroenterol Hepatol 2003;1:28–35.
30. Fiocchi C. Inflammatory bowel disease: Etiology and pathogenesis. Gastroenterology 1998;115:182–205.
31. Podolsky DK. Inflammatory bowel disease. N Engl J Med 2002;347:417–29.
32. van Dullemen HM, van Deventer SJ, Hommes DW, et al. Treatment of Crohn's disease with anti-tumor necrosis factor chimeric monoclonal antibody (cA2). Gastroenterology 1995;109:129–35.
33. Hanauer SB. Medical therapy of ulcerative colitis. Gastroenterology 2004;126:1582–92.
34. Targan SR, Hanauer SB, van Deventer SJ. A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. N Engl J Med 1997;337:1029–35.
35. Hanauer SB, Feagan BG, Lichtenstein GR. Maintenance infliximab for Crohn's disease: The ACCENT I randomised trial. Lancet 2002;359:1541–9.
36. Rutgeerts P, Sandborn WJ, Feagan B, et al. Infliximab for induction and maintenance therapy for ulcerative colitis. N Engl J Med 2005;353:2462–76.
37. Sester U, Sester M, Heine G, et al. Strong depletion of $CD14^+CD16^+$ monocytes during haemodialysis treatment. Nephrol Dial Transplant 2001;16:1402–8.
38. Griveas I, Visvardis G, Sakellariou G, et al. Biocompatibility study based on differential sequestration kinetics of $CD14^+CD16^+$ blood monocyte subsets with different dialyzers. Ren Fail 2006;28:493–9.
39. Carracedo J, Merino A, Nogueras S, et al. On-line hemodiafiltration reduces the proinflammatory $CD14^+CD16^+$ monocyte-derived dendritic cells: A prospective, crossover study. J Am Soc Nephrol 2006;17:2315–21.
40. Fingerle-Rowson G, Angstwurm M, Andreesen R, et al. Selective depletion of $CD14^+CD16^+$ monocytes by glucocorticoid therapy. Clin Exp Immunol 1998;112:501–6.
41. Dayyani F, Belge KU, Ziegler-Heitbrock L. Mechanism of glucocorticoid-induced depletion of human $CD14^+CD16^+$ monocytes. J Leukoc Biol 2003;74:33–9.
42. Weber C, Belge KU, von Hundelshausen P, et al. Differential chemokine receptor expression and function in human monocyte subpopulations. J Leukoc Biol 2000;67:699–704.
43. Saniabadi AR, Hanai H, Fukunaga K, et al. Therapeutic leucocytapheresis for inflammatory bowel disease. Transfus Apher Sci 2007;37:191–200.
44. Lee A, Whyte M, Haslett C. Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators. J Leukoc Biol 1993;54:283–8.
45. Meagher LC, Cousin JM, Seckl JR, et al. Opposing effects of glucocorticoids on the rate of apoptosis in neutrophilic and eosinophilic granulocytes. J Immunol 1996;156: 4422–8.
46. D'Arrigo C, Candal-Couto JJ, Greer M, et al. Human neutrophil Fc receptor mediated adhesion under flow: A hallow fiber model of intravascular arrest. Clin Exp Immunol 1994;100:173–9.
47. Hiraishi K, Takeda Y, Saniabadi A, et al. Studies on the mechanisms of leukocyte adhesion to cellulose acetate beads: An in vitro model to assess the efficacy of cellulose acetate carrier-based granulocyte and monocyte adsorptive apheresis. Ther Apher Dial 2003;7:334–40.
48. Marzocchi-Machado CM, Alves CM, Lucisano-Valim YM. Fcgamma and complement receptors: Expression, role and co-operation in mediating the oxidative burst and degranulation of neutrophils of Brazilian systemic lupus erythematosus patients. Lupus 2002;11:240–8.
49. Ross GD, Vetvicka V. CR3 (CD11b, CD18): A phagocyte and NK cell membrane receptor with multiple ligand specificities and functions. Clin Exp Immunol 1993;92: 181–4.
50. Muto S, Vetvicka V, Ross GD. CR3 (CD11b/CD18) expressed by cytotoxic T cells and natural killer cells is upregulated in a manner similar to neutrophil CR3 following stimulation with various activating agents. J Clin Immunol 1993;13:175–84.
51. Sulica A, Morel P, Metes D, et al. Ig binding receptors on human NK cells as effector and regulatory surface molecules. Int Rev Immunol 2001;20:371–414.

CONFLICT OF INTEREST

Guarantor of the article: Hiroyuki Hanai, M.D., Ph.D.

Specific author contributions: Hiroyuki Hanai designed the study and wrote the manuscript; Takayuki Iida, Ken Takeuchi, Fumitoshi Watanabe, Masami Yamada, Masataka Kikuyama, Yasushi Maruyama, Yasushi Iwaoka, Kazuhisa Hirayama, and Seiji Nagata performed the treatment and provided investigation samples; Ken Takeuchi carried out the laboratory assays.

Financial support: This study is an outcome of the authors' clinical research activities and was done without any external financial support.

Potential competing interests: None.

Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 368 (2008) 923–929
BBRC
www.elsevier.com/locate/ybbrc

Proteasomal degradation of Atoh1 by aberrant Wnt signaling maintains the undifferentiated state of colon cancer

Mikayo Aragaki [1], Kiichiro Tsuchiya [1], Ryuichi Okamoto, Sanae Yoshioka, Tetsuya Nakamura, Naoya Sakamoto, Takanori Kanai, Mamoru Watanabe *

Department of Gastroenterology and Hepatology, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan

Received 2 February 2008
Available online 12 February 2008

Abstract

Atoh1 plays a crucial role in intestinal cell differentiation. We have demonstrated that its human homolog Hath1 protein is targeted by the Wnt-GSK3 axis, resulting in the proteasomal degradation in human colon cancer. However, the contribution of Hath1 degradation to the undifferentiated state of colon cancer remains unknown. In this study, we demonstrated that both constitutive expression of mutant Hath1 and stabilization of Hath1 protein by a GSK3 inhibitor in colon cancer cells increased the expression of MUC2 known as a representative function of differentiated goblet cells. This means that Hath1 protein degradation may be required for maintaining the undifferentiated state of colon cancers, and that GSK3 inhibitors have potential for use in cancer therapy.

Keywords: Atoh1; Hath1; Proteasomal degradation; Differentiation; Wnt; GSK3β; Colon cancer

Atoh1 is a bHLH transcriptional factor that plays a critical role in terminal cell differentiation of intestinal epithelium, dorsal interneuron in the spinal cord, granule cells in the cerebellum, and inner hair cells in the auditory systems [1–4]. The mouse homolog of Atoh1, Math1, is expressed in the central nervous system and intestine in embryos but confined to the intestine in adults [5]. In Math1-deficient mice, loss of Math1 induces depletion of secretory cells such as Paneth cells, enteroendocrine cells and goblet cells, indicating that continuous expression of Atoh1 is required for proper differentiation and maintenance of epithelial homeostasis in intestine throughout the life span [4]. The human homolog of Atoh1, Hath1, is also strongly expressed in normal colon and small intestine [6,7], suggesting that it likewise contributes to the terminal differentiation of intestinal epithelial cells.

Dysregulation of Hath1 expression is thought to induce various diseases of the intestinal tracts. Loss of Hath1 protein has been observed in colorectal cancer, where Wnt signaling is constitutively activated by the truncated mutation of the adenomatous polyposis coli gene (APC); and Hath1 mRNA expression was found to be down-regulated in some colon cancer tissues compared with normal colon [6,7]. Thus, repression of Hath1 mRNA in colon cancer might promote maintenance of the undifferentiated state. However, not all colorectal cancers show low expression of Hath1 mRNA [6–8]. We have reported that Hath1 protein was lost in colorectal cancer even in the presence of Hath1 mRNA [7].

To clarify this anomaly in the expression of Hath1protein and mRNA in colon cancer, we have examined the regulation of Hath1 protein stability in colon cancer-derived cell lines. Our previous study demonstrated that Hath1 protein was actively degraded by the ubiquitin–proteasome system via Wnt signaling that switched the target of

Abbreviations: APC, Adenomatous polyposis coli; GSK3β, glycogen synthase kinase 3β.

* Corresponding author. Fax: +81 3 5803 0262.
E-mail address: mamoru.gast@tmd.ac.jp (M. Watanabe).

[1] Both authors equally contributed to this work.

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.02.011

GSK3β from β-catenin to Hath1 in colon cancer cells [7]. Aberrant Wnt signaling by truncated APC has been highlighted as the most critical trigger for carcinogenesis in colon, being observed in approximately 80% of patients with colorectal cancer [9,10]. Although it has been supposed that the accumulated β-catenin protein resulting from the aberrant Wnt signaling induced carcinogenesis and maintained the undifferentiated state of colon cancer, we have found that the Wnt signaling induced not only the continuous expression of β-catenin protein but also proteasomal degradation of Hath1 protein. Moreover, the inactivation of Wnt signaling by forced expression of the full-length APC gene in colon cancer cells gave rise to both β-catenin protein degradation and Hath1 protein stabilization, resulting in the cell differentiation toward goblet cells [7].

These results raise questions of whether is more effective in maintaining the undifferentiated state of colon cancer, uncontrolled expression of β-catenin or proteasomal degradation of Hath1, and whether the expression of Hath1 protein alone has the potential to promote the differentiation of intestinal epithelial cells even with β-catenin accumulation promoting the proliferating state.

In this study, we aimed to elucidate the effect of Hath1 protein stability on the undifferentiated state in human colon cancer. We found that the mutant Hath1 protein in which serine residues were replaced with alanine was stably expressed in human colon cancer cells with the aberrant Wnt signaling, resulting in the induction of differentiation toward goblet cells. We also demonstrated that treatment with a GSK3 inhibitor stabilized Hath1 protein and directed the colon cancer cells toward goblet cells, suggesting that GSK3 inhibitors have potential for use in a new therapeutic approach for the majority of patients suffering from colorectal cancers.

Materials and methods

DNA constructs. Expression plasmids used for this study were generated as reported previously [7].

Cell culture and creation of transient and stable cell lines. Human colon adenocarcinoma-derived SW480, DLD-1 cells, and human embryonic kidney-derived 293T cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. In all experiments 1×10^6 cells were grown in 6-cm dishes and were transiently transfected by using TransIT transfection reagent (Mirus, Madison, WI) according to the manufacturer's protocol with 4 μg of expression plasmids. Transfected cells were cultured under the usual conditions or in the presence of 10 μmol/L MG132 (Calbiochem) or 100 mmol/L LiCl (Sigma–Aldrich). Stable cell lines in DLD-1 were constructed with the Tet-On system with tet-repressor, pcDNA4-Flag Hath1 or Flag SA Hath1 plasmids. For selection, both blasticidin (7.5 μg/ml) and zeocin (750 μg/ml) were added for WT and SA cells. Hath1 gene expression was induced by cultivation in the presence of doxycycline for 12 h or more. After cultivation with doxycycline, MG132 (10 μmol/L) was added to the media and cultivation was continued for 8 h.

Western blot analysis. Cells were extracted with 1% sodium dodecyl sulfate (SDS)-containing radioimmunoprecipitation assay (RIPA) buffer as previously described [7]. The supernatants were removed and quantitated using protein assay reagent (Pierce, Rockford, IL). Preparations of 50 μg or 100 μg of proteins were separated in 12% SDS–polyacrylamide gels, transferred to polyvinylidene difluoride (PVDF) membranes according to standard procedures. The membranes were immunoblotted with anti-Flag M2 (Sigma Chemical Co., St. Louis, MO), anti-β-catenin (BD Biosciences Pharmingen), and anti-USF2 (Santa Cruz Biotechnology), then incubated with the secondary antibodies.

Luciferase assays. 293T cells were transiently transfected with 10 ng of *Renilla* luciferase reporter plasmid pRL-TK-Luc (Promega) along with 100 ng of either E-box-Luc or MUC2-Luc reporter plasmid and 100 ng of the expression plasmids. The data are represented as total relative light units (RLU) on histograms showing the average ± SEM of triplicate determinations.

Real-time PCR. SW480 cells were transiently transfected as indicated previously, then stimulated with 100 mmol/L LiCl for 8 h. DLD-1cells with the Tet-On system were stimulated with doxycycline for 5 d. Total RNA was isolated using TRIzol reagent (Invitrogen). Aliquots of 1 μg of total RNA were used for reverse transcription (Qiagen). Quantitative polymerase chain reaction was carried out using lightcycler (Roche) in triplicate to measure mRNA expression of Mucin2 (MUC2), Hath1, c-myc, and CDX2. The following Hath1 and CDX2 specific primers were used: Hath1 forward primer 5′-GCC CAA ATC TAC ATC AAC GCC-3′; Hath1 reverse primer 5′-TTG CCC GCG CCC CCT TCA TAG-3′; CDX2 forward primer 5′-CGG CTG GAG CTG GAG AAG G-3′; and CDX2 reverse primer 5′-TCA GCC TGG AAT TGC TCT GC-3′. Primers for MUC2 and c-myc were as described previously [7].

Immunocytochemistry. DLD-1 Tet-On cells were incubated in MG132 for 8 h following stimulation by doxycycline for 12 h, were fixed with 2.0% paraformaldehyde and were permeabilized with 0.5% Triton-X in PBS. Anti-Flag M2 antibody diluted with PBS was utilized to detect Flag-tagged Hath1. Incubation with anti-mouse IgG HRP-linked whole antibody (Amersham Biosciences) as the secondary antibody followed.

Results

A mutant Hath1 protein showed enhanced protein stability while maintaining its transcriptional activity in colon cancer cells

To determine the contribution of Hath1 protein stability to cell differentiation of colon cancer, we first attempted to express Hath1 protein stably in colon cancer cells. We have previously confirmed the protein stability of various mutant Hath1 constructs in colon cancer cells and found the critical region for Hath1 degradation to be the 54th and 58th serine residues of Hath1 protein [7]. The mutants represented in Fig. 1A were as follows. N1 and N5 are N-terminal deletion mutants of Hath1 preserving or excluding the critical serine residues, respectively. SA is a mutant in which the 54th and 58th serine residues are replaced with alanine residues (Fig. 1A). Wild-type (WT) and mutants of Hath1 transiently transfected into 293T cells with inactive Wnt signaling were confirmed to express all proteins stably (Fig. 1B). When transfected into the human colon cancer cell line with active Wnt signaling, both WT and N1 were degraded by the ubiquitin–proteasome system, while both N5 and SA were stably expressed even in the absence of the proteasome inhibitor MG132, as previously found (Fig. 1C). Although it has been reported that Hath1 binds to E-box sequences to activate the transcription of target genes [5], the critical region for the transcriptional activity of Hath1 remains unknown. We therefore per-

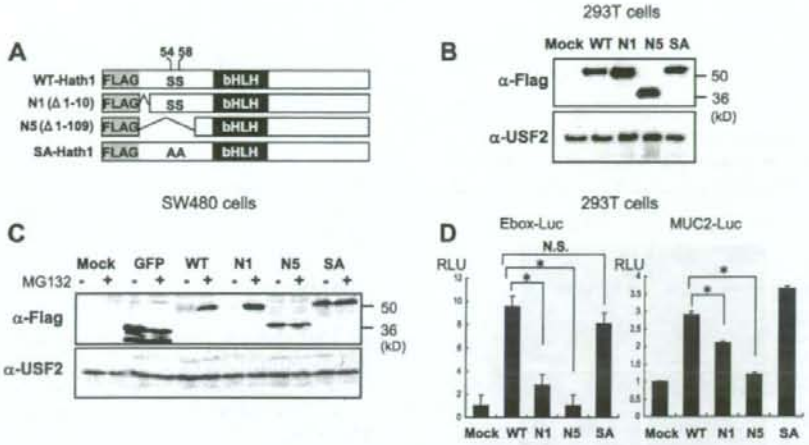

Fig. 1. Hath1 protein induced by alanine-substituted mutant showed enhanced stability while maintaining its transcriptional activity. (A) Schematic representation of various Flag-tagged Hath1 mutants used in this study. The N-terminal deleted regions of each mutant are designated by amino acid numbers. Serine residues at positions 54 (S54) and 58 (S58) are indicated. (B) 293T cells in which Wnt signaling is inactive were transfected with two of the N-terminal deletion mutants and a substituted mutant of Hath1 in addition to the WT. (C) SW480 cells were transfected by the same procedure as (B). WT and N1 mutant preserving the critical regions for protein degradation were detected only in the presence of MG132. N5 and SA lacking the critical regions expressed the proteins without the reagent. (D) 293T cells were co-transfected with of each of the WT and Hath1 mutants and either E-box Luc or MUC2 Luc. $^{*}P < 0.05$.

formed the luciferase assay with the Hath1 mutants in 293T cells (Fig. 1D). SA showed equivalent transcriptional activity to the wild-type, while N1 and N5 showed significantly reduced transcriptional activity, implying that the critical region for the transcriptional activity lies at the N-terminus of Hath1. Moreover, previous studies have noted a close association of Hath1 with MUC2 gene expression [6–8] and the transactivation of MUC2 promoter by Hath1 [8]. We found that SA and WT Hath1 showed equivalent transcriptional activity of MUC2, while N1 and N5 lost this activity (Fig. 1D).

Stable expression of SA Hath1 increases its transcriptional activity

Since only the SA mutant of Hath1 was stably expressed while maintaining its transcriptional activity in colon cancer cells, we considered that SA Hath1 was suitable to assess whether Hath1 has the potential to promote differentiation in colon cancer. We then constructed a cell line stably expressing the SA Hath1 protein through the Tet-On system in which expression of a target gene is induced by doxycycline. Expression of WT Hath1 protein was not induced by doxycycline alone, while SA was expressed regardless of treatment with MG132 (Fig. 2A). Moreover, immunofluorescence analysis revealed that SA Hath1 protein was expressed in the nucleus even in the absence of MG132 (Fig. 2B). Next, we examined the E-box-dependent transcriptional activity of SA Hath1 in colon cancer cells. Luciferase assay showed that the transcriptional activity of SA Hath1 is notably higher than of WT Hath1, possibly because of the increased amount of protein bound to E-box sequences (Fig. 2C).

Stable expression of SA Hath1 induces MUC2 mRNA on active Wnt signaling

Because the transcriptional activity of SA Hath1 was higher than of WT Hath1 in colon cancer cells, we further investigated whether constitutive Hath1 protein expression could induce differentiation characteristics in colon cancer cells. MUC2 mRNA expression was significantly up-regulated by the stable expression of SA Hath1 with doxycycline stimulation for 5 d, suggesting that SA Hath1 protein might induce the differentiated state of colon cancer cells (Fig. 3A). On the other hand, WT Hath1 was actively degraded by the proteasome system, resulting in the invariable expression of MUC2 mRNA. Next we examined whether MUC2 expression in colon cancer cells was related to the up-regulation of CDX2, since transcription of the MUC2 gene is reported to be induced by CDX2 binding to the MUC2 promoter [11]. Quantitative real-time RT-PCR assay revealed that CDX2 mRNA was not up-regulated, suggesting that MUC2 mRNA up-regulation by stable expression of Hath1 was independent of CDX2 in colon cancer cells (Fig. 3B). Importantly, Wnt signaling in SA Hath1 stable cell line is as active as in naive cells, because the amount of β-catenin and the expression of c-myc, which is one of the target genes of Wnt signaling, did not change in any of the cells (Fig. 3C and D). Together, these results indicate that stable Hath1 protein is itself sufficient to induce MUC2 mRNA without the

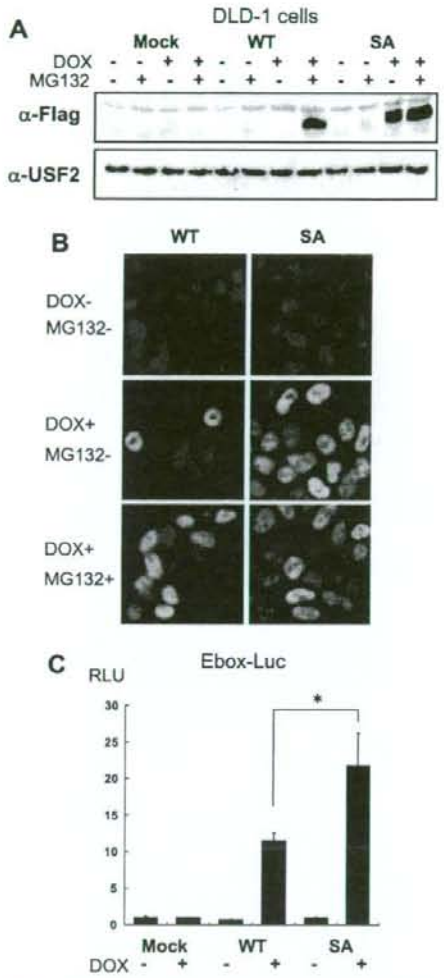

Fig. 2. DLD-1 stable cell line inducible for mutant SA Hath1 expression has increased transcriptional activity. (A) DLD-1 Tet-On cells were stimulated with doxycycline for 12 h before the incubation with MG132. (B) Immunocytochemical analysis of DLD-1 Tet-On cells in which expression from transfected SA Hath1 was performed to detect the SA Hath1 protein in the nucleus with or without MG132. (C) Luciferase assay was performed in DLD-1 Tet-On cells transfected with E-box Luc reporter plasmid. $^{*}P < 0.05$.

influence on constitutive expression of a group of target genes of β-catenin/TCF4 driving cell proliferation.

The GSK3 inhibitor stabilizes Hath1 protein, leading to up-regulation of MUC2 mRNA in colon cancer cells

As results suggested that SA Hath1 protein stably expressed in colon cancer cells was responsible for MUC2 mRNA expression, we investigated whether WT Hath1 stabilized by GSK3 inhibitors is able to induce MUC2 mRNA expression without the repression of Wnt signaling. Hath1 protein was detected in colon cancer cells incubated with a GSK3 inhibitor, LiCl, while the amount of β-catenin protein accumulated in the cells remained unchanged (Fig. 4A). Interestingly, WT Hath1 stabilized by LiCl increases MUC2 mRNA expression, suggesting that the stabilization of Hath1 protein is essential to induce definitive intestinal differentiation without the suppression of Wnt signaling in colon cancer cells (Fig. 4B).

Discussion

The results of this study demonstrate the significance of Hath1 protein stabilization in regulating the differentiation state of human colon cancer. We found that a Hath1 mutant in which serine residues were replaced with alanine (SA Hath1) is capable of stable expression while maintaining its transcriptional activity in colon cancer cells, and the N-terminus of Hath1 is essential for the E-box-dependent transcriptional activity. We demonstrated that the stable expression of SA Hath1 up-regulates its transcriptional activity, possibly through an increase in the amount of protein bound to E-box sequences. This results in increased expression of MUC2 mRNA, even in cells with accumulated β-catenin protein, which gives rise to the proliferated state. We further showed that the stabilization of WT Hath1 protein by a GSK3 inhibitor is adequate for the up-regulation of MUC2 mRNA in colon cancer cells, and propose that degradation of the Hath1 protein might lead to the undifferentiated state in colon cancer.

The significant role of Wnt signaling in healthy and malignant intestinal epithelium has been noted in terms of maintenance for both continuous proliferated and undifferentiated states [12,13]. Active Wnt signaling simultaneously promotes cell proliferation and inhibits differentiation through controlling c-myc and $p21^{CIP/WAF1}$ activity [13]. Our concern has been to find whether suppression of cell proliferation by inactivation of Wnt signaling leads exclusively the differentiated state in colon cancer. Focusing on the role of Hath1 in intestinal cell differentiation and its regulation at protein level, we contribute a new aspect to the establishment of neoplastic characteristics. Our findings in this study first suggest a partial contribution of Hath1 protein to the undifferentiated state in colon cancer. Hath1 protein degradation by active Wnt signaling might cause cell differentiation to halt, giving rise to the undifferentiated state viewed as one of characteristics of colon cancer. Our results are supported by the fact that, although Hath1 protein is expressed in only 7% of nonmucinous carcinomas, both Hath1 and MUC2 are expressed in more than 70% of mucinous cancers which have low malignant potential without APC deletion [8]. However, stabilization of Hath1 protein did not induce other differentiation markers such as phospholipase A2 (sPLA2), isomaltase and chromogranin A (CgA), in spite of the possibility of differentiation toward three secretory lineages